2022 Physical Biosciences Research Meeting

Program and Abstracts

Zoom Virtual Meeting

October 26 – 28, 2022

Chemical Sciences, Geosciences, and Biosciences Divisions

Office of Basic Energy Sciences

Office of Sciences

U.S. Department of Energy

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Forward

This Meeting Book is a record of the 8th biennial meeting of the principal investigators funded by Physical Biosciences program in the Chemical Sciences, Geosciences, and Biosciences (CSGB) Division of the Office of Basic Energy Sciences (BES), U.S. Department of Energy (DOE). CSGB supports fundamental biophysical and biochemical research relevant to DOE's mission areas through 2 core research programs: Photosynthetic Systems and Physical Biosciences. These programs provide foundational knowledge on energy capture, conversion, and storage in complex natural systems to advance energy technologies. These two programs, together with the Solar Photochemistry program, comprise the CSGB Photochemistry and Biochemistry Team, a coordinated group of programs supporting areas of basic research that are central to the science mission of the DOE.

Biology remains capable of complex chemical transformations, electron transfer, and energy management beyond what can be recreated in engineered and synthetic systems currently. These natural processes exhibit enormous structural and chemical diversity and understanding them requires characterization of diverse phenomena including the mechanisms of multielectron catalysis, cofactor and metallocluser biosynthesis, redox tuning of electron transfer within protein and across pathways, and the role of structure, function and conformational change in regulating electron flow. The abstracts in this volume describe research at the forefront of understanding the chemistry, biochemistry, biophysics, and molecular biology that underpin these essential energy relevant processes. The quality and novelty of the research presented here demonstrates the drive, dedication, and talent of the outstanding researchers who make Physical Biosciences an exciting and innovative scientific community.

This meeting aims to distribute recent research accomplishments and foster exchange of scientific knowledge and insights among the community of researchers who make up the Physical Biosciences program. Questions and ideas from meeting participants, the exchange of new results and methodologies, the forging of cooperative studies and collaborations, and opportunities to interact with program managers and staff of the DOE are all welcome and encouraged.

I would like to thank Teresa Crockett in DOE BES along with Linda Severson and Kimberly Olson of the Oak Ridge Institute for Science and Education (ORISE) for their help with planning and execution of meeting logistics. I would also like to thank the rest of the Photochemistry and Biochemistry team program managers - Steve Herbert, Chris Fecko, and Jenny Roizen for their support and advices, and out team lad Gail McLean for her exemplary leadership and dedication. Finally, thank you to all the participants for sharing their time and their hard work.

Kate A. Brown, Program Manager, Physical Biosciences, DOE BES

Agenda

AGENDA

8th Biennial Physical Biosciences Research Meeting

October 26-28, 2022

Note: All Times Below are Eastern!

Wednesday October 26, 2022

12:30 – 1:00 Log-in to Zoom and "settle in." Having trouble? Call 865-951-3259

Session I: Welcome and Team and Physical Biosciences Program Updates

1:00 – 1:30 Welcome and Physical Biosciences Program Update
Kate Brown, Program Manager, Physical Biosciences, DOE-BES

Session II: Keynote Lecture 1

1:30 – 2:30 Multimodal Approaches for Leveraging Domain Knowledge with State-of-the-Art Machine Learning to Engineer Biocatalysts
Frances Arnold, California Institute of Technology

2:30 – 3:00 Break

Session III: Plant Metabolism and Energy Management

Moderator – **Kent Chapman**

3:00 – 3:30 Characterizing Plant-Specific Features of Mitochondrial Respiratory Complexes
James Letts, University of California, Davis

3:30 – 4:00 Molecular Mechanism of Energy Transduction by Plant Membrane Proteins
Michael Sussman, University of Wisconsin

4:00 – 4:30 Exploring the Role of TOR Kinase in the Regulation of Central Metabolism and Lipid Synthesis
Zhiyang Zhai, Brookhaven National Laboratory

4:30 – 4:40 Short Break

Session III: Enzyme Directionality and Electron Flow

Moderator – **David Mulder**

4:40 – 5:10 Tuning Directionality for CO2 Reduction in the Oxo-acid:Ferredoxin Superfamily
Sean Elliot, Boston University

5:10 – 5:40 Understanding Redox Proportioning Through Ferredoxins, Low Potential Iron-Sulfur Proteins Acting as Electrical Hubs to Control Metabolism
Jonathan Silberg, Rice University

5:40 – 6:30 Break

Session IV: Poster Session I

6:30 – 8:30 Posters in Gathertown
Even numbered posters

Thursday October 27, 2022

12:30 – 1:00 Log-in to Zoom and "settle in." Having trouble? Call 865-951-3259

Session V: Keynote Lecture 2

1:00 – 2:00 Living Bio-Nano Systems for Solar Hydrogen Production
Kara Bren, University of Rochester

2:00 – 2:30 Break

Session VI: Mechanisms in CO_2 Chemistry

Moderator – **Hannah Shafaat**

2:30 – 3:00 Methyl-coenzyme M reductase: Understanding assembly and the significance of coenzyme F_{430} modifications
Kylie Allen, Virginia Polytechnic Institute and State University

3:00 – 3:30 Activation of Recombinant Methyl-Coenzyme M Reductase in the Methanogenic Archaeon Methanococcus maripaludis and the Role of Post-Translational Modifications
Evert Duin, Auburn University

3:30 – 4:00 Elucidating the Catalytic Mechanism of Microbial CO2 Fixation
Ritimukta Sarangi, SLAC National Accelerator Laboratory

4:00 – 4:30 Formate Metabolism in Hydrogenotrophic Methanogens
Kyle Costa, University of Minnesota

4:30 – 4:40 Short Break

Session VII: Complex Chemical Synthesis in Plants

Moderator – **Parastoo Azadi**

4:40 – 5:10 The Chemical Wizardry of Terpene Synthases – How Managing Reactive Carbocations Determines Catalytic Outcomes
Mark Lange, Washington State University

5:10 – 5:40 Dimers are Forever - How a Plant Crosslinks Pectin
Malcolm O'Neill, University of Georgia
Vivek Bharadwaj, National Renewable Energy Laboratory

5:40 – 6:30 Break

Session VIII: Poster Session II

6:30 – 8:30 Posters in Gathertown
Odd numbered posters

Friday October 28, 2022

12:30 – 1:00 Log-in to Zoom and "settle in." Having trouble? Call 865-951-3259

Session IX: Keynote Lecture III

1:00 – 2:00 Transformative Biohybrid Diiron Catalysts for C-H Bond Functionalization
Qun Liu, Brookhaven National Laboratory

2:00 – 2:30 Break

Session X: Nitrogen Chemistry

Moderator – **Kyle Lancaster**

2:30 – 3:00 Understanding Nitrogenase Maturation and Activity in Methanogens
Daniel Lessner, University of Arkansas

3:00 – 3:30 Engineering a Functional Equivalent of Nitrogenase for Mechanistic Investigations of Ammonia Synthesis
Yilin Hu, University of California, Irvine

3:30 – 4:00 Allosteric Control of Electron Transfer in Nitrogenase-like Enzymes
Edwin Antony, St Louis University

4:00 – 4:30 Dissimilatory Nitrite Reduction to Ammonium: Catalyzing Multi-Electron Reductions Using a Pentaheme Scaffold
Eric Hegg, Michigan State University
Nicolai Lehnert, University of Michigan

Session XI: Closing Session and Q&A

4:30 – 5:00 What's Next, Where Do We Go From Here? *Program Manager Q and A*
Kate Brown, Program Manager, Physical Biosciences, DOE-BES
Steve Herbert, Program Manager, Photosynthetic Systems, DOE-BES

Posters

Even Posters present Wednesday October 26

Odd Posters present Thursday October 27

1. An Abundant and Diverse New Family of Electron Bifurcating Enzymes with a Non-Canonical Catalytic Mechanism
   Michael W. W. Adams, University of Georgia
2. DOE Center for Plant and Microbial Complex Carbohydrates
   Parastoo Azadi, University of Georgia
3. Tracking Photosynthetic Metabolites Across Bacterial Microcompartments Using Computational Microscopy
   Christoph Benning, Michigan State University
4. Biological free energy transduction is an Achilles heel of mean-field transport theory
   David N. Beratan, Duke University
5. Probing Novel Pathways of Metal Sulfide Acquisition and Trafficking from Minerals
   Eric Boyd, Montana State University
6. Mechanisms and Pathways in Hydrogenase Maturation
   Joan B. Broderick, Montana State University
7. Elucidating the Cellular Machinery for Lipid Storage in Plants
   Kent D. Chapman, University of North Texas
8. Molecular Mechanisms of Plant Cell Wall Loosening
   Daniel Cosgrove, Pennsylvania State University
9. The Fe Protein Cycle Associated with Nitrogenase Catalysis Required that Hydrolysis of Two ATP for Each Single Electron Transfer Event
   Denis Dean, Virginia Polytechnical
   Lance Seefeldt, Utah State University
10. Extracellular Charge Transport in Microbial Redox Chains: Linking the Living and Non-Living Worlds
    Mohamed Y. El-Naggar, University of Southern California
11. Characterization of the archetype from group 4 of the FTR-like family of ferredoxin:thioredoxin reductases prevalent in the domains Bacteria and Archaea
    Greg Ferry, Pennsylvania State University
12. Selecting a new electron transfer pathway for nitrogen fixation uncovers an electron bifurcating-like enzyme involved in anaerobic aromatic compound degradation
    Kathryn R. Fixen, University of Minnesota
13. Directed evolution of aerotolerance in a sulfide-dependent thiazole synthase
    Andrew D. Hanson, University of Florida
14. The air activation of formate dehydrogenase FdsDABG from Cupriavidus nector
    Russ Hille, University of California, Riverside
15. Electron Transport in Bacterial Cytochrome Polymers
    Allon I. Hochbaum, University of California, Irvine
    Daniel R. Bond, University of Minnesota
    Edward H. Egelman, University of Virginia

16. Is the CFe6 Core of the Nitrogenase Catalytic Cofactor a Hemilabile 'Beating Heart' or Stabilizing 'Heart of Steel'
Brian M. Hoffman, Northwestern University
17. Electron Flow and Energy Conversion in Syntrophic Metabolism
Elizabeth A. Karr, University of Oklahoma
18. Mechanism of Photochemical N2 Reduction by Nanocrystal-Nitrogenase Biohybrids
Paul W. King, The National Renewable Energy Laboratory
19. Characterization of a reductive activase from Methanosarcina barkeri
Joseph A. Krzycki, The Ohio State University –
20. Radical-Polar Crossover Mechanism of Ethylene Forming Enzyme and Rational Perturbations of its Two Branch Points
Carsten Krebs, Pennsylvania State University
21. Bioinorganic Chemistry of Nitrification: Structure and Function of Ammonia Monooxygenase
Kyle H. Lancaster, Cornel University
22. Structure and Mechanism of an Electron Bifurcating NiFe-Hydrogenase
Huilin Li, Van Andel Research Institute
23. The Unusual Thermodynamics of a Flavin-Based Electron Bifurcation Enzyme
Carolyn E. Lubner, The National Renewable Energy Laboratory
24. Mechanistic Studies of a Primitive Homolog of Nitrogenase Involved in Coenzyme F430 Biosynthesis
Steven O. Mansoorabadi, Auburn University
25. Archaeal redox biochemistry and post-translational modification
Julie A. Maupin-Furlow, University of Florida
26. Energy conservation, electron transfer and enzymology during methane production by Methanosarcina species
William W. Metcalf, University of Illinois, Urbana Champaign
27. Conformational Gating and Dynamics
Anne-Frances Miller, University of Kentucky
28. Exploring properties of single carboxysomes with an advanced anti-Brownian trap
W. E. Moerner, Stanford University
29. Multidimensional Spectroscopies for Probing Coherence and Charge Separation in Photosystem II
Jennifer P. Ogilvie, University of Michigan
30. Novel microbial based enzymatic CO2 fixation mechanisms: conformational control of enzymatic reactivity
John Peters, Washington State University
31. Enzymology of Methanogenesis: Mechanism of Methyl-Coenzyme M Reductase
Steven W. Ragsdale, University of Michigan
32. Energy Conservation via Aerobic Respiration in a Methanogen
C. S. Raman, University of Maryland
33. Enzymatic Energy Conversion
Simone Raugei, Pacific Northwest Laboratory

34. Generating Nitrogenase Hybrids with Altered Cofactor Compositions
Markus W. Ribbe, University of California, Irvine
35. Missing links in biological methane and ammonia oxidation
Amy C. Rosenzweig, Northwestern University
36. Identification of Novel Extracellular Proteins Up-regulated Curing Extracellular Electron Uptake in Methanosarcina barkeri
Annette Rowe, University of Cincinnati
37. Regulated reductive flow through archaeal respiratory and energy production systems
Thomas J. Santangelo, Colorado State University
38. Structure and function of carbon concentrating machinery
David F. Savage, University of California, Berkeley
39. Developing Molecular Level Understanding of Carbon Monoxide/Acetyl Coenzyme A Synthase Through Model Metalloenzymes
Hannah S. Shafaat, The Ohio State University
40. Characterization of Trehalose 6-Phosphate Binding to SNF1-Related Protein Kinase Catalytic Subunit Alpha: KIN10
John Shanklin, Brookhaven National Laboratory
41. Engineering selenoproteins for enhanced hydrogen production
Dieter Soll, Yale University
42. Where are the Electrons in Iron-Sulfur Clusters?
Daniel L. M. Suess, Massachusetts Institute of Technology
43. Atomic Resolution of Lignin-Carbohydrate Interactions in Native Plant Tissues from Solid-State NMR
Tuo Wang, Michigan State University
44. Expression of recombinant methyl-coenzyme M reductase in Methanococcus maripaludis
William B. Whitman, University of Georgia
45. Elucidating the Biochemical Mechanisms Controlling Secondary Wall Biosynthesis in Plants
Zheng-Hua Ye, University of Georgia

Abstracts

(in alphabetical order)

Hyperthermophilic Multiprotein Complexes for Energy Conservation and Catalysis

Michael W. W. Adams, Principal Investigator
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Overall research goals:

The goal of our research is understand at a mechanistic level multiprotein complexes that carry out novel means of catalysis and energy conservation involving multielectron transfer reactions and electron bifurcation. Some of the complexes under study have the remarkable property of being synthesized (self-assembling) at temperatures near 100°C and are involved in the conversion of high and low potential electrons via electron bifurcation into a wide variety of products. Some of the other complexes are involved in the conversion of low potential reducing equivalents into gaseous end products (hydrogen, H_2, and hydrogen sulfide, H_2S) with the concomitant conservation of energy in the form of ion gradients, while others appear to utilize electron bifurcation to conserve energy.

Significant achievements (2019-2022):

Pyrococcus furiosus (*Pfu*) grows optimally at 100°C and is thought to represent an ancestral life form. *Pfu* obtains carbon and energy for growth by fermenting carbohydrates and producing H_2 gas or by reducing elemental sulfur (S°) to H_2S gas. It also has a respiratory metabolism in which it couples H_2 production by a ferredoxin-dependent, 14-subunit membrane-bound NiFe-hydrogenase (MBH) to sodium ion translocation and formation of a membrane potential that that the organism utilizes to synthesize ATP. MBH is highly homologous to modern day NADH-oxidizing, quinone-reducing Complex I of the aerobic respiratory chain. Addition of S° to growing *Pfu* induces the synthesis of a homologous 13-subunit membrane-bound complex termed MBS, a sulfane sulfur reductase. This respiratory enzyme catalyzes the reduction of internal S-S bonds in polysulfides (S_n^{2-}) but does not itself generate H_2S. The cryo-EM structures of both MBH and MBS were determined by Dr. Huilin Li (Van Andel Research Institute). MBH contains a membrane-anchored NiFe-hydrogenase module that is highly similar structurally to the quinone-binding Q-module of Complex I while its membrane-embedded ion translocation module can be divided into a H^+- and a Na^+-translocating unit. The H^+-translocating unit is rotated 180° in-membrane with respect to its counterpart in Complex I, leading to distinctive architectures for the two respiratory systems despite their largely conserved proton-pumping mechanisms. The Na^+-translocating unit, absent in Complex I, resembles that found in the Mrp H^+/Na^+ antiporter and enables H_2 production by MBH to establish a Na^+ gradient for ATP synthesis. MBS retains all of the structural features thought to be involved in energy transduction in MBH, but contains an additional ion pumping module not present in MBH. This is thought to enable polysulfide reduction by MBS to conserve more energy than proton reduction by MBH.

In addition, we very recently discovered an abundant and diverse class of electron bifurcating (BF-) enzyme family that we term BfuABC. We noticed that the genome of thermophilic bacterium *Acetomicrobium mobile* (*Amob*) contained an unusual NiFe-hydrogenase that appeared to also contain subunits (HydABC) of a BF-FeFe-hydrogenase, an enzyme we discovered more than a decade ago. We purified the *Amob* NiFe-enzyme and showed that it is, indeed, a BF-enzyme (NiFe-BfuABCSL). The cryoEM structure was determined by Dr. Huilin Li, the first for an HydABC-type BF-enzyme, and the arrangement of its iron-sulfur and flavin cofactors showed that it has a new type of bifurcating site. *Pfu* does not contain a Bfu family member but the closely-related hyperthermophile *Thermococcus sibricus* (*Tsib*) does. Termed Nfn-Bfu, the *Tsib* enzyme has been heterologously produced in *Pfu.*

Science objectives for 2022-2023: The specific aims of the proposed research are 1) to determine the unique properties of the bifurcating site in Bfu enzymes and elucidate the mechanism of electron bifurcation, 2) to

characterize unprecedented types of bifurcating Bfu enzymes, including ones that potentially utilize hydrogen peroxide, acetylene and monoterpenes, and ones involved with cellular processes, such as proteolysis, and 3) to characterize membrane-bound ion-translocating Bfu family members that couple electron bifurcation to the formation of chemical gradients and compare their properties with those of *Pfu* MBH.

My project addresses BES cross-cutting priority areas by: This research specifically address several of the overall goals of the Physical Biosciences Program. We are providing "a better understanding of the structure/function, mechanistic and electrochemical properties of enzymes that catalyze complex multielectron redox reactions". We are focusing on "electron bifurcation and critical components that direct and regulate the flow of electrons on larger spatial and temporal scales through energy-relevant pathways."

The ideal collaborator for my project would have expertise in: Protein film electrochemistry

Publications supported by this project [2020-2022]:

1. Haja, D. K, Wu, C.-H., Poole, F. L., Williams, S. D., Jones, A. K. and Adams, M. W. W. (2020) "Characterization of thiosulfate reductase from *Pyrobaculum aerophilum* heterologously expressed in *Pyrococcus furiosus*" *Extremophiles* **24**, 53-62(doi: 10.1007/s00792-019-01112-9)
2. Haja, D. K., Wu, C.-H., Poole, F. L., Ponomarenko, O., George, G. N., and Adams, M. W. W. (2020) "Improving arsenic tolerance of *Pyrococcus furiosus* by the heterologous expression of a respiratory arsenate reductase" *Appl. Environ. Microbiol.* **86**, e01728 (doi: 10.1128/AEM.01728-20)
3. Vansuch, G. E., Wu, C-H., Haja, D. K., Blair, S. A., Chica, B., Johnson, M. K., Adams, M. W. W. and Dyer, R. B. (2020) "Metal-ligand cooperativity in the soluble hydrogenase-1 of *Pyrococcus furiosus*" *Chem. Sci.* **11,** 8572 – 8581 (doi: 10.1039/d0sc00628a)
4. Yang, W., Vansuch, G. E., Liu, Y., Jin, T., Liu, Q., Ge, A., Sanchez, M., Haja, D. K., Adams, M. W. W., Dyer, R. B., Tianquan, L. (2020) "Surface ligand "liquid" to "crystalline" phase transition modulates the solar H_2 production quantum efficiency of CdS nanorod/mediator/hydrogenases assemblies" *ACS Appl. Mater. Interfaces* **12,** 35614-35625 (doi: 10.1021/acsami.0c07820)
5. Straub, C. T., Schut, G. J., Otten J. K., Keller, L. M., Adams, M. W. W. and Kelly R. M. (2020) Modification of the glycolytic pathway in *Pyrococcus furiosus* and the implications for metabolic engineering. *Extremophiles* 24, 511-518 (doi: 10.1007/s00792-020-01172-2)
6. Yu, H., Haja, D. K., Schut, G. J., Wu, C.-H., Meng, X., Zhao, G., Li, H. and Adams, M. W. W. (2020) "Iron-sulfur cluster-catalyzed elemental sulfur reduction gave ancient life energy" *Nature Commun.* **11**,5953 (doi: 10.1038/s41467-020-19697-7)
7. Yu. H., Schut, G. J., Haja, D. K., Adams, M. W. W. and Li, H. (2021) "Evolution of complex I-like respiratory complexes" *J. Biol. Chem.* **296,** 100740 (doi: 10.1016/j.jbc.2021.100740)
8. Feng, X., Schut, G. J., Lipscomb, G. L., Li, H. and Adams, M. W. W. (2021) "CryoEM structure and mechanism of the membrane-associated electron bifurcating flavoprotein Fix/EtfABCX" *Proc. Natl. Acad. Sci. USA* **118,** e2016978118 (doi: 10.1073/pnas. 2016978118)
9. Vali, S. W., Haja, D. K., Brand, R. A., Adams, M. W. W. and Lindahl, P. A. (2021) "The *Pyrococcus furiosus* ironome is dominated by $[Fe_4S_4]^{2+}$ clusters or thioferrate-like iron depending on the availability of elemental sulfur" *J. Biol. Chem.* **296**, 100710 (doi: 10.1016/j.jbc.2021.100710)
10. Haja, D. K. and Adams, M. W. W. (2021) "pH homeostasis and sodium ion pumping by Mrp in *Pyrococcus furiosus*" *Front. Microbiol.* **12,** 712104 (doi.org/10.3389/fmicb.2021.712104)
11. Clarkson, S. M., Haja, D. K. and Adams, M. W. W. (2021) "The hyperthermophilic archaeon *Pyrococcus furiosus* utilizes environmental iron sulfide cluster complexes as an iron source" *Extremophiles* **25**, 249-256 (doi: 10.1007/s00792-021-01224-1)
12. Feng, X., Schut, G. J., Haja, D. K., Poole, F. L., Adams, M. W. W. and Li, H. (2022) "Structure and electron transfer pathways of an electron bifurcating NiFe-hydrogenase" *Science Advs.* **8,** eabm7546 (doi: 10.1126/sciadv.abm7546)
13. Schut, G. J., Haja, D. K., Feng, X., Poole, F. L., Li, H. and Adams, M. W. W. (2022) "An abundant and diverse class of bifurcating enzyme with a non-canonical catalytic mechanism" *Front. Microbiol.* **13,** 946711 (doi: 10.3389/fmicb.2022.946711)

Understanding the biosynthesis and functions of modified F_{430} coenzymes in methanogens and anaerobic methanotrophs

Kylie Allen, Principal Investigator
Justin Lemkul, Emily Mevers, Biswarup Mukhopadhyay, Co-PI(s)
340 West Campus Drive, Department of Biochemistry, Virginia Tech, Blacksburg, VA 24060
Email: kdallen@vt.edu; Website: https://www.theallenlab.com/

Overall research goals:

The focus of our research is the nickel tetrapyrrole prosthetic group known as coenzyme F_{430}, the central catalytic component of methyl-coenzyme M reductase (MCR). MCR catalyzes the final methane-forming step of methanogenesis in methanogenic archaea as well as the initial methane activation step of anaerobic methane oxidation in anaerobic methanotrophs. MCR is the only enzyme with which coenzyme F_{430} is known to function, signifying that nature has evolved a specialized cofactor to catalyze complex methane conversions. Several chemical modifications to F_{430} have been discovered including three different thioether substituents and a vinyl sidechain. We propose that these F_{430} modifications have a role in fine-tuning the MCR active site to enhance catalytic efficiency, influence protein dynamics and stability, and/or to guide the directionality of the MCR reaction.

The specific goals of this project are to define and biochemically characterize the enzymes involved in the biosynthesis of modified F_{430}s as well as confirm the structures and locations of the proposed modifications. We will further provide insights into the growth conditions that promote the production of modified F_{430}s in model methanogens as well as if and how the modifications influence MCR structure and catalysis using *in vivo* and *in silico* approaches. Taken together, this work will uncover new aspects of MCR structure and function that could inform the design of optimized MCR-based catalysts for bioenergy applications.

Significant achievements (2021-2023):

- Growth experiments in *M. maripaludis*, *M. jannaschii*, and *M. acetivorans* demonstrated the production of thioether-containing F_{430} modifications are largely present in the stationary phase of growth and are likely associated with low-energy growth conditions
- Demonstrated that mercaptopropamide-F_{430} (Figure 1) is up to 50% of the total F_{430} pool in acetate-grown *M. acetivorans* and is associated with MCR, thus indicating that the F_{430} modification is physiologically relevant and likely affects the activity of MCR
- Confirmed that M. *jannaschii* produces our originally characterized mercaptopropionate-F_{430} (Allen et al., Appl. Environ. Microbiol., 2014), while *M. maripaludis* produces our more recently discovered amide version (Figure 1) that is also found in *M. acetivorans*
- Demonstrated that mercaptopropamide-F_{430} retains the characteristic 430 nm absorbance peak, revealing that this modification retains the 17^1 keto group as opposed to the proposed cyclized structure of mercaptopropionate-F_{430} (Figure 1)
- Heterologously expressed and biochemically characterized a novel archaeal radical SAM methylthiotransferase involved in tRNA and/or F_{430} modification
- Demonstrated that AhbD from *M. acetivorans* (MA0573), the enzyme involved in heme biosynthesis, can also accept F_{430} as a substrate to generate vinyl-F_{430} (Figure 1) *in vitro*

- Developed protocols for purification of modified F_{430} from methanogen cell extracts, which separates the cofactor from other small molecules as well as unmodified F_{430}- will be used for large scale purification for NMR structure determination
- Completed initial molecular dynamics simulations of MCR from *M. acetivorans,* which required extensive parametrization efforts on the F_{430} cofactor, Ni, coenzyme B, and methyl-coenzyme M
- Initiated the free energy simulations necessary to transform F_{430} into the modified F_{430} derivatives for future molecular dynamics simulations to understand the influence of F_{430} modifications on MCR structure and activity

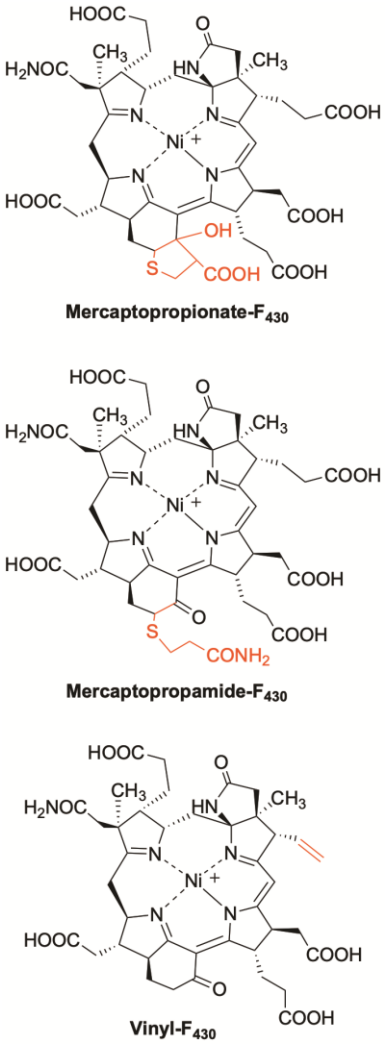


Figure 1. Proposed structures of F_{430} modifications found in methanogens.

Science objectives for 2022-2023:

- Scale up growth of *M. jannaschii* in a 12-liter bioreactor to obtain large amounts of cells for mercaptopropionate-F_{430} purification and subsequent structure determination by NMR spectroscopy
- Identify and characterize putative sulfur insertion enzyme required for methylthio-F_{430} biosynthesis in anaerobic methanotrophs
- Isotope feeding experiments to identify the biochemical precursors to mercaptopropionate(amide)-F_{430}
- Perform polarizable molecular dynamics simulations of MCR with F_{430} compared to modified F_{430} – this will reveal the role of F_{430} modification in potentially re-structuring the MCR active site and influencing catalysis

My project addresses BES cross-cutting priority areas by: providing molecular, physiological, and biophysical insights into modified F_{430} coenzymes that function with the key methane-generating and methane-oxidizing enzyme, methyl-coenzyme M reductase. These fundamental studies could inform the optimization of biochemical pathways and the design of MCR-based biocatalysts for methane production and bioconversion applications.

My scientific area(s) of expertise is/are: anaerobic enzymology, radical SAM enzymes, methanogen culture and genetic manipulation, mass spectral structure determination of new metabolites/coenzymes

The ideal collaborator for my project would have expertise in: structural biology of metalloenzymes and protein complexes, biophysical techniques to assess small molecule-protein interactions and protein-protein interactions, EPR spectroscopy

Publications supported by this project 2021-2023:

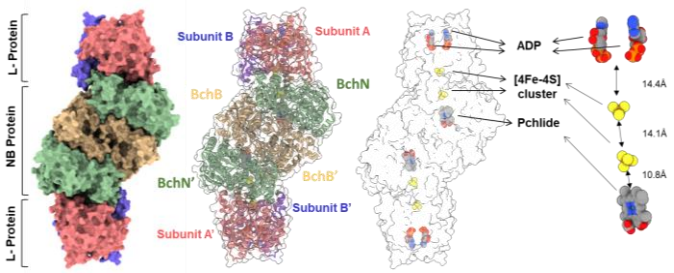
Gendron, A., Allen, K. "Overview of Diverse Methyl/Alkyl-Coenzyme M Reductases and Considerations for Their Potential Heterologous Expression" *Frontiers in Microbiology* (2022) https://doi.org/10.3389/fmicb.2022.867342

Allosteric control of electron transfer in nitrogenase and nitrogenase-like enzymes

Edwin Antony, Principal Investigator
Rajnandani Kashyap, Postdoctoral Research Associate
Department of Biochemistry and Molecular Biology, Saint Louis University School of Medicine, St. Louis, MO 63104
Email: eantony@slu.edu; Website: www.antonylab.org

Overall research goals:

Enzymes that catalyze multi-electron substrate reduction reactions are found throughout nature including several enzymes important for nitrogen fixation and photosynthesis. The mechanisms by which these enzymes keep track of electrons accumulated at the active site and accurately transfer them to the substrate are subjects of intense investigation. The biological design of these enzymes and how they mediate long-range electron transfer over a series of sophisticated metal centers have served as blueprints for the design of bio-inspired catalysts. Knowledge of how long-range electron transfer reactions are catalyzed will be directly applicable to engineer designer enzymes capable of reducing substrates. Many such enzymes that catalyze electron transfer are structurally arranged as higher order oligomers and use long-range allosteric control between subunits as a mechanism to regulate activity in response to cellular needs. However, the mechanistic basis of such control is poorly understood. Our lab focuses on the mechanism of action of nitrogenase and nitrogenase-like enzymes, which function as oligomeric complexes that utilize ATP to coordinate electron transfer. Our work uncovered that both these structurally symmetric, multi-subunit, ATP-utilizing enzymes, function asymmetrically and use ATP binding/hydrolysis to establish asymmetry. Our current efforts focus on investigating the origins of allostery within nitrogenase-like enzymes using single-molecule and cryoEM approaches.



Significant achievements: [2021, 2022, 2023]:

Our efforts to investigate the ATPase coupling of electron transfer in the nitrogenase system revealed an asymmetry and intrinsic negative allostery controlling substrate reduction (Duval et. al. PNAS 2013 and Danyal et. al. PNAS 2016). Since this discovery, we have shown that the principles that govern asymmetric electron transfer in nitrogenase is also conserved in the nitrogenase-like enzyme – dark operative protochlorophyllide oxidoreductase (DPOR). DPOR is a key enzyme in the maturation of bacteriochlorophyll and shares structural homology with nitrogenase. However, they differ in the composition of the metal clusters and a large active site capture protochlorophyllide (Pchlide) and reduces it to chlorophyllide (Chlide). We uncovered that the electron donor protein (BchL) uses a disordered N-terminus as a regulatory mechanism for electron transfer (Corless et. al. JBC 2021). In addition, we revealed that substrate binding induced asymmetry in the system (Corless et. al. JBC 2020). Along the way, we also developed cell lines and methodologies to better overproduced FeS containing enzymes (Corless et. al. JBac 2020 and Corless et. al. Methods Mol.Bio).

Science objectives for 2021-2022:

Develop better anaerobic conditions for single-molecule FRET measurements.

Solve the transition-state structures of the DPOR complex using cryoEM.

Perform HDX-MS experiments to uncover the origins of allostery within DPOR and COR

My project addresses BES cross-cutting priority areas by:

Enzymes capable of reducing complex substrates are of high economic value. More than a third of all known proteins to date are either oxidoreductases or metalloproteins. The diversity of metal cofactors and redox centers in these proteins make them attractive candidates for engineering protein-based biocatalysts, biosensors, and biofuel cells, among other necessary tools for the bioeconomy. The proposed research investigates the mechanism of action of two such nitrogenase-like oxidoreductase enzymes: DPOR and COR, which catalyze key successive multi-electron reductive steps in the biosynthesis of bacteriochlorophyll. Similar to nitrogenase, these enzymes utilize a higher order oligomeric architecture with two identical halves and our work over the past several years has uncovered that the two catalytic halves function in a sequential manner for electron transfer. In addition, several recently discovered nitrogenase-like enzymes showcase novel reduction chemistries in sulfur scavenging (methylthio-alkane reductases) and coenzyme-F430 cofactor biosynthesis (CfbCD complex). These exciting discoveries suggest that the nitrogenase-like family evolved to tackle elemental processes beyond nitrogen and carbon assimilation. Furthermore, the catalytic unit of nitrogenase has been developed into a light-driven, bio-hybrid, nitrogen reduction system and holds the potential to revolutionize fertilizer production. Understanding the workings of these higher order oligomeric catalytic units is imperative to drive such protein-driven catalytic applications.

My scientific area(s) of expertise is/are: Transient-state kinetics, single-molecule fluorescence microscopy, biophysical chemistry, and CryoEM microscopy.

The ideal collaborator for my project would have expertise in: Computational calculations of protein motions. Data collection at DOE cryoEM facility.

Publications supported by this project 2021-2023:

1. E. Corless and E. Antony. Methods for heterologous overproduction of Fe-S proteins. Methods Mol Biol 2353; 69-78 (2021). doi: 10.1007/978-1-0716-1605-5_4.
2. E. Corless, S. M. Saad Imran, M.B. Watkins, J.P. Bacik, J.R. Mattice, A. Patterson, K. Danyal, M. Soffe, R. Kitelinger, L.C. Seefeldt, S. Origanti, B. Bennett, B. Bothner, N. Ando, and E. Antony. The flexible N-terminus of BchL autoinhibits activity through interaction with its [4Fe-4S] cluster and released upon ATP binding. J. Biol. Chem. 296:100107 (2021). doi: 10.1074/jbc.RA120.016278.
3. J. Deveryshetty, and E. Antony. Electrons and Protons: Nitrogenase. EBC. 2:586-595. (2021). doi.org/10.1016/B978-0-12-819460-7.00246-2.
4. J. Deveryshetty, G.R. Sorg, and E. Antony. Dark-operative protochlorophyllide oxidoreductase. EiBC. (2022). doi.org/10.1002/9781119951438.eibc2820

Multimodal Approaches for Leveraging Domain Knowledge with State-of-the-Art Machine Learning to Engineer Biocatalysts

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Overall research goals:

The goal of this project is to advance data-driven biocatalyst engineering toward a future where fuels, materials and chemicals can be produced using rapidly and reliably optimized biological systems. We envision a generational advance in enzyme engineering enabled by fundamentally new data-driven models that incorporate information from multiple sources, including sequences, structures, energetics, (limited) experimental measurements, and more.

Enzymes are nature's catalysts, the protein machines that build the materials and molecules of life from abundant and often renewable resources. By engineering the DNA that encodes an enzyme, we can create new versions that are optimized and adapted to human applications. Enzyme engineering presents an enormous opportunity for the development of new catalysts, but the nearly infinite design space for enzyme engineering is poorly understood. Directed evolution (DE) has driven the success of enzyme engineering for more than 25 years; however, DE remains time-consuming and resource-intensive due to limited strategies and experimental methods for searching the near-infinite design space of enzymes. We are developing new strategies for enzyme engineering that combine advances in protein engineering, machine learning, and physical simulations. Our goal is to create generalizable ML tools and computational frameworks for enzyme discovery and engineering.

Our project is organized into three aims: (1) develop much-needed, robust enzyme sequence-fitness datasets enhanced with structural and energetic properties; (2) create a set of ML tools that can learn effectively from such multimodal data sources, accelerate enzyme engineering, and predict novel enzymatic activities; and (3) develop a computational framework for the design of training sets that minimize uncertainty and facilitate knowledge transfer to different protein/enzyme systems.

Significant achievements: [09/01/2021 - 08/31/2022]:

All the milestones for the first year mark progress toward Aim 1 (Multimodal Enzyme Datasets Tailored for Data-Driven Discovery). We generated single- and double-site-saturation mutant libraries of *Tm*TrpB (the ß subunit of tryptophan synthase) and screened those libraries via competitive growth assays combined with next-generation sequencing. These sequence-function data have been compared to *in vitro* rates of tryptophan formation to validate use of the high-throughput growth assay as a quantitative measure of enzyme activity. Construction of libraries having mutations at three and four sites is underway, and scaling challenges that were identified have been addressed. We have generated homology models of *Tm*TrpB structures using AlphaFold based on multiple sequence alignments (MSAs) with variable maximum allowed sequence identity. We also obtained a crystal structure of TmTrpB9D8* at 2.15 Å resolution. Our method and software for protein variant sequencing was published, and we have developed more robust sequence-fitness pairing software that is being tested in-house.

We have also made progress toward the Aim 2 (Multimodal Protein Representations Enable Enzymatic Activity Predictions) goals by generating physics-based features for enzyme reactivity. Drawing on expertise for the simulation of biological molecules and chemical reactions, we have begun development of a generalized workflow for computational enzyme simulation and characterization. Mimicking the workflow of directed evolution, the computational workflow begins with the parent protein structure, generates structures of mutants, and screens variants via observables from simulations, including molecular dynamics and quantum mechanics simulations. These observables are being made compatible with the machine learning pipeline that is under simultaneous development. The machine learning pipeline incorporates residue-resolution observables from simulations into graph-based structures, including side chain motions and dynamic cross-correlations. The benefit of including this additional information will be assessed relative to sequence-only- and structure-only models. The workflow is designed to contain elements general to any enzyme of interest for future studies.

Finally, we have been developing a multimodal representation learning pipeline that is synergistic with protein structure and sequence information by starting with a publicly available four-site protein binding dataset that resembles our four-site *Tm*TrpB enzyme library under construction.

We have begun work on Aim 3 (Robust Data-Driven Experimental Design); at this stage our work is geared toward laying the foundations for data-driven experimental design. We have started a reading group focused on the topic of uncertainty quantification in machine learning and are outlining approaches that are promising fits to the challenge of data-driven experimental design.

Science objectives for 2022-2023:

Much of our work in the next reporting period will focus on obtaining the experimental sequence-function data for TrpB and using computational chemistry to develop physics-based representations of enzyme variants and developing the workflow for generating these features for a large set of enzyme variants. These efforts will allow us to complete development of an automated workflow for building multimodal datasets with chemical calculations and to progress toward generating physics-based predictions of enzyme activity.

The generalized workflow for enzyme modeling involves preparation of variant structures, which are then passed through several computational workflows. Specifically, the workflow will generate structures of variant proteins with specified point mutations for automated molecular dynamics simulations at biological conditions as well as quantum mechanics calculations of the active site. These data are then condensed into observables, including binding affinities, residue-level motions, and residue-residue cross-correlations. These observables serve as representations to distinguish between protein variants in physically meaningful ways and will be investigated for their ability to inform several goals in the aim of reducing the experimental load of directed evolution studies.

First, the simulation pipelines will be evaluated for their ability to rank-order protein variants for the specific task. For TrpB, for example, this will aim to rank-order variants for their rate of tryptophan synthesis. The rank-ordering from the computational pipeline will be compared to the data from exhaustive experimental screening of variants as described in Aim 1. Second, these representations will be made compatible with graph neural networks being developed currently for structure-only representation-based deep learning models. Similarly, the models' effectiveness before and after the addition of these physics-based features will be evaluated against experimental data. Finally, these data will aid in models aimed at selecting sites for generation of combinatorial libraries.

To aid in selection of protein sites for combinatorial mutagenesis, dynamics simulations will be combined with statistical methods to determine causal relationships between residue motions within the parent protein structure, and causal relationships between sidechain motion with the transitions state of the rate-determining step will be used to rank-order sites. The hypothesis that this rank-ordering will correspond with a rank-ordering of sites important to protein function, and that this is a useful metric by which to select sites for mutation, will be tested by comparison to existing experimental screening data. New experimental data for sites selected by the algorithm will also be collected as needed. TrpB will be used as a model system to develop and evaluate the method, with the intent to identify and test the generalization of the method to new systems in subsequent studies.

My project addresses BES cross-cutting priority areas by:

Our work sits at the interface of biocatalysis, machine learning and computational chemistry. Using computational chemistry, we calculate useful features for machine learning or predict enzyme properties of interest. Combining these calculations with sequence-based enzyme representations, structures, and select enzyme-fitness data enables a multimodal approach for fitness prediction, paving a way toward computationally-driven directed evolution and biocatalyst engineering. We are looking to open new approaches to efficient bioproduction of chemicals, fuels and materials.

My scientific area(s) of expertise is/are: protein engineering, biocatalysis, directed evolution (FHA), machine learning, representation learning (YY), molecular dynamics, quantum chemistry (WAG).

Publications supported by this project 2021-2024:

1. B. J. Wittmann, K. E. Johnston, P. J. Almhjell, F. H. Arnold, "evSeq: Cost-effective amplicon sequencing of every variant in a protein library." ACS Synthetic Biology **11**, 1313 (2022). doi: 10.1021/acssynbio.1c00592

The DOE Center for Plant and Microbial Complex Carbohydrates
at the University of Georgia

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Overall research goals:

The overall goal of our research is to enhance our understanding of rhamnogalacturonan I (RGI), an important and extremely complex pectin polysaccharide present in most plants. RGI is a major component of the pectic polysaccharides in plants, and thus knowledge of the structure and biosynthesis of this polysaccharide is essential to the understanding of the plant cell wall. We are approaching the goal of this research on two separate but interconnected tracks: (1) through the development of analytical methods to determine the structure of RGI and (2) through elucidating the biosynthesis of RGI. The structural studies focus on using permethylation as a means to solubilize insoluble RGI fractions in organic solvents for composition and structure analysis by mass spectrometry and NMR. In addition, we aim to understand the structural variety of RGI from different plant species and tissues, as well as waste industrial plant biomass. A component of our studies includes RGI that is covalently attached to arabinogalactan-protein (AGP). Another part of our efforts is to develop a suite of linkage specific enzymes that can be used to selectively break down RGI, allowing us to gain understanding of how structure contributes to biological function. In addition, these enzymes will be used to generate a library of RGI polymers and oligosaccharides for use as acceptors in studies of glycosyl transferases involved in RGI synthesis. The long-term goals of the RGI biosynthesis studies are to identify the enzymes that add the glycosyl and non-glycosyl substituents during the synthesis of the RGI backbone and side chains. A secondary goal is the application of the methods being developed to the structural characterization of other insoluble or sparingly soluble plant and microbial polysaccharides through our many collaborative efforts of the DOE Center. In addition, we will also disseminate our methods and protocols through number of hands-on workforce training courses/workshops.

Significant achievements

We heterologously expressed a side chain tolerant polysaccharide lyase from family 11 (PL11), RGPL-BT4175, and developed optimal conditions for enzymatic fragmentation of RG-I isolated from Arabidopsis seed mucilage and tissues. We recombinantly expressed, purified, and analyzed four candidate *endo*-β-1,4 galactanases. We showed that an endogalactanase from *Geobacillus stearothermophilus* can readily produce large oligosaccharide products and developed and optimized a method to generate 95% pure galactotetraose for structural and biochemical studies of glycosyltransferases. We have developed and published a method for the glycosyl composition analysis of whole cell wall samples by permethylation and GC-MS.[1] We also applied permethylation to solubilize plant cell walls for NMR structure analysis. An NMR chemical shift database generated from a number of permethylated polysaccharide standards has enabled structure determination of both the major and minor polysaccharides.[2] We developed a modified method for linkage analysis of acidic polysaccharides using O-acetylation and ionic liquids using a modified methylation procedure, increasing recovery by a factor of 10 and 100 for neutral and acidic sugars, respectively. We have discovered that duckweed RGI and apiogalacturonan containing pectic AGPs can form borate diesters with each other and with the pectin RGII, offering an explanation for plant resistance to breakdown in high pH water. We further found that the major RGI released from both citrus peels and sugar beet residues with endopolygalacturonase/pectin methyl esterase after oxalate and carbonate extraction are covalently attached RGI-AGP complexes, and some of the RGIs have bound boron in the form of boric acid monoesters.

We have established a method for mass production of duckweed biomass for pectin isolation from monocots. A library of more than 30 plasmids for the expression of recombinant enzymes specifically involved in RGI deconstruction was assembled and screened for heterologous expression. In addition, we have completed constructs for the heterologous expression of several RGI transferases including putative arabinosyltransferases, β-1,4-galactosyltransferases, and two putative pectin synthesis enzymes from a new glycosyltransferase family 47 subfamily in liverwort. Using a subset of these enzymes, we demonstrate that Arabidopsis thaliana galactosyltransferase 1(AtGALs1) enzymes can transfer azido-functionalized sugar nucleotide donors to selected synthetic plant cell wall oligosaccharides on a glycan array, and determined that AtGALS1 is able to

utilize more sugar donors than was currently known, generating unnatural xylogalactan oligosaccharides that are potential new biomaterials.[3]

Prior to this project, a family of RGI:rhamnosyltransferases (RRT) had been identified, but the identity of the galacturonosyltransferases that incorporate GalA (galacturonic acid) into the RGI backbone was unknown. The identity of the first RGI:galacturonosyltransferase has now been confirmed. We expressed this enzyme in HEK293 cells and completed assays to demonstrate that it is an RGI backbone specific GalA transferase (GalAT). Combining the GalAT and RhaT activities, we demonstrated elongation of RGI backbone oligosaccharides *in vitro*.[4]We established preparatory-scale methods to purify UDP-Rha and RGI oligosaccharide acceptors necessary to complete these studies. We developed a method to identify and quantify the formation of the acyl-enzyme intermediate for polysaccharide O-acetyltransferases.[5]

In order to make our tools and methodologies available to investigators outside of the DOE Center, we are offering hands-on and virtual workforce training courses in glycoscience which have been well received by the scientific community with over 300 registered course participants.

Science priorities for the next year (2022-2023):

1) The database of permethylated polysaccharides will be expanded by acquiring data of additional samples, as well as using computational approaches in collaboration with Goran Widmalm (Stockholm University). We will continue to improve analysis of uronic acid containing polysaccharides by permethylation and by acetylation in ionic liquids. 2) Detailed structural analysis of the RGI-AGPs purified from different plant materials, especially the RGI-AGPs from citrus peels celery, sugar beet, and rice stem, penium, and poplar. We will investigate RG-I assembly on Golgi and plasma membranes by performing structural analysis of RG-I containing structures released from Golgi and plasma membranes. 3) We will scale up tissue production to further build our substrate library for structural elucidation of RGI using NMR and enzyme techniques and evaluate their robustness. A key goal of our gene toolbox project is to optimize enzymes that are able to cleave the RGI backbone in the presence of full side-chain structures, which is necessary for determining fine structure of RGI and implementation of high throughput (HTP)-polysaccharide fingerprinting. 4) Further priorities for year 4 are the production of activated sugar nucleotide donors and saccharide acceptor substrates, determining the role of the *MUCI70/RGGAT1* gene family members in RG-I biosynthesis, and confirming activity of GAUT13 and GAUT14 in elongation of the RG-I backbone with HG, exploring significance in pectin heteropolymer synthesis and patterning.

My major scientific area(s) of expertise is/are:

Carbohydrate structural characterization of plant and microbial polysaccharides by MS, HPLC, and NMR.
Carbohydrate chemistry; plant cell wall pectins and glycoproteins. Pectin and hemicellulose biosynthesis and structure. Characterization of carbohydrate active enzymes. Pectin biosynthesis, structure, and function. Purification of glycosyltransferases and polysaccharides. Biochemical characterization of glycosyltransferase enzyme function.

To take my project to the next level, my ideal collaborator would have expertise in:

Mutagenesis of biomass, targeted enzymatic degradation of plant polysaccharides. Atomic force microscopy, biomechanics and protein crystallography, biological function of carbohydrates.

A subset of publications out of 104 total supported by this project:

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[1] Black, I., Heiss, C., & Azadi, P. (2019). Comprehensive monosaccharide composition analysis of insoluble polysaccharides by permethylation to produce methyl alditol derivatives for gas chromatography/mass spectrometry. *Analytical chemistry*, *91*(21), 13787-13793.

[2] Ikenna E. Ndukwe, Ian Black, Christian Heiss and Parastoo Azadi (2020) Evaluating the Utility of Permethylated Polysaccharide Solution NMR Data for Characterization of Insoluble Plant Cell Wall Polysaccharides. Anal. Chem., *92*(19), 13221-13228.

[3] Colin Ruprecht, Max P. Bartetzko, Deborah Senf, Anna Lakhina, Peter J. Smith, Maria Soto, Hyunil Oh, Daniel Varon Silva, Mads H. Clausen, Kelley W. Moremen, Michael G. Hahn, Breeanna R. Urbanowicz, and Fabian Pfrengle (2020) A Glycan Array-Based Assay for the Identification and Characterization of Plant Glycosyltransferases *Angewandte Chemie*. 2020 May 12. doi: 10.1002/anie.202003105.

[4] Amos R, Atmodjo M, Huang C, Gao Z, Venkat A, Taujale R, Kannan N, Moremen K, Mohnen D. (2022) Polymerization of the backbone of the pectic polysaccharide rhamnogalacturonan I. ResearchSquare

[5] Lunin, V. V.; Wang, H. T.; Bharadwaj, V. S.; Alahuhta, M.; Pena, M. J.; Yang, J. Y.; Archer-Hartmann, S. A.; Azadi, P.; Himmel, M. E.; Moremen, K. W.; York, W. S.; Bomble, Y. J.; Urbanowicz, B. R., (2020) Molecular Mechanism of Polysaccharide Acetylation by the Arabidopsis Xylan O-acetyltransferase XOAT1. *Plant Cell 32* (7), 2367-2382

Photosynthetic Energy Capture, Conversation and Storage: From Fundamental Mechanisms to Modular Engineering

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Overall research goals:

The capture and conversion of sunlight into chemical energy by photosynthesis is the basic biological process driving life on earth. Photosynthesis sustainably provides food, feed, and energy-rich molecules, and it has led to the formation of fossil fuels over geological time. An interdisciplinary and diverse team of 11 lead investigators with complementary expertise, as well as talented scientists at all levels, is exploring photosynthetic processes within the Plant Research Laboratory. Research within the laboratory covers multiple scales of biological organization ranging from photoactive compounds, enzymes, protein complexes, organelles and bacterial micro compartments (BMC), the thylakoid membrane, to the overall integration of photosynthesis in cells and organisms in their environments. The long-term goal of these scientific endeavors is to explore basic mechanisms of energy storage by oxygenic photosynthesis, at scales ranging from primary capture of light energy and carbon fixation to the building and maintenance of the biological solar panels in cyanobacteria, algae, and plants. By increasing our mechanistic knowledge of photosynthesis across scales, we will be better poised to not only improve photosynthetic efficiency and productivity, but also develop photosynthetic modules that can be recombined in innovative ways to produce novel photosynthesis-based products.

This Project integrates complementary strengths of the participants at the MSU-DOE Plant Research Laboratory (PRL) to allow for cross-disciplinary interactions enabling novel approaches that would not be otherwise possible in individual labs. The three Subprojects pursed by the PRL are: A. Primary capture, storage and regulation of light energy, and avoidance of deleterious side reactions; B. Integrating energy supply and demand in the biological solar panel; and C. Characterizing, engineering and integrating subcellular and cellular modules for photosynthetic productivity. In the summary below, we are reporting on progress of recently initiated research under subproject C, led by Josh Vermaas and Daipayan Sarkar, a postdoctoral researcher working in his laboratory.

Science objectives for 2021-2022:

Carboxysomes are bacterial microcompartments, found in cyanobacteria, that locally concentrate carbon dioxide (CO_2) to improve the efficiency of the enzyme RuBisCO, a key step towards photosynthetic carbon fixation by the Calvin-Benson-Bassham (CBB) cycle. The carboxysome shell is made up of proteins that encapsulate RuBisCO and carbonic anhydrase. Carbonic anhydrase converts the soluble bicarbonate to CO_2, increasing the local concentration of CO_2 for RuBisCO. In addition to CO_2, other metabolites such as oxygen (O_2), bicarbonate (HCO_3^-), 3-phosphoglyceric acid (3-PGA) and ribulose-1,5-bisphosphate (RuBP) also need to permeate through the carboxysome shell to efficiently perform CO_2 fixation. Quantifying the permeability of the shell for these metabolites currently remains a challenge and one of the critical design features related to the design of synthetic bacterial microcompartments for metabolic and sustainable bioengineering applications.

Significant achievements:
Leveraging a high resolution cryo-electron microscopy (cryo-EM) structure of a synthetic β-carboxysome shell and new graphics processing unit (GPU)-resident molecular simulation engines, we determine the permeability of photosynthetic metabolites across synthetic carboxysome shell through unbiased molecular simulation at all-atom resolution. We find that the carboxysome itself is not selectively permeable to bicarbonate over carbon dioxide, as originally hypothesized. Instead, the carboxysome shell proteins form a general barrier to maintain the carbon dioxide gradient generated by carbonic anhydrase activity within the carboxysome, compensating for inefficiencies of endogenous RuBisCO, specifically slow turnover rate, and photorespiration. The results of all-atom molecular simulations presented here provide a detailed mechanistic picture, using the shell permeabilities to design smart synthetic bacterial microcompartments and improve photosynthetic efficiency with a wide range of applications in sustainable, metabolic, agricultural, and biomedical engineering.

This subcomponent of the Project addresses BES cross-cutting priority areas by:
The permeability coefficients developed through this research are essential to modeling carbon fluxes around carbon fixation, which in turn connect energy capture from photons to carbohydrate energy storage.

Scientific areas of expertise are: Josh Vermaas has expertise in classical molecular dynamics simulation, particularly when studying permeation and transport phenomena. However, the wider collaborative enterprise at the Plant Research Laboratory features researchers that work at the interface of biophysics, biochemistry, plant biology, and microbiology.

The ideal collaborator for Josh Vermaas would have expertise in: Dr. Vermaas's ideal collaborator would have a mechanistic hypothesis that plays out at the molecular level but are difficult to test through traditional benchtop experiments.

Publications supported by this project 2020-2023:
While this particular subcomponent of the overall project was recently initiated and represents unpublished science, the PRL as a whole has published 89 publications supported by the grant over the past 3 years.

Electron Bifurcation Theory

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Overall research goals:

This project explores the electron-transfer kinetics and mechanisms that underpin electron bifurcation (EB) and electron confurcation reactions. First, we aim to determine how biological EB avoids short-circuiting reactions during the critical electron refilling process. Second, we are developing a general procedural strategy to generate and solve the requisite kinetics equations to describe electron bifurcase function, and to understand the limits of validity of mean-field multi-particle transport theories in the context of EB processes. These studies are intended, ultimately, to produce design principles to allow the realization of bioinspired synthetic EB structures.

Significant achievements, 2021-2022:

Our goal of understanding how reversible EB enzymes avoid short-circuiting during refilling is largely complete, as we have constructed a minimal model that includes refilling and does not short-circuit, relying on the energy landscape of the high- and low-potential redox chains of EB systems. We have also developed and explored mean-field descriptions of EB and related energy transducing enzymes.

Electron bifurcation and mean-field approximations of electron transport kinetics.
The redox states of cofactors along biological hopping networks are statistically correlated due to disequilibrium and interactions between the cofactors. Calculating the exact occupancy statistics associated with biological electron hopping in these networks is very difficult to accomplish, since it is necessary to include all correlation functions to fully describe statistically correlated site occupancies - an exponentially difficult problem as the number of unique redox configurations grows. Neglecting statistical correlations among site occupancies, accomplished by assuming equilibrium and no site interactions, reduces the complexity of the problem dramatically. This approach is useful only to the extent that the mean-field approximation is valid. The mean-field approximation is used routinely in many descriptions of multi-step biological electron hopping network. Yet, the reliability of the mean-ield approximation to describe EB functions and the underlying kinetics of hopping networks was unknown. We have studied the mean-field treatment of electron hopping over the last year in detail, especially in the context of biological EB networks.

Our analysis finds that the mean-field approximation does not predict free-energy transduction in regimes where an exact master equation does predict free-energy transduction. We were surprised by the failure of the mean-field approximation to describe reversible EB. In electron-hopping kinetics, disequilibrium and site-site interactions (including Coulombic interactions) introduce statistical correlations among site occupancies. Thus, it may be expected that the error of the mean-field approximation becomes significant for systems with strong disequilibrium (strong driving forces between electron reservoirs) or strong Coulombic or other interactions between redox cofactors. Electron bifurcation, however, is often near reversible overall, and indeed the short-circuit rates are well-approximated by an expressions that assumes separate redox equilibrium in the high- and low-

potential branches. Furthermore, there are no interactions between the redox cofactors in our modeling. Thus, one might have expected that reversible EB would be well- described by the mean-field approximation.

Science objectives for 2022-2023:

Mean-field approximations of electron hopping master equations.

Our future work will be to further identify features of coupled kinetics that may be attributed primarily to the failure of the mean-field approximation. We hypothesize that slippage, which is a strongly driven reaction, and is intrinsic to transport networks that engage coupled kinetics, introduces substantial statistical correlations to the system. We will explore this hypothesis by investigating whether the introduction of only the correlation functions associated with slippage pathways to the mean-field description can reproduce free energy transduction in near-reversible EB systems. We are also exploring the origins of inverted potentials in electron bifurcating enzymes.

My project addresses BES cross-cutting priority areas by:

This project seeks to understand, at the level of electrons and atoms, the foundational mechanisms of energy capture and conversion via electron transport, especially through mechanism of multi-electron transport as occur in the electron bifurcation pathways of living systems.

My scientific area(s) of expertise is/are: Theoretical biophysical chemistty; electron transfer theory, theory, modeling and simulation; inverse molecular design.

The ideal collaborator for my project would have expertise in: A visioinary synthetic chemist or nanoscientist who is interested in building bio-inspired electron transfer systems that demonstrate electron bifurcation in open systems (coupled to electron reservoirs) based on the bio-inspired theoretical principles developed in this project.

Publications supported by this project 2020-2022:

- J.L. Yuly, "Energy transduction by electron bifurcation," Ph.D. Thesis, *Duke University*, 07/15/2021.
- C.E. Wise, A.E. Ledininaa, J.L. Yuly, J.H. Artza, C.E. Lubner, "The role of thermodynamic features on the functional activity of electron bifurcating enzymes," *BBA - Bioenergetics*, **1862**, 148377 (2021).
- J.L. Yuly, P. Zhang, Peng; X. Ru, K. Terai, N. Singh, D.N. Beratan, "Efficient and reversible electron bifurcation with either normal or inverted potentials at the bifurcating cofactor", *Chem*, **7**, 1870 (2021).
- J.L. Yuly, P. Zhang, D.N. Beratan, "Energy transduction by reversible electron bifurcation", *Curr. Opin. Electrochem.*, **29**, 100767 (2021).

Probing novel pathways of metal sulfide acquisition and trafficking from minerals in model biocatalytic systems

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Overall research goals:

Pyrite (FeS_2) is the most abundant iron sulfide in Earth's crust and is a reservoir of trace elements of national strategic importance including nickel (Ni), cobalt (Co), and molybdenum (Mo). Traditional methods to extract trace elements from pyritic ores employ oxidative leaching that generates acid. We recently showed that methanogens can reductively dissolve FeS_2 through a process that does not generate acid. Further, methanogens assimilate dissolution products to meet their iron (Fe) and sulfur (S) demands, including for biosynthesis of simple and complex metalloclusters. This project seeks to forge new understanding of the mechanisms used by methanogens to reductively dissolve FeS_2 and assimilate, traffic, and bioconcentrate Fe and S. Our data also indicate that methanogens can assimilate Ni, Co, and Mo from pyritic ores to meet biosynthetic demands. Given the growing need for economic and domestic Ni, Co, and Mo sources, this motivates additional experiments to characterize the mechanisms used by methanogens to acquire, traffic and bioconcentrate these metals from FeS_2. A suite of physiological, biochemical, -omic, and spectroscopic approaches are being combined to address these research objectives. Further, using state of the art imaging and computational approaches, we will develop new understanding of the reaction mechanisms, kinetics, and chemical transformations that take place at the surface of FeS_2 during reduction, focusing on molecular interactions at the mineral-water-cell interface. Through this integrated approach, we aim to identify the enzymes and pathways that allow methanogens to bio-mine Fe, Ni, Mo, Co, and S from FeS_2 ore to enable the cost-effective recovery and/or conversion of raw substrate into catalysts for electrochemical bioenergy generation.

Significant achievements: 2019-present:

• ***Genomics.*** A comparative genomics analysis of archaeal methanogens and methanotrophs identified a number of heme auxotrophs that encode heme-dependent enzymes and organisms that can synthesize siroheme but lack known destinations for this cofactor. We also characterized the distribution of iron sulfur storage (IssA) proteins. These informatics data form the basis of much of the work below.

• ***Physiology***. We showed that methanogens prefer to grow using FeS_2 when compared to tradiational sources of Fe and S used to cultivate cells (ferrous iron/sulfide) due to trace metal limitation under the latter condition. We showed that methanogens can synthesize all required metalloclusters to enable a functional molybdenum nitrogenase from FeS_2. We demonstrated that methanogen cells can access and bioaccumulate Ni during growth with Ni-doped FeS_2 as the sole source of Ni, Fe, and S. Lastly, we demonstrated the growth of methanogens on heme as the sole source of Fe.

• ***Computational Chemistry and Geochemistry.*** We have shown that methanogens require direct contact with FeS_2 to reduce it through a yet to be defined mechanism of extracellular electron transport. Our model for FeS_2 reduction suggests initial release of sulfide into solution is concomitant with the precipitation of pyrrhotite ($Fe_{1-x}S$) on the mineral surface. Dissolved Fe, but not S, from $Fe_{1-x}S$ then combines with sulfide in solution to form aqueous iron sulfur clusters (FeS_{aq}) that are neutrally charged and likely passively diffuse into the cell. Atomic scale models of FeS_2 nanoparticles have been built, validated, and are now being subjected to simulations to model surface transformations during reduction.

• ***Omics.*** Experiments showed that growth on FeS_2 versus ferrous iron/sulfide induces major shifts in the carbon and energy metabolism of cells. Consistent with this, metallomics experiments show different

protein destinations for metals (Fe, Mo, Ni, Co, Zn) during growth on FeS_2 versus ferrous iron/sulfide. Data from growth experiments indicates that this is due to sulfide that limits metal availability.

• ***Spectroscopy.*** Experiments (EPR, Mössbauer, UV-Vis, XAS) on purified IssA homologs or on whole cells heterologously expressing these proteins provided insight into the function of this protein. Ion microprobe, XANEs, and microXRD are being applied to samples to define the structure and composition of the mineral that is generated intracellularly. We are also characterizing IssA homolog expressing *E. coli* cells during variable growth states.

• ***Biochemistry***. Several conserved and differentially expressed proteins identified in FeS_2 versus ferrous iron/sulfide-grown cells are being heterologously expressed (*E. coli*) and functionally characterized. This includes DUF2193 and two novel radical SAM domain proteins, including one with what is termed a SPASM domain that have been shown to be involved in post-translational modification of cysteine-rich proteins..

Science objectives for 2022-2023:

- Conduct integrated physiology/geochemistry/spectroscopic experiments to quantify the mobilization, trafficking, and bioconcentration of Ni, Co, and Mo during FeS_2 following reductive dissolution
- Identify novel pathways for heme demetallation allowing for growth of methanogen heme auxotrophs under heme-dependent growth conditions.
- Generate metal-specific proteomes for detecting assimilation of Fe, Ni, Co, and Mo into proteins during FeS_2 reduction; use $^{57}FeS_2$ to temporally track movement of Fe in cell proteome
- Characterize the role of IssA-like homologs in metal sulfide oxidation and sequestration using a combination of heterologous expression, physiological, biochemical, and spectroscopic approaches
- Develop atomic-scale computational models of FeS_2 and heterometal-containing FeS_2 to define the molecular transformations that accompany reduction and dissolution reactions

My project addresses BES cross-cutting priority areas by:

Our research combines physiological, molecular, biochemical, computational, and geochemical approaches with a suite of cutting-edge imaging and spectroscopic physical science techniques to characterize the mechanisms of biological FeS_2 reduction and the proteins involved in Fe/Ni/Co/Mo/S acquisition, trafficking, and storage. Since metal sulfides form key components of metalloenzyme active sites that function in the conversion of light or electrical energy to potential energy in the form of chemical bonds, this work has direct relevance to understanding the underlying physical and chemical principles that govern how microbes capture, convert, and store energy via metalloenzymes (electrocatalysts). Further, our work aims to improve mechanistic understanding at the interface of the physical bio- and geo-sciences (project co-funded by physical biosciences and geosciences) by focusing on how Earth abundant pyrite mineral can be transformed into highly tuned electrocatalysts.

My scientific area(s) of expertise is/are: Physiology, geomicrobiology, bioinformatics, evolution.

The ideal collaborator for my project would have expertise in: Electrochemistry, Mössbauer spectroscopy, archaeal genetics.

Publications supported by this project 2022 only:

1. R. Spietz, D. Payne, R. Szilagyi, E. Boyd. "Reductive biomining of pyrite by methanogens." Trends in Microbiology. **30**, 1072-1083. (2022) [10.1016/j.tim.2022.05.005]
2. K. Steward, D. Payne, W. Kincannon, C. Johnson, M. Lensing, H. Fausset, B. Németh, E. Shepard, W. Broderick, J. Broderick, J. Dubois, B. Bothner. "Proteomic analysis of *Methanococcus voltae* grown in the presence of mineral and nonmineral sources of iron and sulfur. Microbiology Spectrum (2022) **10**, e0189322. [10.1128/spectrum.01893-22]
3. R. Spietz, D. Payne, E. Roden, G. Kulkarni, W. Metcalf, E. Boyd. "Investigating abiotic and biotic mechanisms of pyrite reduction." Frontiers in Microbiology (2022) **13**, 878387. [10.3389/fmicb.2022.878387]

Mechanisms and Pathways in Hydrogenase Maturation

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Overall research goals:

The overall goal of this project is to advance our understanding of the mechanism of biogenesis of the active site metal cluster of [FeFe]-hydrogenase, an efficient catalyst for hydrogen production. The studies discussed in this research proposal will use biochemical and biophysical approaches to elucidate the reactions catalyzed by the three specific hydrogenase maturase enzymes common to all organisms that harbor the [FeFe]-hydrogenase. This research promotes our knowledge of structure/function relationships in complex biological pathways for metal cluster assembly, while additionally contributing to our understanding of how microbes build the protein cofactors that enable them to capture and convert energy. Moreover, as the steps associated with complex metallocofactor biosynthesis are better understood, the knowledge gained will inspire and influence the design of new biomimetic catalysts with applications in biohydrogen technologies.

The active site metal cluster of [FeFe]-hydrogenase, referred to as the H-cluster, is a specialized iron-sulfur cluster consisting of a [4Fe-4S] cubane bridged to a 2Fe subcluster that has three carbon monoxide, two cyanide, and a bridging dithiomethylamine group as ligands. Biogenesis of this specialized cluster requires the combined actions of three specific maturation enzymes, denoted HydE, HydF, and HydG. Two of these enzymes (HydE and HydG) are radical-SAM enzymes and use *S*-adenosylmethionine (SAM) and a redox active [4Fe-4S] cluster to initiate radical chemistry. The third protein, HydF, is a GTPase that functions as a scaffold/carrier during H-cluster assembly. During the last funding period, we have gained new insights into the biochemical and spectroscopic properties of HydG and this has in turn informed our understanding of hydrogenase maturation. We have probed the interactions of individual radical SAM maturase enzymes with HydF in an effort to understand the order of events in hydrogenase maturation. Fundamental studies of the mechanism of radical initiation have also been a focus of the last funding period. Finally, we have developed the first fully-defined system that supports in vitro maturation of the [FeFe]-hydrogenase to an active enzyme, a development that enables much of the work proposed for the coming funding period.

The aim of this project is to develop a molecular-level understanding of the reactions catalyzed by HydE, HydF, and HydG as well as the interactions between the different maturases, in order to clearly delineate the mechanistic chemistry, the order of events, and the protein-protein interactions involved during H-cluster biosynthesis. *Specifically, the current project objectives are 1) to determine the role of iron and HydG during maturation, 2) to provide functional and mechanistic insight into HydE and the other maturases by employing our fully-defined maturation system, and 3) to probe the biological pathway of maturation.* This work stands to reveal fundamentally unique biochemical transformations and will help define new paradigms for complex metal cluster assembly in biology. Further, the work will provide understanding and insight needed to develop biohydrogen catalysts employing the [FeFe]-hydrogenase.

Significant achievements: 2020-2022:

- Photoinduced reductive cleavage of SAM in HydE and HydG resulted in S-CH_3 homolysis to generate a •CH_3 radical trapped in the active site. These results contributed to our understanding of the regioselectivity of the reductive cleavage of SAM as based in the Jahn-Teller effect.
- We have shown that the SAM analog S-adenosyl-L-ethionine (SAE) is a competent co-substrate for HydG catalysis, and produces a •CH_2CH_3 radical upon photolysis.

- We have shown that *C.a.* HydG forms multiple equivalents of the diatomic ligands CO and CN^- in the 'free' state, with no evidence of organometallic synthon formation.
- We have developed a fully-defined in vitro maturation system for the [FeFe]-hydrogenase. By including components of the glycine cleavage system (H-protein and T-protein) as well as serine hydroxymethyltransferase, serine, and ammonium, we could eliminate cell lysate while still achieving nearly full maturation. We used this system to demonstrate that the C of DTMA arises from serine, while the N arises from ammonium under these conditions. These results provide new insights into the missing components previously provided by the absolutely essential cell lysate during maturation.

Science objectives for 2022-2024:

- Delineate the role for added iron during in vitro maturation.
- Identify and test putative iron chaperones that may play roles in maturation.
- Use semisynthetic approaches to better define the roles of glycine cleavage system proteins in maturation.
- Generate and characterize enzymatically-loaded HydF.
- Characterize protein-protein interactions important to maturation.
- Examine the origin of the sulfur atoms of DTMA.

My project addresses BES cross-cutting priority areas by:
Developing a fundamental understanding of the biogenesis of [FeFe]-hydrogenase, one of nature's most efficient hydrogen-producing catalysts, and thereby providing a foundation for development of biohydrogen catalysts.

My scientific area(s) of expertise is/are: bioinorganic chemistry, iron-sulfur clusters, radicals, EPR.

The ideal collaborator for my project would have expertise in: EPR and ENDOR spectroscopy, protein-protein interactions and biophysical approaches to examine them, cryo-EM and other structural approaches.

Publications supported by this project 2020-2022:

1. Stella Impano, Hao Yang, Richard J. Jodts, Adrien Pagnier, Ryan Swimley, Elizabeth C. McDaniel, Eric M. Shepard, William E. Broderick, Joan B. Broderick, and Brian M. Hoffman, "Active-Site Controlled, Jahn-Teller Enabled Regioselectivity in Reductive S-C Bond Cleavage of S-Adenosylmethionine in Radical SAM Enzymes," *J. Am. Chem. Soc.* **2021**, *143(1)*, 335-348. [DOI 10.1021/jacs.0c10925]
2. Stella Impano, Hao Yang, Eric M. Shepard, Ryan Swimley, Adrien Pagnier, William E. Broderick, Brian M. Hoffman, and Joan B. Broderick, "S-Adenosyl-L-ethionine is a catalytically competent analog of S-adenosyl-L-methionine (SAM) in the radical SAM enzyme HydG," *Angew. Chemie* **2021**, *60(9)*, 4666-4672. [DOI 10.1002/anie.202014337]
3. Eric M. Shepard, Stella Impano, Benjamin R. Duffus, Adrien Pagnier, Kaitlin S. Duschene, Jeremiah N. Betz, Amanda S. Byer, Amanda Galambas, Elizabeth C. McDaniel, Hope Watts, Shawn E. McGlynn, John W. Peters, William E. Broderick, and Joan B. Broderick, "HydG, the 'Dangler' Iron, and Catalytic Production of Free CO and CN-: Implications for [FeFe]-Hydrogenase Maturation," *Dalton Trans.* **2021**, *50(30)*, 10405-10422. [DOI 10.1039/D1DT01359A]
4. Adrien Pagnier, Batuhan Balci, Eric M. Shepard, Hao Yang, Douglas M. Warui, Stella Impano, Squire J. Booker, Brian M. Hoffman, William E. Broderick, and Joan B. Broderick, "[FeFe]-Hydrogenase: Defined Lysate-Free Maturation Reveals a Key Role for Lipoyl-H-Protein in DTMA Ligand Biosynthesis," *Angew. Chem. Int. Ed.* **2022**, *61*, e202203413. [DOI 10.1002/anie.202203413]
5. Adrien Pagnier, Batuhan Balci, Eric M. Shepard, William E. Broderick, and Joan B. Broderick, "[FeFe]-Hydrogenase In Vitro Maturation," *Angew. Chem. Int. Ed.* **2022**, *in press*. [DOI 10.1002/anie.202212074]

Elucidating the Cellular Machinery for Lipid Storage in Plants

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Overall research goals:

Our overarching goal is to understand the fundamental biochemical and cellular processes important for compartmentalization of storage lipids in plant tissues, ultimately to enable dramatic increases in the energy storage capacity of plants. Lipids represent a fully reduced form of carbon, and these hydrophobic molecules are a major store of electrons and energy in plant systems. Understanding the mechanisms of their packaging remains an important question in cell biology research. We are addressing our goal through the following three specific research objectives: 1) Perform a detailed assessment of the mechanisms and functional interactions of SEIPIN, LDIP, and LDAP proteins in plants, 2) Identify and characterize new protein players involved in compartmentalization of neutral lipids in plants, and 3) Characterize variants of known LD proteins in the packaging of energy-dense storage lipids.

Significant achievements (2019-2023):

- Demonstrated that a previously unknown hydrophobic protein now termed LDIP (LD-Associated Protein [LDAP]-Interacting Protein) works together with both endoplasmic reticulum (ER)-localized SEIPIN and the LD-coat protein LDAP to facilitate the process of LD formation in plants. Results were published in *Plant Cell* and featured on the cover of the issue (Pyc & Gidda et al., 2021).
- Identified a functional interaction between SEIPIN and an isoform of the organelle-tethering protein VAP27 (vesicle-associated membrane protein [VAMP]-associated protein 27). Results were published in *Plant Cell* (Greer et al., 2020).
- Integrated multiple -omics studies in jojoba (a desert shrub that accumulates wax esters instead of triacylglycerols in seeds) suggest that LDAPs may be key factors for efficient wax-ester packaging. A paper describing these "–omics" resources was published in *Science Advances* (Sturtevant et al., 2020).
- Our group also contributed several reviews including one for the *Annual Review of Plant Biology* that has been accepted and is undergoing copyediting for publication in 2023.

Science objectives for 2022-2023:

We are currently exploring the potential role for the GTP-binding dynamin proteins in LD biogenesis (Objective 2). Exciting new results suggest that specific isoforms of plant dynamins interact with the LD biogenesis proteins, SEIPIN 1, SEIPIN 3, and LDIP, and this contributes to LD dynamics. Two post-doctoral fellows (one at UNT and one at the University of Guelph) are cooperating to make headway on this objective. Dynamins are well known for their roles in membrane trafficking and remodeling, but this will be the first demonstration that members of this large family are involved in lipid storage in plant cells.

My project addresses BES cross-cutting priority areas by: Elucidating how cells synthesize, package and store energy-rich lipid molecules in an aqueous subcellular environment remains our top research priority, and this supports one goal of the BES, Physical Biosciences program-- "to [investigate] the processes that regulate energy-relevant chemical reactions within the cell" (https://science.osti.gov/bes/csgb/Research-Areas/Physical-Biosciences).

My scientific area(s) of expertise is/are: Lipid Biochemistry, Cell Biology, Protein Biochemistry
To take my project to the next level my ideal collaborator would have expertise in:
Structural Biology/ Cryo-Electron Microscopy, Reconstitution of Cellular Systems *in Vitro*

Publications supported by this project 2019-present (11 of 17 listed)**:**

1. **BH Kang, CT Anderson, S Arimura, E Bayer, M Bezanilla, MA Botella, F Brandizzi, TM Burch-Smith, KD Chapman, K Dünser, Y Gu, Y Jaillais, H Kirchhoff, MS Otegui, A Rosado, Y Tang, J Kleine-Vehn, P Wang, BK Zolman** (2022) A glossary of plant cell structures: Current insights and future questions, *Plant Cell*, 34(1): 10–52, https://doi.org/10.1093/plcell/koab247.
2. **P Scholz, KD Chapman, RT Mullen and T Ischebeck** (2022) Finding new friends and revisiting old ones – how plant lipid droplets connect with other subcellular structures. *New Phytologist*. https://doi.org/10.1111/nph.18390. *Tansley Insight*
3. **Busta L, KD Chapman, EB Cahoon** (2022) Better together: Protein partnerships for lineage-specific oil accumulation. *Current Opinion in Plant Biology* 66: 102191. https://doi.org/10.1016/j.pbi.2022.102191.
4. **M Pyc, Gidda SK, Seay D, Esnay N, Kretzschmar FK, Cai Y, Doner NM, Greer MS, Hull JJ, Coulon D, Bréhélin C, Yurchenko O, de Vries J, Valerius O, Braus GH, Ischebeck T, Chapman KD, Dyer JM, Mullen RT** (2021) LDIP cooperates with SEIPIN and LDAP to facilitate lipid droplet biogenesis in Arabidopsis. *Plant Cell* 33(9): 3076–3103. https://doi.org/10.1093/plcell/koab179. **Cover** of issue.
5. **Horn PJ, Chapman KD, Ischebeck T** (2021) Isolation of Lipid Droplets for Protein and Lipid Analysis. In: Bartels D., Dörmann P. (eds) Plant Lipids. *Methods in Molecular Biology*, vol 2295. Humana, New York, NY. https://doi.org/10.1007/978-1-0716-1362-7_16.
6. **Sturtevant D, Aziz M, Romsdahl TB, Corley CD, Chapman KD** (2021) In Situ Localization of Plant Lipid Metabolites by Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry Imaging (MALDI-MSI). In: Bartels D., Dörmann P. (eds) Plant Lipids. *Methods in Molecular Biology*, vol 2295. Humana, New York, NY. https://doi.org/10.1007/978-1-0716-1362-7_24.
7. **Doner NM, D Seay, M Mehling, S Sun, SK Gidda, K Schmitt, GH Braus, T Ischebeck, KD Chapman, JM Dyer, RT Mullen** (2021) Arabidopsis thaliana EARLY RESPONSIVE TO DEHYDRATION 7 Localizes to Lipid Droplets via Its Senescence Domain Frontiers in Plant Science 12: 652. https://doi.org/10.3389/fpls.2021.658961.
8. **MS Greer, Y Cai, SK Gidda, N Esnay, FK Kretzschmar, D Seay, E McClinchie, T Ischebeck, RT Mullen, JM Dyer, KD Chapman** (2020) SEIPIN isoforms interact with the membrane-tethering protein VAP27-1 for lipid droplet formation. *Plant Cell*, 32 (9), 2932-2950, http://www.plantcell.org/content/32/9/2932.full.
9. **T Ischebeck, HE Krawczyk, RT Mullen, JM Dyer, KD Chapman** (2020) Lipid droplets in plants and algae: Distribution, formation, turnover and function. *Seminars in Cell & Developmental Biology*. *In press,* corrected proof is available online https://doi.org/10.1016/j.semcdb.2020.02.014
10. **N Esnay, JM Dyer, RT Mullen, KD Chapman** (2020) Lipid droplet–peroxisome connections in plants. *Contact*, **3**, 2515256420908765 https://doi.org/10.1177/2515256420908765
11. **D Sturtevant, S Lu, Z-W Zhou, Y Shen, S Wang, J-M Song, J Zhong, DJ Burks, Z-Q Yang, Q-Y Yang, AE Cannon, C Herrfurth, I Feussner, L Borisjuk, E Munz, GF Verbeck, X Wang, RK Azad, B Singleton, JM Dyer, L-L Chen*, KD Chapman*, L Guo*** (2020) The genome of jojoba (*Simmondsia chinensis*): a taxonomically-isolated species that directs wax-ester accumulation in its seeds. *Science Advances*, **6 (11)**, eaay3240. DOI: 10.1126/sciadv.aay3240. *joint correspondence.

Molecular Mechanisms of Plant Cell Wall Loosening: Expansin Action

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Overall research goals:

This project concerns the structure and function of expansin proteins. Expansins facilitate plant cell wall enlargement but no enzymatic activity has been detected for these proteins, whose mechanism of wall loosening remains enigmatic. In this funding period we have three major sets of goals.

(A) Refine a molecular dynamics model of *Arabidopsis thaliana* α-EXPANSIN4 (AtEXPA4) and its interaction with cellulose, as a means to develop testable hypotheses of the molecular basis of wall-loosening and stress relaxation by α-expansins. We do not have an experimental structure for any α-expansin, largely because α-expansins have proved recalcitrant to heterologous expression of active protein (we've tested many expression systems). This leads us to the second major goal, where we test the predictions of the molecular dynamics model by complementation in Arabidopsis.

(B) Develop a platform for functional testing of α-expansin mutants. It makes use of a novel CRISPR/Cas9 double mutant we created that is defective for functional EXPA7 and EXPA18 proteins. Root hairs are initiated but fail to elongate. Complementation with a functional *EXPA* gene restores root hair elongation. Our approach therefore is to complement the double mutant using genes with EXPA variants containing amino acid modifications that are hypothesized to be important for EXPA function. We use an EXPA-mCherry chimeric protein that restores root hair growth to the double mutant, as a means to verify appropriate expression of the construct.

(C) Screen an expression library of 200 highly diverse microbial expansin genes for novel activities (binding targets, biophysical and biochemical actions). This exploratory work is based on the hypothesis that the large diversity in microbial sequences reflects a large diversity of functionalities (substrates, physical actions, environmental optima) that evolved in the microbial world. The work is enabled by synthesis of 200 constructs by JGI.

Significant achievements: 2021-2022

For part B: Expansins comprise a superfamily of cell-wall loosening proteins grouped into four families: EXPA, EXPB, EXLA, and EXLB. The largest family, EXPA, consists of 26 genes in Arabidopsis thaliana, stemming from 12 ancient clades (EXPA-I to EXPA-XII). Analysis of their wall-loosening activities has been limited by technical difficulties with heterologous expression of plant expansins in active form. We have developed a way around this impasse, enabling answers to long-standing questions of expansin functional diversity. By CRISPR/Cas9-generated mutation of root-hair specific EXPA-X genes (*EXPA7* and *EXPA18*), we generated a double mutant in which root hairs failed to advance beyond initial bulge formation. This hairless phenotype was complemented by

EXPAs from EXPA-X as well as other EXPA clades (driven by the EXPA7pro), demonstrating an EXPA requirement and family-wide functional equivalence of EXPAs for tip growth of root hairs. In contrast, *EXPB*, *EXLA* and *EXLB* genes did not restore root hair growth. EXPA-mCherry chimeric proteins were functional and localized to the root hair tip. With this root-hair assay and targeted mutation, we demonstrated the functional necessity of two highly conserved EXPA residues. We conclude that EXPAs mediate cell wall loosening for tip growth of root hairs, providing an in-vivo system to probe expansin structure/function relations.

For Part C: From an analysis of NCBI databases we identified 200 microbial expansin genes that were synthesized by JGI as chimeras with green fluorescent protein (GFP) for expression in E. coli. We screened this set of constructs for expression, identifying 109 constructs showed GFP fluorescence in the first round of screen (37 very bright fluorescence, 31 bright, 21 medium, and 19 low fluorescence). We focused further work on the 89 constructs with medium to very bright fluorescence.

Science objectives for 2022-2023:

- Use the *expa7/18* double mutant to test the functional activity of various proteins in the growth hair growth complementation assay. Protein candidates include:
  - (a) site-directed mutants of EXPA7, to test hypotheses of the functions of specific EXPA residues;
  - (b) domain-1, domain-2, and homologs, to test the activity of single expansin domains; and
  - (c) enzymes with potential wall-loosening activities including family-12 endoglucanases and pectinases;
- Test the *in-vitro* activities of selected recombinant microbial expansins in a variety of binding, mechanical and biochemical assays, to identify microbial expansins with outlier activities, potentially of biotech applications in the materials and biofuel fields.

My project addresses BES cross-cutting priority areas by:
We are investigating the unique physical actions of expansin proteins to promote physical slippage of cellulose-cellulose interfaces. This action, whose molecular mechanism is still mysterious, is at the heart of plant cell wall growth and could have practical applications in cellulosic biomass conversion to biofuels and engineering novel 'green' biomaterials with tailored mechanical properties. The work connects to the BES priority themes *Chemistry at Complex Interfaces* as well as *Chemistry in Aqueous Environments* and the *Carbon Negative Shot* (via carbon sequestration in plant cell walls).

My scientific area(s) of expertise is/are: cell wall structure; cellulose; analysis of cell wall proteins; plant growth biophysics.

The ideal collaborator for my project would have expertise in: polymer biophysics; interactions of water with polymers, particularly polysaccharides.

Publications supported by this project 2021-2022:

1. Monschein, M., Ioannou, E., Amin, L.A.K.M.A.L., Varis, J.J., Wagner, E.R., Mikkonen, K.S., Cosgrove, D.J., and Master, E.R. (2022). Loosenin-like proteins from *Phanerochaete carnosa*; impact both cellulose and chitin fiber networks. bioRxiv, 2022.2007.2001.498415. 10.1101/2022.07.01.498415.
2. Guo, K., Huang, C., Miao, Y., Cosgrove, D.J., and Hsia, K.J. (2022). Leaf morphogenesis: the multifaceted roles of mechanics. Mol Plant *15*, 1098-1119. 10.1016/j.molp.2022.05.015.
3. Cosgrove, D.J. (2022). Building an extensible cell wall. Plant Physiol *189*, 1246-1277. 10.1093/plphys/kiac184.
4. Behar, H., Tamura, K., Wagner, E.R., Cosgrove, D.J., and Brumer, H. (2021). Conservation of endo-glucanase 16 (EG16) activity across highly divergent plant lineages. Biochem J *478*, 3063-3078. 10.1042/BCJ20210341.

Formate metabolism in hydrogenotrophic methanogens

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Overall research goals:

We are characterizing the metabolism of hydrogenotrophic methanogens. These organisms generate CH_4 as a product of metabolism and catalyze the terminal steps in the degradation of organic matter in anoxic environments. Specifically, we are investigating electron flow and energy conservation during the oxidation of formate, an alternate electron donor with an underappreciated role in methanogenesis.

Hydrogenotrophic methanogens are thought to prefer H_2 as an electron donor for CO_2 reduction to CH_4. However, in addition to H_2, many can also utilize electron donors such as formate, ethanol, secondary alcohols, or carbon monoxide. We seek to understand how these alternative electron donors integrate into the core methanogenic pathway. The terminal step of methanogenesis is carried out by heterodisulfide reductase (Hdr) and catalyzes the exergonic reduction of a heterodisulfide of two methanogenic cofactors: CoM-SH and CoB-SH. This exergonic reaction is coupled to the reduction of ferredoxin through flavin-based electron bifurcation (FBEB) (Fig. 1A). H_2 oxidation coupled to the FBEB reaction of Hdr is well documented, but how donors such as formate feed into this reaction is poorly understood. In previous work, we found that, in several methanogens, formate dehydrogenase (Fdh) can associate with Hdr to catalyze the FBEB reaction. Fdh is also responsible for the reduction of coenzyme F_{420}, the electron carrier needed for reduction of methenyl- carbon to methyl- carbon in methanogenesis. How Fdh coordinates these various activities and how they are regulated is the subject of the current funding period.

Finally, methanogens often grow syntrophically in close association with bacterial partners. The exchange of H_2 or formate between these organisms is essential for growth. However, it remains difficult to quantify the relative contributions of these compounds to growth, a question we seek to address given our interest in formate metabolism. Using mutant strain of *Methanococcus maripaludis* will assess nutrient exchange in syntrophic communities.

Significant achievements:

We developed a genetic system for *Methanoculleus thermophilus* (publication 1) and leveraged this advance to epitope tag and purify Hdr protein complexes from this organism (publication 2). We found that Hdr from *M. thermophilus* required formate as an electron donor for activity and only purified with a formate dehydrogenase. These data suggest that this organism may specialize in formate rather than H_2 metabolism, highlighting an underappreciated role for formate as an electron donor in methanogens. These results raised the question of the source of formate in cultures where H_2 was the only supplied electron donor. We found that whole cells of *M. thermophilus* catalyze a reversible formate hydrogenlyase activity; this activity produces the formate required for Hdr activity. Many members of the Methanomicrobiales lack genes encoding Hdr associated hydrogenases, but encode several copies of formate dehydrogenase. Our results suggest that formate metabolism is widespread in this group. Other methanogens such as *M. maripaludis* are also capable of formate-dependent Hdr activity. Using purified Fdh-Hdr protein complexes from *M. maripaludis*, we found that formate-dependent F_{420} reduction proceeds to completion before the initiation of the FBEB reaction (Fig. 1B).

In the environment, most methanogens likely grow in close association with syntrophic bacterial partners. During syntrophy, interspecies exchange of H_2 or formate is essential for sustaining the community. Due to the importance of formate as an electron donor to Hdr, we hypothesized that interspecies formate exchange may be important during syntrophic growth under certain conditions. To

test this, we paired *M. maripaludis* and *Syntrophotalea carbinolica* grown under conditions of ethanol, ethanolamine and propanediol oxidation. Using mutant strains, we found that formate exchange accounted for the majority of electron flux between these organisms (publication 4).

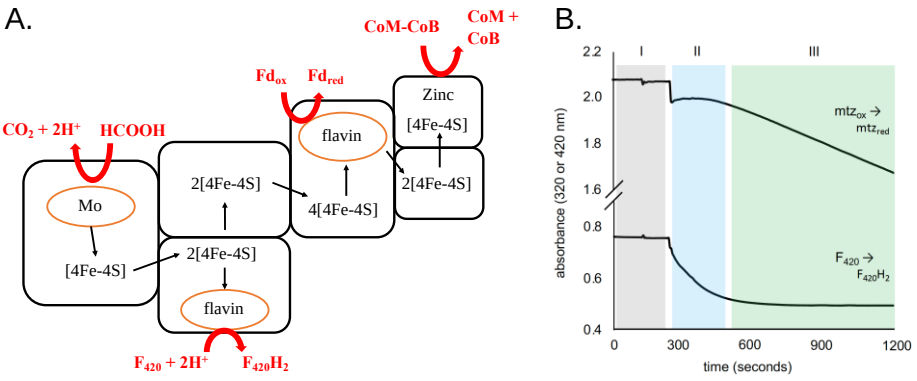

Figure 1. Formate dependent Hdr activity. **A.** Diagram of reactions catalyzed by Fdh-Hdr protein complexes. **B.** Formate dependent F_{420} reduction (activity shown in phase II, blue) and FBEB activity (phase III, green). Metronidazole (mtz) was used in place of ferredoxin (Fd) to easily track activity. F_{420} reduction proceeds to completion (abs 420nm) before FBEB activity begins (abs 320nm).

Science priorities for 2022-2023:

- Determine the activities of various isoforms of Fdh present in methanogens that grow with formate as an electron donor.
- Fdh-Hdr catalyzes several reactions: reduction of CoM-S-S-CoB and ferredoxin (FBEB) and reduction of coenzyme F_{420}. We hope to determine the structural or biochemical properties of Fdh that controls electron flow towards these competing reactions.
- Characterize environmental factors that contribute to the switch between H_2 and formate metabolism in hydrogenotrophic methanogens.

My major scientific area(s) of expertise is/are: Microbial physiology and metabolism. Anaerobic microbiology. Genetics and molecular biology.

To take my project to the next level, my ideal collaborator would have expertise in: Structural biology and biochemistry of O_2 sensitive enzymes. Electron paramagnetic resonance spectroscopy. Electrochemistry.

Publications supported by this project (2020-present):

1. D. Fonseca, M.F. Abdul Halim, M. Holten, K.C. Costa "Type IV-like pili facilitate transformation in naturally competent archaea." (2020) *J Bacteriol*. 202:e00355-20.
2. M.F. Abdul Halim, L. Day, K.C. Costa "Formate dependent heterodisulfide reduction in a Methanomicrobiales archaeon." *Appl Environ Microbiol* (2021) 87:e02698-20.
3. L.A. Day, K. C. Costa "Complete genome sequence of the secondary alcohol-utilizing methanogen *Methanospirillum hungatei* strain GP1." *Microbiol Resour Announc.* (2021) 10:e00708-21.
4. L.A. Day, E.L. Kelsey, M.F. Abdul Halim, K.C. Costa "Interspecies formate exchange drive syntrophic growth of *Syntrophotalea carbinolica* and *Methanococcus maripaludis*." *Appl Environ Microbiol* (2022) in press.
5. F.J. Arriaza-Gallardo, S. Schaupp, Y-C. Zheng, M.F. Abdul Halim, H. Pan, J. Kahnt, G. Angelidou, N. Paczia, X. Hu, K.C. Costa, S. Shima "The function of two radical-SAM enzymes, HcgA and HcgG, in biosynthesis of the [Fe]-hydrogenase cofactor." (in review)
6. X.J. Kang, A.J. Zmuda, K.B. Hillmann, K.C. Costa, T.D. Niehaus. "A universally conserved metabolite repair enzyme removes a strong inhibitor of the TCA cycle." (in review)

Activation of Recombinant Methyl-Coenzyme M Reductase in the Methanogenic Archaeon *Methanococcus maripaludis* and the Role of Post-Translational Modifications

Evert Duin, Auburn University
Barny Whitman, University of Georgia

Methanogens, or methane-producing archaea, are obligate anaerobes that form methane as a major product of their energy metabolism. They catalyze 1-2 % of the carbon cycle on Earth and are responsible for most of the methane in the Earth's atmosphere. The methanogens are also closely related to the archaea that anaerobically consume methane (called ANME). The methyl-coenzyme M reductase (or MCR) is a key enzyme in both the formation and anaerobic oxidation of methane. The prosthetic group of this unique enzyme is the nickel tetrapyrrole, coenzyme F_{430}. When active, the metal must be in the Ni(I) oxidation state. Because the redox potential of the F_{430}Ni(II)/F_{430}Ni(I) couple is near -650 mV, the stability of the Ni(I) prosthetic group is critical for maintaining enzyme activity. In addition, to F_{430}, MCR contains 4-5 post-translational modifications (PTM), depending on the organism. The functions of these PTMs are not clear, and they might affect protein stability, catalysis, or both.

Protein expression. A long-term goal of this project is to create a system that distinguishes between MCR structural components important for catalysis, activation and/or enzyme stability. The ready availability of active recombinant enzyme will be an important tool for these studies. Different tagging and expression strategies were explored to optimize purification and yield of active MCR. As a result of these experiments, recombinant MCR can now be produced with full coenzyme F_{430} content, methanococcal PTMs, and levels of about 5 % of the total cellular protein. This system was also successful for the expression of the other proteins important to our studies, including methanococcal MCR-like genes from uncultured archaea. While both recombinant methanococcal and ANME-2 MCR assembled with host MCR forming hybrid complexes, the tested ANME-1 MCR and ethyl-coenzyme M reductase (ECR) only formed homogenous complexes. However, the levels of protein made were very low.

In vitro activation of the recombinant MCR. Active MCR can be generated in whole cells. The activation, however, is lost quickly during purification, which usually entails two precipitation steps and ion-exchange chromatography. A strain has been developed that contains a second copy of MCR with a Strep tag provides a rapid, one-step purification procedure. This strain will be used to further characterize MCR activation in vitro. For instance, MCR mutations will be created with substitutions of specific amino acid residues. The effects on activation, catalysis and enzyme stability can then be evaluated independently. Similarly, recombinant enzyme lacking the PTMs will be produced by expression of the recombinant MCR in mutants with deletions of the modification genes, such as *mmpX* (see below). Thus, the role of PTMs in activation and catalysis can be evaluated.

Characterization of the activation proteins. An activation complex has been purified from *Methanothermobacter marburgensis* that contains the hydrogenase:heterodisulfide reductase complex as well as McrC and several methanogenesis marker proteins. The exact composition is not clear since the procedure requires four column chromatography steps, and some proteins may have been lost or some fortuitous contaminants might remain. To gain additional evidence for

the composition of the activation complex, pull-down experiments were performed in *M. maripaludis*. Individual components were expressed with Strep tags. Upon purification, proteins that copurified were identified by mass spectroscopy. These experiments identified interactions between MCR, McrC, A2, Mmp3, 7, and 17. Only a weak interaction with Hdr was found. With tagged HdrB (expressed from the wild-type *hdr* operon), Hdr, hydrogenase, and formylmethanofuran dehydrogenase were pulled down when cells were grown on H_2. These experiments confirmed that both the Hdr complex and a marker protein complex are involved in the activation of MCR. Likewise, using the tagged recombinant McrC may also provide a means of rapidly purifying the marker protein complex involved in activation.

Characterization of the Mmp10. Mmp10, the product of the *mmpX* gene, is a SAM-dependent methyltransferase required for the 5-C-(S)-methyl-Arg PTM of Mcr. Mmp10 from *M. maripaludis* was found to be cobamide-dependent. Two competing groups in the field produced data from enzyme expressed in *E. coli* with added cobalamin instead of cobamide. Although EPR of the native protein shows the presence of an axial (side on) N ligand, this ligand was not found in the published crystal structure. From this observation, we conclude that *E. coli* may not correctly assemble some coenzymes in methanococcal proteins and is probably not a good expression host for some archaeal proteins.

Tuning Directionality for CO_2 Reduction in the Oxo-acid:ferredoxin Superfamily

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Overall research goals:

The oxo-acid:ferredoxin oxidoreductase (OFOR) enzyme superfamily represents one of the best examples to study the reversible transformation of CO_2. Members of the family are responsible for both oxidizing oxo-acids such as pyruvate, as in a PFOR, to produce electrons that are taken up in a ferredoxin (Fd) pool, and yield acetyl-CoA. However, OFORs must also operate in the reductive direction, such as the OGOR enzyme that produces oxo-glutarate from CO_2 and succinyl-coA, taking up electrons from Fd proteins. How nature biases oxidation *versus* reduction is not well understood for the OFOR superfamily. We have hypothesized that the rate-determining steps of catalysis involving electron transfer (ET) may be responsible, at the level of specific unimolecular rate constants or the macroscopic redox potential. Our research goals are to (a) determine the impact of redox potential of internal "wiring" of an OFOR enzyme upon catalytic rate constants and (b) monitor the impact of redox partners (Fds) of diverse potential upon the same. As our project has continued, increasing focus has fallen on the roles of specific (or non-specific) Fd proteins, which have tunable redox potentials that themselves act as the determinants of the 'bias' or directionality of catalytic chemistry.

Significant achievements: [2021-present]:

• We examined the Fd-dependent properties associated with catalysis. In the context of both a synthetic library of single, double and triple mutants of a given set of homologous Fd proteins, we have examined the impact of Fd mediator potential on the ability to support oxidative versus reductive catalysis. Our first paper, now published in ACS-Catalysis, describes these relationships for a 'canonical' oxidative PFOR and its ortholog associated with CO_2 reduction.

• We continue to make exciting measurements of the direct electrochemistry of OFOR family members giving us access to the direct redox potentials of the 3 x [4Fe-4S] cluster bearing OFORs for the first time. We have achieved this with the enzymes from *Chlorobaculum tepidum* and *Moorella thermoaceticum*. In collaboration with Prof. William Metcalf (UIUC) we have also address a methanogenic PFOR, and its Fd-bearing subunit independently. These data are critical, as they illustrate the impact of binding of a Fd subunit to the entirety of a multi-polypeptide enzyme.

• We have initiated a combination of EPR and stopped flow optical experiments on the characterization of kinetically important species in the reverse TCA cycle OFOR family member KorAB. This enzyme we have previously reported on in the prior granting period, and now we have been able to move into time resolved measurements of the generation of its radical and reduced iron-sulfur cluster intermediates. Our analysis suggests that the timescales of this chemistry, in the oxidative direction is very, very slow compared to canonical PFORs, suggesting that the strategy for biasing CO_2 reduction in the reverse TCA cycle may be slowing down the oxidative direction, versus speeding up the reductive direction.

• Additionally, in a collaborative mode, we have begun working with the *Geobacter* nitrite reductase enzyme with Prof. Eric Hegg and Prof. Nicolai Lehnert. In these experiments, we have engage in the electrochemical characterization of the monomeric NrfA enzyme from *Geobacter*. We have elucidated the redox potentials of the hemes (published in 2021), and we are now examining its electrocatlytic behavior.

Science objectives for 2022-2023:

- The most pressing objectives are to complete our pre-state analysis of KorAB, and submit the nearly completed work on the redox potentials of OFOR superfamily iron-sulfur clusters. The last remaining piece of that puzzle is the assignment of the clusters, which we are in the process of achieving through our ability to mutagenize the environment around each of the 4Fe-4S clusters in the *Cholorbaculum* and *Magnetococcus* cases.
- Leveraging our success on wild-type *Mm*KorAB integration with semi-conductor nanorods with different nano-structured light collectors, in our continued collaboration with Prof. Gordana Dukovic.
- Complete our efforts on mapping the relationship of Ferredoxin redox potentials and binding kinetics to other OFOR members.

My project addresses BES cross-cutting priority areas by:
Our work describes foundational principles that govern the reversibility of OFOR enzymes, which are associated with either the generation or the capture of CO_2. Thus, we are actively examining the role that redox cofactors play in tuning reactivity. We have investigated the interrelationships between multi-electron redox catalysis in carbon and nitrogen transformations, and this theme will continue on in our work.

My scientific area(s) of expertise is/are: Metalloenzymology, and Electrochemistry/electrocatalysis.

The ideal collaborator for my project would have expertise in: Anaerobic synthetic biology; ability to make site-directed mutants in Wood-Ljungdahl Pathway or rTCA-cycle bearing micro-organisms; anaerobic facilities for studying protein-protein interactions.

Publications supported by this project 2021-present:

1. B. Li, P. Steindel, N. Haddad, S. J. Elliott, "Maximizing (Electro)catlaytic CO_2 Reduction with a Ferredoxin-based Reduction Potential Gradient", *ACS Catalysis* **11** (7):4009-4023 (2021).
2. S.J. Elliott. "Bioenergetics Theory and Components | Iron Sulfur Proteins", in *Encyclopedia of Biological Chemistry*, 3rd Ed., *2*: 53-65 (2021). https://doi.org/10.1016/B978-0-12-819460-7.00320-0
3. V.S. Alfaro, J. Campeciño, M .Tracy, S.J. Elliott, E.L. Hegg, N. Lehnert. "Elucidating Electron Storage and Distrubtion within the Pentaheme Scaffold of Cytochrome *c* Nitrite Reductase", *Biochemistry*, **60**(23): 1853-1867 (2021).

Extracellular Charge Transport in Microbial Redox Chains:
Linking the Living and Non-Living Worlds

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Overall research goals:

The overarching goal of our work is to understand the fundamentals, limits, and prevalence of extracellular electron transport (EET) conduits that link microbial metabolism to solid-state electrodes. A biophysical understanding will enable energy conversion at hybrid biomolecular/synthetic interfaces. The objectives of the current grant (2019-2023) are to:

(1) Quantify the diffusive dynamics of bacterial outer-membrane multiheme cytochromes through *in vivo* single molecule tracking on cell surfaces and membrane nanowires. The results directly test the hypothesis that protein dynamics facilitate a collision-exchange mechanism of inter-protein electron transport over micrometer length scales.

(2) Electrochemically characterize the process of *inward* EET into cells with emphasis on the role of cytochromes in facilitating electron uptake. The overarching goal is to shed light on cathode-microbe EET, which remains poorly understood relative to its microbe-anode counterpart.

(3) Develop solid-state physics and electrochemical techniques to measure inter-species electron transport in anaerobic oxidation of methane consortia where multiheme cytochromes are hypothesized to link methanotrophic archaea and sulfate-reducing bacteria.

Significant achievements: 2019-2023 (with 1 year no cost extension due to COVID-19):

Goal 1. We have completed this project, which resulted in a recent publication (Chong *et al.* PNAS, 119, 2022) with the title '*Single molecule tracking of bacterial cell surface cytochromes reveals dynamics that impact long-distance electron transport*'. After successfully engineering the *Shewanella oneidensis* MR-1 decaheme cytochromes MtrC and OmcA to allow quantum dot labeling, we used single molecule imaging and tracking to quantify their lateral diffusive dynamics. We observed confined diffusion behavior for both cytochromes along cell surfaces (diffusion coefficients D_{MtrC} = 0.0192 ± 0.0018 μm^2/s, D_{OmcA} = 0.0125 ± 0.0024 μm^2/s) and the membrane nanowires. We found that these dynamics can trace a path for electron transport via overlap of cytochrome trajectories, consistent with the long-distance conduction mechanism. Based on these measurements, we performed kinetic Monte Carlo simulations that account for both electron hopping and the physical diffusion of cytochromes. The simulations revealed significant electron conduction along cellular membranes and membrane nanowires, with magnitudes that can explain experimental measurements of the apparent electron diffusion coefficient and electrical redox conductivity in bacterial biofilms.

Goal 2. Much of our mechanistic understanding of the EET process is derived from studies of transmembrane cytochrome complexes and extracellular redox shuttles that mediate *outward* EET to anodes and external electron acceptors. In contrast, there are knowledge gaps concerning the reverse process of *inward* EET from external electron donors to cells. Recent evidence of oxidation of extracellular iron by an outer membrane cytochrome (Cyc2) common to iron oxidizing bacteria, and a possible role for extracellular soluble iron as an EET mediator from *S. oneidensis* to anodes, motivated us to test whether soluble iron can also play a role in inward EET from cathodes to *S. oneidensis*. We electrochemically confirmed enhanced electron uptake by *S. oneidensis* in the presence of exogenously added $FeCl_2$. Electron uptake from the cathode is coupled to fumarate reduction by the fumarate reductase FccA, and outer membrane cytochromes play an important role in the inward EET pathway, as indicated by the significantly reduced current levels in cytochrome-deficient mutants. We also demonstrated a heterologous expression approach for analyzing the electrochemical activity of Cyc2

from two Fe(II)-oxidizing bacteria (*Mariprofundus ferrooxydans* PV-1 and *Acidithiobacillus ferrooxidans*) in cytochrome-deficient mutants of our model EET organism *Shewanella*. The stage is now set for more detailed characterization of this conduit.

Goal 3. Recent imaging, genomic/transcriptomic studies, and modeling suggest that the syntrophy within anaerobic oxidation of methane (AOM) consortia is based on direct electron transport via large cytochrome complexes expressed by both anaerobic methanotrophic archaea (ANME) and sulfate-reducing bacteria (SRB). Yet direct electron transport and electrochemical evidence were lacking. We developed a new experimental apparatus for solid-state conduction, electrochemical measurements, and microscopy of ANME-1, ANME-2a and ANME-2c consortia using sediment-free enrichment cultures, in collaboration with Victoria Orphan's group. Solid-state conductance was confirmed for different lineages of ANME-SRB consortia. This dry conductance diminished within a day upon exposure to high temperature or air, suggesting biomolecular origin. Cyclic voltammetry of dried and live consortia revealed a redox component in different ANME-SRB consortia. Electrochemical generator-collector measurements demonstrated electron transport over micrometer scale in the consortia, which is sufficient to link the metabolisms of multiple cells. Taken together, our findings establish that ANME-1 and ANME-2 consortia contain intrinsic and redox-active conductive components capable of directly transporting electrons between cells to enable direct inter-species electron transfer.

Science objectives for 2022-2023:

- Complete and publish two studies focused on inward extracellular electron transport (manuscript in review) and redox conduction in AOM consortia (manuscript in preparation).
- Continue characterizing the electrochemical activity of Cyc2 expressed in *Shewanella oneidensis* mutants, to test direct and/or mediated electron uptake facilitated by this porin-cytochrome conduit.

My project addresses BES cross-cutting priority areas by:
Supporting programmatic BES interests in understanding the biophysical processes of energy capture, transduction, and conversion by biological systems. The knowledge gained through studies on electron transport, extended redox reactions outside cells, and biotic-abiotic charge transport can be harnessed for biological (e.g. microbial) energy capture and conversion technologies, as well bio-hybrid, bio-inspired, and biomimetic clean energy production.

My scientific area(s) of expertise is/are: Biological electron transport, *in vivo* microscopy, scanning probe measurements, bioelectrochemistry, nanoscience.

The ideal collaborator for my project would have expertise in: Structural biology, Ultrafast time-resolved measurements.

Publications supported by this project (2019-2022):

1. K. Abuyen and M.Y. El-Naggar. *Soluble iron enhances extracellular electron uptake by Shewanella oneidensis MR-1*, in review at ChemElectroChem, 2022
2. G.W. Chong, S. Pirbadian, Y. Zhao, L.A. Zacharoff, F. Pinaud, M.Y. El-Naggar. *Single molecule tracking of bacterial cell surface cytochromes reveals dynamics that impact long-distance electron transport*, Proceedings of the National Academy of Sciences of the United States of America, 119 (19), e2119964119, 2022
3. L.A. Zacharoff and M.Y. El-Naggar, *The Electrical frontier of biofilms*, Physical Biology, 18 051501, 2021, part of the Roadmap on emerging concepts in the physical biology of bacterial biofilms
4. D.A Phillips, L.A. Zacharoff, C.M. Hampton, G.W. Chong, A.P. Malanoski, L.A. Metskas, S. Xu, L.J. Bird, B.J. Eddie, G.J. Jensen, L.F. Drummy, M.Y. El-Naggar, S.M. Glaven, *A Prokaryotic Membrane Sculpting BAR Domain Protein*, eLife, 10:e60049, 2021
5. S.N. Gavrilov, D.G. Zavarina, I.M. Elizarov, T.V. Tikhnova, N.I. Dergousova, V.O. Popov, J.R. Lloyd, M.Y. El-Naggar, S. Pirbadian, K.M. Leung, F.T. Robb, M.V. Zakhartsev, O. Bretschger, E.A. Bonch-Osmolovskyaya, *Novel extracellular electron transfer channels in a Gram-positive thermophilic Bacterium*, Frontiers in Microbiology, 11:597818, 2021
6. A.R. Rowe, S. Xu, E. Gardel, A. Bose, P. Girguis, J.P. Amend, M.Y. El-Naggar, *Methane-linked mechanisms of electron uptake from cathodes by Methanosarcina barkeri*, mBio, e02448-18, 2019
7. A. Karbelkar, A.R. Rowe, M.Y. El-Naggar, *An Electrochemical Investigation of Interfacial Electron Uptake by the Sulfur Oxidizing Bacterium Thioclava electrotropha ElOx9*, Electrochimica Acta, 324, 134838 2019

Enzyme Complexes of Model Acetotrophic Methanogens

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Overall research goals:

Acetate-utilizing methane-producing anaerobes (acetotrophic methanogens) account for two-thirds of the methane produced in anaerobic microbial food chains converting complex renewable biomass to methane (biomethanation) as a carbon-neutral biofuel. The long-term goal of this study is to understand the biochemical mechanisms that lead to the conversion of acetate to methane so that they can guide improvements in the efficiency and reliability of biomethanation of renewable biomass as an alternative to fossil fuels. Until now, only two genera of acetotrophic methanogens have been described (Methanosarcina and Methanosaeta).

Although much is known regarding one-carbon transformations leading from the methyl group of acetate to methane, there is a rudimentary understanding of electron transport processes coupled to energy conservation. The project aims to advance an understanding of redox reactions and the enzymes and proteins that direct and regulate the flow of electrons in acetotrophic methanogens employing biochemical, biophysical, and molecular approaches.

Significant achievements: [2020-2022]:

- Characterization of the archetype from group 4 of the FTR (AFTR)-like family of ferredoxin:thioredoxin reductases prevalent in the domains *Bacteria* and *Archaea*.
- Original discovery of identification of $[4Fe\text{-}4S]^{1+}$ cluster exhibiting a mixture of $S = 7/2$ and classical $S = 1/2$ unique intermediate during catalytic reaction of AFTR.
- Mechanistic understanding of the heterodisulfide reductase B2 (HdrB2) of the coenzyme F420 electron bifurcating complex HdrA2B2C2.

Science objectives for [2021-2022]:

- Atomic resolution structure of the acetyl-CoA decarbonylase/synthase and coenzyme F420 electron bifurcating complex HdrA2B2C2 multienzyme complex.
- Mechanistic understanding of the heterodisulfide reductase D of the membrane heterodisulde reductases complex (HdrDE)
- Elucidation of the molecular mechanism of formation of unprecedented S=7/2 $[4Fe\text{-}4S]^{1+}$ cluster intermediate in the AFTR.

My project addresses BES cross-cutting priority areas by:

Acetotrophic methanogens contribute two-thirds of the nearly one billion metric tons of methane produced annually by anaerobic microbial food chains transforming renewable biomass to methane (biomethanation). This proposal addresses the Physical Biosciences Program of the FOA. The overall objective of this study is to provide a better understanding of aceticlastic methanogenesis, which will lead to improved bioreactor designs with improved rates and reliability of biomethanation.

My scientific area(s) of expertise is/are: Biochemistry, spectroscopy and physiology of anaerobic microbes.

The ideal collaborator for my project would have expertise in: Advanced spectroscopic methods, cryo-electron microscopy, x-ray crystallography.

Publications supported by this project (2021-2023):

1. Thomas Giunta, Jeanine Ash, Jabrane Labidi, Douglas Rumble III, Tina Treude, Sebastian Krause, Rachel Harris, Divya Prakash, James Gregory Ferry, Mojhgan Haghnegahdar, Edwin Schauble, Barbara Sherwood Lollar, Edward D. Young "Reversibility controls on extreme methane clumped isotope signatures from anaerobic oxidation of methane" Geochimica et Cosmochimica Acta (under review).
2. Yuanxu Song, Ling Li, Kaifeng Du, Fanping Zhu, Chao Song, Xian-Zheng Yuan, Mingyu Wang, Shuguang Wang, James G Ferry, Zhen Yan (2022). "Unraveling quinoproteins-based extracellular electron transport in humus-dependent respiratory growth of Methanosarcina acetivorans" Research Square.
3. William E.Balch and James G.Ferry (2021). The Wolfe cycle of carbon dioxide reduction to methane revisited and the Ralph Stoner Wolfe legacy at 100 years. Advances in Microbial Physiology , Volume 79, 2021, Pages 1-23
4. Ferry JG (2020) *Methanosarcina acetivorans*: A model for mechanistic understanding of aceticlastic and reverse methanogenesis. Front. Microbiol. 11(1806). 10.3389/fmicb.2020.01806.
5. Prakash D, et al. (2019) Structure and function of an unusual flavodoxin from the domain *Archaea*. Proc. Natl. Acad. Sci. U. S. A. 116(51):25917-25922.
6. Prakash D, Chauhan SS, & Ferry JG (2019) Life on the thermodynamic edge: Respiratory growth of an acetotrophic methanogen. Sci. Adv. 5(8):1-6.
7. Yan Z & Ferry JG (2018) Electron bifurcation and confurcation in methanogenesis and reverse methanogenesis. Front. Microbiol. 9:1-10. 10.3389/micb.2018.01322.
8. Prakash D, et al. (2018) Towards a mechanistic and physiological understanding of a ferredoxin:disulfide reductase from the domains *Archaea* and *Bacteria*. J. Biol. Chem. 293:9198-9209.
9. Holmes DE, *et al*. (2018) Electron and proton flux for carbon dioxide reduction in *Methanosarcina barkeri* during direct interspecies electron transfer. Front. Microbiol. 9:1-11.
10. Yan Z, Wang M, & Ferry JG (2017) A Ferredoxin- and F420H2-dependent, electron-bifurcating, heterodisulfide reductase with homologs in the domains *Bacteria* and *Archaea*. mBio 8:e02285-02216. doi: 02210.01128/mBio.02285-02216.

Uncovering determinants of electron transfer insulation

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Overall research goals:

Insulation of electron transfer pathways is critical to maximizing both natural and designed electron flow. Natural biological electron transfer pathways must be insulated to maximize energy yield, minimize interactions with competing electron transfer pathways, and prevent the formation of damaging free radicals. Synthetic electron transfer pathways need to be incorporated into existing cellular metabolism and insulated from pathways that could compete for electrons and reduce the overall output of the synthetic pathway. Despite the obvious importance of ETI in controlling electron flow, the factors that establish ETI are not well understood, making it difficult to predict or exploit insulation in both natural and designed electron flow. We have found evidence of ETI between the electron transfer pathways for nitrogen fixation and anaerobic aromatic compound degradation in the anoxygenic phototroph, *Rhodopseudomonas palustris*. Moreover, we found that a single amino acid substitution in the electron-bifurcating-like enzyme involved in anaerobic aromatic compound degradation and the ferredoxin involved in nitrogen fixation is sufficient to overcome ETI and form a new electron transfer pathway to nitrogenase. This proposal aims to understand how these amino acid substitutions alter the properties of these proteins to enable electron transfer and gain a fundamental understanding of the determinants of ETI.

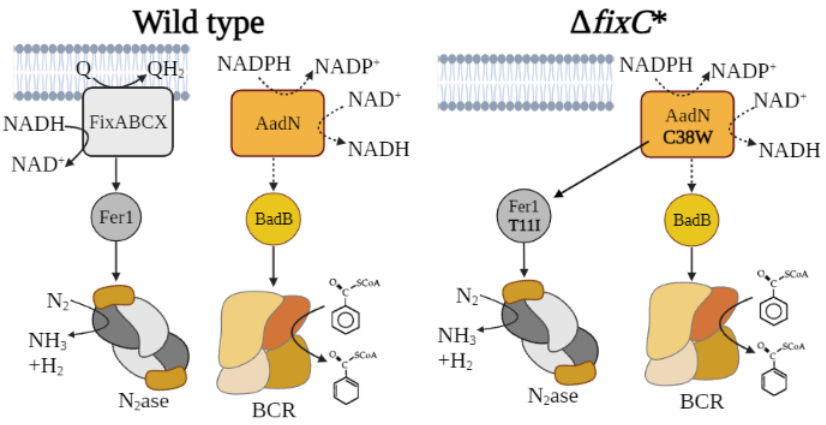

Figure 1. Current model of electron transfer to nitrogenase (N_2ase) and benzoyl-CoA reductase (BCR) in *R. palustris*. Electron transfer to BCR is incompatible with nitrogen fixation in the wild type, but the C38W substitution in AadN and T11I substitution in Fer1 enable the electron transfer pathway for benzoate degradation to support nitrogen fixation. The hypothesized activity of AadN shown in dotted lines is inferred based on its similarity to NfnSL from *Pyrococcus furiosis*.

Significant achievements: 2022-2024:

Ferredoxins (Fds) and flavodoxins (Flds) are small protein electron carriers that transfer a single electron from an electron donor to an electron acceptor. In particular, Fds have specialized over evolutionary time to associate with specific partner proteins, allowing Fds to selectively shuttle electrons to specific pathways. Key factors such as the structure and charge of the Fd binding surface, regulation of Fd abundance, and the reduction potential of the Fd all affect which partner proteins interact with a Fd. In theory, these properties can be altered to enable a Fd to interact with a new partner protein(s) to re-route electron flow, but this remains a challenge for rational design since these properties are ill-defined for many Fds. However, Fds can mediate electron transfer in biological reactions essential for growth, making it possible to select mutations that would allow a Fd and a new partner protein to interact.

One such reaction is biological nitrogen fixation, which is catalyzed by the enzyme nitrogenase. Nitrogenase reduces atmospheric dinitrogen into ammonia and hydrogen using large amounts of ATP and low potential electrons delivered by Fd or Fld. In the purple nonsulfur bacterium,

Rhodopseudomonas palustris, electron transfer to nitrogenase requires the FixABCX complex, which couples the oxidation of NADH to the reduction of quinone and a Fd or Fld using flavin-based electron bifurcation (Fig. 1). *R. palustris* Fix^- mutants have a severe growth defect under nitrogen-fixing conditions, despite *R. palustris* encoding multiple Fd-reducing enzymes including PFOR and FNR that play a role in electron transfer to nitrogenase in other diazotrophs. *R. palustris* also encodes six 2[4Fe-4S] Fds, with the primary electron donor to nitrogenase being the Fd, Fer1 (Rpa4631). Since *R. palustris* encodes multiple Fds and Fd-reducing enzymes, *R. palustris* in which FixABCX is inactive can be leveraged to select for mutations that would enable a new electron transfer chain (ETC) to nitrogenase.

Using an *R. palustris* Δ*fixC* strain, we isolated a suppressor mutant that restored growth of this strain under nitrogen-fixing conditions. We found two mutations in the suppressor strain were required to restore nitrogenase activity in the absence of a functional FixABCX complex (Fig. 1). One mutation was in the gene encoding Fer1, while the second was in an uncharacterized gene renamed *aadN.* Sequence and genetic analysis revealed that the protein encoded by this gene is a homolog of a flavin-based electron bifurcating NAD^+-dependent Fd:NADPH oxidoreductase (Nfn), and we found it is required for anaerobic aromatic compound degradation in *R. palustris* (Fig. 1). The data herein support a model where a new ETC for nitrogenase formed between components of two endogenous ETCs and provides a system that can be used to study the determinants of selective electron transfer.

Science objectives for 2021-2022:

1. Determine if AadN has NAD(P)H:Fd oxidoreductase activity, and if the C38W substitution alters stability or activity of AadN
2. Purify Fer1 and variants of Fer1 and determine their reduction potential

My project addresses BES cross-cutting priority areas by:
Energy-relevant metabolisms generate electron-rich compounds that are released from microbes. Many anaerobic bacteria and archaea have metabolisms that are well suited to this because to grow, these organisms have evolved energy-conserving pathways that allow them to efficiently release reduced compounds such as CH_4, H_2, butanol, and ethanol with little energy input. Electron bifurcation is a mechanism of anaerobic energy conservation that couples an exergonic reaction with the endergonic reduction of a low-potential protein electron carrier such as Fd. Low-potential Fd serves as a captured form of potential energy. Understanding how to generate and harness this stored energy is critical for enabling control of electron flow to metabolic modules that release energy-rich compounds and for designing bioinspired catalysts. Using the anoxygenic phototroph R. palustris, we have developed a system that will allow us to understand electron flow between metabolic modules and potentially how new connections can be made to tap into energy-conserving pathways.

My scientific area(s) of expertise is/are: bacterial genetics, systems biology.

The ideal collaborator for my project would have expertise in: EPR and electrochemistry.

Publications supported by this project 2022-2024:

1. Nathan M Lewis, Abigail Sarne, Kathryn R Fixen. (2022). Selecting a new electron transfer pathway for nitrogen fixation uncovers an electron bifurcating-like enzyme involved in anaerobic aromatic compound degradation bioRxiv 2022.10.07.511188; doi: https://doi.org/10.1101/2022.10.07.511188

Understanding the mechanism and properties of catalytic THI4 proteins

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Overall research goals:

The THI4 thiazole synthases of plants, fungi, and many prokaryotes are suicide enzymes that self-inactivate after a single catalytic cycle because they destroy an essential active-site Cys residue to obtain the sulfur needed for the thiazole ring. The resulting fast protein turnover of these suicide THI4s makes them extremely energetically expensive to operate. Intriguingly, non-suicidal – i.e., catalytic – THI4s have been found in hyperthermophilic, anaerobic archaea from high-sulfide (HS^-) environments; these THI4s have no active-site Cys and instead use HS^- as sulfur donor. Our pilot data indicated that catalytic THI4s with no active-site Cys ('non-Cys' THI4s) also occur in mesophilic prokaryotes from low-HS^- environments having low levels of O_2. Our long-term goal is therefore to define features that allow non-Cys THI4s to operate as efficient catalysts in air and at low HS^- levels like those in plant cells. If better understood, these THI4s could enable design of energy-conserving replacements for plant THI4s. Specific goals are:

1. Screen diverse bacterial non-Cys THI4s for O_2 sensitivity and operation at low HS^- concentration
2. Use continuous directed evolution to lower O_2 sensitivity/raise HS^- affinity; sequence evolved genes
3. Model structures of THI4s with contrasting O_2 sensitivities/HS^- affinities to predict residues involved
4. Determine/model crystal structures of contrasting THI4s and of THI4s before/after directed evolution
5. Use the structure/sequence data to design features needed for catalytic function at high O_2/low HS^-
6. Ultimately, implement these features in suicidal THI4s, analyze outcomes, and evolve further

Significant achievements: 2019-2023:

Goal 1. Screen diverse bacterial non-Cys THI4s for O_2 sensitivity and operation at low [HS^-]: We completed a diversity survey of prokaryotic non-Cys THI4 sequences, tested 26 representatives for *in-vivo* activity in *E. coli*, and chose a subset for continuous directed evolution (see Publication 2 below).

Goal 2. Apply directed evolution to lower O_2 sensitivity/raise HS^- affinity: We modified the yeast OrthoRep continuous directed evolution system and successfully used it to evolve two THI4s from the subset above (MhTHI4 and SfTHI4) for O_2-tolerance. Six beneficial mutations were identified (e.g. **Fig. 1**), of which two recapitulate features in natural, weakly O_2-tolerant THI4s. Structure modeling of these and other beneficial mutations indicated that they could help exclude O_2 from the catalytic Fe(II) center or increase enzyme flexibility (Publications 1 and 3 and submitted Publication 7 below).

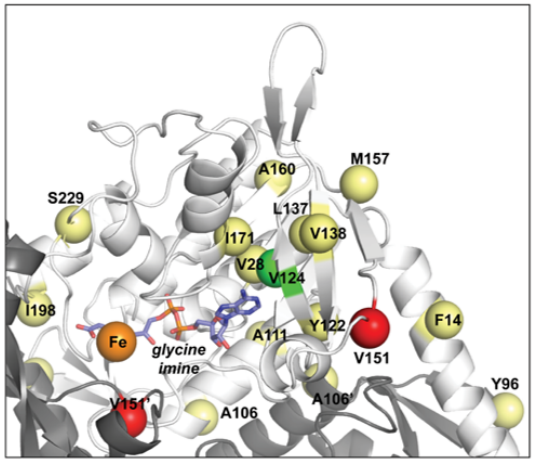

Fig. 1. Structure model of MhTHI4 showing 15 mutations in relation to the active site of one monomer. Two mutations with a beneficial effect (V124) or a deleterious effect (V151) are respectively colored green and red; other mutations are in yellow.

Goal 3. Model structures of THI4s with contrasting O_2 sensitivities/HS^- affinities
Modeling the structures of MhTHI4 and SfTHI4 mutants improved by directed evolution, in combination with comparative genomic analysis, indicated that V124A and Y122C in MhTHI4 are associated with natural O_2-tolerance (see Goal 2). V124A is a fairly common natural variant. Most simply, if A at position 124 confers greater O_2-tolerance than V, then V124 should be prevalent in low-O_2 organisms and A124 in high-O_2 ones. This is the observed pattern. Similarly, changing Y122 to C creates, with C121, a CC motif near the metal center that mimics one in the aerobic suicide THI4 of certain fungi.

Goals 4 & 5. Determine crystal structures of contrasting THI4s
Diffraction-quality crystals were obtained for CaTHI4 from *Caldanaerovirga acetigignens* (which can metabolize low levels of O_2) and TmTHI4 from *Thermotoga maritima* (which cannot metabolize O_2). Diffraction data were collected on a beamline of the Life Sciences Collaborative Access Team facility, Argonne National Laboratory Advanced Photon Source (APS-ANL). The solved structures, along with our TaTHI4 structure (Publication 2 below), constitute an informative natural cline in O_2 adaptation.

Science objectives for 2021-2022:

- Continue to evolve MhTHI4 and SfTHI4 for O_2-tolerance and to interpret outcomes
- Evolve MhTHI4 and SfTHI4 for ability to operate at low HS^- concentrations
- Complete interpretation of CaTHI4 and TmTHI4 structures

My project addresses BES cross-cutting priority areas by: Finding how catalytic THI4s manage to function in the presence of O_2 and at low HS^- levels, with the broader aim of understanding catalytic THI4s well enough to design ones that can function in plants, and thereby increase plants' net capture of light energy.

My scientific area(s) of expertise is/are: Plant and microbial metabolic biochemistry; synthetic biology.

The ideal collaborator for my project would have expertise in: Enzyme and structural biochemistry, with capacity to determine protein structures by cryo-EM. Mark A. Wilson (Univ. Nebraska-Lincoln) is ideal in these respects; we have begun collaborating (see submitted Publications 6 and 7 below).

Publications supported by this project (2019-2023):

1. J.D. García-García, J. Joshi, J.A. Patterson, L. Trujillo-Rodriguez, C.R. Reisch, A.A. Javanpour, C.C. Liu, A.D. Hanson, "Potential for applying continuous directed evolution to plant enzymes: an exploratory study." Life (Basel) **10**, 179 (2020). DOI: 10.3390/life10090179.
2. J. Joshi, Q. Li, J.D. García-García, B.J. Leong, Y. Hu, S.D. Bruner, A.D. Hanson, "Structure and function of aerotolerant, multiple-turnover THI4 thiazole synthases." Biochem. J. **478**, 3265 (2021). DOI: 10.1042/BCJ20210565.
3. J.D. García-García, K. Van Gelder, J. Joshi, U. Bathe, B.J. Leong, S.D. Bruner, C.C. Liu, A.D. Hanson, "Using continuous directed evolution to improve enzymes for plant applications." Plant Physiol. **188,** 971 (2022). DOI: 10.1093/plphys/kiab500.
4. R.S. Molina, G. Rix, A.A. Mengiste, B. Álvarez, D. Seo, H. Chen, J.E. Hurtado, Q. Zhang, J.D. García-García, Z.J. Heins, P.J. Almhjell, F.H. Arnold, A.S. Khalil, A.D. Hanson, J.E. Dueber, D.V. Schaffer, F. Chen, S. Kim, L.Á. Fernández, M.D. Shoulders, C.C. Liu, In vivo hypermutation and continuous evolution. Nat. Rev. Methods Primers. **2**, 36 (2022). DOI: 10.1038/s43586-022-00119-5.
5. U. Bathe, B.J. Leong, K. Van Gelder, G.G. Barbier, C.S. Henry, J.S. Amthor, A.D. Hanson, "Respiratory energy demands and scope for demand expansion and destruction". Plant Physiol. (2022, submitted).
6. J. Joshi, J.S. Amthor, D.R. McCarty, C.D. Messina, M.A. Wilson, A.H. Millar, A.D. Hanson, "Why cutting respiratory CO_2 loss from crops is possible, practicable, and prudential." Mod. Agric. (2022, submitted)
7. K. Van Gelder, E.R. Oliveira-Filho, J.D. García-García, Y. Hu, N. Smith, M.A. Wilson, S.D. Bruner, A.D. Hanson, "Directed evolution of aerotolerance in a sulfide-dependent thiazole synthase". ACS Synth. Biol. (2022, submitted).

Dissimilatory Nitrite Reduction to Ammonium: Catalyzing Multi-Electron Reductions Using a Pentaheme Scaffold

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Overall research goals:

Pentaheme cytochrome *c* nitrite reductase (NrfA) catalyzes the remarkable six-electron reduction of NO_2^- to NH_4^+. Currently, there are several unanswered questions concerning the precise molecular mechanism of NrfA and the exact electron flow through this intriguing enzyme. The long-term goals of this project are to (*i*) ascertain the precise enzymatic mechanism of NrfA, (*ii*) elucidate the strategy for the storage and flow of electrons, and (*iii*) determine how the flow of electrons is regulated. The specific objectives of this proposal are to investigate key NrfA protein-protein interactions (including interactions with its physiological donor NrfH), characterize NrfH, and initiate mechanistic studies on NrfA to determine some of the intermediates in the reaction cycle. To accomplish these objectives, we are employing a synergistic combination of biochemical, kinetic, spectroscopic, and electrochemical methods to trap and interrogate reaction intermediates. We are focusing our studies on the NrfA enzyme from *Geobacter lovleyi*, a DNRA bacterium identified for its environmental relevance. As time permits, we will also interrogate communications between NrfA pairs in the NrfA-NrfA dimer and between NrfA and its redox partner, NrfH. Successful completion of this project will provide insight into how NrfA stores and regulates the flow of electrons, and it will also lay the foundation for subsequent detailed mechanistic studies to ascertain how this unique pentaheme enzyme orchestrates the challenging multi-electron and multi-proton reduction of NO_2^- to NH_4^+.

Significant achievements (2021-2022):

- After interrogating many expression vectors, growth conditions, expression tags, and bacterial hosts, we developed a heterologous expression system for *G. lovleyi* NrfH in *Shewanella oneidensis* MR-1, and a purification protocol that involves the use of Strep-tag II affinity and size exclusion chromatography.
- We performed spectroscopic characterizations of NrfH using UV-Vis absorption spectroscopy and demonstrated the successful reduction of the protein via a methyl viologen-mediated assay.
- We initiated cloning of linked NrfA-NrfA and NrfA-NrfA* dimers (where NrfA* is a heme variant of NrfA) to study the transfer of electrons between the monomeric units.
- We established a chemical reduction method using Ti(III) citrate to sequentially add electrons to the fully oxidized NrfA protein, 1 equivalent at a time. The step-by-step reduction of the hemes was then followed using UV-Vis absorption and EPR spectroscopy. EPR spectral simulations were used to elucidate the sequence of heme reduction within the pentaheme scaffold of NrfA and identify the signals of all five hemes in the EPR spectra.

- We employed non-turnover voltammograms to ascertain the reduction potentials for each heme, which were observed in a relatively narrow range from +10 mV (heme 5) to −226 mV (heme 3) (vs the standard hydrogen electrode). E_m values were −226, −180, −152, −66, and +10 mV.
- We used quantitative analysis and simulation of the EPR data to demonstrate that hemes 4 and 5 of NrfA are reduced first (before the active site heme 1) and serve the purpose of an electron storage unit within the protein.
- To probe the role of the central heme 3, we generated an H108M NrfA variant where the reduction potential of heme 3 is shifted positively (from −226 to +48 mV). The H108M mutation significantly impacts the distribution of electrons within the pentaheme scaffold and the reduction potentials of the hemes, reducing the catalytic activity of the enzyme to 1% compared to that of the wild type. We propose that this is due to heme 3's important role as an electron gateway in the wild-type enzyme.

Science objectives for 2021-2022:

The specific goals of this 3-year research proposal are to:

Goal 1: Characterize key NrfA protein-protein interactions

A. Determine how NrfH alters the activity of NrfA
B. Ascertain the impact of NrfA-NrfA dimerization on activity

Goal 2: Determine the flow of electrons to the active site

A. Determine the spectroscopic and electrochemical properties of *G. lovleyi* NrfH
B. Probe the effect of NrfA dimerization on communication between subunits

Goal 3: Elucidate the detailed mechanism of NO_2^- reduction

A. Clarify the role of the conserved Arg residue near the active site in this subclass of NrfA enzymes
B. Characterize some of the intermediates in the reaction cycle via stopped-flow methods

My project addresses BES cross-cutting priority areas by:

The proposed work aligns well with the Reaction Pathways in Diverse Environments fundamental research theme outlined by the Chemical Sciences, Geosciences, and Biosciences Division, and it synergizes well with both the Charge Transport and Reactivity and the Chemistry in Aqueous Environments themes. In addition, our work is directly relevant to the BES – Physical Biosciences core mission to "further our understanding of the ways plants and non-medical microbes capture, convert, and store energy." Consistent with the Physical Biosciences vision, our work will "provide a better understanding of the structure/function, mechanistic, and electrochemical properties of enzymes that catalyze complex multielectron redox reactions."

My scientific area(s) of expertise is/are: Hegg: Role of metals in biological systems; mechanistic enzymology. Lehnert: spectroscopy and simulation, quantum-chemical calculations.

The ideal collaborator for my project would have expertise in: protein film voltammetry to assess the redox potentials of the various hemes in both WT *G. lovleyi* NrfA and amino-acid variants. To address this issue, we established a collaboration with Professor Sean Elliott at Boston University.

Publications supported by this project [2020-2022]:

1. J. Campeciño, S. Lagishetty, Z. Wawrzak, V. Sosa Alfaro, N. Lehnert, G. Reguera, J. Hu, E.L. Hegg, "Cytochrome *c* nitrite reductase from the bacterium *Geobacter lovleyi* represents a new NrfA subclass." J. Biol. Chem. 295 (2020). [DOI: 10.1074/jbc.RA120.013981]
2. V. Sosa Alfaro, J. Campeciño, M. Tracy, S.J. Elliott, E.L. Hegg, and N. Lehnert, "Elucidating Electron Storage and Distribution within the Pentaheme Scaffold of Cytochrome c Nitrite Reductase (NrfA)." Biochemistry **60**, 23 (2021). [DOI: 10.1021/acs.biochem.0c00977]

DE-SC0010666 Mechanistic Studies of Energy-Relevant Molybdenum Enzymes

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Alexandre, Postdoctoral Research Associate
Sheron Hakopian and Steve Ortiz, Graduate students
Dimitri Niks, Senior Research Associate
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Overall research goals:

1. Compare and contrast the rapid reaction kinetic behavior of three different formate dehydrogenases, following the rate of reduction of the enzyme by formate by stopped-flow spectrophotometry and by freeze-quench EPR, including site-directed mutagenesis studies of specific highly conserved amino acid residues in the enzymes' active sites.

2. Test the recent suggestion on the basis of computational studies that a specific covalent intermediate exists in the oxidation of formate to CO_2 by ENDOR spectroscopy. This will be done with an eye to elucidating any difference in the reactivity of the selenocysteine- and cysteine-containing proteins.

3. Examine all four enzymes by X-ray crystallography, with the aim of resolving a longstanding controversy in the literature as to whether the (seleno)cysteine ligand to the molybdenum dissociates in the course of the catalytic sequence.

Significant achievements: 2018-2022:

- Characterization of the molybdenum-containing DMSO reductase substituted with tungsten
- Identification of hydride transfer in the reaction mechanism of formate dehydrogenases and related enzymes
- Demonstration of bulk conversion of CO_2 to formate by FdsDABG formate dehydrogenase
- Determination of the reduction potentials of the FdsDABG formate dehydrogenase (in collaboration with Sean Elliott, Department of Chemistry, Boston University)
- Determination of the reduction potentials of *O. carboxidovorans* CO dehydrogenase (in collaboration with Paul Bernhardt, Department of Chemistry, University of Queensland)
- Crystallographic characterization of the FdsBG fragment of *C. necator* formate dehydrogenase
- Characterization of O_2-inactivation of *C. necator* formate dehydrogenase
- Characterization of a tungsten-containing formate dehydrogenase from *D. vulgaris* Hildenborough (in collaboration with C.S. Raman, Department of Pharmaceutical Sciences, University of Maryland, Baltimore)

Science objectives for 2021-2022:

Continue with kinetic and structural characterization of FdhF from *E. coli* and *P. atrosepticum.*

My project addresses BES cross-cutting priority areas by:
…identifying those principles utilized by biological systems to catalyze the reduction of CO_2 to formate at ambient temperature and pressure.

My scientific area(s) of expertise is/are: enzymology; rapid-reaction kinetics; electron paramagnetic resonance spectroscopy as applied to metalloenzymes.

The ideal collaborator for my project would have expertise in: electrochemistry; microbial physiology.

Publications supported by this project 2018-2022:

Pacheco, J. Niks, D., Haffner, N., & **Hille, R.** (2018) Tungsten-substituted DMSO reductase from *Rhodopacter sphaeroides* exhibits a W(V) species with an unusually intense UV/visible absorption spectrum. *J. Biol. Inorg. Chem.* **23**, 295-301.

Niks, D., and **Hille R.** (2018) Molybdenum- and tungsten-containing formate dehydrogenases and formylmethanofuran dehydrogenases: structure, mechanism and cofactor insertion. *Protein Sci.*, DOI 10.1002/pro.3498.

Yu, X., Niks, D., Ge, X., Liu H., **Hille, R**., and Mulchandani, A. (2019) Synthesis of Formate from CO_2 Gas Catalyzed by an O_2-tolerant NAD-Dependent Formate Dehydrogenase and Glucose Dehydrogenase. *Biochemistry* **58**, 1861-1868.

Walker, L.M., Li, B., Niks, D., **Hille, R.** and Elliott, S.J. (2019) Deconvolution of reduction potentials of formate dehydrogenase from *Cupriavidus necator*, *J. Biol. Inorg. Chem.***24**, 889-898.

Kalimuthu, P., Petitgenet, M., Niks, D., Dingwall, S., **Hille, R.**, and Bernhardt, P.V. (2020) The oxidation-reduction and electrocatalytic properties of CO dehydrogenase from *Oligotropha carboxidovorans*. *Biochim. Biophys. Acta* **1861**, #148118.

Young, T., Niks, D., Hakopian, S., Tam, T.K., Yu, X., **Hille, R.** and Blaha, G. (2020) Crystallographic and kinetic analyses of the FdsBG subcomplex of the cytosolic formate dehydrogenase FdsABG from *Cupriavidus necator*. *J. Biol. Chem.*, **295**, 6570-6585.

Hakopian, S., Niks, D., and **Hille, R.** (2022) Air inactivation of formate dehydrogenase FdsDABG from *Cupriavidus necator*. *J. Bioinorg. Chem.* **231**, DOI 10.1016/j.jinorgbio.2022.111788.

Graham, J., Niks, D., Zane, G., Gui, Q., Hom, K., **Hille, R.** Wall, J., and Raman, C.S. (2022) How a formate dehydrogenase responds to oxygen: unexpected O_2-insensitivity of an enzyme harboring tungstopterin, selenocysteine and [4Fe-4S] clusters. *ACS Catalysis* **12**, 10449-10471.

Electron Transport in Polymerized Cytochrome Appendages

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Overall research goals:

In anoxic environments, from aquatic sediments to the human gut, respiring bacteria routinely transfer electrons micron-scale distances beyond their outer membranes to distant and insoluble terminal electron acceptors. Despite its central role in global geochemical cycling and potential for inspiration of bioelectronic materials and interfaces, the mechanism enabling such long-distance electron transfer and between-cell conductivity remains a topic of active debate. One organism long studied as a model of this process, *Geobacter sulfurreducens*, uses filamentous, electrically conductive appendages to respire by transferring spent electrons from cells to extracellular terminal oxidants. One such filament was recently revealed to be polymerized cytochromes of the *G. sulfurreducens* OmcS. Such well-ordered cytochrome filaments are novel structures in biology, and polymerized OmcS is the best-characterized example of protein supramolecular aggregates that support long-range electronic conduction.

Our long-term research goals are to:

(1) Reveal the mechanism facilitating long range electron transport in polymerized cytochrome filaments.
(2) Define biosynthetic pathways enabling assembly of conductive extracellular cytochrome filaments.
(3) Identify homologous structures enabling redox conduction in *G. sulfurreducens* and other environmental bacteria.

This project will focus on the distinctive electron transport mechanisms and structures inherent to newly discovered filaments composed entirely of the cytochrome OmcS, using a cross-disciplinary combination of biochemistry, molecular biology, spectroscopy, electrochemistry, solid-state transport, and cryo-electron microscopy (EM) methods.

Significant achievements: 2019-2023:

- We discovered that mutations in one fiber-forming cytochrome upregulate the production of other cytochrome fibers. These results call into question the conclusions of extensive prior genetic studies that knocked out OmcS in *G. sulfurreducens*, which must be re-considered given that other conductive cytochrome fibers were likely present.
- We determined the atomic-scale structure of two new cytochrome polymer appendages in *G. sulfurreducens*, OmcE and OmcZ, the 2nd and 3rd cytochrome polymer structures after OmcS.
- We found that the axial heme packing in OmcS and OmcE are nearly identical despite negligible protein sequence identity, suggesting a structure-directing role for heme (Fig. 1A).
- We found, through a meta-analysis of Protein Databank structures, that the arrangement of heme pairs found in OmcS and OmcE is largely conserved in multiheme *c*-type cytochromes across domains of life (Fig. 1B).
- We found that the heme arrangement in OmcZ is mostly similar to OmcS and OmcE with the exception of two heme pairs for which the spatial packing lies outside the major conserved clusters of most multiheme *c*-type cytochromes. This observation suggests a distinct evolutionary origin of OmcZ as compared to OmcS and OmcE (Fig 1AB).
- We found that OmcE is the only cytochrome of OmcS/E/Z that is required for soluble Fe(III) reduction, suggesting its key role in extracellular electron transfer.

- We confirmed the published structure of the *G. sulfurreducens* Type-IV pilus (T4P) in a non-overexpressing strain. This structure directly contradicts the long-standing hypothesized T4P structure proposed to be responsible for electronic conductivity in *G. sulfurreducens* biofilms.

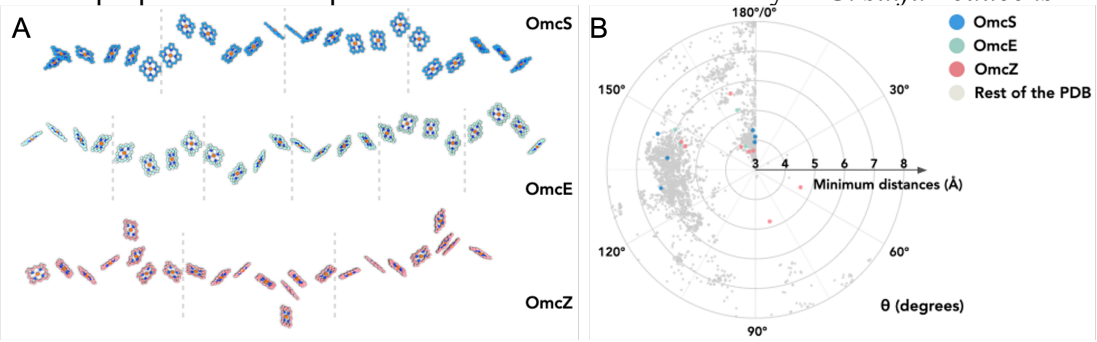

Figure 1. (A) The arrangement of heme along the fiber axis of cytochromes OmcS, OmcE, and OmcZ polymers secreted by *G. sulfurreducens*. (B) The rotation angle and distance between the porphyrins, of neighboring heme pairs shows conserved arrangements of heme in OmcS, OmcE, OmcZ, and across multiheme cytochrome in the PDB. Two heme pairs, both including a highly solvent exposed heme in OmcZ, are notable exceptions to these trends.

Science objectives for 2022-2023:

- Confirmation of expression of mutant OmcS. Currently, some mutants either enrich other cytochrome filament expression (e.g. OmcE) or express filaments that bundle, precluding cryo-EM analysis. We aim to improve protein mass spectrometry sample preparation and more comprehensive cytochrome mutant backgrounds to aid in confirming expression of mutant OmcS filaments.
- Comparison of the reduction potentials and biochemical reactivity of OmcS, OmcE and OmcZ polymers vs. monomers of the same cytochromes. These studies can provide insight into the electron transfer and physiological role of polymerized cytochromes.
- Comparison of electronic conductivity in OmcS vs. OmcE vs. OmcZ. Differences in conductivity or lack thereof in light of our structure insights into these three fibers will establish structure-function relationships in cytochrome polymers.

Our project addresses BES cross-cutting priority areas by:
Next generation energy storage and hydrogen production: our studies reveal how to efficiently move electrons across space through biomolecules and inspire new bio- and biohybrid energy devices. *Biotechnology*: our projects broadens our understanding of how electrical energy flows in and out of cells, providing insights that can improve efficiencies of existing biotechnology processes and enable new energy inputs/outputs for next generation biotechnology processes.

Our scientific area(s) of expertise is/are: Functional genomics, molecular biology and analytical chemistry, cryo-electron microscopy and computational image analysis, charge transport processes, electrochemistry.

The ideal collaborator for our project would have expertise in: analytical or numerical modeling of charge transfer over long distances; methods for studying charge transfer dynamics.

Publications supported by this project 2019-2023:

1. ‡ Wang, F., ‡ Mustafa, K., Suciu, V., Joshi, K., Chan, C.H., Choi, S., Su, Z., Si, D., Hochbaum, A.I.*, Egelman, E.H.*, Bond, D.R.* (2022) Cryo-EM structure of an extracellular *Geobacter* OmcE cytochrome filament reveals tetrahaem packing. *Nat. Microbiol.*, *7*, 1291-1300. https://doi.org/10.1038/s41564-022-01159-z
2. Wang, F., Chan, C.H., Suciu, V., Mustafa, K., Ammend, M., Si, D., Hochbaum, A.I.*, Egelman, E.H.*, Bond, D.R.* (2022) Structure of Geobacter OmcZ filaments suggests extracellular cytochrome polymers evolved independently multiple times *eLife* 11:e81551 https://doi.org/10.7554/eLife.81551

Towards the Mechanism of N_2 Fixation by Nitrogenase

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Overall research goals:

Nitrogenases are the microbial enzymes responsible for the biological reduction of N_2 to two molecules of NH_3. However, in addition to the physiological substrate, N_2, nitrogenase also has the ability to reduce small carbon-based molecules, such as acetylene (C_2H_2), CO_2, and CO. In particular, both wild type and remodeled Mo-nitrogenase can reduce CO_2 to formate ($HCOO^-$), methane (CH_4), and in doing so generate longer chain hydrocarbons (C2-C4). Thus, nitrogenase is a single catalyst that offers an ideal system both to deduce underlying catalytic principles and to explore alternative means of both producing ammonia as fertilizer and hydrocarbons as fuels. An overarching aim of the work of our research Team, involving Seefeldt, Dean, and Raugei, is to gain molecular level insights into the mechanism of nitrogenase catalysis. Our role is to carry out advanced paramagnetic resonance measurements that are uniquely capable of characterizing nitrogenase enzymatic intermediates trapped by Seefeldt in constructs developed by Dean, and to combine the insights we gain with computational visualizations of our findings by Raugei.

Significant Achievements:

The past year has seen the publication of six peer reviewed publications (see below). We focus on the one that probably is of broadest interest, while best highlighting the value of the Team approach.

^{13}C ENDOR Characterization of the Central Carbon Within the Nitrogenase Catalytic Cofactor Indicates the CFe_6 Core is a Stabilizing 'Heart of Steel': Substrates and inhibitors of Mo-dependent nitrogenase bind and react at Fe ions of the active-site FeMo-cofactor [7Fe-9S-C-Mo-homocitrate] contained within the MoFe protein α-subunit. The cofactor contains a CFe_6 core, a carbon centered within a trigonal prism of six Fe, whose role in catalysis is unknown. Targeted ^{13}C labeling of the carbon by Dean enables ENDOR spectroscopy to sensitively monitor the electronic properties of the Fe-C bonds *and* the spin-coupling scheme adopted by FeMo-cofactor metal-ions. This report compares $^{13}CFe_6$ ENDOR measurements on states trapped by Seefeldt: *(i)* wild-type protein resting state (E_0; α-Val^{70}) to those of *(ii)* α-Ile^{70}, *(iii)* α-Ala^{70} substituted proteins; *(iv)* crystallographically characterized CO-inhibited 'hi-CO' state; (*v*) E_4(4H) Janus intermediate, activated for N_2 binding/reduction by accumulation of 4[e^-/H^+]; (*vi*) E_4(2H)* state containing doubly-reduced FeMo-cofactor without Fe-bound substrates; (*vii*) propargyl alcohol reduction intermediate having allyl alcohol bound as a ferracycle to FeMo-cofactor Fe6. All states examined, both S = ½ and 3/2, exhibit near-zero ^{13}C isotropic hyperfine coupling constants, ^{C}a = [-1.3 ↔ +2.7] MHz. Density functional theory computations and Natural Bond Order analysis of the C-Fe bonds by Raugei show this occurs because a (3spin-up/3spin-down) spin-exchange configuration of CFe_6 Fe-ion spins produces cancellation of large spin-transfers to carbon in each Fe-C bond. Previous X-ray diffraction plus DFT indicate that trigonal-prismatic geometry around carbon is maintained with high precision in all these states. The persistent structure and Fe-C bonding of the CFe_6 core indicates it does not provide a functionally dynamic (hemilabile) 'beating heart' - instead it acts as 'a heart of steel', stabilizing the structure of the FeMo-cofactor active site during nitrogenase catalysis.

Science objectives for 2022-2023:

Work in the coming year will continue to advance our understanding of the N_2 activation mechanism at the active site of all three nitrogenases. This objectives include:

- Continue our progress towards trapping all nine intermediates in the full nitrogenase catalytic cycle and characterizing them through use of EPR and ENDOR spectroscopies. This effort involves not only the Mo-nitrogenase, but also the V- and Fe-nitrogenases, with low/high electron flux conditions emphasizing early/late states, and mutant forms selected to emphasize individual states.
- Selectively label with ^{13}C the central C for all three nitrogenase isozymes (Mo, V, Fe) and trap new substrate intermediates. Analysis of these trapped states by ^{13}C-ENDOR, combined with determination of the hyperfine couplings to the cofactor heterometals by ^{95}Mo and ^{57}Fe ENDOR plus EPR will characterize both the bonding in the C-Fe_6 core and the exchange-coupling schemes among the seven cofactor metal ions across the reaction pathway.

My project addresses BES cross-cutting priority areas by:
This project provides mechanistic understanding of the reduction of the diatomic molecules N_2, as well as CO_2 and CO, which are central to energy priorities.

My scientific area(s) of expertise is/are: Development and application of advanced paramagnetic resonance methods, such as ENDOR in support of the determination of metalloenzyme mechanism.

The ideal collaborators for my project would have expertise in: microbiogy, moleclular biology and biochemistry, which would enable the preparation of isotopically labelled enzymes and variants for paramagnetic resonance studies, plus experts in computational chemistry, with theory and experiment providing complementary insights into then catalytic mechanism of metalloenzymes.

DOE-Supported Publications

(1) Lukoyanov, D. A.; Yang, Z.-Y.; Pérez-González, A.; Raugei, S.; Dean, D. R.; Seefeldt, L. C.; Hoffman, B. M. 13C ENDOR Characterization of the Central Carbon within the Nitrogenase Catalytic Cofactor Indicates That the CFe_6 Core Is a Stabilizing "Heart of Steel". *J. Am. Chem. Soc.* **2022**. DOI: 10.1021/jacs.2c06149.
(2) Lukoyanov, D. A.; Harris, D. F.; Yang, Z. Y.; Perez-Gonzalez, A.; Dean, D. R.; Seefeldt, L. C.; Hoffman, B. M. The One-Electron Reduced Active-Site FeFe-Cofactor of Fe-Nitrogenase Contains a Hydride Bound to a Formally Oxidized Metal-Ion Core. *Inorganic chemistry* **2022**, *61* (14), 5459-5464. DOI: 10.1021/acs.inorgchem.2c00180.
(3) Sinhababu, S.; Radzhabov, M. R.; Telser, J.; Mankad, N. P. Cooperative Activation of CO_2 and Epoxide by a Heterobinuclear Al–Fe Complex via Radical Pair Mechanisms. *J. Am. Chem. Soc.* **2022**, *144* (7), 3210-3221. DOI: 10.1021/jacs.1c13108.
(4) Valdez-Moreira, J. A.; Beagan, D. M.; Yang, H.; Telser, J.; Hoffman, B. M.; Pink, M.; Carta, V.; Smith, J. M. Hydrocarbon Oxidation by an Exposed, Multiply Bonded Iron(III) Oxo Complex. *ACS Central Science* **2021**, *7* (10), 1751-1755. DOI: 10.1021/acscentsci.1c00890.
(5) Perez-Gonzalez, A.; Yang, Z. Y.; Lukoyanov, D. A.; Dean, D. R.; Seefeldt, L. C.; Hoffman, B. M. Exploring the Role of the Central Carbide of the Nitrogenase Active-Site FeMo-cofactor through Targeted ^{13}C Labeling and ENDOR Spectroscopy. *Journal of the American Chemical Society* **2021**, *143* (24), 9183-9190. DOI: 10.1021/jacs.1c04152.
(6) Yang, Z. Y.; Jimenez-Vicente, E.; Kallas, H.; Lukoyanov, D. A.; Yang, H.; Martin Del Campo, J. S.; Dean, D. R.; Hoffman, B. M.; Seefeldt, L. C. The Electronic Structure of FeV-cofactor in Vanadium-Dependent Nitrogenase. *Chem Sci* **2021**, *12* (20), 6913-6922. DOI: 10.1039/d0sc06561g.

Engineering a Functional Equivalent of Nitrogenase for Mechanistic Investigations of Ammonia Synthesis

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Overall research goals:

The overarching goal of this project is to use NifEN of *Azotobacter vinelandii* as a mutational platform to construct partially defective or fully functional MoFe protein mimics for mechanistic investigations of ammonia synthesis by nitrogenase. Genetic methods (mutagenesis and homologous recombination) will be used to strategically reconstruct defective or functional mimics of MoFe protein, and biochemical (metal and enzymatic assays) and spectroscopic (EPR and XAS/EXAFS analyses) methods will be employed to monitor and analyze the (re)construction process. Success in generating partially defective nitrogenase variants on a NifEN template will facilitate capture of the reaction intermediates of N_2 reduction for mechanistic investigations of nitrogenase; whereas success in generating an active nitrogenase equivalent on a NifEN template will enable identification of all functional determinants for the catalytic activity of nitrogenase and provide a proof-of-concept for minimizing the essential *nif* gene set for future transgenic expression of nitrogenase via synthetic biology.

Significant achievements (2020-2022):

- **Heterologous expression and engineering of the nitrogenase cofactor biosynthesis scaffold NifEN.** We have successfully expressed and engineered NifEN of *Azotobacter vinelandii* (*Av*NifEN) in *Escherichia* coli. Our biochemical and spectroscopic analyses demonstrate the integrity of the heterologously expressed NifEN in composition and functionality and, additionally, the ability of an engineered NifEN variant to mimic NifDK in retaining the matured cofactor at an analogous cofactor-binding site. This is an important step towards the overall goal of our proposed project.

- **Establishing the requirement of P-cluster formation for NifDK expressed in *E. coli*.** We have successfully co-expressed NifDK with IscS, IscU, NifZ, NifH, and NifM in *E. coli*. Of the proteins co-expressed with NifDK, IscS and IscU provide small [4Fe4S] building blocks for the formation of a P-cluster, and NifH donates electrons for the reductive coupling of two [4Fe4S] clusters into an [8Fe7S] P-cluster. Additionally, NifM serves as a peptidyl-prolyl *cis–trans* isomerase for the functionalization of NifH; whereas NifZ serves as a chaperone to facilitates the maturation of the P-cluster in NifDK. Our XAS/EXAFS, EPR, and activity analyses confirmed the presence of a fully assembled P-cluster in the NifDK protein co-expressed with these maturation components in *E. coli.* We will use this knowledge to generate mature P-clusters in our proposed, NifEN-based mimics of NifDK.

- **Expression of NifEN of *Methanosarcina acetivorans* (*Ma*NifEN) in *E. coli*.** In parallel to our proposed mutational work on *A. vinelandii* NifEN (*Av*NifEN), we also plan to use natural NifEN variants from other organisms that already have certain key features of a functional MoFe protein in place to perform our proposed genetic manipulations. Along this line of effort, we have successfully expressed *Ma*NifEN that carries the complete complement of P-cluster ligands and, therefore, only requires additional genetic manipulations to restore the M-cluster site. In addition, we have confirmed that *Ma*NifEN is capable of binding the L-cluster, a biosynthetic precursor of the mature M-cluster.

Science objectives for 2022-2023:

- Restoring a catalytically active M-cluster site of NifEN that enables at least some N_2-reducing activity.

My project addresses BES cross-cutting priority areas by:

Our efforts not only contribute to a better understanding of the mechanism of ammonia synthesis by nitrogenase, but also have the long-term potential in developing energy-efficient strategies for nitrogenase-based ammonia synthesis and generating modified enzymes with improved efficiency in ammonia or hydrogen production.

My major scientific area(s) of expertise is/are: Molecular biology, structural biology, biochemistry, bioinorganic chemistry.

The ideal collaborator for my project would have expertise in: Mössbauer spectroscopy.

Publications supported by this project:

1. Kang W, Lee CC, Jasniewski AJ, Ribbe MW, Hu Y. (2020) Structural evidence for a dynamic metallocofactor during N_2 reduction by Mo-nitrogenase. **Science** *368*(6497):1381-1385. doi: 10.1126/science.aaz6748.
2. Hu Y, Ribbe MW. (2020) Special issue on nitrogenases and homologous systems. **Chembiochem** *21*(12):1668-1670. doi: 10.1002/cbic.202000279.
3. Solomon JB, Lee CC, Jasniewski AJ, Rasekh MF, Ribbe MW, Hu Y. (2020) Heterologous expression and engineering of the nitrogenase cofactor biosynthesis scaffold NifEN. **Angew Chem Int Ed Engl** *59*(17):6887-6893. doi: 10.1002/anie.201916598.
4. Jasniewski AJ, Lee CC, Ribbe MW, Hu Y. (2020) Reactivity, mechanism, and assembly of the alternative nitrogenases. **Chem Rev** *120*(12):5107-5157. doi: 10.1021/acs.chemrev.9b00704.
5. Newcomb MP, Lee CC, Tanifuji K, Jasniewski AJ, Liedtke J, Ribbe MW, Hu Y. (2020) A V-nitrogenase variant containing a citrate-substituted cofactor. **Chembiochem** *21*(12):1742-1748. doi: 10.1002/cbic.201900654.

Electron Flow and Energy Conservation in Syntrophic Metabolism

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Overall research goals:

The objectives of the proposed work are: (1) to perform biochemical characterization of FeSOR/EMO; and (2) continued functional and structural characterization of the Acs1 from *S. aciditrophicus*. Full-length, truncations, and other variants of FeSOR/EMO homologues from *S. aciditrophicus* and *S. wolfei* will be overexpressed in *Escherichia coli* and be characterized for their enzymatic activity and structural properties. We will use homology modeling/docking and X-ray crystallography to determine the position of amino acid side chains in the *Sa*Acs1 active site when ATP is bound. Additionally, X-ray crystallography will be utilized to identify the conformation of *Sa*Acs1 in the presence and absence of ATP and/or acetyl-coenzyme A (CoA). Furthermore, additional amino acid substitutions will be utilized to determine the role of amino acids important for interactions with ATP in the active site of *Sa*Acs1.

Significant achievements: 2021-2022:

We have obtained two full-length structures (3.24 Å and 2.2 Å) of *Syntrophus aciditrophicus (Sa)* Acs1 with acetyl-AMP in the ATP/AMP binding pocket of the active site in the adenylate-forming conformations. Collaborators at UCLA obtained 2.64 Å structure of the large domain of *Sa*Acs1 in the thioester-forming conformation (determined by position of W317 that blocks the CoA tunnel in the adenylate-forming conformation). At the opening of the CoA binding pocket, there is a loop (position unchanged in the two conformations) that has two well-conserved positively charged amino acids (*Se*Acs R191, R194) that are known to interact with the nucleotide end of the CoA molecule. In some Acs proteins, the R194 equivalent is a lysine. Nevertheless, in all Acs structures available in the PDB, the relative positions of these two side chains and the loop region are highly conserved. The positions of both the loop and the side chains does not vary between the two conformations (adenylate-forming and thioester-forming) nor does it differ when CoA is bound in the pocket. In *Se*Acs, R191 and R194 are positioned so that R191 interacts with the adenine ring and R194 interacts with the phosphate on the 3' carbon of the ribose. However, the corresponding residues in *Sa*Acs1 (R199 and K202, respectively) are shifted 9.3 and 7.5 Å, respectively.

Site-directed mutagenesis was utilized to introduce alanine amino acid substitutions into key active site residues in *Sa*Acs1. Enzymatic assays were performed in the AMP/acetyl-CoA forming direction and the ATP/acetate-forming direction. *Sa*Acs1^{S273A} and *Sa*Acs1^{R536A} variants showed no activity in either enzymatic direction. S273 is in a highly mobile ATP binding loop. The *Sa*Acs1^{W421A} demonstrated ~90% lower activity in both reaction directions. However, alanine substitutions at G395 or T420 gave varying results depending on the reaction direction. In the AMP/acetyl-Co-A forming direction, *Sa*Acs1^{G395A} and *Sa*Acs1^{T420A} variants only showed a reduction in the activity of 53% or 36%, respectively. Activity in the ATP-forming direction was more dramatically impacted with the *Sa*Acs1^{G395A} and *Sa*Acs1^{T420A} variants showing a reduction in the activity of 90% and 80%, respectively. In conjunction with W421, G395 and T420 encircle the adenine ring of the AMP/ATP in the binding pocket. Increased impact on the ATP-forming activity of the enzyme in the G395A and

T420A variants may suggest that when operating in the ATP-forming direction, this region of the active site has an altered shape that cannot be accommodated when either of these amino acids is altered.

Science objectives for 2022-2023:

- Obtain an *Sa*Acs1 full-length structure in the thioester-forming conformation with CoA or acetyl-CoA bound
- Obtain an *Sa*Acs1 full-length structure in the adenylate-forming conformation with non-hydrolyzable ATP bound
- High-level expression of FeSOR/EMO under the control of a low phosphate-inducible promoter in *Methanococcus maripaludis*

My project addresses BES cross-cutting priority areas by:

Understanding how organisms conserve energy at low thermodynamic driving forces is needed to understand how microorganisms in energy-limited environments persist. The free energy changes involved in syntrophic metabolism are close to the minimum free energy needed to maintain biological activity. We found that a model syntroph, *S. aciditrophicus,* uses a novel substrate-level phosphorylation reaction to make ATP, which operates close to thermodynamic equilibrium.

My scientific area(s) of expertise is/are: anaerobic microbiology, structural biology, molecular biology, protein expression

The ideal collaborator for my project would have expertise in: microbial physiology, protein computational modeling, enzymology

Publications supported by this project 2021-2022:

1. Dinh, D., E. Moore, L.M. Thomas, N. Wofford, M.J. McInerney, E.A. Karr. The Structure (and Functions) of Putative 3-hydroxypimelyl-CoA Dehydrogenase (Hcd1) from *Syntrophus aciditrophicus* strain SB. PDB ID: 7SUB, In preparation.
2. Dinh, D., S. Yaghoubi, L.M. Thomas, N. Wofford, M.J. McInerney, E.A. Karr. Functional and Structural Characterization of AMP-forming Acetyl-CoA Synthetase (Acs1) from *Syntrophus aciditrophicus* strain SB. In preparation.

Mechanism of Photochemical N_2 Reduction

Paul W. King, Principal Investigator
Gordana Dukovic, David W. Mulder, John W. Peters, Lance C. Seefeldt, Co-PI(s)
Bryant Chica, Effie Kisgeropoulos, Greg Vansuch, Mark Willis, Postdoctoral Research Associates
National Renewable Energy Lab, Golden CO, 80401, USA.
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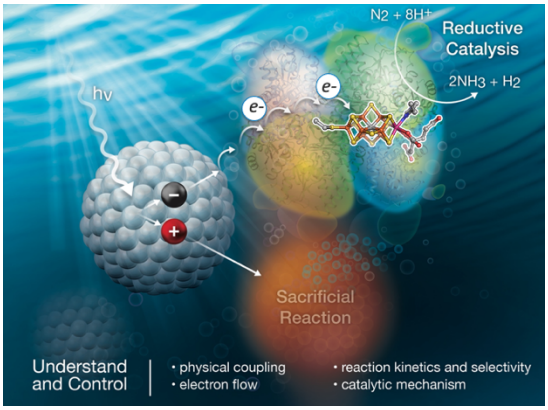
Overall research goals:

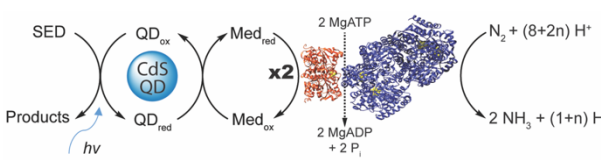
The overall goal of this core program is to understand the physical properties and mechanisms of the photochemical reduction of dinitrogen (N_2) to ammonia (NH_3) in nanocrystal-nitrogenase complexes.



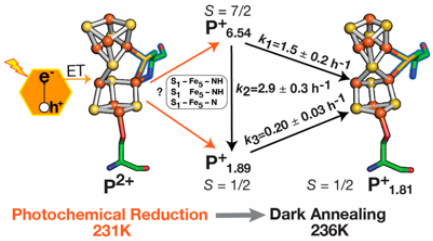
Activation and reduction of N_2 is one of the most energy demanding and difficult chemical reactions. This core program uses nanocrystalline materials to photochemically activate electron transfer directly to the catalytic MoFe protein component of nitrogenase, circumventing the requirement for ATP-dependent electron transfer by the nitrogenase Fe protein. Tunability of nanocrystal light-harvesting combined with exquisite flux dependency of MoFe protein catalysis is enabling new insights and understanding of requirements for N_2 activation and reduction by MoFe protein. The aims of the program include understanding the physical interactions between nanocrystals and MoFe protein that are influential to electron transfer, using light to control electron flux and formation of chemical intermediates to gain insights into unresolved aspects of transport and the catalytic mechanism of N_2 reduction. Overall, the core program goal is to develop a functional photochemical N_2 reduction framework to advance understanding of nanocrystals as electron transfer components of photocatalytic systems and to elucidate mechanistic details of nitrogenase catalyzed N_2 reduction.

Significant achievements: [2022]:

- The kinetics of electron transfer from CdS nanorods to nitrogenase MoFe protein were determined using ultrafast transient absorption spectroscopy. Kinetic modeling determined that two equally important factors limit electron transfer efficiency; (1) the extent of binding, and (2) the competitiveness of electron transfer with electron relaxation pathways in the nanocrystals. Sensitivity of electron transfer kinetics to nanorod structure is most likely due to changes in the electron potential.
- Light-driven N_2-to-NH_3 reduction was shown for CdS quantum dot (QD) and nitrogenase biohybrid, where electrons from the CdS QD were delivered by an electron transfer mediator through the Fe protein to MoFe protein. A quantum yield for conversion of absorbed photons to ammonia of 16% was achieved. A rationale for choosing an electron transfer mediator was established (Figure above).



- Electron transfer in nitrogenase is mediated by the P cluster, which cycles through transitions in structural, redox and magnetic properties during transfer. Low-temperature, light controlled electron transfer in nanocrystal:nitrogenase biohybrids was used to monitor spin-state populations of the P cluster during reduction by EPR, and to formulate a model of transitions in P cluster magnetic states during electron transfer (Figure on the right).



- A microscale thermophoresis was developed to measure the affinity and stoichiometry of nanocrystal binding to MoFe protein. Thus far, dissociation constants (K_d) values range from 100-900 nM indicating strong binding, with Hill coefficients of ~n=2-4. Nanocrystals that vary in physical properties are being tested for effects on K_d and Hill coefficients to understand properties that control binding, and the relationship of binding to electron transfer and reactivity of nitrogenase.

Science objectives for 2023-2024:

- **Establish the relationship between nanocrystal:nitrogenase complexation and reactivity.** Nanocrystals that vary in composition, structure, and size, and MoFe proteins with altered surface functional groups will be used to systematically understand the molecular interactions that control photoexcited electron transfer and efficiency.
- **Understand nanocrystal properties that control electron transfer in nanocrystal:nitrogenase complexes**. Identify the properties controlling the rate constant, k_{ET}, and the quantum efficiency, QEET, of electron transfer with the aim to understand what properties are most critical and why and to further understand the relationship of k_{ET} and QEET to MoFe protein reactivity.
- **Understand role of electron flux in nitrogenase reactivity.** Light and temperature control of electron flux to nitrogenase with trapping of catalytic intermediates will be combined with ^{1}H-NMR quantification of $^{15}NH_3$ production to develop a kinetic framework within the Lowe-Thorneley model of N_2 reduction to understand the flux influence on the catalytic mechanism.

My project addresses BES cross-cutting priority areas by: Understanding the material properties and reaction mechanisms of molecular systems that control the conversion of solar energy into chemical energy.

My scientific area(s) of expertise is/are: Mechanistic studies of electron transfer and catalysis in enzymes that catalyze reduction-oxidation reactions.

The ideal collaborator for my project would have expertise in: Photoelectrochemistry.

Publications supported by this project [2018-2022]:

1. J.K. Utterback, J.L. Ruzicka, H. Hamby, J.D. Eaves, G. Dukovic. "Temperature-Dependent Transient Absorption Spectroscopy Elucidates Trapped-Hole Dynamics in CdS and CdSe Nanorods." J. Phys. Chem. Letts. **10**, 2782 (2019). DOI:10.1021/acs.jpclett.9b00764.
2. Brown, K.A., King, P.W. "Coupling biology to synthetic nanomaterials for semi-artificial photosynthesis." Photosyn. Res. **143**, (2), 193 (2020). doi.org/10.1007/s11120-019-00670-5.
3. J.K. Utterback, J.L. Ruzicka, H.R. Keller, L.M. Pellows, G. Dukovic. "Electron Transfer from Semiconductor Nanocrystals to Redox Enzymes." Ann. Rev. Phys. Chem. **71**, 335 (2020). doi:10.1146/annurev-physchem-050317-014232.
4. B. Chica, H. Kallas, J.L. Ruzicka, D.W. Mulder, K.A. Brown, J.W. Peters, L.C. Seefeldt, G. Dukovic, P.W. King. "Defining the Intermediates Nitrogenase MoFe Protein During N2 Reduction Under Photochemical Electron Delivery by CdS Quantum Dots." J. Am. Chem. Soc. **142**, (33), 14324 (2020). doi:10.1021/jacs.0c06343.
5. K.A. Brown, J.L. Ruzicka, H. Kallas, B. Chica, D.W. Mulder, J.W. Peters, L.C. Seefeldt, G. Dukovic, P.W. King. "Excitation-Rate Determines Product Stoichiometry in Photochemical Ammonia Production by CdS Quantum Dot-Nitrogenase MoFe Protein Complexes." ACS Catal. **10** (19), 11147 (2020). doi.org/10.1021/acscatal.0c02933.
6. J.L. Ruzicka, L.M. Pellows, H. Kallas, K.E. Shulenberger, O.A. Zadvornyy, B. Chica, K.A. Brown, J.W. Peters, P.W. King, L.C. Seefeldt, and G. Dukovic. "The Kinetics of Electron Transfer from CdS Nanorods to the MoFe Protein of Nitrogenase." J. Phys. Chem. C **126**, 8425 (2022). doi:10.1021/acs.jpcc.2c02528.
7. B. Chica, J. Ruzicka, L.M. Pellows, H. Kallas, E. Kisgeropoulos, G.E. Vansuch, D.W. Mulder, K.A. Brown, D. Svedruzic, J.W. Peters, G. Dukovic, L.C. Seefeldt, P.W. King, "Dissecting Electronic-Structural Transitions in the Nitrogenase MoFe Protein P-Cluster during Reduction." J. Am. Chem. Soc. **144**, 5708 (2022). doi:10.1021/jacs.1c13311.
8. A. Badalyan, Z.-Y. Yang, M. Hu, T.L. Liu, L.C. Seefeldt. "Tailoring electron transfer pathway for photocatalytic N_2-to-NH_3 reduction in a CdS quantum dots-nitrogenase system." Sustain. Energy Fuels **6**, 2256 (2022). doi:10.1039/D2SE00148A.

Production of Ethylene from the Common Metabolite, 2-Oxoglutarate, by the Ethylene-Forming Enzyme (EFE)

Carsten Krebs, Principal Investigator
J. Martin Bollinger, Jr.; Amie K. Boal, Co-PI(s)
Evan Burke, PhD Candidate; Rachelle Copeland, PhD Graduate; Shengbin Zhou and Katherine M. Davis, Postdoctoral Research Associates
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Overall research goals:

Ethylene-forming enzyme (EFE) from *Pseudomonas syringae* is an ambifunctional iron(II)- and 2-(oxo)glutarate-dependent (Fe/2OG) oxygenase. In its major reaction, EFE fragments 2OG to ethylene and three equivalents of CO_2, a four-electron oxidation that differs radically from the outcomes of other members of this large enzyme family. Its secondary reaction conforms to the usual *modus operandi*: oxidative decarboxylation of 2OG to succinate is coupled to hydroxylation of C5 of L-arginine, which is a required activator of the major pathway. We aim to define the structural and mechanistic bases for the complex reactivity of EFE. In particular, we seek to identify the branch point between the two pathways and elucidate the mechanism of the unusual multiple-electron and multiple-bond fragmentation reaction leading to ethylene.

Significant achievements: (2016-2022)

We have shown that, consistent with precedent, L-Arg hydroxylation proceeds via an iron(IV)-oxo (ferryl) intermediate. Owing to the large kinetic isotope effect (^{2}H-KIE) on hydrogen-atom transfer ($k_H/k_D \sim 20$), the presence of deuterium at C5 of L-Arg slows decay of the ferryl intermediate, allowing it to accumulate to > 11% of the total iron. The observed insensitivities of (i) the relative yields of the two products and (ii) more than half the reaction flux of a single turnover (as monitored by stopped-flow absorption spectrophotometry) to C5 deuteration imply that the detected ferryl complex is not on the major, ethylene-producing pathway. Thus, the branch point must be earlier in the reaction sequence.

We solved x-ray crystal structure of the anoxically prepared EFE•Fe(II)•2OG•L-Arg reactant complex and the EFE•Fe(II)•NHA•L-Arg complex, wherein NHA (*N*-hydroxysuccinamate) is a mimic of the persuccinate intermediate. The persuccinate complex is a good candidate for the branchpoint of the ethylene-generating and arginine-oxidizing pathways. Thus, we consider the geometric and electronic structure of this complex as potentially insightful for rationalizing the reactivity.

The Asp191→Glu substitution, amounting to simple insertion of a methylene unit into an iron-coordinating amino acid, markedly shifts the partition ratio toward the hydroxylation pathway and permits greater ferryl accumulation. The nearly complete abolition of ethylene production by this nearly conservative substitution, as well as its much less pronounced effect on ferryl-mediated L-Arg oxidation, imply that the unusual primary reaction has more rigid stereoelectronic requirements than the canonical pathway leading to formation of the ferryl complex and L-Arg oxidation.

We determined the ethylene fragmentation to be stepwise radical in nature by synthesis of a chiral 2-oxoglutarate substrate 3R,4S-[$^2H_2$]-2OG and observing, by Fourier-transform infrared (FTIR) spectroscopy, that the reaction with EFE produces a mixture of *cis* and *trans*-[$^2H_2$]-ethylene. This proves the existence of intermediate states with free rotation about the C3-C4 bond, of which we favor a propionate-3-yl radical.

Furthermore, reactions using isotopically labelled 2OG (1-$^{13}C_1$-2OG) and oxygen ($^{18}O_2$) showed that an oxygen atom from O_2 is appended to C1 during the reaction. Unlike all other Fe/2OG oxygenases, C1 of 2OG is not decarboxylated but forms a carbonate species with one oxygen atom from O_2. This

observation proved that bifurcation from the consensus Fe/2OG mechanism occurs very early on: the dioxygen unit of the initial superoxo-iron(III) adduct, which typically attacks C2 of 2OG leading to oxidative decarboxylation and generation of the Fe(II)-peroxysuccinate, can instead formally insert into the C1-C2 bond leading to a Fe(II)-succinylperoxycarbonate complex. This species can create a succinyl radical by O-O bond homolysis, which provides a rationale for how EFE accesses a radical manifold leading to the aforementioned propionate-3-yl radical.

We also discovered partially fragmented ω-hydroxyacids products from the EFE reaction, implicating a second branchpoint wherein ethylene formation by polar concerted Grob fragmentation competes with elimination of carbon dioxide to form 3-hydroxypropionate (3HP). In wild-type EFE, a small amount 3HP is observed along with the other products. This is greatly exacerbated by a variety of C4-substitutions: reactions containing any of a variety of C4-substituted 2OG analogs (4,4-F_2-2OG, (4R)-F-2OG, (4R)-Methyl-2OG, (4L)-hydroxy-2OG, 4-hydroxy-4-methyl-2OG) showed formation of the corresponding ω-hydroxyacids instead of alkene products. The results are most compatible with our hypothesis of a polar Grob fragmentation which has strict positioning requirements of antiperiplanarity between fragmenting groups.

Enzyme substitutions rationally designed from this mechanistic hypothesis can indeed drive reaction flux to either outcome. Firstly, enzyme variants were made with larger hydrophobic residues capable of steric interactions with the presumptive Fe(II)-2-carboxyethylcarbonate intermediate. As intended the A198L substitution markedly impaired the production of ethylene in favor of 3-hydroxypropionate from the native 2OG co-substrate. In a complementary approach, we then sought smaller amino acid substitutions to minimize the steric interactions that we believe caused ω-hydroxyacids formation rather than olefination from 2OG analogs. Two of these substitutions (L206A, L206V) yielded the novel alkene products propylene and isobutylene. These results simultaneously forward our mechanistic hypotheses and demonstrate the potential of EFE as a platform enzyme for renewable synthesis of valuable short-chain chemical feedstocks.

Science objectives for 2022-2023:

Verify that the EFE product complex is a ferrous bicarbonate species using freeze-quench Mössbauer spectroscopy. Crystallography of stable analogs of reaction mimics and computational studies to provide insight into how the unusual reaction with oxygen is promoted by electronic and structural features of the EFE active site. Kinetic studies using arginine analogs to better understand how this substrate serves as an activator for the ethylene forming pathway. Continued testing of new EFE variants that promote greater 3HP formation by disrupting Grob fragmentation, or tolerate 2OG substitutions leading to new alkene products.

My project addresses BES cross-cutting priority areas by:

Our approach involves a combination of biochemical, analytical, kinetic, spectroscopic, crystallographic, and computational methods to obtain a comprehensive picture of the molecular mechanism of EFE.

My scientific area(s) of expertise is/are: Enzyme reaction mechanisms, transient enzyme kinetics, biophysics, transition-metal/dioxygen chemistry.

The ideal collaborator for my project would have expertise in: Metabolic engineering, enzyme evolution.

Publications supported by this project [Click to Enter Years of Current Grant/FWP, e.g. 2012-2014]:

1. Copeland, R. A.; Davis, K. M.; Shoda, T. K. C.; Blaesi, E. J.; Boal, A. K.; Krebs, C.; Bollinger, J. M. An Iron(IV)–Oxo Intermediate Initiating l-Arginine Oxidation but Not Ethylene Production by the 2-Oxoglutarate-Dependent Oxygenase, Ethylene-Forming Enzyme. *J. Am. Chem. Soc.* **2021**, *143* (5), 2293–2303
2. Copeland, R. A.; Zhou, S.; Schaperdoth, I.; Shoda, T. K. C.; Bollinger, J. M.; Krebs, C. Hybrid Radical-Polar Pathway for Excision of Ethylene from 2-Oxoglutarate by an Iron Oxygenase. *Science (80).* **2021**, *373* (6562), 1489–1493

Transmethylation reactions during methylotrophic methanogenesis in methanogenic Archaea

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Overall research goals:

Methanogenesis from methylated amines in *Methanosarcina* spp. requires the prerequisite formation of methyl-CoM, the direct methane precursor. During our DOE sponsored project, we have shown that enzyme systems have evolved for the methylation of CoM with trimethylamine (TMA), dimethylamine (DMA), and monomethylamine (MMA). Each methyltransferase preferentially binds TMA, DMA, or MMA as substrate to methylate a cognate corrinoid binding protein. These distinct yet homologous corrinoid proteins are then demethylated by a single methyltransferase to methylate CoM. The three types of methylamine methyltransferases genes are not homologous, yet each contains an in-frame amber codon. We have shown this amber codon stands for pyrrolysine, an atypical genetically encoded amino acid, as well as how this residue is biosynthesized and genetically encoded. Pyrrolysine is essential for efficient catalysis by the TMA methyltransferase, and homologs of the methyltransferase lacking the residue are instead methyltransferases for larger methylamines, such as glycine betaine. The active sites of glycine betaine and the TMA methyltransferases appear to orient the methyl groups in nearly identical space in their homologous TIM barrel for transfer as methyl cations to the homologous cognate corrinoid protein.

In order for the corrinoid protein of the TMA methyltransferase to accept methyl cations, it must be in the highly reducing Co(I) state and return to this state after donating the methyl group to the CoM methylase. Adventitious oxidation inactivates methyl transfer, which is rescued by the ATP dependent reductive activation of the Co(II)-corrinoid protein by an FeS protein, RamA. RamA will be the focus of our work in the upcoming project period. Our structural models of the corrinoid protein-RamA complex have us to two heuristic hypotheses of how the FeS protein RamA couples ATP hydrolysis to the low potential reduction of the inactive Co(II)-TMA corrinoid protein, MttC. We have devised experiments that test both hypotheses while we characterize basic traits of the protein's activity. We have established collaborations to solve the structure of RamA in complex with MttC; and measure the < -700 mV potential of the Co(II)/Co(I) states . The ASKHA ATP binding domain of RamA is most similar to kinases, and we will determine if RamA carries out ATP:P_i exchange reactions, or phosphorylates itself or MttC during the catalytic cycle. Finally, we have evidence that an uncharacterized and soluble H_2 dependent system exists in *M. barkeri* which provides reducing power to RamA. Our preliminary data indicates RamA exists in complex with unknown proteins in cell extracts, and using flexible approaches we will identify proteins and cofactors required for RamA reduction with H_2. If this effort is successful, it will lead to a defined ATP dependent biological system for reducing a very low potential metal cofactor with H_2.

Significant achievements: 7/31/22-8/1/25:

As the current grant period has just begun we summarize the achievements of the last grant period. During that time we:

- established that monovalent cations significantly increased RamA activity.
- established that RamA, unlike some bacterial homologs, has typical M-M kinetics and a much lower Km for ATP than bacterial homologs.
- established that replacement of a serine residue in RamA that ligated the cobalt in RaCo, a bacterial homolog of RamA, did not significantly change RamA kinetics arguing for a non-essential role for this residue in the overall mechanism of reductive corrinoid activase.

- established that the amount of ATP hydrolyzed by RamA to Co(II) corrinoid protein reduced was 1:1.
- established that RamA functions as a monomer and forms a complex with one monomer corrinoid protein
- established that the ATPase activity of RamA is gated by the reduction state of the corrinoid protein and the presence of the Co(II)corrinoid protein substrate.
- Established that the average potential of the two Fe_4S_4 clusters of RamA is -390 mV.

Science objectives for 2022-2023:

- We have begun working to identify the protein(s) which mediates electron transfer to RamA from hydrogen. This is the last unknown component of the methylamine methyltransferase systems and if successful it will resolve a novel system by which ATP fuels low potential reductions with a higher potential reductant.
- We will test if RamA carries out a Pi:ATP exchange reaction under under various conditions.
- We will synthesize low potential dyes to measure the <-700 mV potential of the corrinoid protein Co(II)/Co(I) protein.

My project addresses BES cross-cutting priority areas by:
examining a novel ATP dependent system for achieving low potential reductions with higher potential electron donors.

My scientific area(s) of expertise is/are: microbiology and molecular biology of anaerobic bacteria and archaea; particularly the biochemistry of their enzymes related to one carbon metabolism.

The ideal collaborator for my project would have expertise in: structural and spectroscopic characterization of iron-sulfur and/or corrinoid proteins..

Publications supported by this project 2022-2025:

1. Li J, Kang PT, Jiang, R, Lee JY, Soares, JA, Krzycki, JA, Chan, MK. 2022. Insights into pyrrolysine function from structures of a trimethylamine methyltransferase and its corrinoid protein complex. Comm. Biol. In press.
2. Jiang, R., and J.A. Krzycki. 2023. Effect of pyrrolysine substitutions on the activity of MttB, the trimethylamine methyltransferase. J. Biol. Chem. (in preparation).
3. Huening KA, Jiang R, Krzycki JA. 2020. Kinetic and substrate complex characterization of RamA, a corrinoid protein reductive activase from Methanosarcina barkeri. FEMS Microbiol Lett. 2020. 367(17):fnaa128. doi: 10.1093/femsle/fnaa128. PMID: 32840570.

Bioinorganic Chemistry of Nitrification: Structure and Function of Ammonia Monooxygenase

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Overall research goals:
Nitrification is a primary metabolism whereby microorganisms derive total energy for life from the oxidation of nitrogen- rather than carbon-based fuels. Despite the ubiquity of nitrifying organisms across practically all ecosystems, major gaps persist in understanding the fundamental biochemical steps comprising nitrification. Case in point, molecular level understanding of the very first step of nitrification remains elusive. This step, in both ammonia oxidizing bacteria (AOB) and archaea (AOA), is the conversion of ammonia (NH_3) to hydroxylamine (NH_2OH) by the multimeric integral membrane copper enzyme ammonia monooxygenase (AMO). No AMO has ever been purified in an active form. Some inferences may be drawn from homology, as AMO is a member of the copper membrane monooxygenase family, whose constituent enzyme particulate methane monooxygenase (pMMO) has been studied extensively, and for which numerous structures and copious reactivity data are available.

The goal of this project is to fill this major and persistent gap in understanding of the nitrogen cycle by solving the structure and studying the reactivity of the AMO family. The immediate goal is to obtain a purified and, ideally, active AMO. Three approaches are being pursued. One is to use *Mycobacterium smegmatis* as a recombinant expression host for AMO. A second approach is to reconstitute AMO from individually produced subunits. A third, recent approach is to increase yields of AOB biomass through manipulation of bacterial quorum sensing.

Significant achievements: 2020–2022:

- We have expressed all three subunits of *Nitrosomonas europaea* AMO (amoA, amoB, amoC) as fusion proteins to maltose binding protein (MBP). Routine expression quantities are 1 mg/L. For convenience, we refer to these proteins as 3MT-X where X = the amo subunit. To date, recombinant expression of the integral membrane subunits (A, C) of a copper membrane monooxygenase have never been reported.

- We have inserted 3MT-A and 3MT-C proteins into lipid bilayer nanodiscs (NDs), enabling us to keep them in buffered solution.

- We have cleaved and purified amoC from MBP both in NDs as well as in detergent.

- We have obtained cryo-electron microscopy (EM) data for ND-solubilized 3MT-C.

- We have obtained electron paramagnetic resonance (EPR) data for detergent-solubilized 3MT-A and 3MT-C.

- We have observed association of 3MT-A, B, and C, indicating that *in vitro* assembly of the AMO complex should occur.

- We have constructed a vector for expression of FLAG-tagged *N. europaea* AMO in *M. smegmatis*.

Science objectives for 2022-2023:

- Assemble AMO from constituent subunits into a ND-solubilized holoprotein complex.
- Obtain cryo-EM structures for all three subunits in MBP-tagged and isolated forms to determine whether they retain structural homology to pMMO subunits when not assembled into the complete protein complex.
- Purify *N. europaea* AMO from *M. smegmatis*.
- Obtain serial cryogenic focused ion beam scanning EM data for *N. europaea* to determine whether AMO forms arrays in intracellular membranes akin to recent findings for pMMO in methanotrophs.
- Identify growth additives that influence AOB quorum sensing towards improving biomass yields for native protein isolation and purification.

My project addresses BES cross-cutting priority areas by:
First and foremost, the project seeks to fill major gaps in understanding the biogeochemical nitrogen cycle that were identified as priority research areas in the 2016 DOE Nitrogen Activation Workshop. More broadly, this project advances other priorities, including the development and application of cryoEM and its use to study hierarchical systems—concerning the latter, we are sequentially studying AMO structure from the individual subunit level to assembled complexes and then to array structures in cell membranes. Additionally, understanding of selective ammonia oxidation advances understanding toward use of ammonia as a fuel and/or storage medium for hydrogen.

My scientific area(s) of expertise is/are: Inorganic spectroscopy, biochemistry, inorganic/organometallic synthesis, electronic structure calculations.

The ideal collaborator for my project would have expertise in: Microbiology, ideally specialized insights into bacterial quorum sensing.

Publications supported by this project 2020–2023:

1. None. An article that combines a review of AMO research with structural homology and AlphaFold predictions is in preparation.

Understanding Selectivity in Terpene Synthases Unique Mechanisms to Generate Precursors for Biocrude and Specialty Chemicals

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Overall Research Goals:

Terpenoid oils and resins accumulated in plants (and sometimes microbes) are characterized by a high volumetric energy density and high degree of reduction, and are thus viable "biocrude" feedstocks for fuels in the diesel and kerosene range. Furthermore, many specialty chemicals are also based on terpenoid backbones, including polymers (e.g., rubber), solvents (e.g., limonene), and diverse small molecules (e.g., menthol). Terpenoids thus have the potential to serve as chemical feedstocks in a non-food bioeconomy based on carbon and energy capture, allocation, conversion and storage by plants, which is directly in line with the research mission of the DOE-BES program. This proposal aims to unravel the mechanistic basis for selectivity in the sophisticated enzymes, termed monoterpene synthases, that catalyze the formation of cyclic hydrocarbons as the first committed step in monoterpene biosynthesis. Such knowledge will allow us to infer the mechanistic underpinnings of how plants produce highly complex, reduced chemical scaffolds.

Significant Achievements (2020-2022):

- **Identification and characterization of novel terpene synthases**. Terpene synthases convert a prenyl diphosphate of a specific chain length to the first pathway-specific (often cyclic) intermediate in the biosynthesis of a specific class of terpenoids. These enzymes are critical determinants of terpenoid chemical diversity, which is an important issue for the development of biocrude and sustainable specialty chemicals. We performed in silico analyses of recently sequenced genomes to identify candidate genes with potentially novel functions (focusing on monoterpene (C10) synthases or MTSs). Candidates were then expressed heterologous in *E. coli*, the corresponding recombinant proteins purified, and apparently homogeneous fractions analyzed in *in vitro* assays. We characterized terpene synthases involved in the formation of γ-terpinene (monocyclic hydrocarbon), α-terpineol (monocyclic alcohol), 1,8-cineole (bycyclic ether), (-)-bornyl diphosphate (bicyclic pyrophosphate), and (-)-β-pinene (bicyclic hydrocarbon).
- **Identification of determinants for the formation of monocyclic and bicyclic products in MTSs**. Using two well-characterized MTSs as models, (−)-limonene synthase (LMNS) and (+)-bornyl diphosphate synthase (BPPS), we implemented an iterative approach that involves comparative atomistic simulations and experimental testing of wild-type enzymes and thirty-six variants to identify the mechanistic underpinnings of selectivity. Free energy simulations indicate that a common reaction intermediate, the α-terpinyl cation (ATC), preferentially adopts one of two different conformations in LMNS and BPPS, thus leading to the formation of monocyclic monoterpenes in the former and bicyclic products in the latter. An assessment of the ATC binding free energy in LMNS, BPPS, and variants revealed that nonbonded interactions with active site residues explain the propensity of the ATC to assume a favored conformation that is consistent with the experimentally determined reaction outcome. The free energy of the ATC in different environments (active sites of LMNS, BPPS, and variants) correlates strongly with the ratio of monocyclic to bicyclic products in model MTSs.

Science Objectives for 2020-2021:

- **Test the hypothesis that the stereochemical outcome of reactions catalyzed by MTSs is determined by substrate binding.** We will be using a combination of experimental (analyzing functions of selected mutants) and computational (performing MD simulations) approaches to assess if the confirmation of the bound substrate is responsible for determining which stereoisomer is produced.
- **Test if selective stabilization of carbocations downstream of the ATC explains formation of bicyclic products by MTSs.** Once again, we will be employing using a combination of experimental (analyzing functions of selected mutants) and computational (performing MD simulations) approaches to compare the free energies of various carbocation intermediates downstream of the ATC (terpinene-4-yl, pinyl, camphyl, and thujyl cations) in MTSs that generate different products.
- *Taken together, these efforts will result in novel insights into how product outcomes are determined in MTSs, and we are beginning to develop models to predict product specificity.*

To take my project to the next level, my ideal collaborator would have expertise in:

- We are currently working with Simone Raugei and Hoshin Kim from PNNL, which has enabled us to make progress in an area that requires unique computational expertise and resources. They continue to be our ideal collaborators.

Publications supported by this project (2020-2022):

(Note: Support by DOE-BES was acknowledged in all publications listed below)

1. Kim H., Srividya N., Lange I., Huchala E.W., Ginovska B., Lange B.M., Raugei S. (2022) Determinants of selectivity for the formation of monocyclic and bicyclic products in monoterpene synthases. ***ACS Catal.*** 12, 7453-7469. https://pubs.acs.org/doi/full/10.1021/acscatal.2c01836
2. Srividya N., Lange I., Richter J.K., Wüst M., Lange B.M. (2022) Selectivity of enzymes involved in the formation of opposite enantiomeric series of p-menthane monoterpenoids in peppermint and Japanese catnip. ***Plant Sci.*** 314, 111119. https://www.sciencedirect.com/science/article/pii/S0168945221003150
3. Vining K.J., Pandelova I., Lange I., Parrish A.N., Lefors A., Kronmiller B., Liachko I., Kronenberg Z., Srividya N., Velasco J.A., Lange B.M. (2020) Chromosome-level *Mentha longifolia* L. genome assembly reveals gene organization underlying disease resistance and essential oil traits. ***G3*** 12, jkac112. https://academic.oup.com/g3journal/article/12/8/jkac112/6584825
4. Liu L., Yin M., Lin G., Wang Q., Zhou P., Dai S., Sang M., Lange B.M., Liu C., Wu Q. (2021) Integrating RNA-seq with functional expression to analyze the regulation and characterization of genes involved in monoterpenoid biosynthesis in Nepeta tenuifolia Briq. ***Plant Physiol. Biochem.*** 167, 31-41. https://pubmed.ncbi.nlm.nih.gov/34544008/
5. Chen Z., Vining K.J., Qi X., Yu X., Zheng Y., Liu Z., Fang H., Li L., Bai,Y., Liang C., Li W. , Lange B.M. (2021) Genome-wide analysis of terpene synthase gene family in Mentha longifolia and catalytic activity analysis of a single terpene synthase. ***Genes*** 12, 518. https://www.mdpi.com/2073-4425/12/4/518
6. Srividya N., Lange I., Lange B.M. (2020) Determinants of enantiospecificity in limonene synthases. ***Biochemistry*** 59, 1661-1664. doi: https://doi.org/10.1021/acs.biochem.0c00206

Understanding Nitrogenase Maturation and Activity in Methanogens

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Overall research goals:

The overall goal of the renewed research project is to determine the factors that control the maturation and activity of nitrogenase in methanogens. Nitrogenase is a metalloenzyme system found only in bacteria and archaea where it functions in biological nitrogen fixation (diazotrophy) by reducing dinitrogen (N_2) to ammonia (NH_3). All diazotrophs possess molybdenum (Mo)-nitrogenase that contains iron-sulfur (Fe-S) clusters, including the Mo-containing active site cofactor (FeMo-co). Some diazotrophs also contain alternative nitrogenases where vanadium (V) or Fe replace Mo in FeMo-co. Nitrogenase is important for bioenergy research since it directly catalyzes the production of biofuels (e.g., H_2), it serves as a model to understand complex metallocofactor biogenesis, and functional production in plants would alleviate the burden to synthesize fertilizers from fossil fuels. Previous results indicate that the assembly, activity, and regulation of nitrogenase in methanogens is distinct from bacteria, which may provide alternative avenues to optimize and/or develop metalloenzyme-based energy production strategies. The long-term goals of this research project are to use genetically tractable *Methanosarcina acetivorans*, which contains Mo-, V-, and Fe-nitrogenases, as a model to 1) determine the regulation and catalytic abilities of methanogen nitrogenases, 2) determine the factors involved in cofactor assembly and maturation in methanogen nitrogenases, and 3) determine how electron transfer to nitrogenase is integrated with methanogenesis.

Significant achievements (2018-2022)

- Related to goal 1: We used genetic and biochemical approaches to determine the functional importance and properties of nitrogenases in *M. acetivorans*.
  - We determined that only Mo-nitrogenase (Nif) is produced in *M. acetivorans* grown in Mo-replete medium, whereas all three nitrogenases are simultaneously produced when grown in Mo-deplete medium (a result unique to *M. acetivorans*).
  - We developed a CRISPRi repression system to control gene expression in *M. acetivorans* and used the system to demonstrate that expression of Mo-nitrogenase is required for diazotrophy under all conditions, including Mo-deplete.
  - V-nitrogenase and/or Fe-nitrogenase were shown to be required for Mo-independent diazotrophy (i.e., Mo-nitrogenase is not functional in the absence of Mo).
  - We developed strains and protocols to purify *M. acetivorans* Mo-nitrogenase.
  - We demonstrated that purified NifDK and recombinant NifH form active Mo-nitrogenase *in vitro* and generated the first EPR spectra for methanogen NifDK.
- Related to goal 2: We used genetic and biochemical approaches to ascertain the role of components of the ISC and SUF Fe-S cluster biogenesis systems and NifB to the maturation of nitrogenases in *M. acetivorans*.
  - We determined that *M. acetivorans* contains a functional minimal ISC system and that loss of the system impacts cysteine-dependent diazotrophy by *M. acetivorans*.
  - We determined that the minimal SUF system that is universally conserved in methanogens is not essential and not required for diazotrophy by *M. acetivorans*.

  - The gene encoding NifB, the radical SAM enzyme required for maturation of all three nitrogenases in bacteria, was shown to be essential to the viability of *M. acetivorans*, indicating nitrogenase maturation is deeply integrated into methanogen physiology.
- Related to goal 2: We used genetic, physiological, and biochemical approaches to assess how diazotrophy effects electron flux in *M. acetivorans*.
  - We demonstrated that Vht hydrogenase is required for hydrogen cycling resulting in a branched electron transport system during nitrogen fixation by non-hydrogenotrophic *M. acetivorans*.
  - Affinity purification of NifDK was used to identify potential electron donor partners to nitrogenase.

Science priorities for the next year (2022-2023)

- Use genetics and proteomics to determine why Mo-nitrogenase is required for nitrogen fixation in the absence of Mo.
- Determine the biophysical/catalytic properties of purified *M. acetivorans* Mo-nitrogenase.
- Determine what function NifB plays outside of nitrogenase maturation (i.e., why is NifB essential to *M. acetivorans*?).
- Use affinity purification of nitrogenase and crosslinking proteomics to identify:
  - Nitrogenase Fe-S cluster biogenesis and maturation proteins.
  - Nitrogenase redox partner proteins.

My project addresses BES cross-cutting priority areas by: This project aims to provide new fundamental knowledge on how the simple and complex Fe-S cofactors in nitrogenase are synthesized in methanogens, the catalytic properties of methanogen nitrogenases, and how electron flux is directed to nitrogenase within the constraints of methanogenesis. This knowledge could be used in the long term to design new chemical processes using methanogens and/or nitrogenase to produce, convert, and/or store energy resources, which is a core mission of BES.

My major scientific area(s) of expertise is/are: Methanogen genetics, biochemistry, physiology, Fe-S cluster proteins, redox proteins.

The ideal collaborator for my project would have expertise in: Proteomics**,** Bioenergetics, Structural Biology

Publications supported by this project:

1. A.E. Dhamad, D.J. Lessner, "A CRISPRi-dCas9 system for archaea and its use to examine gene function during nitrogen fixation by *Methanosarcina acetivorans*." ***Appl. Environ. Microb.*** **86**, 21 (2020) DOI: https://doi.org/10.1128/AEM.01402-20
2. T. M. Deere, D. Prakash, F. H. Lessner, E. C. Duin, D. J. Lessner. "*Methanosarcina acetivorans* contains a functional ISC system for iron-sulfur cluster biogenesis." ***BMC Microbiology*** **20**, 323 (2020). DOI: 10.1186/s12866-020-02014-z.
3. J.M. Hoerr, A.E. Dhamad, T.M. Deere, M. Chanderban, D.J. Lessner, "Vht hydrogenase is required for hydrogen cycling during nitrogen fixation by the non-hydrogenotrophic methanogen *Methanosarcina acetivorans*." ***bioRxiv*** in revision, DOI: https://doi.org/10.1101/2021.10.12.464174
4. M. Chanderban, C.A. Hill, A.E. Dhamad, D.J. Lessner, "*Methanosarcina acetivorans* simultaneously produces molybdenum, vanadium, and iron-only nitrogenases in response to fixed nitrogen and molybdenum depletion" ***bioRxiv*** in revision, DOI: https://doi.org/10.1101/2021.06.03.447018

Characterizing Plant-Specific Features of Mitochondrial Respiratory Complexes

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Overall research goals:

Detailed structural and functional knowledge of plants' mitochondrial electron transport is crucial to understand the bioenergetic budget of vascular plants, yet it is an area that remains largely unexplored. The overall aim of this research is to characterize the molecular mechanisms and atomic structures of the energy-converting complexes and supercomplexes of plant mitochondria, with an emphasis on aspects that are unique to plants. We will use biophysical and biochemical approaches to:
Aim 1: Examine plant-specific functions of CI and its assembly intermediate CI*.
Aim 2: Examine conserved and plant-specific features of respiratory supercomplexes.
Aim 3: Determine the structure and molecular mechanisms of plant CII.

Significant achievements: 2021-2022:

A) For specific aim 1 we were able to show that using the assembly factor GLDH to purify the CI assembly intermediate CI* from plant mitochondria is likely to succeed. We generated histidine and GFP tagged GLDH constructs (His-GFP-GLDH and GLDH-GFP-His). Starting with His-GFP-GLDH we then express the construct in *E. coli* and purified it using nickel affinity chromatography and size exclusion chromatography. On a small scale we then mixed the purified fusion construct with plant mitochondrial membranes that had been solubilized with different detergents. After incubating the His-GFP-GLDH with the detergent solubilized membranes we added HisTrap nickel resin to specifically capture the GLDH construct and any potential binding partners and incubated with the resin while tumbling for ~30 minutes, followed by three rounds resin washes and further tumbling. The protein was then eluted from the resin using imidazole buffer. Samples of the starting material, wash supernatants and elution fractions were then run on a Blue Native (BN)-PAGE gel and subjected to a CI in-gel activity assay. This assay stains the gel specifically where NADH dehydrogenase activity is localized and for our plant mitochondrial samples generally stains three bands corresponding to CI*, CI and SC I+III_2. The results of the experiment were that in the case of the DDM detergent a band corresponding to the size of the CI* was observed in the elution fraction. However, this band was weak, and bands of the same size could also be seen in the wash fractions and in the unbound fraction indicating incomplete and low affinity binding. No significant isolation of CI* was observed for the other detergents tested. Although, only weak binding to the GLDH construct was demonstrated in this experiment it provides evidence that the general approach of using GLDH to purify CI* is a viable strategy for the isolation of this sub-assembly. We are currently preparing our second GLDH construct for testing.

B) For specific aim 2 we have optimized an isolation protocol for SC I+III_2 from *V. radiata* mitochondria using digitonin extraction and SC stabilization in amphipathic polymers (amphipols) followed by sucrose gradient ultracentrifugation and fractionation. Using this protocol, we have optimized cryogenic electron microscopy (cryoEM) grid preparation and collected a dataset that has resulted in the reconstruction of the plant respiratory SC I+III_2 to ~3.2 Å resolution. These data provide many insights, showing a plant specific subunit on the intermembrane space side of the inner mitochondrial membrane that is not conserved in other known supercomplex structures. Conversely, unexpected interactions between the complex III_2 mitochondrial processing peptidase domain and the membrane arm of complex I are also observed. Although distinct in plants these interactions are analogous to those seen in mammals and ciliates indicating a conserved interaction site. In addition, we also obtained a ~5 Å reconstructions of the plant SC I+III_2 in alternative conformations lacking specific subunits of the "ferredoxin bridge domain" in CI.

C) For specific aim 3 to identify conditions for the solubilization of intact plant complex II, we have been screening extraction conditions of *V. radiata* mitochondrial membranes using lipid/detergent/polymer mixtures using a combination of Blue Native PAGE in-gel activity assays, which report on the size of the extracted complexes compared to a digitonin control and quantitative spectroscopic activity assays, which allow us to measure the fraction of functional complexes extracted from the membrane. Thus far we have screened 19 different detergents in the presence or absence of different lipid mixtures. Despite the large number of conditions screened thus far (over 43 conditions including the detergent lipid mixtures) only digitonin appears able to stabilize the intact plant complex II in solution. However, when we try to purify the complex in digitonin or replace the digitonin with amphipathic polymers to stabilize the complex, it rapidly degrades and precipitates. Thus we are now screening mixtures of detergent lipids and amphipathic polymers to determine whether we can trap the complex in solution at the extraction step by co-extracting potential stabilizing lipids and trapping them in a polymer belt. For this approach we are using styrene maleic acid polymers as wells as other commercially available amphipathic polymers (amphipols) with different carbon chain lengths and with different chemical/charge characteristics.

Science objectives for 2022-2023:

During the next reporting period we plan on achieving the following goals related to this project:

1) Develop a full isolation protocol for CI* using GLDH as bait. This will involve testing the alternative GLDH-bait construct and redesigning the construct based on these preliminary results. We will also replace the His tag on the GLDH construct with the higher specificity/affinity Strep tag. This should allow for a one step purification of CI* from plant mitochondrial membrane extracts. Once purified we will develop a protocol for reconstitution of CI* into liposome which will allow for the examination of its H^+ pumping activity.
2) Produce an ~3.0 Å near atomic reconstruction of the plant SC I+III_2, build and refine an atomic model for the SC and publish a manuscript based on these results.
3) Test additional conditions for the stabilization of plant complex II in solution with the goal of purifying the intact complex for structural and functional characterization. Specifically, over the next funding period we will continue to screen detergent/lipid/polymer conditions using BN-PAGE in-gel activity assays to assess the stability of the complexes. If we are unable to find conditions using the mixtures.

My project addresses BES cross-cutting priority areas by:

This work directly advances the aims of DoE BES Physical Biosciences by providing an "unprecedented architectural and mechanistic understanding" of "the structure/function, mechanistic and electrochemical properties of enzymes that catalyze complex multielectron redox reactions." Our long-term goal is to obtain an atomic mechanistic and structural understanding of all the components of plants' canonical and alternative mitochondrial electron transport chains.

My scientific area(s) of expertise is/are: Membrane protein biochemistry, single particle cryogenic electron microscopy, mitochondrial electron transport chain.

The ideal collaborator for my project would have expertise in: Plant genetics and molecular biology.

Publications supported by this project 2021-2022:

1. Meyer, H.E., Letts, J.A., Maldonado, M. (2022) Structural insights into the assembly and the function of the plant oxidative phosphorylation system. New Phytologist; doi:10.1111/nph.18259
2. Maldonado, M., Abe, K.M., Letts, J.A. (2022) A Structural Perspective on the RNA Editing of Plant Respiratory Complexes. *J. Mol. Sci.*, *23*, 684.

Structure and electron transfer pathways of an electron-bifurcating NiFe-hydrogenase

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Overall research goals:

Our overarching goal is to understand at the molecular and chemical level the mechanism of electron transport and energy conservation in the microbial world. We forge close collaborations within the DOE Physical Biosciences Program, in particular with Dr. Michael Adams, and use the cryo-EM approach to solve atomic or near atomic resolution structures of key protein complexes involved in energy conservation. The enzyme complexes to be studied are derived from hyperthermophilic microorganisms such as *Pyrococcus furiosus* and *Thermotoga maritima*, that thrive at temperatures from 80°C to 100°C. Our work in the previous funding cycle focused on two membrane-bound complexes MBH and MBS that convert low potential reducing equivalents into ion gradients across the membrane. In the current cycle, we aim to understand the functional diversification of the novel BfuABC family of electron bifurcation complexes.

Significant achievements for 2020-2022:

We focused on the NiFe hydrogenase complex ABCSL (HydABCSL) from anaerobic bacterium *Acetomicrobium mobile*. HydABCSL belongs to one group of flavin-based electron bifurcating (FBEB) enzymes that contain HydABC module. The bifurcation site and the molecular mechanism of the specific group had not been revealed. We were able to resolve the high-resolution cryo-EM structure of an apo and an FMN/NAD-binding state for HydABCSL.

We demonstrated that HydABCSL is a dimer of pentamer in the native state (**Fig. 1A**). The flavin FMN is most likely the bifurcation site and is surrounded by a unique arrangement of iron sulfur clusters. The mid-potential electrons from H_2 oxidation (E°' = -420 mV) by the NiFe-containing module (HydLS) are relayed by iron-sulfur clusters in HydA to the FMN in HydB for electron bifurcation (**Fig. 1B**). NAD and FMN are closely stacked such that the high-potential electrons can be easily transferred to NAD (E_m = -320 mV). The high-energy, low-potential electrons are first transferred to the HydC-C1 [2Fe-2S] cluster and this transfer requires a conformational change to bring C1 cluster in close contact with FMN in a bifurcation ready state. The electrons are then transferred to the uniquely coordinated HydB-B2 [2Fe-2S] cluster, and this transfer requires the HydC C-terminal domain to rotate 24º, bringing the C1 and B2 iron-sulfur clusters within electron transfer distance in an energy transducing state (**Fig. 1C)**. Finally, the low potential electrons are transferred to the external electron acceptor, the iron sulfur redox protein ferredoxin (E_m ~ - 500 mV) which binds to HydB. This work clarifies the electron bifurcation mechanism for a large group of FeFe- and NiFe-hydrogenases underlying many essential functions in anaerobic microorganisms.

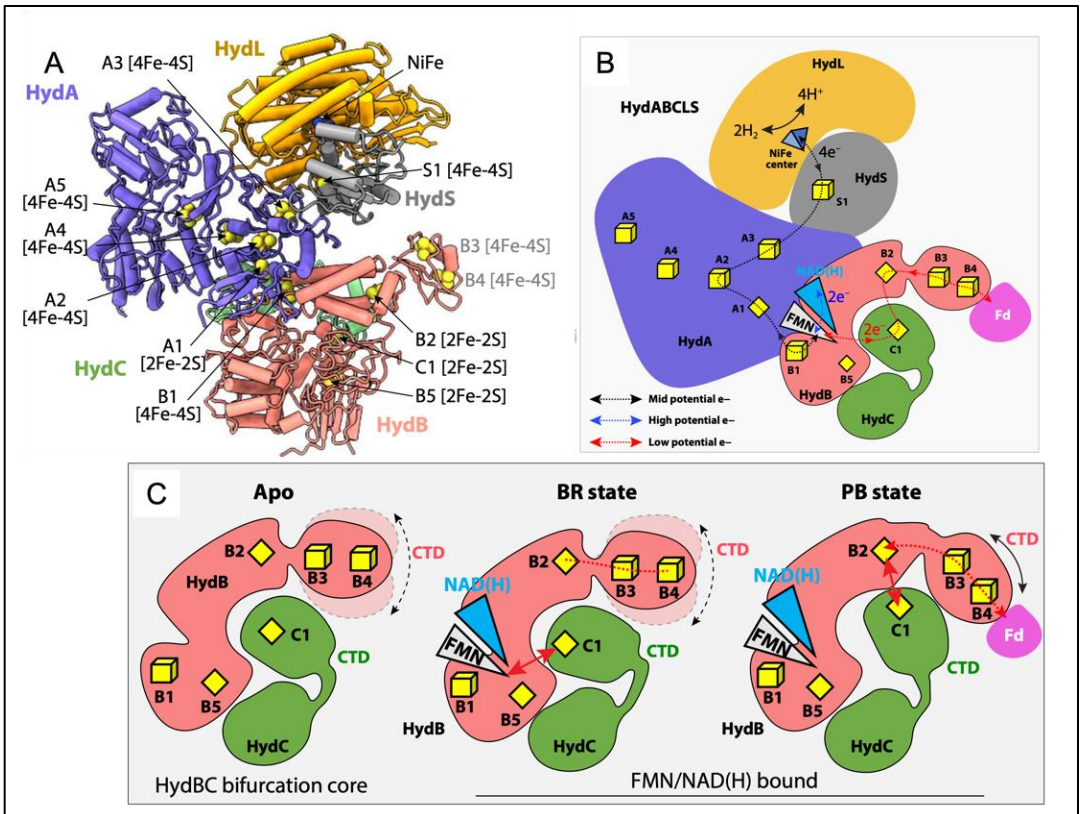

Figure 1. (A)The pentamer structure of NiFe-hydrogenase HydABCSL; (B) Proposed electron pathway for the electron bifurcating and (C) the protein conformational change accompanied.

Science objectives for 2021-2022:

- [We are focusing on structural and mechanistic understanding of the novel BfuABC family of metal-containing electron bifurcation protein complexes.

My project addresses BES cross-cutting priority areas by:

Our lab focuses on understanding the molecular mechanism of the flavin based electron bifurcation, aiming to explore its potential in bioenergy applications.

My scientific areas of expertise are:

Protein biochemistry, cryo-EM structural biology, and other biophysical methods

The ideal collaborator for my project would have expertise in: biochemist and microbiologist in energy metabolism, particularly in electron bifurcation.

Publications supported by this project (2022-2025):

1. Feng X, Schut GJ, Haja DK, Adams M, Li H. (2022). "Structure and electron transfer pathways of an electron-bifurcating NiFe-hydrogenase." Science Advances, 8(8), eabm7546.
2. Schut GJ, Haja DK, Feng X, Poole FL, Li H, Adams M. (2022). "An abundant and diverse new family of electron bifurcating enzymes with a non-canonical catalytic mechanism." Frontiers in Microbiology, 13, 946711.
3. Yu H, Haja DK, Schut GJ, Wu CH, Meng X, Zhao G, Li H, Adams MWW. Structure of the respiratory MBS complex reveals iron-sulfur cluster catalyzed sulfane sulfur reduction in ancient life. Nat Commun. 2020, 23;11(1):5953.

Transformative Biohybrid Diiron Catalysts for C-H Bond Functionalization

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Overall research goals:

The functionalization of C-H bonds in hydrocarbons, such as naturally abundant alkanes from crude oils, in a selective and environmentally benign manner is a grand challenge of chemical manufacturing. C-H bonds in alkanes are inert, and their chemical activation and functionalization in industrial manufacturing requires heat, pressure, and use of precious transition-metal catalysts. Due to similar dissociation energies of different C-H bonds in an alkane, it is also challenging to achieve selective functionalization of bonds at specific positions. Our primary objective is to advance science to enable catalytic biohybrid systems to replace high temperature/pressure catalysis for C-H functionalization with precious metals by low-temperature electrochemically driven biocatalysts using earth abundant iron for selective C-H functionalization tunable to produce a variety of products.

We will employ a multi-disciplinary approach of structural biology, biochemistry, theoretical chemistry and computational modeling, electrochemistry, artificial intelligence and machine learning (AI/ML), and in operando X-ray characterization of catalysts.

(1) Investigate the structure and function of the nonheme diiron monooxygenase biocatalysts to understand structural determinants for substrate specificity, activity, selectivity, and electron transfer reactions.

(2) Employ quantum chemical calculations, computational modeling, X-ray absorption spectroscopy, and AI/ML to reveal the underlying mechanistic details of the biocatalytic C-H bond functionalization and to guide the design and development of tunable transformative biohybrid catalysts.

(3) Elucidate the electron transfer and redox processes for transformative biohybrid catalysis through electrochemically driven synthesis.

Our proposed research will advance clean energy technologies and low carbon manufacturing by laying the scientific foundations for transformative manufacturing of high-value chemical feedstocks from naturally abundant and chemically inert alkanes with unprecedented selectivity, atom and energy efficiency, sustainability, and net zero-carbon release.

Significant achievements: (2022):

- Determined the first structure of an alkane omega monooxygenase electron-transfer biocatalyst complex which provides an insightful structural basis for the realization of a novel catalytic system for C-H bond activation and functionalization.
- Demonstrated C-H activation driven electrochemically in a biofuel cell or electrolyzer.

Science objectives for 2022-2023:

- Production and selection of biocatalysts of desired substrate selectivity through directed evolution.
- Structural investigation of selected biocatalysts.
- Creation of electron-transfer complexes to study the electron transfer process, development of electrodes for biohybrid catalysts.
- Quantum chemical modeling of catalytic reactions and intermediates.

- Collection of experimental and computational X-ray absorption spectra of biocatalysts, development and training of AI/ML models.

My project addresses BES cross-cutting priority areas by:
Research on biohybrid diiron catalysis to advance clean energy technologies and low carbon electrochemically-driven chemical manufacturing.

My scientific area(s) of expertise is/are: Structural biology, membrane proteins, cryo-electron microscopy.

The ideal collaborator for my project would have expertise in: Synthetic biology, high-throughput & automation.

Publications supported by this project

Elucidating the Mechanistic Determinants of Flavin-Based Electron Bifurcation

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Overall research goals:

Electron bifurcation is a biological mechanism to drive a thermodynamically unfavorable redox reaction through direct coupling with an exergonic reaction. The overarching goal of this project is to develop a fundamental understanding of how electrons are controlled, temporally, spatially, and energetically, in the novel flavin-based electron bifurcating (FBEB) class of enzymes. Specifically, our work focuses on investigation of the FBEB enzyme NADH-dependent ferredoxin:$NADP^+$ oxidoreductase (Nfn, Fig. 1). The objective of this project is to delineate the physical and electronic determinants of flavin electron bifurcating sites to generate a detailed, mechanistic framework that leads to a robust understanding of how biocatalysts transform electrochemical potential into chemical bonds. This is being addressed in three aims to: (I) Elucidate the physical features responsible for tuning of bifurcating flavin cofactors. (II) Investigate the impact of spatial configuration and coupling between bifurcating flavin cofactors and the initial acceptors of bifurcated electrons. (III) Understand how bifurcating enzymes impart independent control of the two bifurcated electrons.

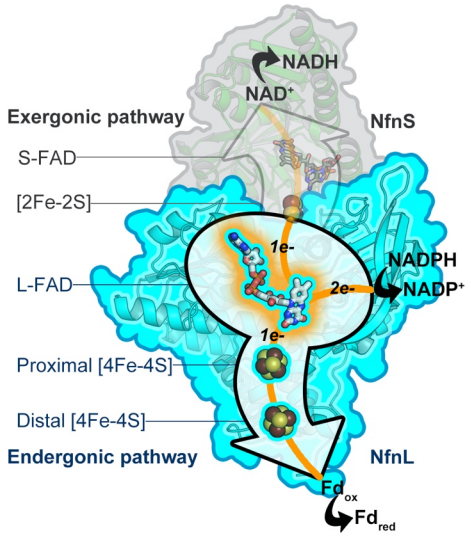

Fig. 1. Nfn from *Pyrococcus furiosus* (*Pf*) (PDB 5JFC, NfnL: large subunit in blue, NfnS: small subunit in grey). Following oxidation of NADPH, the bifurcating flavin, L-FAD (center), performs two one-electron transfers, first to the site-differentiated [2Fe-2S] of NfnS, then to the site-differentiated proximal [4Fe-4S] cluster of NfnL. The [2Fe-2S] is oxidized by S-FAD, which catalyzes the reduction of NAD^+ to NADH following two rounds of bifurcation. The proximal NfnL cluster reduces the distal [4Fe-4S], which subsequently reduces one equivalent of ferredoxin (Fd) for each NADPH oxidized.

Significant achievements (2019-2022):

Over the preceding year, our work has focused on salient aspects of each of the three project aims:

- Defining the thermodynamic landscape at the bifurcation site: We were able to measure and assign all three reduction potentials of the bifurcating cofactor, representing the first time that this has been achieved for a bifurcating cofactor. Our work revealed an unusually negative redox regime for Nfn, while reframing the current understanding of bifurcating energy landscapes and underscoring the mechanistic diversity utilized by these systems.
- Investigating how site-differentiated ligation of iron-sulfur cluster cofactors affects the electronic coupling with the bifurcating site and results in alteration of electron distribution among the two energy transfer pathways of Nfn: One important, and unexpected, outcome of our analysis of the site-differentiated [4Fe-4S] cluster in NfnL was a dramatically increased flux through the low potential branch in both the bifurcation and confurcation directions. The results point to a role for the site-differentiated ligand in facilitating coupling of the branches and fidelity of the electron bifurcation reaction.

- Probing the accessory flavin thermodynamics to generate an understanding of how electrons are managed during the two turnovers that are required to complete one full bifurcation cycle: We have assessed the reduction potentials for the accessory flavin, S-FAD, showing that it also possesses inverted potentials. These properties likely afford S-FAD the ability to significantly regulate electron flux across the whole enzyme.

Science objectives for 2021-2022:

- Perform biochemical activity studies on a variant where the site-differentiated [2Fe-2S] cluster of NfnS is altered to the canonical all-Cys ligation, to understand how this unique cofactor impacts the initiation of electron bifurcation.
- Develop a biophysical understanding for how the altered thermodynamic and electronic properties of the site-differentiated [4Fe-4S] and [2Fe-2S] clusters contribute to the overall effect on electron bifurcation activity and electron partitioning.
- Assess how the conserved Arg residue nearby the bifurcating flavin affects the thermodynamics and stability of L-FAD as well as the binding interaction with NADPH.

My project addresses BES cross-cutting priority areas by:
Understanding the functional principles that govern flavin-based electron bifurcation will constitute a critical advance in our ability to develop improved processes in chemical catalysis and will address the significant knowledge gaps in controlling electrons and matter.

My scientific area(s) of expertise is/are: Ultrafast optical spectroscopy, biophysical and biochemical analyses of redox enzymes and photosynthetic systems.

The ideal collaborator for my project would have expertise in: Mass spectrometry, Cryo-EM, MCD, Resonance Raman.

Publications supported by this project 2019-2022:

1. C. E. Wise, A. E. Ledinina, C. E. Lubner, "Site-Differentiated Iron-Sulfur Cluster Ligation Affects Flavin-Based Electron Bifurcation Activity." *Metabolites*, 12(9), 823 (2022). DOI: 10.3390/metabo12090823
2. H. Wu, M. D. Pun, C. E. Wise, B. R. Streit, F. Mus, A. Berim, A. Islam, D. A. Gang, J. L. DuBois, C. E. Lubner, C. E. Berkman, B. M. Lange, J. W. Peters, "The pathway for coenzyme M biosynthesis in bacteria." *Proc. Natl. Acad. Sci. U.S.A.*, 119 (36) e2207190119 (2022). DOI: 10.1073/pnas.2207190119
3. C. E. Wise, A. E. Ledinina, D. W. Mulder, K. J. Chou, J. W. Peters, P. W. King, C. E. Lubner, "An Uncharacteristically Low-Potential Flavin Governs the Energy Landscape of Electron Bifurcation." *Proc. Natl. Acad. Sci. U.S.A.*, 119 (12) e2117882119 (2022). DOI: 10.1073/pnas.211788211
4. C. E. Wise, A. E. Ledinina, J. L. Yuly, J. H. Artz, C. E. Lubner, "The role of thermodynamic features on the functional activity of electron bifurcating enzymes." *Biochim. Biophys. Acta – Bioenergetics*, 1862, 148377 (2021). DOI: 10.1016/j.bbabio.2021.148377
5. A. Dey, F. A. Houle, C. E. Lubner, M. Sevilla, W. J. Shaw, "Introduction to (photo)electrocatalysis for renewable energy." *Chem. Commun.*, 57, 1540-1542 (2021). DOI: 10.1039/D0CC90530E
6. J. L., Yuly, P. Zhang, C. E. Lubner, J. W. Peters, D. N. Beratan, "Universal Free Energy Landscape Produces Efficient and Reversible Electron Bifurcation." *Proc. Natl. Acad. Sci. U.S.A.*, 117 (35) 21045-21051 (2020). DOI: 10.1073/pnas.2010815117
7. C. E. Lubner, J. L. Yuly, P. Zhang, D.N. Beratan, J. W. Peters, "Electron Bifurcation: Progress and Grand Challenges." *Chem. Commun.* 55, 11823 (2019). DOI: 10.1039/c9cc05611d

Mechanistic Studies of a Primitive Homolog of Nitrogenase Involved in Coenzyme F430 Biosynthesis

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Overall research goals:

Methyl-coenzyme M reductase (MCR) is the key enzyme of methanogenesis and anaerobic methane oxidation. To carry out these reactions, MCR utilizes a unique Ni-containing tetrapyrrole, coenzyme F430. This project aims to investigate the mechanism of a primitive homolog of nitrogenase (CfbCD) that catalyzes a crucial step in the coenzyme F430 biosynthetic pathway. This unprecedented reaction, which converts Ni-sirohydrochlorin *a*,*c*-diamide to 15,17^3-seco-F430-17^3-acid, involves a 6-electron reduction of the isobacteriochlorin ring system, cyclization of the *c*-acetamide side chain to form a γ-lactam ring, and the formation of 7 stereocenters. The overall goals of this research include elucidating the structure and mechanism of this novel two-component metalloenzyme.

Significant achievements: 2017-2022

- A more stable and active version of CfbCD from *Methanosarcina thermophila* was identified and successfully expressed in and purified from the heterologous host *Escherichia coli*.
- Ferredoxin (Fd) and ferredoxin-NADP$^+$ reductase (FNR) from spinach together were found to reduce CfbC using NADPH and support CfbCD catalysis *in vitro*.
- Compatible vectors were constructed to coexpress the *cfb* genes together with those for MCR formation, maturation, and activation in *E. coli*.
- A protocol for the enzymatic synthesis and purification of Ni-sirohydrochlorin *a*,*c*-diamide was optimized and its extinction coefficient ($\varepsilon = 4.3 \times 10^4$ M^{-1}cm^{-1} at 590 nm) was determined.
- UV-visible spectrophotometry, electron paramagnetic resonance (EPR) spectroscopy, and iron/sulfide analyses are consistent with both CfbC and CfbD coordinating a single [4Fe-4S] cluster at their respective homodimeric interfaces.
- EPR analysis of reduced CfbC shows it contains a mixture of low- and high-spin [4Fe-4S]$^{1+}$ clusters. Potentiometric redox titrations of nucleotide-free CfbC gave a mid-point potential of approximately -270 mV vs. SHE, while ATP-binding caused a shift in the potential to below -500 mV.
- Analysis of CfbD using size-exclusion chromatography shows that it exists as a mixture of oligomerization states (homodimers and homotetramers) in solution.
- The formation of an initial product of the CfbCD reaction that slowly converts to 15,17^3-seco-F430-17^3-acid was confirmed. Additionally, a paramagnetic intermediate/product was detected using EPR spectroscopy and the structure of this species is currently being investigated using a combined spectroscopic and computational approach.
- Density functional theory (DFT) was applied to gain insight into the energetic preference and likely regiochemical course of electron-coupled proton addition to the isobacteriochlorin ring system of Ni-sirohydrochlorin *a*,*c*-diamide during the CfbCD catalytic cycle.
- AlphaFold was used to obtain structural models of CfbC, CfbD, and their complexes. Mg-ATP, Ni-sirohydrochlorin *a*,*c*-diamide, and [4Fe-4S] clusters were then docked to these structures and the systems were prepared for molecular dynamics (MD) simulations to investigate the quaternary and active site structures of CfbCD.
- Preliminary cryo-electron microscopy experiments were performed and are being optimized in collaboration with Dr. Catherine Drennan's laboratory to gain insight into the high-resolution structure of the CfbCD complex.

- Sequence similarity network (SSN) and genome neighborhood network (GNN) analyses were performed, which suggest that the nitrogenase superfamily is much larger and more diverse then previously appreciated. The data suggest that the largely uncharacterized Group IV members of the superfamily may play novel roles in metal acquisition, carbon fixation, and/or sulfur metabolism.

Science objectives for 2021-2022:

The specific goals of this project include the determination of: 1) the identity of the immediate product of the CfbCD reaction, 2) the structure, conformational dynamics, and oligomerization state changes of CfbCD, and 3) the source, order, and stereochemistry of proton additions during CfbCD catalysis.

My project addresses BES cross-cutting priority areas by:
combining biophysical, biochemical, molecular biological, and computational approaches to better understand the structure/function and mechanistic properties of a two-component metalloenzyme catalyzing a multielectron redox reaction important for the biosynthesis of coenzyme F430. This important metallocofactor is used by methanogenic/methanotrophic archaea in biological pathways that capture/convert carbon (coupled to hydrogen utilization/generation) that have applications to enable clean energy.

My scientific area(s) of expertise is/are: enzymology, biophysics (e.g., EPR spectroscopy), functional genomics, computational biochemistry (e.g., DFT, MD).

The ideal collaborator for my project would have expertise in: methanogen genetics, metabolic pathway engineering.

Publications supported by this project (2017-2022):

1. Mansoorabadi, S. O., Zheng, K., and Ngo, P. D. (**2017**) Biosynthesis of Coenzyme F430 and the Post-Translational Modification of the Active Site Region of Methyl-Coenzyme M Reductase, in *Metalloprotein Active Site Assembly* (Johnson, M. K. and Scott, R. A., Eds.), John Wiley & Sons, Ltd, West Sussex, UK.
2. Zheng, K. (**2018**) A Study of Methyl-coenzyme M Reductase Maturation: Coenzyme F430 Biosynthesis and Post-translational Modifications, Ph.D. Dissertation, Auburn University, Auburn, AL.
3. Owens, V. L. (**2019**) Functional Genomics Investigation of Siroheme and Heme d_1 Biosynthesis, Ph.D. Dissertation, Auburn University, Auburn, AL.
4. Ghebreamlak, S. M. and Mansoorabadi, S. O. (**2020**) Divergent Members of the Nitrogenase Superfamily: Tetrapyrrole Biosynthesis and Beyond, *ChemBioChem 21*, 1723-1728. doi:10.1002/cbic.201900782.
5. Li, S., Isiorho, E. A., Owens, V. L., Donnan, P. H., Odili, C. L., and Mansoorabadi, S. O. (**2021**) A noncanonical heme oxygenase specific for the degradation of *c*-type heme, *J. Biol. Chem. 296*, 100666. doi: 10.1016/j.jbc.2021.100666.
6. Li, S. (**2021**) Novel Enzymes and Catalytic Mechanisms in Tetrapyrrole Metabolism: a Nitrogenase Homolog and a Noncanonical Heme Oxygenase, Ph.D. Dissertation, Auburn University, Auburn, AL.
7. Donnan, P. H. and Mansoorabadi, S. O. (**2022**) Broken-Symmetry Density Functional Theory Analysis of the Ω Intermediate in Radical *S*-Adenosyl-L-methionine Enzymes: Evidence for a Near-Attack Conformer over an Organometallic Species, *J. Am. Chem. Soc. 144*, 3381-3385. doi: 10.1021/jacs.2c00678.
8. Patrick H. Donnan (**2022**). Computational Studies of Dinoflagellate Luciferase and Radical *S*-Adenosyl-L-Methionine Enzyme Catalysis, Ph.D. Dissertation, Auburn University, Auburn, AL.

Post-translational modifications in archaeal redox biology

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Overall research goals:

Post-translational modifications (PTMs) are of interest in biotechnology and metabolic engineering to expand the molecular toolbox available for control of enzymatic activity, protein stability, and intra- and intermolecular protein interactions. To maximize this potential, advanced understanding of the role PTMs have in regulating cellular responses and altering protein structure and function is needed.

The long-term objective of this project is to discern how PTMs regulate metabolic pathways and responses of archaea to environmental cues such as redox potential shifts. Emphasis is on the PTMs of sampylation (an archaeal form of ubiquitination termed ubiquitin-like (Ubl) ligation for simplicity of this grant review) and lysine acetylation in the halophilic archaeon Haloferax volcanii. We find lysine acetylation and Ubl ligation share overlapping protein targets and that these PTMs are generally more abundant when H. volcanii cells are challenged with oxidant. This PTM response is robust and provides an interesting perspective in archaeal redox biology.

Significant achievements: [(07/15/2022-07/14/2025]:

- The profile and abundance of the lysine acetylome is found altered when cells grown on glycerol are exposed to hypochlorite.
- Genes associated with lysine acetylation and deacetylation impact the profile of the lysine acetylome and the biology of the archaeal cell.
- SILAC-based LC-MS/MS analysis is found useful in identifying redox dependent shifts in the abundance specific proteins, lysine acetylated proteins, and occupancy of the PTM on a global scale.
- 2Fe-2S ferredoxin, glycerol kinase and acyl-CoA synthetases are major targets of lysine acetylation in the haloarchaeon and are selected for downstream analysis of how lysine acetylation regulates cell function.
- Ferredoxin reductase is found modified by lysine acetylation and Ubl-modification and has been purified to further examine of how these PTM systems may interact and regulate redox flux.

Science objectives for 2022-2023:

Aim 1: Identify and quantify lysine acetylome shifts during redox stress and determine the impact of deacetylases, Ubl ligation and proteasomes. *Hypothesis*: Quantitative analysis of the lysine acetylome during HOCl stress will provide a global perspective in archaeal redox biology. The *Δsir2* and Ubl ligation mutants are predicted to have an increased occupancy of acetylated lysine residues, particularly during oxidant challenge. Mutation of the conserved active site residues and zinc-finger motif of Sir2 are hypothesized to have a similar effect. The *Δhda1* mutation will stabilize certain lysine acetylation sites; however, its influence on the oxidant-dependent shifts of the lysine acetylome will be modest.

Aim 2: Determine the protein substrates of Pat1 and Pat2 and how these single GNAT (Gcn5-related N-acetyltransferase) domain proteins are regulated. *Hypothesis:* Pat1 and Pat2 catalyze the addition of acetyl-/acyl-groups to specific lysine residues. Reactive oxygen/nitrogen species (ROS/RNS) modify the N-terminal Cys-rich motif of Pat1 and alter its lysine acetyltransferase activity, while Pat2 activity is sequestered by UspA binding.

Aim 3: Examine how lysine acetylation and Ubl ligation may influence electron transfer between a [2Fe-2S]-type ferredoxin (Fdx) and a Fdx reductase homolog. *Hypothesis*: Oxidant challenge impacts the PTM

occupancy of lysine residues at the intersubunit interface of a Fdx and Fdx reductase homolog. This in-turn alters electron transfer between NAD(P)H and Fdx which influences downstream biological pathways related to redox homeostasis.

My project addresses BES cross-cutting priority areas by:
This project supports the DOE missions in "alternative energy, global carbon cycling, and biogeochemistry" as well as "fundamental understanding regarding the mechanisms non-medical microbes (archaea) use to transduce and/or store energy". Archaea mediate key steps in global nutrient cycling and have extremophilic properties of fundamental interest to the design of biocatalysts to transduce and store energy. Here we propose to study PTM processes of archaea that control metabolic flux to transduce and/or store energy. Archaea are desired as microbial platforms to produce renewable fuels and chemicals but are poorly understood in their use of PTMs to control metabolism and stress responses. Here our focus is on the archaeal Ubl ligation and lysine acetylation systems that appear widespread in this domain of life. Both PTM systems are redox controlled and impact the ability of archaea to survive oxidative challenge. Ubl ligation regulates metabolic enzyme activity and triggers the rapid turnover of proteins by energy-dependent proteasomes in archaea. Design of methods to control PTM systems associated with redox balance would advance use of archaea as microbial platforms for metabolic engineering. This project also holds promise to provide an evolutionary insight into PTM systems that may relate to all domains of life.

My scientific area(s) of expertise is/are: microbial cell biology, metabolism, post-translational modifications, regulatory mechanisms, and bioconversion.

The ideal collaborator for my project would have expertise in: electrochemistry of biomolecules including those with Fe-S clusters and flavin-cofactors; structural biologist who analyzes 'unusual' proteins (*e.g.*, salt-loving proteins from haloarchaea have a highly acidic shell and function in environments of low water activity including organic solvents).

Publications supported by this project [(07/15/2022-07/14/2025]:

1. Mondragon, P, S Hwang, L Kasirajan, R Oyetoro, A Nasthas, E Winters, RL Couto-Rodriguez, A Schmid, and JA Maupin-Furlow. 2022. TrmB family transcription factor as a thiol-based regulator of oxidative stress response. *mBio* 13(4):e0063322. doi: 10.1128/mbio.00633-22.
2. Zhang H, Gong X, Zhao Q, Mukai T, Vargas-Rodriguez O, Zhang H, Zhang Y, Wassel P, Amikura K, Maupin-Furlow J, Ren Y, Xu X, Wolf YI, Makarova KS, Koonin EV, Shen Y, Söll D, Fu X. 2022. The tRNA discriminator base defines the mutual orthogonality of two distinct pyrrolysyl-tRNA synthetase/tRNAPyl pairs in the same organism. Nucleic Acids Res. 50(8):4601–15. doi: 10.1093/nar/gkac271.
3. Couto-Rodriguez, R.L.1, Gal, D.1, McMillan, L.J.1, Koh, J., Chen, S., Maupin-Furlow, J.A. 2022. Quantitative mass spectrometry by SILAC in *Haloferax volcanii*. In: Ferreira-Cerca, S. (Ed) Archaea. Methods in Molecular Biology, vol 2522. Humana, New York, NY. https://doi.org/10.1007/978-1-0716-2445-6_16
4. Maupin-Furlow, JA. 2022. HvJAMM1 desampylase. In Handbook of Proteolytic Enzymes. 4th Edition. N Rawlings and DS Auld (Eds). Elsevier.
5. Hepowit, N and JA Maupin-Furlow. 2022. Application of archaea in deubiquitinase-like enzyme discovery and activity assay. In "Deubiquitinases", Methods in Molecular Biology book series. Maupin-Furlow and Edelmann (Ed) Springer Nature (in press).
6. Joshi, JB, JA Maupin-Furlow, S Uthandi. 2022. Microbial elicitors: A positive or negative modulator of plant defense. In "Mitigation of Plant Abiotic Stress by Microorganisms: Applicability and Future Directions". Elsevier (in press).
7. Priyadharshini R., JB Joshi, L Kasirajan2, JA Maupin-Furlow and S Uthandi. 2022. Enzymatic saccharification technologies for biofuel production: Current status and prospects. In "Microbial Biotechnology for Renewable and Sustainable Energy" S Uthandi and RK Sani (Eds). Springer-Nature (in press).
8. Egan, MS, K Hogan, J Maupin-Furlow and M Pohlschroder. 2022. The best of both worlds: discovery-driven learning through a lab-seminar approach. J. Microbiol. Biol. Educ. (in press).

Energy conservation, electron transfer and enzymology during methane production by *Methanosarcina* species

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Overall research goals:

The long-term goal of the proposed research is to develop a comprehensive understanding of the enzymes, electron carriers and metabolic pathways that comprise the energy-conserving electron transport network of methane-producing Archaea. Using the genetically tractable *Methanosarcina acetivorans* as a model organism, we will focus on two unsolved questions that lie at the center of methanogenic metabolism. The first addresses the biosynthesis, essentiality and mechanistic function of the extensive post-translational modifications found in methyl-coenzyme M reductase (MCR), an enzyme that plays a central role in anoxic production and consumption of methane. The second examines how low potential electrons are partitioned between donors and acceptors within the cell using small iron-sulfur proteins known as ferredoxins. The planned studies on the post-translational modification of methyl-coenzyme M reductase (MCR) will include identification of the genes required for the installation of post-translational modifications, determination of the viability of mutants that lack these post-translational modification genes, physiological characterization of all viable mutants, and structural and biochemical characterization of unmodified MCR derivatives. Our studies of the ferredoxin-dependent electron-transfer networks in *Methanosarcina* will entail examination of the viability of mutants lacking each of the thirteen *Methanosarcina* Fd-encoding genes, establishing the protein interaction network for each ferredoxin, characterizing the metal content and redox properties for each ferredoxin, and determining the structures for each ferredoxin via protein crystallography, cryoelectron micropscopy or solution NMR. Our specific aims for the current funding period are: (1) biochemically characterize MCR derivatives with and without the post-translational modifications found in most methanogens; (2) identify genes required for the synthesis of remaining post-translationally modified MCR residues and characterize of mutants that lack these genes; and (3) elucidate the ferredoxin-dependent electron-transfer networks in *Methanosarcina*.

Significant achievements: 2020-2022

- Identification and biochemical characterization of the radical SAM enzyme responsible for the methyl-glutamine modification in MCR
- We have determined the modification state of MCR from of *Methanobacterium formicicum, Methanosprillum hungatei and Mathanomassillicoccus luminyensis*.
- Determined the crystal structures of MCR from *Methanobacterium formicicum and Methanosprillum hungatei.*
- Constructed and characterized mutants lacking the ferredoxins required for saturation of membrane lipids in *M. acetivorans,* revealing a surprising lack of phenotypic consequences for strains with almost completely unsaturated membrane lipids.
- Developed a system for mass cultivation of M. acetivorans, setting the stage for biochemical characterization of MCR and its unmodified derivatives.
- Conducted pilot studies on reductive activation of MCR

Science objectives for 2021-2022:

- Phenotypic characterization of *M. acetivorans* strains with methylglutamine modified MCR

- Develop a protocol for reductive activation of MCR from *M. acetivorans*
- Biochemical characterization of modified MCR variants
- Establish the post-translational modification of phylogenetically diverse MCRs by mass-spectromtery
- Identify the genes required for the installation of the remaining post-translational modifications using a comparative genomic approach.

My project addresses BES cross-cutting priority areas by: Our experimental approach combines genetic, biochemical and biophysical approaches to develop a holistic understanding of the processes that govern the flow and partioning of electrons used for both anabolic and catabolic reactions that are required for growth of strictly anoxic, methane producing microorganisms.

My scientific area(s) of expertise is/are: Genetic analysis of diverse microorganisms, Energy conservation in anaerobes, Natural product metabolism.

The ideal collaborator for my project would have expertise in: EPR spectroscopy, electrochemical characterization of enzymes.

Publications supported by this project 2020-2022:

1. Nayak, D.D., A. Liu, N. Agrawal, R. Rodriguez-Carerro, S.-H. Dong, D.A. Mitchell, S.K. Nair and William W Metcalf. 2020. Functional interactions between post-translationally modified amino acids of methyl-coenzyme M reductase in Methanosarcina acetivorans. PLOS Biol. 18**:**e3000507. PMID: 32092071, PMC7058361.
2. Spietz, R.L., Payne, D., Kulkarni, G. Metcalf, W.W., Roden, E.E. and E.S. Boyd. 2022.Investigating abiotic and biotic mechanisms of pyrite reduction. Front. Microbiol. 13:878387. PMID: 35615515, PMC9124975.
3. Loyd, C.T., D.F. Iwig, B. Wang, M. Cossu, W.W. Metcalf, A.K. Boal, S.J. Booker. 2022. Discovery, structure and mechanism of a tetraether lipid synthase. Nature **609:**197-203. PMID: 35882349, PMC9433317.
4. Thomsen, J., K. Weidenbach, W.W. Metcalf and R.A. Schmitz. 2022. Genetic methods and construction of chromosomal mutations in methanogenic Archaea. Methods Mol. Biol. **2522:**105-117. PMID: 36125745

Gating Electron Transfer in Biological Energy Storage and Conversion

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Hugh O'Neill, Co-Investigator
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Overall research goals:

1. Establish the starting ('resting') conformation of a bifurcating ETF (Bf ETF) in solution.
2. Determine which catalytic events are coupled to conformational change.
3. Elucidate the bifurcating conformation, which we propose is formed when Bf ETF is complexed with its high-energy electron acceptor FixX (now to be replaced by flavodoxin).

Significant achievements: [Sep. 2020-Aug.2022]:

1- A crystal structure of the flavodoxin that is the low-E° partner of FixAB (=EtfAB) of *Rhodopseudomonas palustris*.

The recent CryoEM structure of FixABCX solved by Li and Adams[1] confirmed my earlier prediction of a complex structure resembling that of mitochondrial quinone reductase, which includes a domain homologous to FixC as well as one homologous to FixX[2]. The implication of the Li/Adams structure was that FixX is not the low-E° electron acceptor of ETF but functions on the high-E° electron transfer path. Thus, conformational effects of association with the low-E° partner should investigate complexes between ETF and ferredoxin/flavodoxin, NOT FixX (our aim 3). Therefore, we pivoted and established baseline studies of the flavodoxins (Flds) associated with our ETFs under study. We are pleased to report a crystal structure of this Fld, of inherent interest and a critical prerequisite for the computational studies proposed. The structure was solved by Oak Ridge National Lab (ORNL) postdoc Dr. Ahmad Ansari based on protein provided by our DOE-supported project. A manuscript is underway reporting this and the progress described in item 2, below.

2- Development of NMR probes of conformation and complex formation.

Study of conformational change in flavoproteins is complicated by the responsiveness of flavins to electromagnetic radiation. Published crystal structures abound with evidence that photoreduction occurred during data collection, preventing attribution of the resulting structure to a defined oxidation state. To determine whether flavin oxidation state changes trigger conformational responses, a non-perturbative probe is required. Hence the power of our small angle neutron scattering approach (below) and NMR. We are pleased to report ^{19}F NMR studies of the low-E° acceptor Fld, demonstrating a fully resolved spectrum, assigning all the resonances and revealing previously unknown internal dynamics. Moreover these were found to be preserved in the Fld of a second strain of *R. palustris*, and therefore possibly general to Flds cognate to Bf ETF.

3- Development of a new Bf ETF system for SANS.

In the past year, ORNL was able to reopen for visiting scholars and the DOE-supported student S. Khan was able to collect SANS and SAXS data in collaboration with ORNL scientists Dr. W. Leite and Dr. L. Heroux. They determined that the *R. palustris* EtfAB (*Rpa*ETF) we had proposed is not favorable for SANS due to rapid aggregation. This may be symptomatic of the fact that this protein associates with its partners FixCX *in vivo*, so we decided to test different Bf ETFs that occur as as the EtfAB dimer in solution. We thank colleagues Prof. R. Hille (U.C. Riverside), Prof. W. Buckel and Dr. N. Chowdhury (Univ. Frankfort, Germany) for generously providing expression systems for the Bf ETFs of *Acidaminococcus fermentans* (*Afe*ETF) and *Megasphaera elsdenii* (*Mel*ETF). Both constructs yielded twice as much protein upon overexpression as our *Rpa*ETF system, and increased protein partitioning into the soluble phase vs. the *Rpa*ETF.

Figure 1 shows virtually ideal SAXS scattering from the *Afe*ETF, so we are now advancing to SANS with this protein. Guinier plots of the three ETFs (panels B) yield the radii of gyration R_g for each of the proteins from the slope of a straight-line fit. A straight line was not a good fit for *Rpa*ETF (blue oval), but when one was imposed, the obtained R_g was 45 - 60 Å, larger than that expected for the EtfAB heterodimer. In welcome contrast, both *Afe*ETF and *Mel*ETF data adhered well to straight lines and the obtained R_gs are 27.7 Å and 28.2 Å (±0.5 Å), respectively, in adequate agreement with the molecular mass of ETF. The linearity of the data indicates monodispersity, which was confirmed by the independence of scattering intensity I(q) on the scattering vector q in the low-q region (panels A). Thus, the I(q) vs. q plot for *Afe*ETF displays an ideal horizontal line at low q indicative of monodispersity, whereas that of *Rpa*ETF displays a line sloping to higher values at lower q symptomatic of presence of aggregates. Also note the strong upward curvature of the *Rpa*ETF data at low q^2 in panel B (blue oval) that is absent from *Afe*ETF. Elimination of this behavior is a critical step forward for our project.

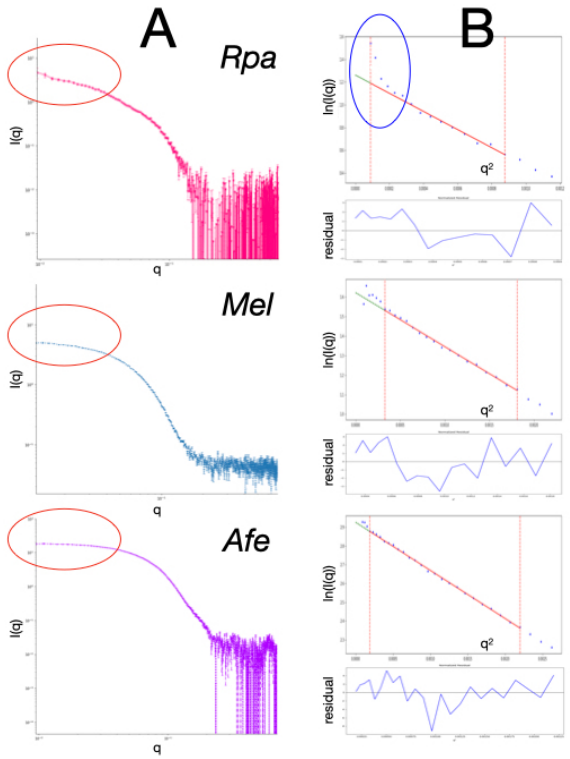

Figure 1. SAXS results for three ETFs: *Rpa*ETF, *Mel*ETF and *Afe*ETF. Panels A: SAXS scattering intensity profiles (note use of log scales). Panels B: Guinier plots showing linear regimes where the slope is proportional to R_q^2 (hydrodynamic radius^2)

1. Feng, X.; Schut, G. J.; Lipscomb, G.; Li, H. Y.; Adams, M. W. W. *Proc. Natl. Acad. Sci. USA* **2021,** *118* (2), e2016978118.
2. Zhang, J. X.; Frerman, F. E.; Kim, J.-J. P. *Proc Natl Acad Sci USA* **2006,** *103* (44), 16212-16217.

Science objectives for 2022-2023:

1. SANS as a function of flavin oxidation state, including apparatus and protocols for stabilizing desired oxidation state for handling required for SANS data collection.

2. Description of the resting state of Bf ETF in solution in terms of the open and the closed conformations observed crystallographically. Comparison of observed scattering profiles with those predicted by extant structures of EtfAB will reveal whether the data are best explained by the open conformation, the closed conformation, or and intermediate/ensemble representing both.

3. Shifting the conformation of ETF between open and closed using substrate analogs and partner proteins.

My project addresses BES cross-cutting priority areas by:
Achieving control over matter at a molecular level: our studies will obtain signatures revealing which conformation is present in solution, under a given set of conditions; our studies varying oxidation state and bound substrates/partners will test ways to produce a desired conformational state on demand.

My scientific area(s) of expertise is/are: Spectroscopy: NMR, and absorption, CD, fluorescence of flavins including quantum chemical computations of these for flavins in protein sites.

The ideal collaborator for my project would have expertise in: transient absorption spectroscopy, direct electrochemistry or electrode-immobilized enzymes.

Publications supported by this project 2020-2023:

1. Khan, S.A., Ansari, A. and Miller, A.-F. (2022) ^{19}F NMR reveals dynamics not evident in crystal structure of long-chain flavodoxin" Oral presentation, Southeast Enzyme Chemistry Conference, 23 Apr. 2022, Atlanta GA.
2. Khan, S.A., Ansari, A. and Miller, A.-F. (2022) ^{19}F NMR reveals dynamics not evident in crystal structure of long-chain flavodoxin" Poster, ASBMB National Meeting, 2-5 Apr. 2022, Philadelphia PA.

Conformational and Chemical Dynamics of Single Proteins in Solution by Suppression of Brownian Motion

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Overall research goals:

The primary objective of this project is to gain mechanistic insight into photosynthetic light harvesting and carbon fixation by directly observing the variation in behavior between individual copies of photosynthetic and energy-related biomolecules. Single-molecule measurements have been firmly established as a cutting-edge technique for elucidating mechanistic details of numerous biological processes. Unfortunately, the immobilization of single biomolecules on surfaces is often undesirably perturbative and introduces extraneous heterogeneity. To address this issue, we have constructed a microfluidic device, the Anti-Brownian ELectrokinetic (ABEL) trap, which uses microscopy and real-time feedback forces to counteract Brownian motion and trap a single molecule in solution. Because of these capabilities, the ABEL trap is particularly powerful for the extended study of the photodynamics of single photosynthetic pigment-protein complexes. However, the ABEL trap requires fluorescence emission from a single molecule for localization and feedback application, but feedback is lost when the molecule blinks or inevitably photobleaches. To overcome the fluorescence requirement for feedback, we recently developed the Interferometric Scattering ABEL (ISABEL) trap, which applies positional feedback on an unlabeled nanoscopic particle based on its interferometric scattering signal. In addition to the information about the particle's refraction properties provided by the scattering signal, this advance removes the need for bright and continuous fluorescence and opens up many possibilities for separate reporter-based fluorescence excitation and detection schemes decoupled from the trapping feedback mechanism. Our goal is to use this capability to study single copies of the important carbon-fixation organelle in bacteria, the carboxysome.

An important second thrust of this work is to develop cryogenic single-molecule fluorescence spectroscopy correlated with cryogenic electron tomography. It is frequently the case that single-molecule fluorescence spectroscopy experiments, like those conducted by the ISABEL and ABEL traps, results in the observation of heterogeneous photophysical states. These optical states are often hypothesized as arising from specific structural (or dynamic) heterogeneity in the sample. By correlating cryogenic single-molecule fluorescence spectroscopy with structure from cryogenic electron tomography we will ultimately be able to identify the physical structures which produced specific optical properties at the single-particle level. This approach gives us the opportunity to observe correlations and underlying mechanisms that would otherwise be left to speculation.

Significant achievements: 2021-2023:

As an overview of the current grant period, we have added fluorescence excitation and detection optics to the ISABEL trap to expand our ability to monitor various fluorescent sensor readouts from trapped molecules and particles. We are utilizing this capability to study the physical and chemical properties of single carboxysomes, the nanoscopic bacterial compartment responsible for carbon concentration and fixation. Additionally, we have completed the proof-of-principle demonstration of cryogenic correlative single-molecule spectroscopy and electron tomography using core-shell quantum dots as a model system. This initial study yielded exciting new observations and has given us the confidence to consider our correlative approach for the photosynthetic PSI-IsiA supercomplex in collaboration with D. Bennett at SMU. Ongoing work seeks to include additional fluorescence readouts to add more dimensions of information extracted from trapped molecules and particles.

In collaboration with the Savage Lab at Berkeley, we have completed multiple new types of measurements on the physical and chemical properties of single carboxysomes, the bacterial microcompartment responsible for carbon fixation. By approximating each carboxysome as a core-shell particle and modeling its scattering signal,

we can estimate the mass of each particle and assemble histograms for the distribution of carboxysome masses.

Moreover, with an internal fluorescent reporter protein roGFP2, we can also measure the redox environment within each carboxysome. Finally, we completed a proof-of-principle demonstration of cryogenic correlative light and electron microscopy with roGFP2 in a model bacterium, enabling us to visualize the redox environment of a nanoscopic biological domain. Ongoing work seeks to measure single-carboxysome translational diffusion and chemical transport kinetics to test long-standing hypotheses on the function of the carboxysome shell.

Science objectives for 2022-2023:

Examining the relation between the mass and protein number in each carboxysome for different tagged proteins could help elucidate models of carboxysome composition and assembly. We can also correlate these mass and loading measurements with measurements of the internal chemical environment, described further below. The future possibility of measuring translational diffusion constants in the ISABEL for each trapped particle should also enable us to measure the external sizes of carboxysomes, allowing for more precise measurements of loading density.

We plan to use redox-sensing carboxysomes to directly measure the permeation kinetics of small reductant molecules into the carboxysome. Such measurements could quantitatively test the current view that the shell architecture acts simultaneously to enhance the influx of necessary anions (e.g. HCO_3^-) and to limit the escape of neutral species, particularly CO_2.[7, 10, 15] Trapping single particles in solution ensures that the entire surface area of each carboxysome is accessible to these small molecules, and it allows us to remove contributions from large aggregates or freely diffusing roGFP2 reporter proteins. With the improved measurements of carboxysome size described above, these studies will allow us to directly measure effective permeabilities of different small molecule species across the carboxysome shell.

My project addresses BES cross-cutting priority areas by:

This project develops new instrumentation to uncover the structural and chemical dynamics of biological photosynthetic and carbon fixation machinery. By expanding the types of observables we can measure for each complex, we uncover the inherent variability between individuals, which helps us determine the principles for their function in the native organism and in engineered systems. With this knowledge, we can envision engineered and optimized nanoscale biological reactors for carbon fixation into small reduced carbon species or other chemical fuels.

My scientific area(s) of expertise is/are: single-molecule and -particle spectroscopy, microscopy, trapping of biomolecules in solution, instrument development.

The ideal collaborator for my project would have expertise in: Molecular biology, biochemistry, nanoscience, nanoscale biological structures.

Publications supported by this project 2020-2023:

1. W. Carpenter, A Lavania, J. Borden, L. Oltrogge, D. Perez, P. Dahlberg, D. Savage, W. Moerner, "Ratiometric Sensing of Redox Environments Inside Individual Carboxysomes Trapped in Solution." J. Phys. Chem. Lett. **13**, 4455 (2022). [https://doi.org/10.1021/acs.jpclett.2c00782].
2. A. Squires, Q. Wang, P. Dahlberg, W. Moerner, "A Bottom-Up Perspective on Photodynamics and Photoprotection in Light-Harvesting Complexes Using Anti-Brownian Trapping." J. Chem Phys **2022**, 070901 (2022). [https://doi.org/10.1063/5.0079042].
3. A. Lavania, W. Carpenter, L. Oltrogge, D. Perez, J. Borden, D. Savage, W. Moerner, "Exploring Masses and Internal Mass Distributions of Single Carboxysomes in Free Solution Using Fluorescence and Interferometric Scattering in an Anti-Brownian Trap." bioRxiv (2022). [https://doi.org/10.1101/2022.08.23.505029].
4. D. Perez, P. Dahlberg, J. Wang, A. Sartor, J. Borden, L. Shapiro, W. Moerner, "Identification and Demonstration of roGFP2 as an Environmental Sensor for Cryogenic Correlative Light and Electron Microscopy." J. Struct. Biol. **2022**, 107881 (2022). [https://dio.org/10.1016/j.jsb.2022.107881].

Multidimensional Spectroscopies for Probing Coherence and Charge Separation in Photosynthetic Reaction Centers

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Overall research goals:

The primary motivation of the proposed work is to *elucidate the design principles critical for our fundamental understanding of photosynthesis and for our ability to mimic PSII's remarkable properties*. This goal requires developing new approaches to connect structure to function; a particularly challenging task in PSII where the overlapping electronic transitions of the many pigments obscure their individual roles in the system's electronic excited states and charge separation process. With the overarching goal of improving our understanding of the structure-function relationship in the PSII RC, we propose parallel studies of spectroscopically simpler systems including PSII grown under far red light, model dyads and other reaction centers including the purple bacterial reaction center (BRC) and the heliobacterial reaction center (HbRC). In these studies we address the following open questions:

1) How can we test and refine structure-based exciton models of photosynthetic reaction centers?

2) How can we relate structure to charge separation mechanisms in photosynthetic reaction centers?

3) Do electronic-vibrational resonances enhance photosynthetic energy transfer and charge separation?

Significant achievements: (2022-2025):

Vibronic Coherence and Coherence transfer in the Bacterial Reaction Center (1)

We have performed two-dimensional electronic spectroscopy (2DES) experiments spanning the Q_y region on the bacterial reaction center (BRC) from purple bacteria, revealing hidden excitonic and vibronic structure. Through analysis of the coherent dynamics of the BRC we resolved specific coherent signatures that allowed us to definitively assign the energy of the upper exciton P_+ of the special pair in agreement with our previous work. We show that the coherent signatures in the 2DES data clearly reveal the presence of this state due to vibronic coupling within the BRC and rapid energy transfer to the lower exciton state. We support our assignment by simulations of coherent dynamics of a reduced excitonic model of the BRC. The simulations also identify nonsecular vibronic coherence transfer processes that are generally neglected in standard models of photosynthetic energy transfer and charge separation. In addition, our analysis of the coherent dynamics reveals multiple

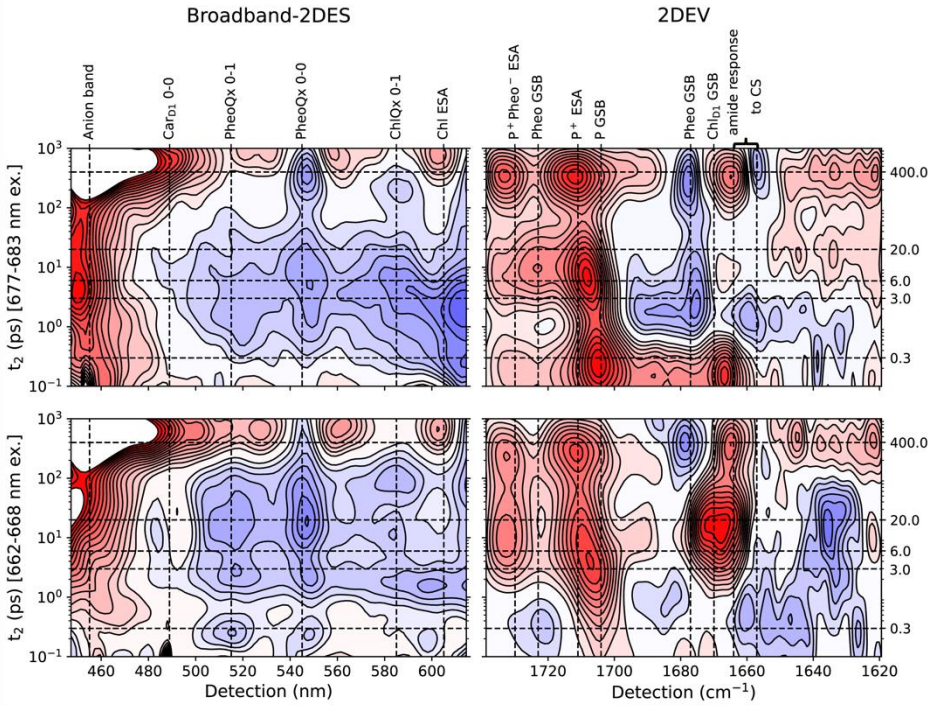

Figure 1 Lifetime density maps of broadband-2DES (left) and 2DEV (right) of the PSII RC at different excitation frequency regions: 677-683 nm (top) and 662-668 nm (bottom). Blue features indicate a rise of ground state bleach or decay of excited state absorption, while conversely, red indicates decay of ground state bleach or rise of excited state absorption.

resonances between key intramolecular pigment vibrations and excited state energy gaps in the BRC. The functional significance of such electronic-vibrational resonances and the identified nonsecular coherence transfer processes for photosynthetic energy conversion remains an open question. This work was recently published in Science Advances.(*1*)

Charge Separation in the Photosystem II Reaction Center Resolved by Multispectral Two-Dimensional Electronic Spectroscopy(2)

In order to extract the richest information content from the system with simultaneous high frequency and temporal resolution, we utilized Fourier transform 2D spectroscopy employing a combination of probes in the visible and mid-infrared region. Previous 2DES experiments of the PSII-RC by our group and others have focused on the Q_y region where the severe overlap of the excitons complicates the interpretation of the data and hinders tests of the exciton model and charge separation mechanism. While exciting within the crowded Q_y region is key to unraveling the sequence of events that proceed from the population of specific Q_y excitonic states, it is critical to obtain pigment-specific spectroscopic signatures to distinguish between distinct charge separation pathways. Previous transient absorption spectroscopy employing broadband probes have exploited the improved spectral separation of Chl and Pheo Q_x transitions, and the formation of distinct anion absorption bands for this purpose. Additionally, the C=O keto and ester vibrational bands of Chl and Pheo have been shown to be sensitive to the protein environment and to charge separation. We combined 2D electronic excitation with a mid-IR probe (2DEV spectroscopy) with broadband 2DES, exciting the Q_y band to interrogate its excitonic structure and initiate charge separation. We probed over the visible and mid-IR regions to access a unique combination of pigment-specific spectroscopic markers and use lifetime density analysis (see Figure 1) and global target analysis with simultaneous fitting of the broadband-2DES and 2DEV data to test models of charge separation. We observe and characterize delocalized excitonic and charge transfer states, as well as trap states that are responsible for slower phases of charge separation. The combination of visible and mid-IR probes reveal a highly delocalized charge separation process in which $Pheo_{D1}$ acts as the primary electron acceptor, with Chl_{D1} and P_{D1} acting in concert as the primary electron donor. This mechanism unites the distinct Chl_{D1} and P_{D1} pathways that have been proposed based on lower-dimensional measurements that probed a subset of the transitions considered in our multispectral study.

Science objectives for 2021-2022:

- Study model dyads to assess the importance of electronic-vibrational resonances on energy transfer
- Perform 2DES studies of PSII grown in far-red-light to understand how the modified pigment content affects the excitonic structure and charge separation process
- Use the full multispectral 2D dataset to improve the excitonic model and test models of the charge separation mechanism of PSII and the BRC

My project addresses BES cross-cutting priority areas by:

The award addresses BES priorities for developing transformational experimental tools that can be applied beyond the scope of this project. The experimental work supported in this project also furthers the advancement of theoretical descriptions of energy transfer and charge separation.

My scientific area(s) of expertise is/are: Multidimensional spectroscopy and microscopy of photosynthetic systems.

The ideal collaborator for my project would have expertise in: Electronic structure and quantum chemical calculations, simulations of spectroscopic signals. Biochemist with the ability to make site-directed mutants.

Publications supported by this project [Click to Enter Years of Current Grant/FWP, e.g. 2012-2014]:

1. V. R. Policht, A. Niedringhaus, R. Willow, P. D. Laible, D. F. Bocian, C. Kirmaier, D. Holten, T. Mančal, J. P. Ogilvie, Hidden vibronic and excitonic structure and vibronic coherence transfer in the bacterial reaction center. *Science Advances* **8**, eabk0953 (2022).
2. H. H. Nguyen, E. Maret, Y. Song, C. F. Yocum, J. P. Ogilvie, Charge Separation in the Photosystem II Reaction Center Revealed by Multipsectral Multidimensional Spectroscopy. (arXiv:2209.09992 [physics.chem-ph]).

Cell-type Specific Pectins in Plant Cell Walls: Structure, Interaction and Function

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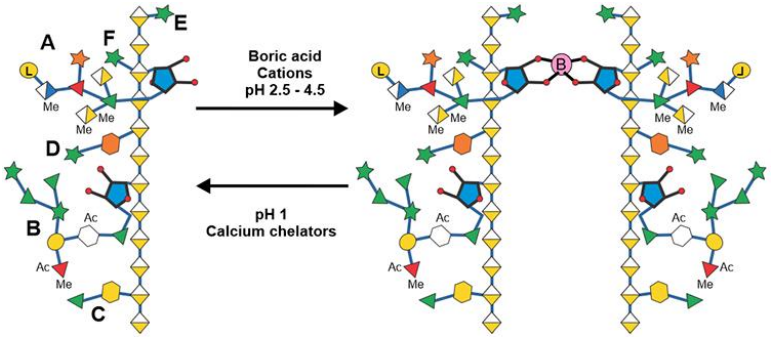
Overall research goals:

Rhamnogalacturonan II (RG-II) is a low molecular weight (~5 KDa) yet structurally complex domain of the pectin present in the primary cell walls of vascular plants (see Figure). Virtually all of this RG-II exists as a borate cross-linked homodimer, which is believed to be required to form a functional pectin network in the wall. Plants unable to form the dimer have abnormal walls and low productivity.



RG-II has a galacturonan backbone substituted with four oligosaccharides (A, B, C and D) and two monosaccharides (E and F) (Figure).This primary structure is largely conserved, although naturally occurring RG-II glycoforms exist. Nevertheless, the relationship between the glycosyl sequence of RG-II and its ability to form a borate crosslinked dimer is not understood.

Our research goals are (i) to determine how the glycosyl sequence of RG-II determines its ability to form a dimer and (ii) use experimental and computational methods to determine the conformation and dynamics of the RG-II monomer and dimer. Ultimately, we wish to exploit the principles of borate crosslinking to develop bio-based polymers with tunable properties.

Significant achievements: [2017-2022]:

RG-II provides a unique opportunity to study the structure-property-function relationship of a polysaccharide since our studies with mutant plants suggest that its ability to form a dimer is diminished by modifying its glycosyl sequence. We exploited the discovery that the bacterium *Bacteroides thetaiotaomicron* (Bt) produces a suite of enzymes that fragment RG-II (Ndeh et al [2017] Nature 544: 65) to generate a library of RG-II glycoforms. These glycoforms together with naturally occurring glycoforms were used to show that:

- Dimer formation *in vitro* is sensitive to changes in the glycosyl sequences of chains A and B.
- The rate of dimer formation is tunable since it may be increased or decreased by the simultaneous modification of chains A and B.
- The dimer may adopt a structure that is largely resistant to the Bt enzymes

We have combined our experimental efforts with molecular modeling studies to:

- Unravel the reaction mechanism of borate cross-linking with apiose
- Develop accurate computational protocols for predicting ^{13}C and ^{1}H chemical shifts in apio-borate complexes
- Evaluated distinct arrangements of sidechains on the galacturonan backbone using molecular dynamics simulations to corroborate experimental NMR data on the 3-D structure of the RG-II monomer

We are developing and using catalytically inactivated Bt glycanses as probes for the specific detection of RG-II in the cell wall and in the cells of different plant tissues.

Science objectives for 2022-2023:

- Determine the role of acetyl esters in dimer formation
- Determine the effects on dimer formation of removing chains C and D
- Determine the mechanism by which aryl boronates inhibit dimer formation
- Develop inactivated Bt enzymes as probes that specifically recognize the monomer or the dimer
- Develop biophysical metrics from molecular simulations of the RG-II monomer that better correlate structure with dimerization propensity
- Develop molecular models of the borate esterified RG-II monomer and dimer to gain further insight into the mechanisms of dimerization

Our project addresses BES cross-cutting priority areas by:
combining biochemical and spectroscopic data with molecular and quantum mechanical modeling to understand the cation-catalyzed borate ester-crosslinking of a biopolymer. Such studies have relevance to BES-funded Biomolecular Materials, Catalysis Science, Computational and Theoretical Chemistry; BER-funded BRC research in plant cell walls; and OS-funded research in ASCR and the High-Performance Computing Initiative. Our research is relevant to the biomaterials community as it will allow us to better understand the complex polymer/polymer interactions that give rise to the mesoscale properties of these materials

Our scientific area(s) of expertise is/are: Cell wall structure and function, polysaccharide chemistry and molecular biology (CCRC), Molecular modelling (quantum mechanics and molecular dynamics) of complex carbohydrates, biopolymers and enzymes (NREL).

The ideal collaborator for our project would have expertise in: (i) Solid state NMR spectroscopy. We believe that recent developments in high-resolution ssNMR of cell walls will provide information on the architecture and properties of walls that lack the borate crosslink. (ii) Data-driven machine learning methods to analyze large datasets generated from molecular simulations to develop robust structure-property-function relationships.

Publications supported by this project 2017 - current:

1. O'Neill, MA., Black, I., Urbanowicz, B, Bharadwaj, V, Crowley, M., Koj, S. Pena MJ (2020) Locating methyl-etherified and methyl-esterified uronic acid residues in the plant cell wall pectic polysaccharide rhamnogalacturonan II. SLAS Technol 25:329
2. Bharadwaj. VS, Crowley, MF., Peña, MJ, Urbanowicz, B O'Neill, M (2020) Mechanism and Reaction Energy Landscape for Apiose Cross-Linking by Boric Acid in Rhamnogalacturonan II. J. Phys. Chem. B 124: 45
3. Barnes, WJ, Koj, S., Black, IM, Aracher-Hartmann, SA., Urbanowicz, BR., Pena, MJ, O'Neill, MA (2021) Protocols for isolating and characterizing polysaccharides from plant cell walls: a case study using rhamnogalacturonan II. Biotechnol Biofuels 14 142
4. Bharadwaj,VS. Westawker, LP, Crowley, MF (2022) Towards Elucidating Structure–Spectra Relationships in Rhamnogalacturonan II: Computational Protocols for Accurate ^{13}C and ^{1}H Shifts for Apiose and Its Borate Esters. Front Mol Biosci. 8: 756219.

Novel microbial based enzymatic CO_2 fixation mechanisms: conformational control of enzymatic reactivity

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Overall research goals:

The broad, long-term goal of the research is to provide insights into the mechanism of novel carboxylation and electron transfer reactions that use large scale conformational changes to control reactivity. These generate unstable intermediates that need to be either directed down a certain reaction pathway or protected from side reactions such as decomposition reactions in aqueous solvents. In many systems, reactivity is controlled through large scale conformational change triggered by specific reaction events.

Three project aims each probe a different fundamental mechanism of conformational control of reactivity. The first aim investigates how 2-ketopropyl-coenzyme M oxidoreductase/carboxylase (2-KPCC) couples the binding of substrate with conformational changes that encapsulate 2-ketopropyl coenzyme M (2-KPC), allowing the enzyme to discriminate substrate electrophiles, CO_2 and protons, from water. The second aim examines how acetone carboxylase (AC) couples ATP binding and reaction of the substrates, acetone and bicarbonate, with large conformational changes which protect ATP hydrolysis and the delivery of reactive intermediates to a remote metal site. The final aim investigates how conformational changes gate electron flow in FixABCX, an electron bifurcating member of the Etf family of enzymes.

Significant achievements (2021-2022):

The mechanism of 2-ketopropyl coenzyme M oxidoreductase carboxylase (2-KPCC)

2-KPCC catalyzes the reductive cleavage of S-S bonds and carboxylation of 2-KPC to produce acetoacetate and CoM in the final step of the epoxypropane carboxylation pathway in *Xanthobacter autotrophicus*. During this grant period, we have made significant progress in investigating how the 2-KPCC structure promotes carboxylation of substrate. Structural, spectroscopic, and catalytic data show that through amino acid substitution in the active site, 2-KPCC differentiates itself from canonical DSOR enzymes promoting carboxylation and the formation of acetoacetate. We also have revealed that biosynthesis of CoM required for 2-KPCC catalysis, occurs by evolutionarily distinct biosynthetic pathways in bacteria and archaea. The five-step pathway in bacteria involves the addition of sulfite, the elimination of phosphate, decarboxylation, thiolation, and the reduction to affect sequential conversion of phosphoenolpyruvate to CoM. The work reveals new reactivities for members of large aspartase/fumarase and 5'-phosphate-dependent enzyme families.

The mechanism of ATP-dependent acetone carboxylation by acetone carboxylase (AC)

AC converts acetone and bicarbonate into acetoacetate, thereby incorporating both substrates into biomass. During this grant period, we have investigated the discrepancy between the metal identity in two highly similar ACs from *X. autotrophicus* and *Aromatoleum aromaticum* and the conformational changes observed in the crystal structures solved with various ligand bounds states. Previous studies suggested the ACs from *X. autotrophicus* and *A. aromaticum* require different metals for catalysis, Mn(II) and Fe(II), respectively, which we verified using recombinant ACs. Swapping the metals renders each protein inactive. Using *X. autotrophicus* AC, we have uncovered residues critical to the conformational changes triggered by AMP, and important for production of acetoacetate. Manuscripts describing these results and new insights are in preparation.

FixABCD conformational dynamics

During nitrogen fixation, *Azotobacter vinelandii*, generates low potential electrons in the form of reduced ferredoxin (Fd) and flavodoxin (Fld) using two distinct mechanisms via the enzyme complexes Fix and Rnf1. Fix has a unique mechanism of action called flavin based electron bifurcation which couples the exergonic reduction of quinone with the endergonic reduction of Fd/Fld. Rnf1 uses the proton motive force to provide the additional energy required for the reaction. Using complementary physiological studies and thermodynamic

modeling, we have demonstrated that Fix is favored for electron transport to nitrogenase under oxygen-limited conditions. Rnf1is presumed to maintain reduced Fd/Fld production for nitrogenase under standard conditions (oxygen excess). We have recently optimized the purification of FixABCX protein complex from *A. vinelandii* cells and are deconvoluting the spectral contributions of the individual cofactors and the determination of reduction potentials to define the energy landscape of FixABCX.

Pipeline for conformational dynamics studies

Conformationally flexible proteins are often incompatible with X-ray crystallography and Cryo-EM, resulting in fundamental gaps in our understanding of the structure-function relationship for many interesting enzymes. Given our interest in conformational change in catalysis, we have developed a pipeline involving next generation protein structure prediction, small angle x-ray scattering (SAXS) and a suite of mass spectrometry (MS) techniques that refine protein structure models and allow extrapolation to alternative conformations. Enzymes are first poised in specific conformational states. Through SAXS analysis, we can determine particle dimensions and shape. When coupled to MS chemical crosslinking, surface labeling, and limited proteolysis, we derive information about residue proximity and solvent accessibility. Hydrogen-deuterium exchange MS and normal mode analysis complement this approach by providing data on dynamic regions of the protein complexes. Two manuscripts are in preparation that describe aspects of these integrated approaches to examine protein conformation changes mapped onto enzymatic catalytic cycles.

Science priorities for the next year (2022-2023):

- Examine protein conformational changes in the AC catalytic cycle and residues that regulate the changes
- Probe the mechanism of AC and the role of the metal ion and metal coordination in catalysis
- Define the reduction potentials and energy landscape of bifurcation in FixABCX
- Map the FixABCX conformational states on the catalytic cycle probing electron transfer gating

Our major scientific area(s) of expertise is/are: Protein structure, function and enzyme mechanism

To take my project to the next level, my ideal collaborator would have expertise in: We would be very interested in collaborators interested in applying our pipeline for examining difficult structural targets in multiple conformational states.

Publications supported by this project: 2021-2022:

1. H.H. Wu, M.D. Pun, C.E. Wise, B.R. Streit, F. Mus, A. Berim, W.M. Kincannon, A. Islam, S.E. Partovi, D.R. Gang, J.L. DuBois, C.E. Lubner, C.E. Berkman, B.M. Lange, J.W. Peters, "The pathway for coenzyme M biosynthesis in bacteria." Proc Natl Acad Sci U S A. **19**,36 (2022).doi: 10.1073/pnas.2207190119.
2. A.B. Alleman, A. Garcia Costas, F. Mus, J.W. Peters, 2022. "Rnf and Fix have specific roles during aerobic nitrogen fixation in *Azotobacter vinelandii*."Appl. Environ. Microbiol. **88**,17 (2022).doi:10.1128/aem.01049-22.
3. J.R. Mattice, K.A. Shisler, J.L. DuBois, J.W. Peters, B. Bothner, "A catalytic dyad modulates conformational change in the CO_2-fixing flavoenzyme 2-ketopropyl coenzyme M oxidoreductase/carboxylase." J Biol Chem. **298**,5 (2022).doi: 10.1016/j.jbc.2022.101884.
4. C.E. Wise, A.E. Ledinina, D.W. Mulder, K.J. Chou, J.W. Peters, P.W. King, C.E. Lubner, "An uncharacteristically low-potential flavin governs the energy landscape of electron bifurcation." Proc Natl Acad Sci U S A., **119**,12 (2022).doi: 10.1073/pnas.2117882119.
5. A.B. Alleman, F. Mus, J.W. Peters, "Metabolic model of the nitrogen-fixing obligate aerobe *Azotobacter vinelandii* predicts its adaptation to oxygen concentration and metal availability." mBio **12**, 6 (2021).doi: 10.1128/mBio.02593-21.
6. G.A. Prussia, K.A. Shisler, O.A. Zadvornyy, B.R. Streit, J.L. DuBois, J.W. Peters, "The unique Phe-His dyad of 2-ketopropyl coenzyme M oxidoreductase/carboxylase selectively promotes carboxylation and S-C bond cleavage." J Biol Chem. **297**,2 (2021).doi: 10.1016/j.jbc.2021.100961.
7. J.W, Peters, O. Einsle, D.R. Dean, S. DeBeer, B.M. Hoffman, P.L. Holland, L.C. Seefeldt. Comment on "Structural evidence for a dynamic metallocofactor during N2 reduction by Mo-nitrogenase" Science **371** (2021).doi: 10.1126/science.abe5481.

Enzymology of Methanogenesis: Mechanism of Methyl-Coenzyme M Reductase

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Overall research goals:

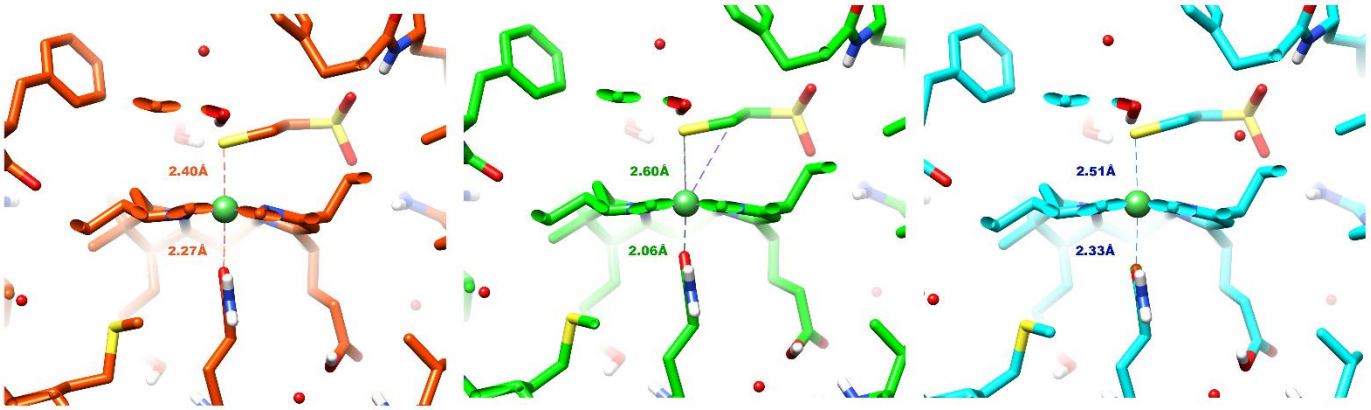
Our overall research goals are to address gaps in our understanding of the catalytic mechanism of Methyl-Coenzyme M Reductase (MCR), the key enzyme in methanogenesis and methane oxidation. We will uncover the active structures as well as the intermediates of MCR presented in the three aims below:

1. Determine the X-ray and neutron diffraction structures of the active Ni(I) state of MCR in the absence and presence of substrates. We have developed methods to grow large crystals of active Ni(I) MCR that are suitable for neutron diffraction and small 20–80-micron sized crystals for serial femtosecond X-ray Free Electron Laser (XFEL) studies. As a proof of concept, we have published the first room temperature structure of MCR using this XFEL method.
2. Investigate our hypothesis that forward and reverse methanogenesis proceed via long-range electron transfer from Ni(I)-sulfonate complexes of MCR with methyl-SCoM and CoMSSCoB using kinetic, spectroscopic, and computational approaches. Furthermore, XFEL studies allow the addition of HSCoB to a Ni(I) bound CH_3SCoM crystal us a Drop-On-Demand substrate delivery system to track structural changes along the reaction pathway in real time. We expect to observe important steps in the catalytic cycle, where the mechanistic CH_3-S bond cleavage occurs, and how the heterodisulfide CoBSSCoM is formed.
3. Generate and characterize stable counterparts of the CoBS• thiyl and CoMSSCoB•- anion radical intermediates. We are synthesizing CoB analogs that should allow detection of these proposed intermediate in the MCR reaction cycle via spectroscopic (UV-visible, EPR, XAS, MCD), kinetic (steady-state and transient) and computational methods.

Significant achievements: (2021 – 2022)

Using serial femtosecond XFEL crystallography, we have proved the capability of our method to structurally analyze MCR. This characterization was published in the Journal of Inorganic Biochemistry as the first room temperature structure of inactive Ni(II) MCR (Fig 1 Left: PDB, 7SUC). This structure provided a fully occupied inhibitor CoM present along the purification process of the enzyme as well as the native substrate CoB although 5 rounds of 10x diluted buffer exchange of MCR was performed. This provided the conclusion that the oxygen degraded Ni(II)-inactive state of the enzyme promotes a "locked-in" state where substrates may not be exchanged. To study the Ni(I) active MCR, well-diffracting crystals of all sizes ranging from 2mm for neutron diffraction to 20-80 microns for XFEL have been obtained using an anaerobic crimped batch-without oil method. This method has provided two XFEL structures on Ni(I) MCR enzyme samples resulting in new unit cell distributions that differ from all Ni(II) structures of MCR (Fig 1 Middle and Right). This Ni(I) sample structure is surprisingly bound by the inhibitor CoM where the S-Ni distance is 2.6Å but is supported by past X-ray Absorption Spectroscopy (XAS) resulting in a non-coordinated CoM outside of the 2.5Å detectable limit of XAS. To study the proposed Ni-sulfonate binding mode, we have synthesized MeCoM labeled with exchangeable O^{17} on the sulfonate functional group to understand how the molecular and electronic structure of the paramagnetic Ni(I) is perturbed compared to the native substrate MeCoM. In

this experiment, it was found that the Ni(I) site had no discernable difference in its electronic structure of the O^{17}- compared to O^{16}-labeled MeCoM. While this appears to negate our Ni(I)-sulfonate binding hypothesis, it supports a markedly different MeCoM binding mode consistent with the proposed Ni(I) XFEL structure and with X-ray absorption studies of Ni(I)-MCR, in which the Ni lacks the S of (Me)CoM as an upper axial ligand. This is in contrast to all Ni(II)-MCR structures.



Ni(II)- MCR 7SUC (1.9Å) Ni(I)-Unit Cell 1 (1.85Å) Ni(I)- Unit Cell 2 (1.75Å)

Science objectives for 2022-2023:

- Validate the proposed Ni(I) active MCR Structure
- Obtain structures of methoxy-CoM and butane sulfonate-bound MCR
- Structurally and electronically elucidate the intermediates of the MCR pathway by.
- Characterize the CoBS(dot) and the methyl radical by EPR and ENDOR spectroscopy

My project addresses BES cross-cutting priority areas by:
Studying electron transfer and synthesis and metabolism of biofuels in the protein MCR, which is relevant to the design of new technologies benefitting from highly specific and efficient flows of energy at the molecular scale.

My scientific area(s) of expertise is/are: Bioinorganic chemistry, Enzyme mechanisms, anaerobic microbiology and biochemistry, Enzyme kinetics, Spectroscopy.

The ideal collaborator for my project would have expertise in: Cutting edge methods in crystallography, neutron diffraction, computational chemistry, and organic synthesis.

Publications supported by this project [Click to Enter Years of Current Grant/FWP, e.g. 2012-2014]:

1. Patwardhan, A.; Sarangi, R.; Ginovska, B.; Raugei, S.; Ragsdale, S. W. Nickel-Sulfonate Mode of Substrate Binding for Forward and Reverse Reactions of Methyl-SCoM Reductase Suggest a Radical Mechanism Involving Long-Range Electron Transfer. *J Am Chem Soc* **2021**, *143* (14), 5481–5496. https://doi.org/10.1021/jacs.1c01086.
2. Ohmer, C. J.; Dasgupta, M.; Patwardhan, A.; Bogacz, I.; Kaminsky, C.; Doyle, M. D.; Chen, P. Y.; Keable, S. M.; Makita, H.; Simon, P. S.; Massad, R.; Fransson, T.; Chatterjee, R.; Bhowmick, A.; Paley, D. W.; Moriarty, N. W.; Brewster, A. S.; Gee, L. B.; Alonso-Mori, R.; Moss, F.; Fuller, F. D.; Batyuk, A.; Sauter, N. K.; Bergmann, U.; Drennan, C. L.; Yachandra, V. K.; Yano, J.; Kern, J. F.; Ragsdale, S. W. XFEL serial crystallography reveals the room temperature structure of methyl-coenzyme M reductase. *J. Inorg. Biochem.* **2022**, *230*, 111768, DOI: 10.1016/j.jinorgbio.2022.111768

Unusual Redox Biochemistry of Energy Conservation in Bacteria, Archaea, and Syntrophic Consortia

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Overall research goals:

We are exploring novel energy-relevant redox biochemical mechanisms in prokaryotes. Aerobic bioenergetics serves as both an underlying theme and launch point. Specifically, we are investigating structure-function relationships in membrane-integral supramolecular energy-transducing machines to understand how these metalloprotein assemblies control electron flow in biological systems. Results from our work inform biological carbon capture and bio-inspired catalyst design as well.

Significant achievements: [2019-2022]:

- We have discovered and characterized a bacterial metallo-formate dehydrogenase (FDH2) that functions under normal atmospheric conditions in the presence of 21% O_2. Our work dispels the established paradigm that mFDHs cannot operate in air due to extreme O_2 sensitivity of their metal centers. FDH2 is exceptional because it: (a) reduces benzyl viologen under fully aerobic conditions; (b) concurrently catalyzes both FDH and formate oxidase (FOX) reactions; and (c) generates stoichiometric amounts of hydrogen peroxide (H_2O_2) from O_2 without releasing superoxide ($O_2^{\bullet-}$). Notably, whereas all previously characterized mFDHs required purification in the presence of an inhibitor (*e.g.,* nitrate or azide) or isolated in an inactivated state, FDH2 can be obtained without any of these complications. ^{1}H and ^{17}O-NMR measurements (in collaboration with Ad Bax, NIH) have shown that for each mole of formate oxidized by FDH2, one mole of H_2O_2 is produced. Similarly, NMR results reveal that O_2 and cytochrome *c* (Cyt *c*) are simultaneously and stoichiometrically reduced following formate oxidation. We have advanced an aerobic electron bifurcation mechanism to rationalize our findings (**Figure 1**). Here, the tungsten cofactor (Wco) accomplishes both formate oxidation and O_2 reduction. Consequently, the [4Fe-4S] clusters can mediate electron transfer (ET) to Cyt *c* even in the presence of O_2.

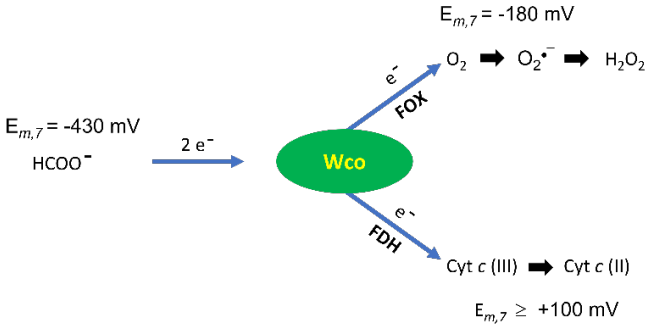

Figure 1. Proposed mechanism for the concomitant reduction of O_2 and Cyt *c* by FDH2.

- We have identified and characterized the native redox partner of FDH2. It contains 11 *c*-hemes, thus garnering the name undecaheme Cyt *c* (Uhc). Furthermore, in collaboration with Hartmut Michel (Max Planck Institute, Frankfurt), we have solved the 2.8 Å resolution cryo-EM structure of FDH2 in complex with Uhc (**Figure 2**). This complex supplies electrons for the 4e$^-$ reduction of O_2 to H_2O catalyzed by the unusual membrane-integral *c*-heme-dependent cytochrome *cbd* oxidase (CydCBD or CydAcA'; 17 transmembrane segments). Altogether, the five subunits form a supercomplex, hereafter referred to as a hardwired respirasome – it is self-sufficient, coupling periplasmic formate oxidation to O_2 reduction in the membrane, resulting in the generation of a proton motive force (PMF) that drives cellular ATP production. Currently, it is believed that bacteria lack respirasomes outside of CICIII$_2$CIV (comprised of respiratory complexes I, III and IV in a 1:2:1 ratio), which is common to both mitochondria and select prokaryotes (e.g., *Paracoccus denitrificans*). Our discovery offers incontrovertible direct proof for the existence of specialized respirasomes that function in energy conservation without involving membrane-embedded quinones as electron carriers.

- As proof-of-principle for the diversity of bacterial hardwired respirasomes, we have empirical evidence on hand that some systems couple thiosulfate or sulfide oxidation to O_2 reduction. These results have direct implications for gaining insights into energy homeostasis of cable bacteria (CB), which achieve ET over centimeter distances, as well as the fastidious and slow-growing chemolithoautotrophs, such as the aerobic nitrite-oxidizing bacteria (NOB). Both CB and NOB are extraordinary in that they lack heme-copper oxidase (HCO) family of O_2 reductases altogether. Instead, they encode genes for one or more atypical cytochrome *bd* oxidase (CydBD)-like systems whose function remains unknown.

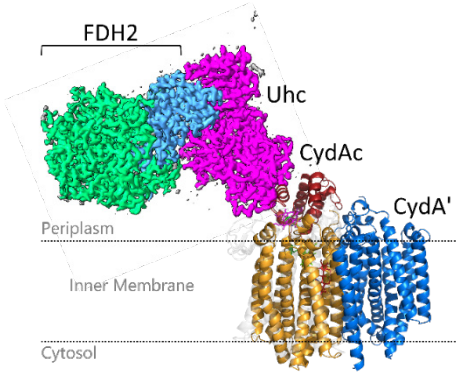

Figure 2. Hardwired respirasome. Cryo-EM structure of FDH2-Uhc complex docked on to AlphaFold v2.1 predicted CydAcA' dimer.

- We have demonstrated that some methanogens use CydBD to enable aerobic respiration. Using diffuse transmittance spectroscopy (DTS), we have shown for the first time that CydBD is natively expressed in whole cells of an obligately methyl-reducing methanogen, *Methanimicrococcus blatticola* (Mb). When Mb is sparged with H_2 gas, *d*-heme reduction ensues, uncovering a new respiratory circuit. In addition to purifying and characterizing native CydBD from stationary phase *M. blatticola* cells, we have used a synthetic biology approach to co-reconstitute Mb-CydBD and *Escherichia coli* ATP synthase into menaquinone-7-enriched proteoliposomes. Subsequently, we have shown that coupling quinol oxidation to O_2 reduction in the latter results in a PMF, which drives ATP production. Through chemical biology, we have identified a quinone-binding site in Mb-CydBD. Collectively, these findings underscore that methanogenic archaea utilize and benefit from both quinones and O_2. Please visit our poster for additional details.

Science objectives for 2021-2022:

- Investigate the ability of FDH2 to catalyze aerobic CO_2 reduction reaction (CO2RR).
- Completing the cryo-EM structure determination of a hardwired respirasome in collaboration with Keiichi Namba, Japan.
- Probe alternate ET mechanisms that facilitate energy conservation in syntrophic consortia

My project addresses BES cross-cutting priority areas by: (a) Seeking fundamental insights into the foundational science of biological CO_2 removal vis-à-vis aerobic CO2RR by FDH2. (b) Discover unconventional biotechnology-relevant materials for gleaning molecular-level understanding of the redox biochemistry occurring in metalloenzyme active sites, and (c) Enabling innovative approaches for deciphering biochemical and biophysical mechanisms that underpin microbial energy capture.

My scientific area(s) of expertise is/are: Redox biochemistry of membrane-integral metalloprotein assemblies, Mechanistic enzymology, Bioinorganic chemistry, Structural biology, Whole cell systems biology, Biochemistry of heme, iron-sulfur clusters and redox mediators, Microbial respiration.

The ideal collaborator for my project would have expertise in: Whole cell electromicrobiology, Single-molecule super-resolution imaging, Tungsten Mössbauer Spectroscopy.

Publications supported by this project [2019 - 2022]:

1. Graham, J.E., Niks, D., Zane, G.M., Gui, Q., Hom, K., Hille, R., Wall, J.D., Raman, C.S. (2022) How a Formate Dehydrogenase Responds to Oxygen: Unexpected O_2 insensitivity of an Enzyme Harboring Tungstopterin, Selenocysteine, and [4Fe–4S] Clusters. ***ACS Catal.*** 12: 10449–10471. https://doi.org/10.1021/acscatal.2c00316
2. Raman, C. S., Graham, J.E. (2022) Formate/Air Biofuel Cell and Method for Producing It. **U.S. Patent Application** Number: 63/298,307.

Enzymatic Energy Conversion

Simone Raugei, Principal Investigator
Bojana Ginovska, Marcel Baer, Lance C. Seefeldt, Co-PIs
Hoshin Kim, Conrad Johnston, Suman Samantray, Postdoctoral Research Associates
Pacific Northwest National Laboratory
Email: simone.raugei@pnnl.gov

Overall research goals:

The Physical Biosciences program at PNNL aims to provide a better understanding of the core principles employed by enzymes to control the flow of energy and matter to achieve remarkable specificities, efficiencies, and catalytic rates. The program integrates state-of-the-art theory and computation with experimental efforts across the U.S. Department of Energy's Basic Energy Sciences Physical Biosciences community to fill critical gaps in knowledge about how enzymes orchestrate spatial and temporal events to direct electrons, protons, and substrates for selective conversions and allosteric regulation.

Our research is divided into three general themes to fill outstanding gaps in knowledge and explore transferability of concepts learned: understand how dynamic confinement imposed by the enzyme scaffold controls specificity and selectivity (Theme 1); understand how electrons and protons are delivered and accumulated at the active site (Theme 2); and understand how mechanical energy and electro- or thermochemical energy are interconverted in biomolecules (Theme 3).

Significant achievements: [Years of Current Funding: 2020-2023]:

Characterization of the Chemical Nature of the Central Carbon in the Nitrogenase FeMo-Cofactor. Substrates and inhibitors of Mo-dependent nitrogenase bind and react at Fe ions of the active-site FeMo-cofactor contained within the MoFe protein α-subunit. The cofactor contains a CFe_6 core, a carbon centered within a trigonal prism of six Fe, whose role in catalysis is unknown. Targeted ^{13}C labeling of the carbon (by Seefeldt and Dean, VirginiaTech) enabled ENDOR spectroscopy (Hoffman, Northwestern University) to sensitively monitor the electronic properties of the Fe-C bonds for several reaction intermediates and inhibited states, and showed that all exhibit near-zero ^{13}C isotropic hyperfine coupling constants. Density functional theory analysis of the C-Fe bonds showed this occurs because of a (3spin-up/3spin-down) spin-exchange configuration of CFe_6 Fe-ion spins that produces cancellation of large spin-transfers to carbon in each Fe-C bond. The persistent structure and Fe-C bonding of the CFe_6 core indicates it does not provide a functionally dynamic (hemilabile) 'beating heart' - instead it acts as 'a heart of steel', stabilizing the structure of the FeMo-cofactor during catalysis.

Selectivity Determinants in Monoterpene Synthases. Monoterpenes are key metabolites in mediating plant–plant communication and the interaction between plants and other organisms. The first step in monoterpene biosynthesis is catalyzed by monoterpene synthases (MTSs). MTSs share a common structure and convert the same prenyl diphosphate precursor to acyclic, monocyclic, and/or bicyclic products. Using two MTSs as models—(4*S*)-(-)-limonene synthase (LMNS) and (+)-bornyl diphosphate synthase (BPPS)—we implemented an iterative approach that involves comparative atomistic simulations and experimental testing (Lange, Washington State University) of wild-type enzymes and selected variants to identify the mechanistic underpinnings of selectivity. Atomistic simulations indicated for the first time that a common reaction intermediate, the α-terpinyl cation (ATC), preferentially adopts one of two different conformations in LMNS and BPPS, thus leading to the formation of monocyclic monoterpenes in the former and bicyclic products in the latter. An assessment of the ATC absolute binding free energy in LMNS, BPPS, and selected variants revealed that non-bonded interactions with active site residues explain the propensity of the ATC to assume a favored conformation that is consistent with the experimentally determined reaction outcome. The integration of atomistic simulations with experimental validation allowed us to assign specific functions to active site residues for controlling the ratio of monocyclic to bicyclic products in model MTSs.

Regioselectivity Mechanism of Desaturases. Desaturases catalyze regiospecific dehydrogenation of fatty acids, creating a carbon–carbon double bond with the concomitant reduction of dioxygen to water. We studied the first steps in the substrate delivery by the acyl carrier protein (ACP) to the castor Δ^9, ivy Δ^4, and thunbergia Δ^6 desaturases. Atomistic simulations showed a varying degree of local dynamics and structural variability

depending on the acyl-chain size, suggesting that substrate-specific changes in ACP structure and dynamics have a crucial impact on the desaturase enzymatic activity. Docking simulations of ACP to castor Δ^9-desaturase and ivy Δ^4-desaturase suggested that ACP–desaturase interactions could lead to a preferential selection between the motifs. Key residues were computationally identified that regulate the orientation of the ACP to the thunbergia Δ^6 desaturase. In silico mutant studies of castor Δ^9 and thunbergia Δ^6 single out a steric clash in the C-terminal regions of the castor that prevents a Δ^6 favorable orientation between the ACP recognition helix and helix X in the desaturase. Chimeras suggested by computations and expressed by Shanklin at Brookhaven National Laboratory validated these findings by converting a castor Δ^9 into a Δ^6 by mutating the identified key residues and swapping the C-terminal end between the castor and thunbergia desaturases.

Science objectives for 2022-2023:
Understand how dynamic confinement imposed by the enzyme scaffold controls specificity and selectivity.

My project addresses BES cross-cutting priority areas by:
Gathering fundamental understanding to predict, manipulate, and design biochemical processes that underpin innovations for energy conversion.

My scientific area(s) of expertise is/are: Computational Biophysics and Biochemistry.

The ideal collaborator for my project would have expertise in: experimental characterization of enzymatic mechanisms and protein redesign. In this regard, we are already fruitfully collaborating with John Shanklin (BNL), Steven Ragsdale (U. Michigan), M. Lange (WSU), John Peters (WSU), Brian Hoffman (Northwestern University), and Yi Lu (U. Texas, Austin/PNNL).

Publications supported by this project (2020-present)

1. D. A. Lukoyanov, Z.-Y. Yang, A. Pérez-González, S. Raugei, D. R. Dean, L. C. Seefeldt, B. M. Hoffman. *13C ENDOR Characterization of the Central Carbon within the Nitrogenase Catalytic Cofactor Indicates That the CFe6 Core Is a Stabilizing "Heart of Steel"*. J. Am. Chem. Soc. Published online on 9/27/2022, DOI: 10.1021/jacs.2c06149.
2. H. Kim, I. Srividya, I. Lange, E. W. Huchala, B. Ginovska, B. M. Lange, S. Raugei. *Determinants of Selectivity for the Formation of Monocyclic and Bicyclic Products in Monoterpene Synthases.* ACS Catal. 12 (2022) 7453.
3. J. E. Guy, Y. Cai, M. D. Baer, E. Whittle, J. Chai, Y. Lindqvist, S. Raugei, J. Shanklin. *Regioselectivity mechanism of the Thunbergia alata Δ6-16:0-acyl carrier protein desaturase*. Plant Phys. 88 (2022) 1537.
4. A. Patwardhan, R. Sarangi, B. Ginovska, S. Raugei, S. W. Ragsdale. *Nickel–Sulfonate Mode of Substrate Binding for Forward and Reverse Reactions of Methyl-SCoM Reductase Suggest a Radical Mechanism Involving Long-Range Electron Transfer*. J. Am. Chem. Soc. 143 (2021) 5481.
5. D. Tzeli, S. Raugei, S. S. Xantheas. *Quantitative Account of the Bonding Properties of a Rubredoxin Model Complex* $[Fe(SCH_3)_4]^q$, $q = -2, -1, +2, +3$. J. Chem. Theory Comput. 17 (2021) 6080.
6. Q. Huang, M. Tokmina-Lukaszewska, L. E. Johnson, H. Kallas, B. Ginovska, J. W. Peters, L. C. Seefeldt, B. Bothner, S. Raugei. *Mechanical Coupling in the Nitrogenase Complex*. PLOS Comp. Biol., 17 (2021) e1008719.
7. M. D. Baer, J. Shanklin, S. Raugei. *Atomistic insights on Structure and Dynamics of Spinach Acyl Carrier Protein with Varying Substrate Length*. Biophys. J. 17 (2021) 3841.
8. D. A. Lukoyanov, Z. Yang, D. R. Dean, L. C. Seefeldtd, S. Raugei, B. M. Hoffman. *Electron Redistribution within the Nitrogenase Active Site FeMo-cofactor During Reductive Elimination of* H_2 *to Achieve* $N{\equiv}N$ *Triple-Bond Activation*. J. Am. Chem. Soc., 142 (2020) 21679.
9. L. C. Seefeldt, Z.-Y. Yang, D. A. Lukoyanov, D. F. Harris, D. R. Dean, S. Raugei, B. M. Hoffman. *Reduction of Substrates by Nitrogenases*. Chem. Rev. 120 (2020) 5082.
10. J. H. Art, O. A. Zadvornyy, D. W. Mulder, S. M. Keable, A. E. Cohen, M. W. Ratzloff, S. G. Williams, B. Ginovska, N. Kumar, J. Song, S. E. McPhillips, C. M. Davidson, A. Y. Lyubimov, N. Pence, G. J. Schut, A. K. Jones, S. M. Soltis, M. W. W. Adams, S. Raugei, P. W. King, J. W. Peters. *Tuning catalytic bias of hydrogen gas producing hydrogenases*. J. Am. Chem. Soc. 141 (2020) 1227.

Light-driven activation of small molecules by nitrogenase hybrids

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Overall research goals:

Nitrogenase is a versatile metalloenzyme that is capable of ambient activation and reduction of a wide range of small molecules, such as N_2, CO, and CO_2. In this project, we propose to generate a light-driven electron transport system for small molecule activation based on nitrogenase proteins and their homologs from phototrophic bacteria, taking advantage of the similarities and distinctions among these homologous proteins that can be utilized to combine key elements with different functionalities and distinct locations to harvest light and drive reduction of substrates at the nitrogenase cofactor center. Success in generating light-driven nitrogenase hybrids for small molecule activation will facilitate time-resolved mechanistic investigation of nitrogenase-catalyzed reactions.

Significant achievements (up to 2021):

Our work in the last period focused on establishing the feasibility of generating nitrogenase hybrids with altered cofactor compositions. Specifically, we targeted the heterometal and organic ligand of the cofactor and examined the impact of alterations of these components on the reactivities of nitrogenase toward N_2 and CO. With respect to the heterometal, we generated a V-nitrogenase hybrid containing the cofactor of the Mo-nitrogenase (*i.e.*, M-cluster) and, *vice versa*, a Mo-nitrogenase hybrid containing the cofactor of the V-nitrogenase (*i.e.*, V-cluster) via combined *in vivo* and *in vitro* approaches.[1,2] Comparative biochemical and spectroscopic analyses of these variants with their respective native counterparts provided a first look into the "weighted" contributions of protein environment and cofactor properties to the substrate-reducing activities of nitrogenase, thereby shedding important light on the structural determinants that are crucial for nitrogenase catalysis. With respect to the organic ligand, we generated citrate-substituted M- and V-clusters, respectively, in the Mo- and V-nitrogenases via genetic manipulations.[3,4] Biochemical and spectroscopic analyses of these hybrids revealed a disparate impact of citrate substitution on N_2- and CO-reduction by nitrogenase, pointing to a crucial role of homocitrate in nitrogenase catalysis and the possibility to tune product profiles of nitrogenase reactions via organic ligand substitution. Together, our studies in the last grant period have established the feasibility and utility of the hybrid approach for nitrogenase systems and, together with the strategies we developed for the heterologous expression of nitrogenase proteins and their homologs (including NifEN, NifDK and BchNB) in *E. coli* and the preliminary results we obtained on these heterologously expressed proteins, they have laid an important foundation for the proposed research that centers on creating nitrogenase-based hybrids via a "swapping" of structural elements between the homologous, yet distinct nitrogenase proteins and homologs.

Science objectives for 2022-2024:

Based on the sequence and structural comparisons of NifDK, NifEN and BchNB and the preliminary data collected on them, we hypothesize that each of the three proteins can be used as a mutational template to combine their key catalytic components and create an electron transport pathway from Pchlide → [4Fe4S] or P-cluster →

L- or M-cluster to enable light-driven catalysis at the nitrogenase cofactor site. Specifically, we hypothesize that we can create a hybrid for the light-driven reduction of "simple" substrates at the surface exposed L-cluster site by introducing the "fishing pole" of NifEN into BchNB for L-cluster attachment or, conversely, by introducing the Pchlide-binding "pocket" of BchNB into NifEN. We further theorize that we can enhance the electron flow to the L-cluster and broaden the substrate profile by recreating the P-cluster site in the BchNB variant carrying the "fishing pole" or in the NifEN variant carrying the Pchlide-binding "pocket". Finally, we hypothesize that we can create a hybrid for light-driven catalysis of more "complex" substrates at the buried M-cluster site by introducing the Pchlide-binding "pocket" of BchNB into NifDK. We will tackle this research in a collaborative effort, with the genetic, biochemical and biophysical work performed by our groups at UC Irvine, and the ENDOR/ESEEM/EDNMR, XAS/EXAFS and XFEL analyses performed in collaboration with the Britt group (UC Davis), the Hodgson/Hedman group (Stanford) and the Yachandra/Yano/Kern group (LBL).

My project addresses BES cross-cutting priority areas by:

Our efforts not only contribute to a better understanding of the mechanism of nitrogenase, but may also provide a proof-of-concept for developing cost-efficient strategies that harness light energy for the production of useful chemical commodities like ammonia and hydrocarbons. As such, our proposed research addresses the DOE mission of providing answers to America's energy and environmental challenges through transformative science and technology solutions.

My major scientific area(s) of expertise is/are: Bioinorganic chemistry

The ideal collaborator for my project would have expertise in: Mössbauer spectroscopy.

Publications supported by this project:

1. Rebelein JG, Lee CC, Newcomb M, Hu Y, Ribbe MW. Characterization of an M-Cluster-Substituted Nitrogenase VFe Protein. mBio. 2018 Mar 13;9(2):e00310-18. doi: 10.1128/mBio.00310-18.
2. Lee CC, Tanifuji K, Newcomb M, Liedtke J, Hu Y, Ribbe MW. A Comparative Analysis of the CO-Reducing Activities of MoFe Proteins Containing Mo- and V-Nitrogenase Cofactors. Chembiochem. 2018 Apr 4;19(7):649-653. doi: 10.1002/cbic.201800035.
3. Newcomb MP, Lee CC, Tanifuji K, Jasniewski AJ, Liedtke J, Ribbe MW, Hu Y. A V-Nitrogenase Variant Containing a Citrate-Substituted Cofactor. Chembiochem. 2020 Jun 15;21(12):1742-1748. doi: 10.1002/cbic.201900654.
4. Liedtke J, Lee CC, Tanifuji K, Jasniewski AJ, Ribbe MW, Hu Y. Characterization of a Mo-Nitrogenase Variant Containing a Citrate-Substituted Cofactor. Chembiochem. 2021 Jan 5;22(1):151-155. doi: 10.1002/cbic.202000598.
5. Jasniewski AJ, Lee CC, Ribbe MW, Hu Y. Reactivity, Mechanism, and Assembly of the Alternative Nitrogenases. Chem Rev. 2020 Jun 24;120(12):5107-5157. doi: 10.1021/acs.chemrev.9b00704.
6. Hu Y, Ribbe MW. Special Issue on Nitrogenases and Homologous Systems. Chembiochem. 2020 Jun 15;21(12):1668-1670. doi: 10.1002/cbic.202000279.

Missing links in biological methane and ammonia oxidation

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Overall research goals:

This project focuses on biochemical and functional characterization of proteins encoded by the extended pMMO/AMO operons. The overarching hypothesis is that these proteins play an important role in biological methane and ammonia oxidation, and as such, represent "missing links" in our understanding of these enzymes. The operons include the three genes encoding the enzyme subunits, *pmoB* (or *amoB*), *pmoA* (or *amoA*), and *pmoC* (or *amoC*). Directly adjacent to *pmoB*/*amoB* is a gene denoted *pmoD*/*amoD* followed by three genes encoding putative copper transport proteins, *copC, copD,* and *pmoF*. These four genes are coregulated with the pMMO genes in a copper-dependent fashion. CopC and PmoF belong to periplasmic copper chaperone families, and CopD is a putative copper importer. PmoD does not belong to any known protein family and homologs are only found in methane- and ammonia-oxidizing bacteria. PmoD consists of an N-terminal periplasmic cupredoxin domain followed by a predicted transmembrane helix. The isolated periplasmic domain forms a homodimer bridged by an unusual Cu_A-like site. Importantly, genetic disruption experiments show that PmoD is critical for methanotroph growth during pMMO-utilizing conditions, suggesting that it is indeed a missing link. However, its function and the specific role of the observed Cu_A-like site remain unknown. The project goals are divided into two specific aims. The first aim addresses whether the Cu_A-like site forms in vivo and its relation to the growth defect observed in the Δ*pmoD Methylosinus* (*Ms.*) *trichosporium* OB3b strain. The goal of the second aim is to determine why disruption of the *pmoD* gene impairs cell growth under pMMO-utilizing conditions. Each aim incorporates both in vitro and in vivo approaches.

Significant achievements: (2019-2022):

- We characterized the formation and decay of the PmoD Cu_A site using stopped-flow and electron paramagnetic resonance (EPR) spectroscopies (Fig. 1) and probed its electronic configuration and coordination via advanced paramagnetic resonance spectroscopies.
- We performed a bioinformatic analysis of PCu_AC domain-containing PmoF proteins, identified three classes in methanotrophs, PmoF1, PmoF2, and PmoF3, and showed that PmoF1 and PmoF2 bind only Cu(II), unlike typical PCu_AC domains, which bind a single Cu(I) ion.
- We determined crystal structures of PmoF1 and PmoF2, revealing an N-terminal histidine brace $HX_{10}H$ Cu(II) coordination motif (Fig. 2) resembling motifs in CopC proteins and lytic polysaccharide monooxygenases. This finding provides new insight into the biological significance of histidine brace coordination.

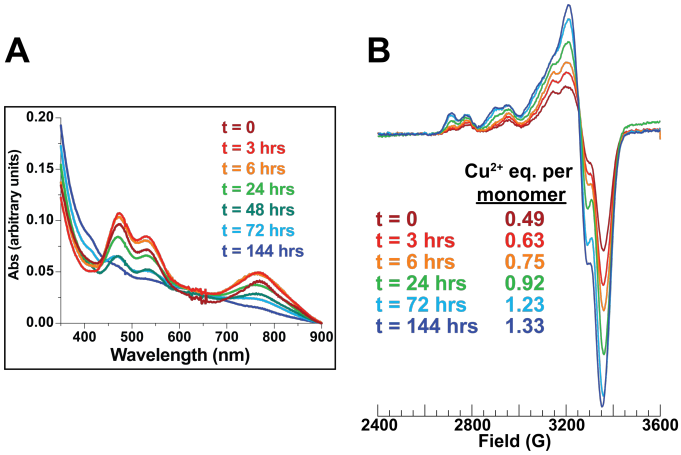

Fig. 1. Decay of the PmoD Cu_A site at 4 °C observed in parallel by (A) optical and (B) CW X-band EPR spectroscopies..

- We established a heterologous expression system for full-length PmoD, including the transmembrane domain.
- We completed the first steps toward establishing a genetic manipulation system for *Methylocystis* (*Mc.*) sp. strain Rockwell.

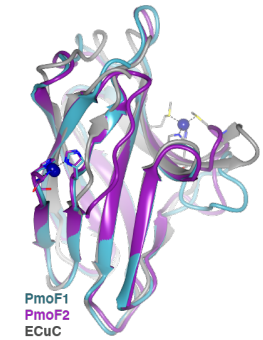

Fig. 2. Superposition of PmoF1 and PmoF2 with the homolog ECuC from *S. lividans*.

Science objectives for 2021-2022:

- We will purify full-length PmoD, evaluate its copper binding properties, determine whether it forms the previously observed Cu_A site, and pursue crystallization and structure determination. It may be necessary to reconstitute full-length PmoD into bicelles or nanodiscs to stabilize its transmembrane helix.
- We will investigate potential interactions between full-length PmoD and pMMO as well as between PmoD and PmoF, and explore the possibilities of copper or electron transfer between PmoD and pMMO.
- We will create a Δ*pmoD* strain of *Mc.* sp. Rockwell and determine its phenotype via growth assays. In parallel, we will generate a Cys41Ser variant, which will disrupt Cu_A site formation, and assess its phenotype.

My project addresses BES cross-cutting priority areas by:
Elucidating active site chemistry and redox reactivity, regulation of energy relevant reactions in methane-oxidizing bacteria

My scientific area(s) of expertise is/are: bioinorganic chemistry, structural biology, copper proteins, biological methane oxidation.

The ideal collaborator for my project would have expertise in: cryoelectron tomography (cryoET), molecular dynamics simulations of membranes and membrane proteins.

Publications supported by this project 2019-2022:

1. Fisher, O. S.; Sendzik, M. R.; Ross, M. O.; Lawton, T. J.; Hoffman, B. M.; Rosenzweig, A. C. PCu_AC domains from methane-oxidizing bacteria use a histidine brace to bind copper. *J. Biol. Chem.* **2019**, *294*, 16351-16363.
2. Ross, M. O.; Fisher, O. S.; Morgada, M. N.; Krzyaniak, M. D.; Wasielewski, M. R.; Vila, A. J.; Hoffman, B. M.; Rosenzweig, A. C. Formation and electronic structure of an atypical Cu_A site. *J. Am. Chem. Soc.* **2019**, *141*, 4678-468
3. Fisher, O. S.; Kenney, G. E.; Ross, M. O.; Ro, S. Y.; Lemma, B. E.; Batelu, S.; Thomas, P. M.; Sosnowski, V. C.; DeHart, C. J.; Kelleher, N. L.; Stemmler, T. L.; Hoffman, B. M.; Rosenzweig, A. C. Characterization of a long overlooked copper protein from methane- and ammonia-oxidizing bacteria. *Nat. Commun.* **2018**, *9*, 4276.

Identifying a novel pathway for extracellular electron uptake in *Methanosarcina barkeri* using shotgun proteomics

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Overall research goals:

The biochemical diversity of electron transfer reactions in microbial systems is reshaping our understanding of the metabolism, ecology, and utility of microbes for energy related applications. Recently, members of the *Methanosarcinales*, a lineage of the cytochrome-containing methanogens, were shown to participate in direct interspecies electron transfer (DIET) with organisms such as *Geobacter metallireducens*. This work highlighted an electron transfer mode between species that required the extracellular electron transfer (EET) pathways known in *Geobacter* and pointed to an unidentified mode of extracellular electron uptake in *Methanosarcina barkeri*. To uncover a potential mechanism for DIET from the methanogen perspective, we characterized the electrochemical activity of *Methanosarcina barkeri*, and confirmed the ability of *M. barkeri* to perform electron uptake from electrodes. We were able to link cathodic current to quantitative increases in methane production. The underlying mechanisms identified in this work include: 1) a recently proposed association between cathodes and non-cell associated extracellular enzymes (e.g., hydrogenases) that can facilitate current generation through the formation of reduced and diffusible methanogenic substrates (e.g., hydrogen) and 2) a lower voltage hydrogen-independent electron uptake pathway that facilitates cathodic activity coupled to methane production. This second feature remained in electrochemical experiments after minimizing the contributions of non-cell associated extracellular enzymes (via washing cells) and using a mutant that lacked methane-linked hydrogenases (provided by William Metcalf, UIUC). This points to a novel and extracellular-enzyme-free mode of electron uptake able to facilitate cathodic current generation at redox potentials lower than -498 mV vs. a standard hydrogen electrode (over 100 mV more reduced than the observed hydrogenase midpoint potential under these conditions). This work seeks to identify the proteins potentially involved in this novel electron uptake mechanism.

To identify the proteins involved in *M. barkeri*-EET, we used shotgun proteomic techniques to: 1) identify the extracellularly exposed proteins of electrode grown *M. barkeri*, and 2) compare differential protein abundances between electrode and traditional methanogenic growth conditions (e.g., methanol). Using a biotin labeling and streptavidin purification technique to target proteins in *M. barkeri* that contain a surface exposed amine group, we identified 20 high confidence proteins in *M. barkeri* as having an extracellular component. Ten of these proteins were annotated as unknown, or unknown function. Four of the proteins identified shared homology over at least a third of the protein length to S-layer proteins. Three proteins with DUF1699 domains, which are highly conserved in the *Methanosarcinales*, were also highlighted. Only on protein with predicted redox activity was identified. To determine which genes were differentially regulated under electrochemical conditions, compared with traditional growth conditions (on methanol), we compared proteomes from these growth conditions using an isotopic labeling approach, tandem mass tagging of TMT.

Significant achievements: Year 2, no cost extension

Over the past two years, we have completed the proteomic experiments and analysis proposed for Aims 1 and 2 of our grant. For **Aim 1** specifically, we have labelled, purified, and identified proteins that are extracellular in *M. barkeri*. We analysed both traditionally grown and electrochemically grown cells for these experiments in addition to open circuit controls. Non labelled control samples were also analysed to control for non-specific binding to streptavidin. We identified 20 proteins as being extracellular using their identification from labelled only samples (and not in unlabelled controls) and containing at least two high confidence peptides.

For **Aim 2**, we have completed two rounds of differential proteins expression experiments (TMT) with triplicate electrochemically grown cells, methanol grown cells, and cells exposed to an open circuit condition. Our first set of experiments identified 7 and 22 proteins, as being up-regulated under EC condition compared with methanol grown and open circuit conditions respectively (at a p-value of 0.05). However, we were challenged in these experiments with poor reproducibility in our open circuit conditions compared with our other samples, which may have affected the confidence in differential protein abundances with these groups. As such we, repeated this set of experiments, and focused on better quantifying and assessing quality and reproducibility (by protein gel electrophoresis) prior to analysis. This resulted in more within replicate reproducibility, and more total proteins identified as differentially expressed in this experiment. In addition, a greater number of proteins were identified as being up regulated under electrochemical conditions compared with methanol grown (41 proteins) and open circuit conditions (8 proteins). Within the group of 41 proteins identified as up regulated relative to methanol grown conditions, three redox active proteins of unknown function were identified. One is a ferredoxin proteins, and the other two are iron-sulfur binding proteins, one of which was identified in our extracellular protein experiments. Due to the potential for redox activity and the extracellular nature of this protein, we are planning to target his protein for future genetic and biochemical experiments. A cell surface protein that was identified in our extracellular protein experiments was also enriched under electrochemical conditions and is another important candidate for genetic experiments.
We are currently in the process of addressing **Aim 3** which has been a challenge due to both experimental and personnel issues. Aim 3 depends on reproducing co-cultures of *Geobacter* and *Methanosarcina* based on previously published studies that demonstrate direct interspecies electron transfer, or DIET. Our first experimental challenge was being unable to get growth of our *Methanosarcina barkeri* strain in the published co-culture medias. As such we had to re-design the media to obtain consistent growth of *M. barkeri* which entailed using a different mineral mixture than the reported mixture and adding higher concentrations of both Mg^{2+} and Ca^{2+} salts. We are on our second attempt to generate successful co-cultures using the published protocols. Our first attempt did not produce methane beyond that amount that we would expect based on growth with Acetate from Ethanol only. We are working on optimizing the appropriate ratios of *Geobacter* and *Methanosarcina* to add to co-cultures, based on discussions with our collaborator Amelia Rotaru from Southern Denmark University. The focus of this last year of no cost extension will be completing the co-culture electrochemistry we proposed in Aim 3. My student Linda Vu, changed from a PhD to a master's degree and is currently on a leave of absence and has been unable to work on this project. However, out postdoc in the lab Saranya Sriram is taking over the work associated with Aim 3.

Science objectives for 2021-2022:

1)Culture co-cultures of *Geobacter* and *Methanosarcina* that are performing direct interspecies electron transfer, using carbon cloth as a conductive material.

2) Investigate the electrochemical properties of co-cultures under turnover and non-turnover conditions.

3) Seed IDAs with *Geobacter* and *Methanosarcina* co cultures and measure conductance properties including formal potential for electron transfer.

4) Pursue collaboration that will allow us to investigate the genetic effects of removing candidate genes involved in electron uptake in *M. barkeri*.

My project addresses BES cross-cutting priority areas by:
The overarching goal of this project is to identify the underlying proteins involved in extracellular electron uptake in *M. barkeri.* Our current evidence points to a novel (non-cytochrome based) mechanism for reducing power to be coupled to CO_2 reduction, which has important implication for both physiology of the environmentally and technologically important microbes. One important example is the implications for this work to technologies like electorsynthesis, which could serve to provide a platform for carbon neutral fuels production.
My scientific area(s) of expertise is/are: Microbiology, Anaerobic Microbiology, Electromicrobiology, Physiology, Proteomics, Electrochemistry .

The ideal collaborator for my project would have expertise in: Methanogen genetics, biophysical measurements of electron transfer in microbial systems.

Publications supported by this project 2020-2022:
We expect two publications to result form this work, in 2023.

Regulated reductive flow through archaeal respiratory and energy production systems

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Overall research goals:
The energy production strategies supporting growth of hyperthermophilic Archaea push the known limits of energy conversation mechanisms and likely mimic the energy transaction strategies present in early life on Earth. Many advances in our understanding of these systems have emerged from the genetically-tractable order *Thermococcales* wherein the roles of individual enzymes, soluble and membrane-bound respiratory complexes, regulatory factors, and competing and complementary catabolic pathways have been probed by a combination of ever-increasingly complex genetic, biochemical, and –omics approaches. ***Our long-term research goal is to determine the interplay, competition and regulation of archaeal energy and redox transactions systems***.

Catabolism of marginal substrates may yield only ~one-tenth of the necessary energy for ATP production. If reduction of even weakly energetic substrates can be coupled to formation of an electrochemical gradient, this gradient can be exploited for ATP production. The *Thermococcales*, a group of heterotrophic, anaerobic, hyperthermophilic marine archaea use a chemiosmotic mechanism involving an electrochemical ion gradient across the cytoplasmic membrane to drive ATP synthesis via an A_1A_0 ATP synthase. This gradient is generated via the action of a multi-subunit membrane-bound NiFe-hydrogenase (termed MBH) or a membrane-bound sulfane-reductase (MBS) which couples ferredoxin-driven reduction of protons or polysulfides (thereby generating H_2 or H_2S, respectively) to H^+ translocation across the cytoplasmic membrane. Ferredoxins (Fds) are critical to this lifestyle, given their redox potentials ($E^{0'}$) are sufficiently low to not only permit reduction of protons to H_2 ($E^{0'}$ = -414 mv), but to drive such a reaction with sufficient excess that energy is available to drive translocation of ions across the membrane.

On-going efforts build on a paradigm shift regarding specific roles of distinct Fd-isoforms in directing electron flux *in vivo* through selective pathways. We aim to establish the rules dictating Fd-interactions, to rationally manipulate electron flux *in vivo* by altering Fd availability and interplay, and to establish the structures and redox potentials of the Fd-isoforms from *T. kodakarensis*.

Significant achievements: [ongoing since 2014; only recent manuscript listed here]:
We determined the essentiality of, and *in vivo* protein interactions made by each Fd. The results obtained convincingly demonstrate distinct, non-overlapping physiological roles for each ferredoxin *in vivo*. Burkhart BW, Febvre HP, Santangelo TJ. 2019. Distinct Physiological Roles of the Three Ferredoxins Encoded in the Hyperthermophilic Archaeon *Thermococcus kodakarensis*. MBio. 10(2). e02807-18.

Our Fd-based studies revealed that a major sink of electrons *in vivo* was lipid production via a mevalonate-based isoprenoid biosynthesis pathway. Many routes of lipid production in archaeal cells have been proposed, and given the ambiguity, we used a combination of genetic techniques and *in vitro* biochemistry to define the pathway of mevalonate production in *T. kodakarensis*. Liman GLS, Hulko T, Febvre HP, Brachfeld AC, Santangelo TJ. A linear pathway for mevalonate production supports growth of Thermococcus kodakarensis. Extremophiles. 2019. s00792-019-01076.

We established the regulation controlling expression of each Fd *in vivo*. Total transcriptomics and protein purifications from cultures grown in the presence and absence of S˚ have established the expression patterns of each ferredoxin. Expression patterns are unique for each locus, with regulation imposed by primary and secondary promoters, locus specific transcription regulators, antisense transcription and local genomic architecture. Jager D, Forstner KU, Sharma CM, **Santangelo TJ**, Reeve JN. Primary transcriptome map of the hyperthermophilic archaeon Thermococcus kodakarensis. BMC Genomics. 2014. 15:684. We are currently investigating the totality of transcription and potential translation regulation imposed on ferredoxin expression *in vivo*. Antibodies have been raised against each ferredoxin for use in quantitative Western blots, and qRT-PCR is used

to monitor RNA levels *in vivo*. The promoter(s) of each ferredoxin have been manipulated to impact RNA and protein levels *in vivo* and to determine the critical expression elements that control expression of each ferredoxin locus. A manuscript is in preparation.

To rationalize the distinct functions of each Fd we aimed to solve the atomic structure of each protein and determine their electrical potentials. The atomic structure of Fd-1 is now completely resolved, and the mid-point electric potentials of Fd-1 and Fd-3 have been determined. Manuscripts are in preparation.

Science objectives for 2021-2022:

Establish how selective Fd interactions with redox active proteins are established in vivo.

Establish the impacts of tethering Fd isoforms to cognate and non-cognate partners in vivo impacts electron flux.

Establish how Fds bring together electron-donating complexes and electron-accepting complexes in vivo.

Generate hybrid Fds to alter partnerships dictating electron flux in vivo.

My project addresses BES cross-cutting priority areas by:
Our efforts directly address the core programs of the BES Physical Biosciences portfolio, namely to elucidate fundamental knowledge pertaining to the biochemical and molecular mechanisms that underlie energy conversion in non-medical microbes. On-going efforst leverage existing techniques and develop new strategies to establish the roles of fundamentally important electron carriers in central energy-related transactions in hyperthermophilic Archaea. The results obtained will be more broadly applicable to the overarching missions of the DOE to establish novel platforms and pathways for next generation biofuels and bio-renewables, as well as greener-whole cell biocatalysts for conversion of renewable biomass to fermentable sugars.

My scientific area(s) of expertise is/are: Microbial (specifically archaeal) physiology; application of genetics approaches to alter metabolism; establishing in vivo protein networks; genetic engineering; elucidating mechanisms underlying gene expression ath the transcription level.

The ideal collaborator for my project would have expertise in: Structural determination of small proteins with metal centers; determination of mid-point electrical potentials of proteinaceous electron carriers.

Publications supported by this project on-going since 2014:

Scott RA, Williams SA, Santangelo TJ. Thermococcus kodakarensis provides a versatile hyperthermophilic archaeal platform for protein expression. Methods in Enzymology. 2021. 659:243-273.

Liman GLS, Stettler ME, Santangelo TJ. Transformation techniques for the anaerobic hyperthermophile Thermococcus kodakarensis. Methods in Molecular Biology. 2022. 2522:87-104.

Burkhart BW, Febvre HP, Santangelo TJ. Distinct physiological roles for the three ferredoxins encoded in the hyperthermophilic archaeon Thermococcus kodakarensis. mBio. 2019. 10(2):e02807-18.

Liman GLS, Hulko T, Febvre HP, Brachfeld AC, Santangelo TJ. 2019. A linear pathway for mevalonate production supports growth of Thermococcus kodakarensis. Extremophiles. 23(2): 229-238.

FWP100593 Elucidating the Catalytic Mechanism of Microbial CO_2 Fixation.

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Overall research goals:

The WL pathway is earth's predominant anaerobic CO_2 sink and is found in strictly anaerobic bacteria, including acetogens and methanogenic archaea where it functions as the major means of energy generation and autotrophic growth. Perhaps most interestingly, the WL pathway is extremely energy-efficient and utilizes only 1 ATP molecule for the reduction of two CO_2's. To achieve CO_2 reduction and functionalization, the WL pathway elegantly brings together a complex array of metalloenzymes, which precisely use/generate, transport and functionalize CO_2 and the otherwise toxic gas molecule, CO to acetyl Coenzyme A (acetyl-CoA). The pathway combines two mechanisms of CO_2 reduction: a sequential six-electron reduction of CO_2 to a methyl group (Methyl branch) and a two-electron reduction of CO_2 to CO (Carbonyl branch). This is followed by the tightly regulated fusion of CO and the methyl group (anaerobic C-C coupling) to form an acetyl group that reacts with CoA (C-S coupling) to form acetyl-CoA. The one-carbon pathway of the Methyl branch in present in all organisms, while the Carbonyl branch, present only in bacteria and archaea performs the remarkable C-C and C-S fusion of Me-, CO and CoA to acetyl-CoA. This is performed by the combined effort of three metalloproteins, the corrinoid iron-sulfur protein (CFeSP), carbon monoxide dehydrogenase (CODH) and Acetyl CoA Synthase (ACS), which strategically form a series of bio-organometallic intermediates (Figure 1).

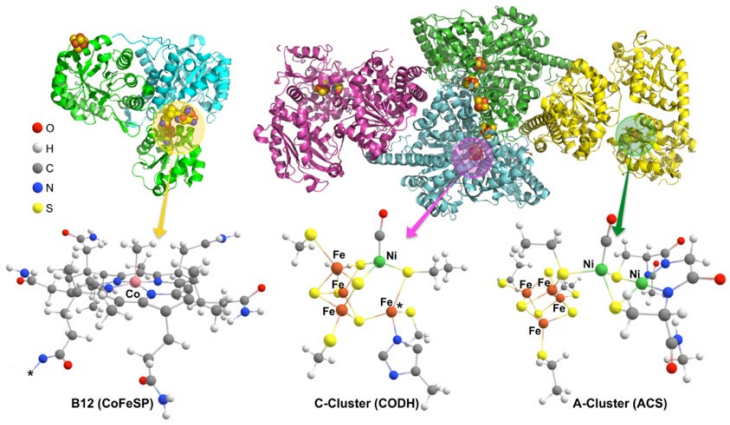

Figure 1. Top (left) Crystal structure of CFeSP (2H9A), containing the corrinoid iron-sulfur protein CFeSP. Top (right) The CODH/ACS crystal structure(1OAO), showing the central dimeric CODH(in blue and green), flanked by two ACS units (pink and yellow). A schematic of the methylated Co center in CFeSP (bottom, left) and the CO bound Ni sites in CODH (bottom, middle) and ACS (bottom, right) are shown.

We propose to investigate the geometric and electronic structure of key enzymatic intermediates formed during the course of anaerobic CO_2 reduction in the Wood-Ljungdahl (WL) pathway, using x-ray spectroscopy techniques and theoretical methods. We have two overarching aims that target structure and function questions about ACS and CODH, respectively:

Aim 1: We will investigate the oxidation state and electronic structure of methylated and acetylated A-cluster in ACS. Using a combination of Fe and Ni based X-ray spectroscopic techniques, we will differentiate between the diamagnetic and paramagnetic mechanisms and understand how the electronic structure tunes catalysis.

Aim 2: We will use Ni K-edge XAS and EXAFS methods to study the geometric/electronic structure changes that occurs at the Ni center due to the dramatic structural change of the C-cluster in CODH upon reversible reduction/oxidation. We will investigate the oxidation state and geometry of the

resting, CO, CN^-, one-reduced and three-electron reduced forms of the C-cluster in CODH using Ni based x-ray spectroscopic and DFT methods.

Significant achievements: [Year 1/FWP100593]:
Ours is a newly funded FWP in the Physical Bioscience portfolio. In 2021, some of the significant achievements (in addition to hiring a post-doctoral fellow) are as follows: we obtained beamtime at the synchrotron facility (SSRL) at SLAC and in collaboration with Prof. Ragsdale, were able to obtain XAS data on the methylated and acetylated intermediates of acetyl-CoA synthase. We also completed DFT calculations to correlate with experimental data. Additionally, we were able to leverage an existing collaboration between Prof. Ragsdale and Prof's Guo and Hoffman to correlate the XAS data with ENDOR and Mossbauer spectroscopy.

Science objectives for 2021-2022:

In the upcoming grant year, we will focus on CODH and plan to engage in two distinct studies. In the first, we will probe the C-cluster of *Desulfovibrio vulgaris* CODH and spectroscopically characterize the electronic changes occurring at the active site in response to oxidation events. In a recent study, the Drennan lab have described large structural shifts in the location of Ni and FCII upon oxidation of the C cluster that appear to be both reversible and protective of the otherwise strictly anerobic function of the enzyme. We plan to test our hypothesis that the large structural changes observed between the oxidized and reduced forms of *dv*CODH result in alterations of the Ni electronic structure, enabling the reversibility of the rearrangement. In the second, we will plan to resolve the disputed electronic structures of catalytic intermediates of CODH. We will perform Ni K-edge EXAFS studies that will serve to resolve structural disputes regarding the crystallographic CO_2-bound C_{Red2} structure. We also propose Ni K-edge XAS and XES measurements to help us elucidate the electronic structure of Ni in the C_{Red1} and C_{Red2} states to directly address the formation of a proposed Ni^0 state. While, we feel reasonably confident in our ability to complete the structural characterization with Ni K-edge EXAFS methods, our ability to perform the XES experiments are subject to access to the oversubscribed experimental facilities that are currently undergoing repairs at SSRL.

My project addresses BES cross-cutting priority areas by:
This project will help us obtain a detailed mechanistic understanding of the metalloenzymes involved in the highly efficient WL pathway. There is a wide interest from technology companies who are combining acetogen metabolism to ferment waste industrial gases from process industries, such as the steel or refining sectors to produce ethanol and other chemicals. With a more detailed understanding of the metabolism of acetogens, carbon could be fixed more efficiently from existing waste streams into everyday products that would otherwise come from fossil resources.

My scientific area(s) of expertise is/are: Synchrotron-based X-ray spectroscopy techniques, density functional theory applications to bioinorganic systems, electronic structure elucidation of inorganic and organometallic compounds.

The ideal collaborator for my project would have expertise in: One of my strongest collaborators in this FWP is Steve Ragsdale who brings years of expertise and leadership in the enzymes involved in the WL pathway. My ideal collaborator is like Steve, a biochemist who is interested in using synchrotron-based x-ray spectroscopy methods tools to understand the electronic and geometric structure transformations at complex metalloenzyme active sites. As a synchrotron scientist, I am uniquely situated to expand the x-ray spectroscopy toolkit to the Physical Biosciences portfolio and am always open to new collaborations!

Publications supported by this project 2021-2024:

Structure and function of carbon concentrating machinery

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Overall research goals:

Cells compartmentalize biochemical reactions as a means of improving pathway rate, yield, and toxicity. This strategy is epitomized in the bacterial microcompartment known as the carboxysome, which acts as part of a physiological strategy known as the CO_2 Concentrating Mechanism to insure fast and efficient CO_2 fixation by the enzyme Rubisco. Microcompartments are simplified, protein-based organelles. Understanding their assembly and function could provide a generalized and modular scaffold for improving the catalysis of engineered biochemical pathways. The carboxysome also plays a central role in the Calvin-Benson-Bassham Cycle - a fundamental pathway in the capture and conversion bioenergy-related molecules – and it is thought that the principles and components of the CO_2 Concentrating Mechanism could be used to improve CO_2 assimilation in many photosynthetic organisms. The overarching goal of our work is therefore to develop a mechanistic understanding of each major component of the bacterial CO_2 Concentrating Mechanism, including the carboxysome, in order to reveal how holistic function emerges from the integration of these individual components. We propose here to investigate three open and critical questions which have arisen through our continued work on the CO_2 Concentrating Mechanism. Firstly, we will investigate how an essential component of the carboxysome, carbonic anhydrase, is incorporated during the assembly process. Secondly, we will investigate the structure and function of the DAB, the inorganic carbon transporter which powers CCM function. Finally, we aim to reconstitute the CCM in new organisms in order to understand the evolution and improvement of CO2 assimilation.

Significant achievements: 2020-2022

- Discovered and characterized the mechanism of loading for the alpha-carboxysome carbonic anhydrase.
- Completed initial functional reconstitution of the entire bacterial CCM in a heterologous host.
- Using reconstitution of CCM to explore evolutionary trajectories.
- Investigating structure-function of the DAB inorganic carbon pump.
- Initiated new research collaborations with the Moerner (spectroscopy) and Metskas (tomography) Labs to investigate the function and structure of single carboxysome complexes.

Science objectives for 2022-2023:

- Continue moving towards biochemical reconstitution of entire carboxysome.
- Carry out single molecule functional studies of carboxysome redox chemistry and permeability.
- Finish structure-function studies of the DAB complex.

My project addresses BES cross-cutting priority areas by:

The goal of our work is to understand and engineer improvements into the biological pathways of carbon capture and transformation. We primary use the tools of biochemistry in these studies but we also use cryoEM and spectroscopy for mechanistic studies as well as high throughput genetics and genome editing for *in vivo* studies.

My scientific area(s) of expertise is/are: biochemistry, protein engineering, genome editing.

The ideal collaborator for my project would have expertise in: We are increasingly applying the principles and components of our system *in planta* and looking for plant biology collaborators.

Publications supported by this project 2020-2022:

1. Prywes N, Phillips NR, Tuck OT, Valentin-Alvarado LE, Savage DF. 2022. Rubisco function, evolution, and engineering. arXiv [q-bioBM]. http://arxiv.org/abs/2207.10773.
2. Lavania AA, Carpenter WB, Oltrogge LM, Perez D, Borden JS, Savage DF, Moerner WE. 2022. Exploring masses and internal mass distributions of single carboxysomes in free solution using fluorescence and interferometric scattering in an anti-Brownian trap. bioRxiv 2022.08.23.505029. https://www.biorxiv.org/content/10.1101/2022.08.23.505029v1.abstract.
3. Flamholz AI, Dugan E, Panich J, Desmarais JJ, Oltrogge LM, Fischer WW, Singer SW, Savage DF. 2022. Trajectories for the evolution of bacterial CO2-concentrating mechanisms. bioRxiv 2022.06.21.497102. https://www.biorxiv.org/content/10.1101/2022.06.21.497102v2
4. Wang RZ, Nichols RJ, Liu AK, Flamholz AI, Banda DM, Savage DF, Eiler JM, Shih PM, Fischer WW. 2022. Evolution of Carbon Isotope Fractionation in Cyanobacteria. bioRxiv 2022.06.22.497258. https://www.biorxiv.org/content/10.1101/2022.06.22.497258v1
5. Metskas LA, Ortega D, Oltrogge LM, Blikstad C, Lovejoy DR, Laughlin TG, Savage DF, Jensen GJ. 2022. Rubisco forms a lattice inside alpha-carboxysomes. Nat Commun 13: 4863. http://dx.doi.org/10.1038/s41467-022-32584-7.
6. Carpenter WB, Lavania AA, Borden JS, Oltrogge LM, Perez D, Dahlberg PD, Savage DF, Moerner WE. 2022. Ratiometric Sensing of Redox Environments Inside Individual Carboxysomes Trapped in Solution. J Phys Chem Lett 13: 4455–4462.
7. Blikstad C, Dugan EJ, Laughlin TG, Liu MD, Shoemaker SR, Remis JP, Savage DF. 2021. Discovery of a carbonic anhydrase-Rubisco supercomplex within the alpha-carboxysome. bioRxiv 2021.11.05.467472. https://www.biorxiv.org/content/10.1101/2021.11.05.467472v1 In revision at PNAS
8. LaFrance BJ, Cassidy-Amstutz C, Nichols RJ, Oltrogge LM, Nogales E, Savage DF. 2021. The encapsulin from Thermotoga maritima is a flavoprotein with a symmetry matched ferritin-like cargo protein. Sci Rep 11: 22810.
9. Flamholz AI, Dugan E, Blikstad C, Gleizer S, Ben-Nissan R, Amram S, et al. Functional reconstitution of a bacterial CO2 concentrating mechanism in Escherichia coli. Elife 2020;9.: https://doi.org/10.7554/eLife.59882.
10. Laughlin, TG, Savage, DF, Davies, KM, 2020. Recent advances on the structure and function of NDH-1: The complex I of oxygenic photosynthesis. Biochim. Biophys. Acta Bioenerg. 148254. doi:10.1016/j.bbabio.2020.148254.

Nitrogenase Reduction of N_2 and CO_2

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Dennis Dean, Principal Investigator (DE-SC0010834)
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Overall research goals:

The research objectives of our joint project are to reveal molecular level insights into the mechanism of nitrogenase catalyzed reduction of N_2 to NH_3 and the reduction of CO_2 and CO to formate and hydrocarbons. Further, we seek to gain insights into the complex array of proteins and steps involved in the installation of the active site metal clusters of nitrogenase. The outcomes of these studies are expected to provide foundational information about the assembly and reactivity of the complex metal clusters of nitrogenase, giving guidance to the design of next generation N_2, CO_2, and CO reduction catalysts.

Significant achievements: 2020-2023:

Progress over the two years of this funding cycle has resulted in 12 peer-reviewed publications (below). Significant progress has been made in both of the two thrusts of the project: insights into the mechanism of substrate reduction at the nitrogenase active site and assembly of the active site. An important advance has come from developing an approach to selectively label the C in the middle of the nitrogenase active site, FeMo-cofactor. By labelling with ^{13}C and trapping substrate reduction intermediates by freezing during turnover, we have been able to apply advanced spectroscopic techniques (^{13}C-ENDOR in collaboration with Brian Hoffman) to gain new insights into the spin states of the C-Fe_6 core of FeMo-co during turnover. We have found that in all of the states trapped so far, the C is acting to stabilize the core. This finding contrasts with the proposal that the C-Fe bonds could be hemi-labile to provide substrate binding sites on Fe atoms. We have also discovered that the alternative nitrogenase isozymes (V- and Fe-based) are EPR active in the odd E states (E_1, E_3, etc.). This finding complements the earlier finding that the Mo-nitrogenase active site is EPR active in the even E states (E_0, E_2, etc.). Thus, we are now in position to examine all of the E states of the catalytic cycle during N_2 reduction, providing important mechanistic insights. We have recently been able to elucidate the stoichiometry of ATP utilization per electron transferred for different oxidation states of the Fe protein (1+ or 0), resolving confusion from earlier studies about the energy transduction mechanism. We have also put forward a new kinetic model for substrate reduction by Mo-nitrogenase, revealing new aspects of the kinetic mechanism. Finally, new insights have been reported on the proteins involved in controlling synthesis and installation of the active sites of all three nitrogenase isozymes (Mo, V, Fe).

Science objectives for 2022-2023:

- Trap early E states (E_1, E_2) in the V-, Fe-, and Mo-nitrogenases under low electron flux conditions and determine the properties of each state using EPR and ENDOR spectroscopy. The goals of these studies are to gain insights into the chemical structures of the early E states in the catalytic cycle.
- Selectively label with ^{13}C the central C for all three nitrogenase isozymes (Mo, V, Fe) and trap substrate intermediates. Analysis of these trapped states by ^{13}C-ENDOR will reveal the spin states of the C-Fe_6 core at different steps in the reaction pathway. These studies are expected to advance our understanding of the mechanism of nitrogenase reduction of substrates.
- Apply the new kinetic model developed for the Mo-nitrogenase to the V- and Fe-isozymes, gaining insights into substrate selectivity among the three enzymes (protons vs N_2).

- Advance the analysis of auxiliary proteins that are associated with, but not essential for, the maturation of the three nitrogenase isozymes.

My project addresses BES cross-cutting priority areas by:
Work in the project is advancing the mechanistic understanding of the metalloenzyme nitrogenase during the reduction of the abundant gas molecules (N_2, CO_2, CO) that are priority energy carrier molecules. These studies are providing insights into transfer of electrons and protons under green conditions to achieve reduced molecules.

My scientific area(s) of expertise is/are: Metalloenzyme mechanism, catalysis, Fe-S clusters, electron transfer, N_2 reduction, bacterial genetics.

The ideal collaborator for my project would have expertise in: Spectroscopic methods for paramagnetic metal clusters such as ENDOR; theory on catalytic mechanism of metalloenzymes.

Publications supported by this project: 2020-2023:

1. Harris, D. F., Jimenez-Vicente, E., Yang, Z.-Y., Hoffman, B. M., Dean, D. R., and Seefeldt, L. C. (2020) CO as a substrate and inhibitor of H^+ reduction for the Mo-, V-, and Fe-nitrogenase isozymes. *J. Biol. Inorg. Chem. 213*, 111278. 10.1016/j.jinorgbio.2020.111278
2. Lukoyanov, D. A., Yang, Z.-Y., Dean, D. R., Seefeldt, L. C., Raugei, S., and Hoffman, B. M. (2020) Electron redistribution within the nitrogenase active site FeMo-cofactor during reductive elimination of H_2 to achieve N≡N triple-bond activation. *J. Am. Chem. Soc. 142*, 21679–21690. 10.1021/jacs.0c07914
3. Seefeldt, L. C., Yang, Z.-Y., Lukoyanov, D. A., Harris, D. F., Dean, D. R., Raugei, S., and Hoffman, B. M. (2020) Reduction of substrates by nitrogenases. *Chem. Rev. 120*, 5082–5106. 10.1021/acs.chemrev.9b00556
4. Pérez-González, A., Jimenez-Vicente, E., Gies-Elterlein, J., Salinero-Lanzarote, A., Yang, Z.-Y., Einsle, O., Seefeldt, L. C., and Dean, D. R. (2021) Specificity of NifEN and VnfEN for the assembly of nitrogenase active site cofactors in *Azotobacter vinelandii*. *mBio 12*, e0156821. 10.1128/mBio.01568-21
5. Pérez-González, A., Yang, Z.-Y., Lukoyanov, D. A., Dean, D. R., Seefeldt, L. C., and Hoffman, B. M. (2021) Exploring the role of the central carbide of the nitrogenase active-site FeMo-cofactor through targeted ^{13}C labeling and ENDOR spectroscopy. *J. Am. Chem. Soc. 143*, 9183–9190. 10.1021/jacs.1c04152
6. Peters, J. W., Einsle, O., Dean, D. R., DeBeer, S., Hoffman, B. M., Holland, P. L., and Seefeldt, L. C. (2021) Comment on "Structural evidence for a dynamic metallocofactor during N_2 reduction by Mo-nitrogenase." *Science 371*, eabe5481. 10.1126/science.abe5481
7. Yang, Z., Jimenez-Vicente, E., Kallas, H., Lukoyanov, D. A., Yang, H., Campo, J. S. M. del, Dean, D. R., Hoffman, B. M., and Seefeldt, L. (2021) The electronic structure of FeV-cofactor in vanadium-dependent nitrogenase. *Chem. Sci. 12*, 6913–6922. 10.1039/D0SC06561G
8. Lukoyanov, D. A., Harris, D. F., Yang, Z.-Y., Pérez-González, A., Dean, D. R., Seefeldt, L. C., and Hoffman, B. M. (2022) The one-electron reduced active-site FeFe-cofactor of Fe-nitrogenase contains a hydride bound to a formally oxidized metal-ion core. *Inorg. Chem. 61*, 5459–5464. 10.1021/acs.inorgchem.2c00180
9. Payá-Tormo, L., Coroian, D., Martín-Muñoz, S., Badalyan, A., Green, R. T., Veldhuizen, M., Jiang, X., López-Torrejón, G., Balk, J., Seefeldt, L. C., Burén, S., and Rubio, L. M. (2022) A colorimetric method to measure in vitro nitrogenase functionality for engineering nitrogen fixation. *Sci Rep 12*, 10367. 10.1038/s41598-022-14453-x
10. Pérez-González, A., Jimenez-Vicente, E., Salinero-Lanzarote, A., Harris, D. F., Seefeldt, L. C., and Dean, D. R. (2022) AnfO controls fidelity of nitrogenase FeFe protein maturation by preventing misincorporation of FeV-cofactor. *Molecular Micro. 117*, 1080–1088. 10.1111/mmi.14890
11. Stappen, C. V., Jiménez-Vicente, E., Pérez-González, A., Yang, Z.-Y., C. Seefeldt, L., DeBeer, S., R. Dean, D., and Decamps, L. (2022) A conformational role for NifW in the maturation of molybdenum nitrogenase P-cluster. *Chem. Sci. 13*, 3489–3500. 10.1039/D1SC06418E
12. Harris, D. F., Badalyan, A., and Seefeldt, L. C. (2022) Mechanistic insights into nitrogenase FeMo-cofactor catalysis through a steady-state kinetic model. *Biochemistry* (*in press*). 10.1021/acs.biochem.2c00415

Developing a molecular level understanding of carbon monoxide dehydrogenase/acetyl coenzyme A synthase through model metalloenzymes

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Overall research goals:

This project will provide mechanistic insight into the bifunctional metalloprotein complex, carbon monoxide dehydrogenase (CODH)/acetyl coenzyme A synthase (ACS), which catalyzes the selective reduction of carbon dioxide to carbon monoxide and generation of new energetic carbon-carbon and carbon-sulfur bonds from one-carbon precursors. These reactions represent valuable targets for carbon dioxide utilization and the selective synthesis of liquid fuels from CO_2. However, we lack a fundamental understanding of how these enzymes work. In this project, we will develop and characterize protein-based models of CODH and ACS as structural, functional, and mechanistic mimics to reveal the biochemical principles by which CODH and ACS operate.

In **Aim 1**, a robust ferredoxin scaffold will be used to construct a site-differentiated nickel-iron-sulfur cluster-containing protein as a model for CODH. Detailed electronic structure characterization will be pursued using complementary spectroscopic tools, and function will be assessed using solution-phase and electrochemical experiments. **Aim 2** will build from prior work within the group that demonstrated electronic and mechanistic parallels between a modified nickel-substituted azurin model and ACS. New variants will be pursued to develop functional models of ACS, allowing resolution of the key protein-derived factors influencing reactivity. Specific contributors to substrate channeling will be investigated in **Aim 3**. The development of functional, robust enzymes that catalyze CO_2 reduction and carbon-carbon bond formation will provide catalyst design guidelines for sustainable generation of liquid fuels from CO_2.

Significant achievements: [2022-2025]:

This project will provide mechanistic insight into the bifunctional metalloprotein complex, carbon monoxide dehydrogenase (CODH)/acetyl coenzyme A synthase (ACS), which catalyzes the selective reduction of carbon dioxide to carbon monoxide and generation of new energetic carbon-carbon and carbon-sulfur bonds from one-carbon precursors. These reactions represent valuable targets for carbon dioxide utilization and the selective synthesis of liquid fuels from CO_2. However, we lack a fundamental understanding of how these enzymes work. In this project, we will develop and characterize protein-based models of CODH and ACS as structural, functional, and mechanistic mimics to reveal the biochemical principles by which CODH and ACS operate.

In **Aim 1**, a robust ferredoxin scaffold will be used to construct a site-differentiated nickel-iron-sulfur cluster-containing protein as a model for CODH. Detailed electronic structure characterization will be pursued using complementary spectroscopic tools, and function will be assessed using solution-phase and electrochemical experiments.

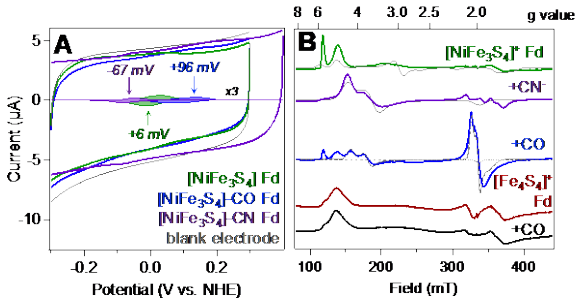
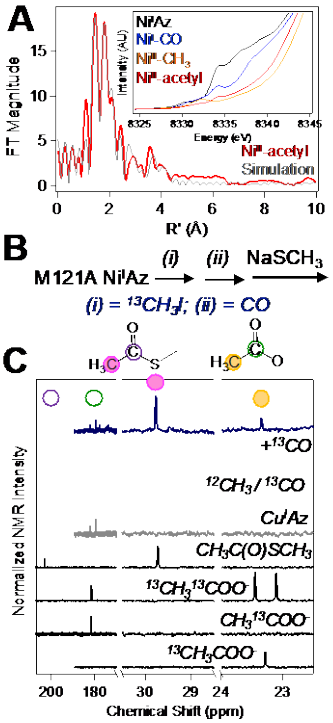


Figure 1. (**A**) Protein film electrochemistry and (**B**) EPR spectra of $[NiFe_3S_4]^+$ Fd under the conditions indicated.

Aim 2 will build from prior work within the group that demonstrated electronic and mechanistic parallels between a modified nickel-substituted azurin model and ACS. New variants will be pursued

to develop functional models of ACS, allowing resolution of the key protein-derived factors influencing reactivity.

Figure 2. (**A**) EXAFS and (*inset*) XANES spectra of Ni^{II}-acetyl Az. EXAFS parameters are consistent with the DFT-optimized structure (*not shown*). (**B**) Reaction scheme and (**C**) ^{13}C NMR spectra showing thioester formation.



Specific contributors to substrate channeling will be investigated in **Aim 3**. The development of functional, robust enzymes that catalyze CO_2 reduction and carbon-carbon bond formation will provide catalyst design guidelines for sustainable generation of liquid fuels from CO_2.

Science objectives for 2021-2022:

Aim 1

- Establish reactivity of NiFd and mutants towards CO and CO_2
- Detailed spectroscopic characterization of NiFd-CO state using X-ray absorption, pulsed EPR, and Mössbauer techniques
- Electronic structure description of NiFd states using BS-DFT and more advanced computational methods
- Express and metallate primary sphere mutants of NiFd

Aim 2

- Probe the impact of covalency on inverted ligand field configuration and reactivity using substitution with unnatural amino acids
- Interrogate electronic structure of nickel-thiolate interaction across states using S K-edge XAS and pulsed EPR techniques

My project addresses BES cross-cutting priority areas by:
Resolving how structural, steric, and electronic factors contribute to catalyst selectivity and efficiency with respect to CO_2 reduction and carbon-carbon bond forming processes. This directly addresses the ***Priority Research Opportunity 2*** laid out in the Report on BES Roundtable on Liquid Solar Fuels: **Control the catalyst microenvironment to promote selective and efficient fuel production**.

My scientific area(s) of expertise is/are: bioinorganic spectroscopy (esp. pulsed EPR, resonance Raman), metalloprotein active site design, protein electrochemistry, and enzymatic redox catalysis.

The ideal collaborator for my project would have expertise in: computational enzyme engineering, designing protein-protein interfaces, modeling gas channels within proteins, and anaerobic protein structure determination.

Publications supported by this project 2021-2022:

1. Lewis, L. C.; Shafaat, H. S. Reversible Electron Transfer and Substrate Binding Support [NiFe3S4] Ferredoxin as a Protein-Based Model for [NiFe] Carbon Monoxide Dehydrogenase. *Inorg. Chem.* **2021**, *60*, 13869–13875 DOI: 10.1021/acs.inorgchem.1c01323.
2. Manesis, A. C.; Yerbulekova, A.; Shearer, J.; Shafaat, H. S. Thioester synthesis by a designed nickel enzyme models prebiotic energy conversion. *Proc. Natl. Acad. Sci. U. S. A.* **2022**, *119*, e2123022119 DOI: 10.1073/pnas.2123022119.

Mechanisms and Regulation of Carbon Allocation and Storage in Plants.

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Jantana Blanford, Zhiyang Zhai, Scientists
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Overall research goals:

We address the Department of Energy (DOE) Basic Energy Sciences mission: "To provide foundational knowledge on processes that convert renewable resources into fuels, chemicals and other energy-enriched products". The BNL group focuses on uncovering foundational mechanisms governing the allocation of photosynthetically fixed carbon within central metabolism of plants, and how that carbon flows into lipid and phenylpropanoid biosynthetic pathways to create the major highly reduced end products: triacylglycerols and lignin. There are parallel goals for both lipids and lignins, first to understand the enzymology and genetics of the two pathways, to control the composition of the final storage compounds and second, to understand the molecular basis for various metabolic feedback regulation within each pathway as a foundation for reengineering plant metabolism to defeat it and thereby accumulate more of the desired storage compounds. Lipids and lignins are highly reduced carbon compounds thus their synthesis are very energy demanding. All plant storage compounds originate from sugars, the levels of which are under tight homeostatic regulation by SnRK1, a conserved sensor-kinase that controls the balance between anabolism and catabolism. SnRK1 controls key regulatory processes related to lipid and lignin synthesis, and each of the subtasks addresses targets that are regulated by this system.

Significant achievements: Current award 2020-2022:

Roles for sugar signaling in the SnRK1-dependent regulation of lipid and phenylpropanoid anabolism were defined. The regulatory network of the transcriptional activator of lipid metabolism WRI1 was expanded from lipid synthesis to central metabolism. The role of cytochrome b5 in supplying electrons to various metabolic partners was defined and a biocatalytic system for evaluating the role of electron transfer chains in P450 system was established. A role for thylakoid remodeling in the regulation of lipid metabolism was established. Structural and computational approaches defined a mechanism for regioselectivity for variant desaturase enzymes. Gravitropism responses were associated with alterations in phenylpropanoid metabolism.

Science objectives for 2022-2023:

Characterization of trehalose 6-phosphate binding to SNF1-related protein kinase catalytic subunit α: KIN10 (see J. Blanford et. al poster). Defining the structural mechanism of inhibition of acetyl-coA carboxylase, the rate limiting activity in lipid synthesis with PII and BADC inhibitors. Explore the molecular mechanism underlying autophagy-mediated lipid droplet remobilization in leaves and determine the role of the core autophagic proteins in lipophagy. Deepen our understanding of the functional association and metabolic roles of different microsomal electron transfer chains and their related redox components in delivering reducing power for phenylpropanoid-lignin biosynthesis. Define the regulation of key metabolic branchpoints in central metabolism mediated by pyruvate kinase and phospho-fructokinase in the control of oil synthesis in developing seeds using lines overexpressing non-plant homologs of these enzymes.

My project addresses BES cross-cutting priority areas by:

Building a detailed knowledge of carbon capture, conversion and storage in plants that lays the foundations for new energy technologies to advance DOE missions in energy and the environment.

Specifically, we focus on the discovery, design, and understanding of biochemical processes to better understand the physical world and harness nature to benefit people and society.

My scientific area(s) of expertise is/are: PIs in this program have expertise in: structure-functions studies on enzyme specificity, biochemistry of sensor kinase regulation of central metabolism and storage of reduced carbon, metabolic modeling, mechanisms of regulation of lipid and phenylpropanoid metabolism and intracellular lipid trafficking.

The ideal collaborator for my project would have expertise in:. Computational theory of electron transfer.

Publications supported by this project -Selected from 2021-2022 publications due to space limitation.

Anaokar, S., Liu, H., Keereetaweep, J., Zhai, Z., and Shanklin, J. 2021. Mobilizing vacuolar sugar increases vegetative triacylglycerol accumulation. Frontiers in Plant Science 12, 1550. https://doi.org/10.3389/fpls.2021.708902

Baer, M.D., Shanklin, J. and Raugei, S. 2021. Atomistic Insight on Structure and Dynamics of Spinach Acyl Carrier Protein with Substrate Length. Biophysical Journal 120: 1–13, https://doi.org/10.1016/j.bpj.2020.12.036

Li, DD., Ni, R., Wang, PP., Zhang, XS., Wang, PY., Zhu, TT., Sun, CJ., Liu, CJ., Lou, HX., Cheng, AX. 2021 Molecular basis for chemical evolution of flavones to flavonols and anthocyanins in land plants. PLANT PHYSIOLOGY https://doi.org/10.1104/pp.20.01185

Wang, B., Zhao, X., Zhao, Y., Shanklin, J., Liu, C-J. 2021. Arabidopsis SnRK1 negatively regulates phenylpropanoid metabolism via Kelch domain-containing F-box proteins. New Phytologist 229: 3345-3359.

Yu, L., Zhou, C., Fan, J., Shanklin, J., Xu, C. 2021.Mechanisms and functions of membrane lipid remodeling in plants. The Plant Journal. https://doi.org/10.1111/tpj.15273

Yu, L., Fan, J., Zhou, C., Xu, C. 2021. Chloroplast lipid biosynthesis is fine-tuned to thylakoid membrane remodeling during light acclimation. Plant Physiology. https://doi.org/10.1093/plphys/kiaa013

Yu, X-H., Cai, Y., Keereetaweep, J., Wie, K., Chai, J., Deng, E., Liu, H., and Shanklin, J. 2021. BADC Proteins mediate hydroxy FA-dependent inhibition of Acetyl CoA Carboxylase. Plant Physiology 185: 892-901.

Zhai, Z., Keereetaweep, J., Liu, H., Xu, C., Shanklin, J. 2021. The role of sugar signaling in regulating plant fatty acid synthesis. Frontiers in Plant Science. https://doi.org/10.3389/fpls.2021.643843

Zhai, Z., Keereetaweep, J., Liu, H., Feil, R., Lunn, J., Shanklin, J. 2021. Expression of a Bacterial Trehalose-6-phosphate Synthase otsA Increases Oil Accumulation in Plant Seeds and Vegetative Tissues. Frontiers in Plant Science DOI: 10.3389/fpls.2021.656962

Zhao, Y., Yu, X., Lam, PY., Zhang, K., Tobimatsu, Y., Liu, CJ_Monolignol acyltransferase for lignin p-hydroxybenzoylation in Populus. 2021 Nature Plants. https://doi.org/10.1038/s41477-021-00975-1.

Kuczynski, C., McCorkle, S., Keereetaweep, J., Shanklin, J., Schwender, J. 2022. An Expanded Role for the Transcription Factor WRINKLED1 in the Biosynthesis of Triacylglycerols during Seed Development. Front Plant Sci doi.org/10.3389/fpls.2022.955589.

Zhao, Y., Yu, X.-H. and Liu, C.-J. 2021. The Inducible Accumulation of Cell Wall-Bound p-Hydroxybenzoates Is Involved in the Regulation of Gravitropic Response of Poplar. Front. Plant Sci. 12:755576.

Guy, J.E., Cai, Y., Baer, M.D., Whittle, E., Chai, J., Yu, X-H., Lindqvist, Y., Raugei, S., and Shanklin, J. 2021. Regioselectivity mechanism of the Thunbergia alata Δ6-16:0-acyl carrier protein desaturase. Plant Physiology, Plant Physiology, 2021; kiab577, https://doi.org/10.1093/plphys/kiab577

Gan, L., Park, K., Chai, J., Updike, EM., Kim, H., Voshall, A., Behera, S., Yu, XH., Cai, Y., Zhang, C., Wilson, M., Mower, JP., Moriyama, EN., Zhang, C., Kaewsuwan, S., Liu, Q., Shanklin, J., Cahoon, EB. 2022. Divergent Evolution of Extreme Production of Variant Plant Monounsaturated Fatty Acids. PNAS 119 (30) e2201160119.

Liu, C.-J. 2022. Cytochrome b5: A versatile electron carrier and regulator for plant metabolism. Front Plant Sci. DOI 10.3389/fpls. 984174.

Understanding redox proportioning through ferredoxins, low potential Fe-S proteins acting as electrical hubs to control metabolism

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Overall research goals:

Ferredoxins (Fd) function as protein electron carriers in biochemical pathways important for energy transduction, with roles ranging from hydrogen and alcohol production to carbon and nitrogen fixation. These low potential (high energy) proteins behave as central energy-conserving redox hubs, serving as conduits between diverse redox donors and acceptors. While it is clear that Fds are abundant across the tree of life, with individual microbes frequently using multiple Fd paralogs to control electron flow, we do not yet fully understand what controls the proportion of electrons relayed by individual protein electron carriers among the diverse oxidoreductases found within cells. Our goal is to elucidate the physicochemical parameters that underlie protein control over electron flow in the cell sufficiently so that we can use protein sequence and structure to anticipate the proportion of electrons that colocalized protein electron carriers deliver to their natural redox partners. We posit that studies that characterize large numbers of natural and non-natural protein-protein interactions involving structurally-diverse protein electron carriers will be critical to establishing the rules that underlie sequence-structure-ET relationships in the diverse proteins that control energy flow across the electron fluxome.

Significant achievements: [2020-2023]:

Monitoring ferredoxin-mediated electron transfer using extracellular electron transfer. Real-time chemical sensing is needed to counter the global threats posed by pollution. We used synthetic biology to develop a living sensor platform that uses a ferredoxin to achieve minute-scale detection times of two different chemicals. *Escherichia coli* was programmed to reduce an electrode in a chemical-dependent manner using a modular, eight-component, synthetic electron transport chain. This strain uses a Fd to transfer electrons between Fd-NADPH reductase (FNR) and sulfite reductase, and it then uses a non-native sulfide quinone reductase (SQR) to drive extracellular electron transfer through the CymA/MtrCAB pathway from *Shewanella*. This strain produced significantly more current upon exposure to thiosulfate, an anion that causes microbial blooms. Incorporating an allosteric Fd switch into the pathway and encapsulation of microbes with electrodes and conductive nanomaterials yielded a living sensor that could detect an endocrine disruptor within two minutes in riverine water, implicating the signal as mass transfer limited. These measurements show how ferredoxin-mediated electron transfer can be directly monitored by measuring extracellular electron transfer.

Like Fds, Flds can support electron transfer to assimilatory sulfite reductases. Flavodoxins (Flds) can couple with an overlapping set of partner oxidoreductases as ferredoxins (Fd), but their ability to couple with many Fd partners remains unclear. To establish whether Flds can support electron transfer to Fd-dependent sulfite reductases (SIRs), we performed bioinformatic analysis to understand the prevalence of organisms that encode both Flds and assimilatory SIRs. Using bioinformatic analysis, we found that a subset of cyanobacterial genomes contain Fld, SIR, and Fd genes. To establish whether cyanobacterial Flds can support electron transfer to Fd-dependent SIRs, we evaluated whether Flds could support growth of an *Escherichia coli* auxotroph that requires ET from a Fd-NADP reductase (FNR) to SIRs for complementation. We show that cyanobacterial Flds can complement the growth of this auxotroph when coexpressed with a non-cognate SIR, including *Synechocystis sp* and *Nostoc sp* Fld. We also performed a systematic peptide insertion with *Synechocystis sp* Fld and identified regions within this protein that retain function following insertion of an octapeptide. These results represent the first

evidence that Flds can support ET to plant and cyanobacterial SIRs, and they provide insight into the mutational tolerance of a Fld when supporting electron transfer from FNR to SIR.

Recombination of ferredoxins reveals differences in the inheritance of thermostability and midpoint potential. To investigate how recombination affects Fds, we created chimeras by recombining distantly-related cyanobacterial and cyanomyophage homologs that present similar midpoint potentials but distinct thermostabilities. Chimeras having a wide range of amino acid substitutions retained the ability to coordinate an iron-sulfur cluster, although their thermostabilities varied with the fraction of residues inherited from each parent. The midpoint potentials of chimeric ferredoxins also differed. However, all of the synthetic ferredoxins exhibited midpoint potentials outside of the parental protein range. Each of the chimeric ferredoxins could also support electron transfer between ferredoxin-NADP reductase (FNR) and sulfite reductase (SIR) in *Escherichia coli*, although the chimeric protein electron carriers required distinct expression levels to support similar levels of cellular electron transfer. These results show how recombination can be used to rapidly diversify the properties of ferredoxins and reveal differences in the inheritance of thermostability and electrochemical properties.

Science objectives for 2022-2023:

Use cellular assays to analyze how protein electron carriers from diverse organisms and with a range of mutations vary in their electron cycling between pairs of donor/acceptor proteins that require ET across distinct midpoint potential ranges. Determine how ET variation in the cellular assays depends upon the biophysical properties of the proteins, such as midpoint potential.

My project addresses BES cross-cutting priority areas by:

providing insight into "energy transfers in proteins, protein complexes, and biohybrid constructs that are relevant to the design of new technologies benefitting from highly specific and efficient flows of energy at the molecular scale."

My scientific area(s) of expertise is/are: metabolism, microbial genetics, protein electron carriers, protein design, redox cofactors, and synthetic biology.

The ideal collaborator for my project would have expertise in: (i) computational docking of electron transfer proteins, (ii) high-throughput biophysical analysis, such as analysis of midpoint potentials, and (iii) quantitative MS for determining intracellular protein concentrations.

Publications supported by this project 2020-2023:

1. J.T. Atkinson, L. Sun, X. Zhang, G.N. Bennett, J.J. Silberg, and C.A. Ajo-Franklin, "Real-time environmental monitoring of contaminants using living electronic sensors." *Nature*, in press (2022) [BioRxiv DOI: 10.1101/2021.06.04.447163v1]
2. J. Bluford, L. Windham, A. Truong, and J.J. Silberg, "Cellular strategies to study and engineer low potential protein electron carriers," Electron Transfer in Biomacromolecules (A. Furst, Ed.) De Gruyter Publishing, in press (2022)
3. I.J. Campbell, J.T. Atkinson, M. Carpenter, D. Myerscough, C.A. Ajo-Franklin, and J.J. Silberg, "Determinants of multiheme cytochrome extracellular electron transfer uncovered by systematic peptide insertion." *Biochem*., **61**, 1337-1350 (2022). [DOI: 10.1021/acs.biochem.2c00148]
4. C.P. Tseng, F. Liu, X. Zhang, P.C. Huang, I.J. Campbell, Y. Li, J.T. Atkinson, C.M. Ajo-Franklin, J.J. Silberg, and R. Verduzco, "Solution-deposited and patternable conductive polymer thin film electrodes for microbial bioelectronics." *Advanced Materials*, 2109442 (2022). [DOI: 10.1002/adma.202109442]
5. I.J. Campbell, D. Kahanda, J.T. Atkinson, N.O. Sparks, J. Kim, C.P. Tseng, R. Verduzco, G.N. Bennett, and J.J. Silberg, "Recombination of 2Fe-2S ferredoxins reveals differences in the inheritance of thermostability and midpoint potential." *ACS Synth Biol*., 9(12): 3245–3253 (2020). [DOI: 10.1021/acssynbio.0c00303]

Engineering Selenoproteins for Enhanced Hydrogen Production

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Overall research goals:

1. Engineering hydrogenases with Sec-coordinated metal clusters
2. Method for making designer selenoproteins in mammalian cells

Significant achievements: (2019-2022):

1. Collaborative studies of Cys to Sec conversions in hydrogenases are continuing with (1) Fraser Armstrong (Oxford University) on [NiFe]-hydrogenases and (2) David Mulder (NREL) on [FeFe]-hydrogenases. We have generated a Sec variant of *E. coli* hydrogenase-1 which shows extraordinary oxygen tolerance over that of its corresponding Cys enzyme. We have also identified some striking effects of Sec insertion on *E. coli* hydrogenase-2 which may answer a fundamental question about the proton path in hydrogen production. We have made progress using *E. coli* as a host to express Sec [4Fe-4S]$_H$ variants of the apo-version of HydA1 from *Chlamydomonas reinhardtii* (CrHydA1), devoid of the [2Fe]$_H$ subcluster (Figure 1). Following artificial reconstitution of the [2Fe2]$_H$ site (collaboration with Tom Rauchfuss, University of Illinois) we can produce active enzyme. We expect this work will provide new information on how ligand coordination can tune H-cluster redox properties, as a strategy to design biomimetic systems.

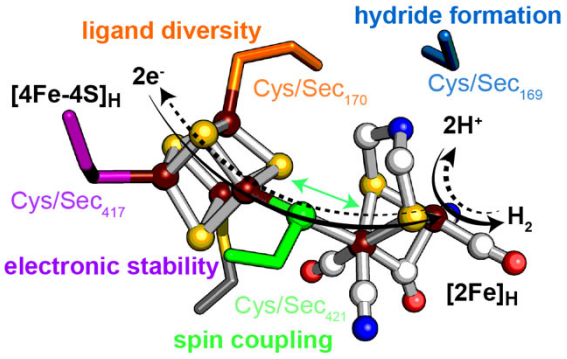

Figure 1. H-cluster active-site of CrHydA1 [FeFe]-hydrogenase which catalyzes the reversible oxidation of H_2. Selective substitution of cysteine (Cys) for selenocysteine (Sec) at different ligand positions will make it possible to probe the underlying tuning mechanisms of the H-cluster.

2. We have developed a versatile and tunable Sec-specific reporter system involving inteins. This milestone is a key factor to facilitate high-throughput screening of Sec translation systems in eukaryotes (e.g., yeast, mammalian cells). Since the intein relies on Sec insertion (not the reporter), the intein can be inserted into any reporter gene in any organism. Our sfGFP_intein results in *E. coli* provided the necessary information to easily transfer the intein into eGFP to screen for Sec insertion in mammalian cells (Figure 2). With this setup, we are equipped with the technology to efficiently develop Sec insertion technology in eukaryotes.

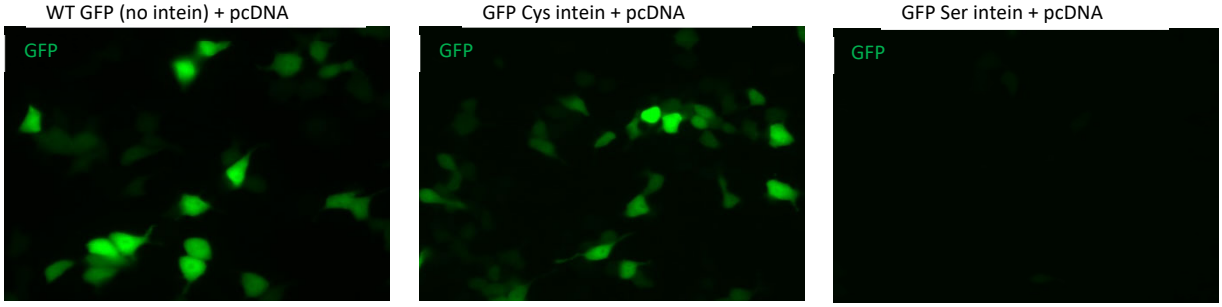

Figure 2. HEK293 cells shows the efficiency and selectivity of the eGFP_intein system. When cysteine (Cys) is present, splicing occurs and shows fluorescence levels similar to wild-type (no intein, WT). In the presence of serine (Ser), intein splicing does not occur resulting in the expression of inactive eGFP reporter and no fluorescence. This technology is the basis for developing an additional simplified and robust mammalian Sec insertion path programmed by UAG.

Science objectives for 2021-2022:

To develop a selenocysteine insertion pathway in yeast and mammalian cells which facilitates Cys to Sec conversion in any protein or enzyme.

My scientific area(s) of expertise is/are: translation and the genetic code.

The ideal collaborator for my project would have expertise in: artificial intelligence

Publications supported by this project (2019-2022):

1. K.S. Hoffman, O. Vargas-Rodriguez, D.W. Bak, T. Mukai, L.K. Woodward, E. Weerapana, D. Söll, N.M. Reynolds, "A cysteinyl-tRNA synthetase variant confers resistance against selenite toxicity and decreases selenocysteine misincorporation." J Biol Chem, **294**:12855. (2019). DOI: 10.1074/jbc.RA119.008219.
2. J.M. Tharp, N. Krahn, U. Varshney, D. Söll, "Hijacking translation initiation for synthetic biology." Chembiochem, **21**:1387. (2020). DOI: 10.1002/cbic.202000017.
3. N. Krahn, J.T. Fischer, D. Söll, "Naturally occurring tRNAs with non-canonical structures." Front Microbiol, **11**:596914. (2020). DOI: 10.3389/fmicb.2020.596914.
4. C.Z. Chung, K. Amikura, D. Söll, "Using genetic code expansion for protein biochemical studies." Front Bioeng Biotechnol, **8**:598577. (2020). DOI: 10.3389/fbioe.2020.598577.
5. C.Z. Chung, C. Miller, D. Söll, N. Krahn, "Introducing selenocysteine into recombinant proteins in *Escherichia coli*." Curr Protoc, **1**:e54. (2021). DOI: 10.1002/cpz1.54.
6. J.M. Tharp, O. Vargas-Rodriguez, A. Schepartz, D. Söll, "Genetic encoding of three distinct noncanonical amino acids using reprogrammed initiator and nonsense codons." ACS Chem Biol, **16**:766. (2021). DOI: 10.1021/acschembio.1c00120.
7. R.M. Evans, N. Krahn, B.J. Murphy, H. Lee, F.A. Armstrong, D. Söll, "Selective cysteine-to-selenocysteine changes in a [NiFe]-hydrogenase confirm a special position for catalsis and oxygen tolerance." Proc Natl Acad Sci USA, **118**:e2100921118. (2021). DOI: 10.1073/pnas.2100921118.
8. O. Vargas-Rodriguez, A.H. Badran, K.S. Hoffman, M. Chen, A. Crnković, Y. Ding, J.R. Krieger, E. Westhof, D. Söll, S. Melnikov, "Bacterial translation machinery for deliberate mistranslation of the genetic code." Proc Natl Acad Sci USA, **118**:e2110797118. (2021). DOI: 10.1073/pnas.2110797118.
9. C.Z. Chung, N. Krahn, A. Crnković, D. Söll, "Intein-based design expands diversity of selenocysteine reporters." J Mol Biol, **434**:167199. (2022). DOI: 10.1016/j.jmb.2021.167199.
10. T. Mukai, K. Amikura, X. Fu, D. Söll, A. Crnković, "Indirect routes to aminoacyl-tRNA: The diversity of prokaryotic cysteine encoding systems." Front Genet, **12**:794509. (2022). DOI: 10.3389/fgene.2021.794509.
11. C.Z. Chung, D. Söll, N. Krahn, "Using selenocysteine-specific reporters to screen for efficient $tRNA^{Sec}$ variants." Methods Enzymol, **662**: 63. (2022). DOI: 10.1016/bs.mie.2021.10.005.
12. J.T. Fischer, D. Söll, J.M. Tharp, "Directed evolution of *Methanomethylophilus alvus* pyrrolysyl-tRNA synthetase generates a hyperactive and highly selective variant." Front Mol Biosci, **9**:850613. (2022). DOI: 10.3389/fmolb.2022.850613.
13. H. Zhang, X. Gong, Q. Zhao, T. Mukai, O. Vargas-Rodriguez, H. Zhang, Y. Zhang, P. Wassel, K. Amikura, J. Maupin-Furlow, Y. Ren, X. Xu, Y.I. Wolf, K.S. Makarova, E.V. Koonin, Y. Shen, D. Söll, X. Fu, "The tRNA discriminator base defines the mutula orthogonality of two distinct pyrrolysyl-tRNA synthetase/$tRNA^{Pyl}$ pairs in the same organism" Nucleic Acids Res, **50**:4601. (2022). DOI:10.1093/nar/gkac271.
14. A. Prabhakar, N. Krahn, J. Zhang, O. Vargas-Rodriguez, M. Krupkin, Z. Fu, F.J. Acosta-Reyes, X. Ge, J. Choi, A. Crnković, M. Ehrenberg, E.V. Puglisi, D. Söll, J. Puglisi, "Uncovering translation roadblocks during the development of a synthetic tRNA." Nucleic Acids Res, **27**:gkac576. (2022). DOI: 10.1093/nar/gkac576.
15. N. Krahn, D. Söll, O. Vargas-Rodriguez, "Diversification of aminoacyl-tRNA synthetase activities *via* genomic duplication." Front Physiol, **13**:983245. (2022). DOI: 10.3389/fphys.2022.983245.
16. L.-T. Guo, K. Amikura, H.-K. Jiang, T. Mukai, X. Fu, Y.-S. Wang, P. O'Donoghue, D. Söll, J.M. Tharp, "Ancestral archaea expanded the genetic code with pyrrolysine." J Biol Chem, **21**:102521. (2022). DOI: 10.1016/j.jbc.2022.102521.
17. K. Meng, C.Z. Chung, D. Söll, N. Krahn, "Unconventional genetic code systems in archaea." Front Microbiol, 13:1007832. (2022). DOI: 10.3398/fmicb.2022.1007832.

Primary and Secondary Sphere Effects on the Valence Isomerism of Iron–Sulfur Clusters

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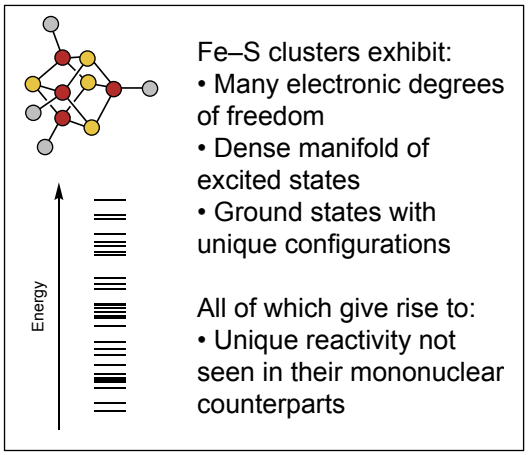
Overall research goals:



Iron–sulfur (Fe–S) clusters are ubiquitous cofactors in energy transduction and energy-relevant catalysis. Underlying their remarkable functions are their unusual electronic structures, notably the presence of a dense manifold of excited states that are thermally populated at ambient temperature. Our goals are to experimentally characterize this landscape of excited states, to quantitatively uncover the influence of the protein polypeptide on tuning the relative energies of these states, and to reveal the impact of these electronic structure considerations on function and reactivity.

Our approach is to parameterize the low-lying excited states in terms of the individual Fe site valences, and thus answer the question: what factors in the primary and secondary coordination sphere control the individual site valences of Fe–S clusters in the ground state and the excited states? In doing so, we endeavor to reveal, for example, why some Fe–S clusters have non-canonical primary-sphere ligands (e.g., site-differentiated clusters in many electron-bifurcating enzymes), to what extent changes in the secondary coordination sphere can impact the electron distribution in a cluster, etc.

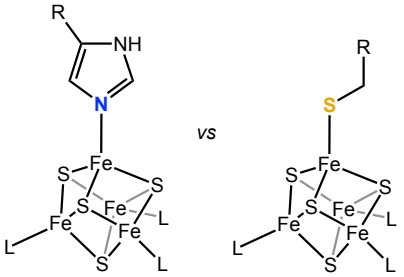
Significant achievements [2020-2022]:

Extending the decades of precedent for using paramagnetic NMR spectroscopy to study the electronic structures of Fe–S proteins, we developed an NMR-based method for (i) identifying the ground state valence configuration of $[Fe_4S_4]^+$ clusters; (ii) experimentally quantifying the energies of alternative valence arrangements; (iii) as a bonus, obtaining the exchange coupling constants describing the magnetic interactions within the clusters. Critical to this analysis was the use of models whose symmetry groups the different valence configurations into two triply degenerate classes (in contrast to a protein environment in which every valence isomer is rigorously inequivalent) while still capturing the key elements of the coordination environment of a protein. With only two sets of valence isomers to consider, the previously intractable fitting problem was made tractable, and the matter of valence isomerism was simplified to modeling a single energy difference: ΔE_{VI}.

This NMR method, which relies on fitting data to model Hamiltonians, was validated with EPR measurements, the interpretation of which requires only the application of Boltzmann statistics and, critically, no assumptions about the clusters' electronic structures. Thus, the EPR measurements provided independent, quantitative validation of both the NMR analysis and our models for the clusters' electronic structures.

We then determined the influence of the primary sphere on the valence isomer landscape in a series of $[Fe_4S_4]^+$ clusters. We found that the primary coordination sphere can have a dramatic influence on ΔE_{VI} (up to nearly 10^3 cm^{-1}) and that the trends in ΔE_{VI} follow trends in the $[Fe_4S_4]^{2+/+}$ redox potential. This illustrates how tuning the donicity of the ligands in the primary coordination sphere results in a reorganization of the valence electrons in the cluster. Most interestingly, we found that swapping a

thiolate for an imidazole ligand (e.g., in a Cys_4 vs Cys_3His environment, the latter of which has been observed in electron-transport chains) repositions the two classes of valence isomers by ~200 cm^{-1}. We are currently working to understand the functional importance of this discovery.



Incorporating an imidazole ligand dramatically alters the valence isomer landscape, favoring localization of Fe^{2+} by ~200 cm^{-1} relative to a thiolate ligand.

In the course of these studies, we made two related discoveries. First, in our efforts to utilize Mössbauer spectroscopy as a complementary tool in these efforts, we observed and studied the facile and dynamic exchange of the Fe centers in Fe–S clusters with exogenous Fe. This provided insights into the dynamics of Fe–S clusters as well as a simple method for ^{57}Fe labeling of Fe–S proteins that could be applied in several Physical Biosciences-supported projects. Second, we discovered and spectroscopically studied a new ground-state electronic structure for Fe–S clusters: one in which an Fe center adopts a low-valent (Fe^{1+}) configuration. This provided new insights into how Fe–S enzymes may convert C_1 molecules to higher-order hydrocarbons and suggests that the rules of Fe–S cluster electronic structure continue to undergo substantial refinement.

Science objectives for 2022-2023:

Our primary objective is to extend the method described above beyond the primary coordination sphere in order to understand the role of the secondary sphere on the valence isomer landscape of $[Fe_4S_4]^+$ clusters. Particularly, we aim to quantitatively relate the number and strength of hydrogen bonds to ΔE_{VI} (the energy difference between the ground and excited valence isomer states).

My project addresses BES cross-cutting priority areas by:

Elucidating how proteins tune the properties of metallocofactors to accomplish efficient electron transfer, particularly in the context of energy-relevant biocatalysis.

My scientific area(s) of expertise is/are: bioinorganic chemistry, transition metal chemistry, spectroscopy.

The ideal collaborator for my project would have expertise in: the theory, kinetics, and structural biology of electron transfer.

Publications supported by this project 2020-2022:

1. Skeel, B. A.; Suess, D. L. M. Molecular Symmetry and Magnetic Resonance as Tools for Quantitatively Mapping the Low-Lying Excited States of Iron-Sulfur Clusters. *In preparation*.
2. Thompson, N. B; Namkoong, G.; Skeel, B. A; Suess, D. L. M. Facile and Dynamic Cleavage of Every Iron–Sulfide Bond in Cuboidal Iron–Sulfur Clusters. *Under revision*.
3. Brown, A. C.; Thompson, N. B.; Suess, D. L. M. Evidence for Low-Valent Electronic Configurations in Iron-Sulfur Clusters. *J. Am. Chem. Soc.*, **144**, 9066-9073 (2022). DOI: 10.1021/jacs.2c01872

Molecular Mechanism of Energy Transduction By Plant Membrane Proteins

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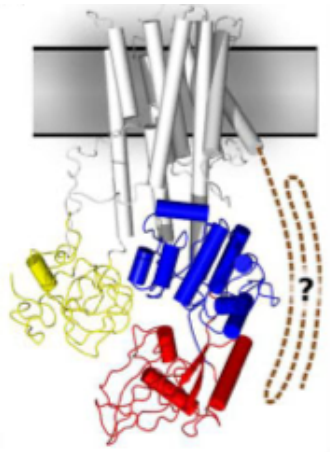
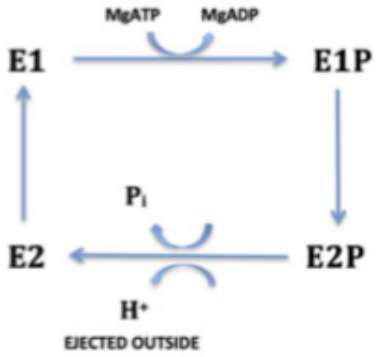
Overall research goals: In eukaryotic cells with a rigid cell wall, i.e. all higher plants and fungi, bioenergetics at the plasma membrane is dominated by a P-type ATPase of M_r=100,000 daltons that is a proton pump. This enzyme is functionally and structurally similar to the more widely known animal enzymes such as ubiquitous plasma membrane Na^+,K^+-ATPase and the sarcoplasmic reticulum Ca^{+2}-ATPase in muscle. Within this group of enzymes, because of its unique stoichiometry of one proton ejected per ATP molecule hydrolyzed, it has a very high reversal potential, i.e. minus 450 millivolts, and in fact, unusually large membrane potentials, e.g. -250 mv can be observed in plants and fungi. In this project my laboratory has been using mass spectrometric based methods, as well as collaborative cryo-EM studies with Huilin Li at Michigan's Van Andel Institute, to elucidate its three-dimensional structure. Of particular interest is how the C terminal 100 amino acids regulate its catalytic functions (i.e. ATP hydrolysis and proton ejection) since this domain appears to be the nexus of regulatory phosphorylation and dephosphorylation events that ultimately control critical biological functions. These include the control of cell elongation that mediates the bending responses of higher plants to gravity and light, as well as the cessation of growth caused by the presence of pathogens. In this year's talk I will present recent developments in the creation and application of 'bottom-up' mass spectrometric technologies involving new covalent labeling and crosslinking reagents that experimentally measure changes in solvent accessible surface area (SASA) at a single amino acid level, as the enzyme passes through its conformational changes during the catalytic cycle. As with all





P-type ATPases, there are four basic conformational states, starting with ATP dependent phosphorylation of an aspartyl group in the nucleotide binding domain, and ends with dephosphorylation of the aspartate and ejection of a single proton (see Figure). A crystal structure for the protein lacking the 100 amino acid water soluble regulatory C-terminal domain has been known for over a decade and provides useful information on specific amino acids involved in ATP hydrolysis and possibly, proton movement. However, since the X ray diffraction data is incomplete and cryo-EM has not yet been successful with the plant enzyme, definitive data on how these two processes occur and especially, how they are coupled to perform energy transduction, is missing.

Significant achievements: Our major achievement obtained at the end of the previous grant period was the discovery that the protein is being regulated, at least in part, by the water-soluble C-terminal 100 amino acids via an inter-molecular, rather than intra-molecular, mechanism. In other words, contrary to prior expectations, we have found that the C- terminus of one pump polypeptide is interacting with N-terminal residues in a second identical polypeptide, in a head-to-tail formation of dimers and higher

order polymers. This discovery was made possible by utilizing heavy isotope (^{15}N) labeling of one cell, and mixing the detergent solubilized protein from that cell, with identical protein derived from a second cell produced with only the ambient, light isotope (^{14}N) lacking the extra neutron. We observed that over a few hours, the pump exchanged polypeptides and with crosslinking reagents, we were able to identify specific amino acids present at the peptide surfaces of the two polypeptides. We observed both dimers and trimers and from this and other mass spectrometric based crosslinking experiments, we created a 'head-to-tail' model for how the proteins were positioned, with potentially important ramifications for how the C-terminal domain may be regulating ATP hydrolysis and/or proton efflux. More recently, we have utilized covalent labeling (e.g. hydroxyl radical footprinting) and crosslinking reagents that include a new functionality, i.e. a 'clickable' chemical handle, such as an azide or alkyne, so that the modified residues and dipeptides can be enriched, providing a much larger amount of information per sample. Furthermore, in collaboration with Huilin Li at the Van Andel Institute, we are using cryoEM to obtain how resolution structural data to identify the specific amino acids within the catalytic center that interact with specific residues within the C terminal regulatory domain. Previous work with the plant enzyme and current studies with the enzyme from fungi indicate that hexameric rings provide the highest resolution data and may represent the enzyme in its most active form. We are exploring various methods needed to obtain the plant enzyme in this conformation, e.g. by utilizing a phosphomimetic T947D mutation together with a small protein known as 14-3-3, that may be acting as a chaperone to facilitate stability of the hexameric rings.

Science objectives for 2021-2022: There are two major objectives for the experiments currently underway. First, as in the past, we are using the plant enzyme AHA2 overexpressed in yeast, but in order to facilitate the formation of hexameric rings suitable for cryoEM, we are co-expressing the 14-3-3 protein chaperone together with specific mutations in the plant enzyme, as well as in the presence of fusicoccin, a compound some fungi secrete to accelerate the rate of elongation of plant cells, via its ability to stabilize the interaction of the 14-4-3 with phosphorylated T947 only in the plant enzyme. Simultaneous with the cryoEM efforts, we are exhaustively utilizing various covalent labeling reagents to obtain experimental measurement of changes in the SASA (Solvent Accessible Surface Area) of specific side chains in amino acids when the enzyme is treated with various substrates and inhibitors. to trap the enzyme in its stable conformers and facilitate the design and interpretation of cryoEM experiments. The mass spectrometric based observations with labeling reagents are performed in solution with protein potentially undergoing series of conformational changes and are thus orthologous, and useful to help design and interpret the cryoEM measurements that require an abundance of one conformer.

My project addresses BES cross-cutting priority areas by: Providing atomic level resolution of a protein that not only creates the highest membrane potential of any energy transducing protein in nature, but also is critical for understanding how its structure and its unique catalytic and regulatory activities satisfies the biological needs of higher plants, in terms of controlling their rates of cell elongation and key developmental biology events.

My scientific area(s) of expertise are: tandem mass spectrometry, protein chemistry, genomic technology development.

The ideal collaborator for my project would have expertise in: cryoEM of energy transducing membrane proteins, and I have already found him, via this DOE BES meeting two years ago (Huilin Li).

Publications supported by this project (2021-2022):

Blackburn MR, Minkoff BB, Sussman MR. Mass spectrometry-based technologies for probing the 3D world of plant proteins. Plant Physiol. 2022 May 3;189(1):12-22. doi: 10.1093/plphys/kiac039. PMID: 35139210; PMCID: PMC9070838.

Atomic Resolution of Lignin-Carbohydrate Interactions in Native Plant Tissues from Solid-State NMR

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Overall research goals:

The energy-rich and carbon-rich plant cell wall is a sophisticated composite of macromolecules. The interactions between the phenolic polymer lignin and polysaccharides have made the biomass recalcitrant to post-harvest processing. This project aims at developing a biophysical toolbox to enable atomic-level investigations of polysaccharides and lignin using intact stems of maize, Arabidopsis, spruce and poplar. We will employ advanced solid-state NMR methods to resolve the roles of electrostatic interactions and covalent linkages in stabilizing lignin-polysaccharide contacts cellulose, xylan, and glucomannan). We will also determine the domain distribution and hydrophobicity heterogeneity of biopolymers as well as their functional relevance. Comparing wild-type samples with lignin-engineered, transgenic plants will uncover the molecular principles involved in biopolymer interactions and supramolecular assembly. The fundamental knowledge will advance our understanding of energy storage in plants, form the foundation for optimizing the utility of lignocellulose for energy and biomaterial, and inspire the rational design of synthetic polymers and composites with tunable structure and properties. The spectroscopic methods established here are widely applicable to many energy-relevant systems such as plants, algae, microbes, as well as carbon-rich materials and synthetic polymers.

Significant achievements (2020-2025):

Over the last year, we have made multiple advances toward the method development and structural elucidation of carbohydrate structure in plants and other photosynthetic organisms.

First, we have compared the nanoscale assembly of lignin and carbohydrates in the plant secondary cell walls and have proposed three comparative models for the secondary cell wall organization and the lignin-carbohydrate interface in the grass, hardwood, and softwood. The function of hemicellulose xylan is found to be conformation-dependent: coating the even surface of cellulose via its flat‐ribbon structure and primarily binding lignin domains via its non-flat conformation using preferential surface contacts stabilized by numerous electrostatic interactions. However, this selectivity is partially compromised in the stems of woody plants, with some of the lignin particles closely packed with the flat-ribbon part of xylan as well as the surface of cellulose fibrils, which is likely forced by molecular crowding in the densely packed stems of trees. Finally, the softwood spruce has the best molecular mixing of biopolymers on the nanoscale, with lignin adapting a dispersive distribution instead of forming nanoparticles. This paper answers to two of the six questions listed as the major goals of this project by validating the central role of electrostatic interactions in stabilizing polymer contacts and estimating the dimension of polymer nanodomains.

Second, we conducted our first exploratory study on another photosynthetic organism, the green microalgae. Using solid-state NMR methods, we developed an innovative protocol for identifying and quantifying the carbohydrate content and composition using intact cells, without any chemical or physical perturbation. We are able to show that starch is the most abundant polysaccharide in a naturally cellulose-deficient strain of this microalga, and this polymer adopts a well-organized and highly rigid structure in the cell. Some xyloses are distributed in both mobile and rigid domains of the algal cell wall, with their chemical shifts partially aligned with the flat-ribbon 2-fold xylan (the

part coating the flat surface of macromolecules such as cellulose) identified in plants. The glycolipids, the major part of algal carbohydrates, are found to be largely mobile. Although demonstrated on a model green microalga Parachlorella beijerinckii, the method is widely applicable to glycan quantification in different photosynthetic organisms, including plants.

Third, we have developed a novel method that relies on the sensitivity-enhancing technique Dynamic Nuclear Polarization (DNP) to eliminate the need for ^{13}C-labeling during the solid-state NMR investigation of photosynthetic biomaterials. We have successfully demonstrated this method on a grass Oryza sativa (rice). The atomic resolution allows us to fully reveal the polymorphic structure of cellulose and xylan in intact stems and further reveal the sophisticated change of molecular motions. We also found that both cellulose and xylan have become more dynamic on the nanosecond and microsecond timescale in a ctl1/ctl2 double mutant. The method will allow for rapid screening and structural determination of carbon-rich biomaterials that are hard to label.

Science objectives for 2022-2023:

We expect that by the end of next fiscal year, we will achieve major advance in the understanding of mannan's structure and function and lignin-carbohydrate bonds *in muro*. Mannan is always difficult to resolve from other carbohydrates in softwood spruce, making it highly challenging to understand the structural function of this carbohydrate. We have coded a new NMR pulse sequence and successfully tested it on softwood. This new experiment provides the needed spectral resolution for unambiguously tracking the signals of mannan. We are now able and planning to investigate the patterns of mannan-cellulose, mannan-xylan, and mannan-lignin interactions in softwood spruce. At the same time, we are optimizing the methods for searching for lignin-carbohydrate bonds in native cell wall samples, which relies on the DNP for enhancing NMR sensitivity to allow for the search of lowly populated bonds like that.

My project addresses BES cross-cutting priority areas by:

Supporting the understanding and predicting matter and energy at the atomic and molecular levels for facilitating the development of novel technologies in sustainable energy and biomaterials.

My scientific area(s) of expertise is/are: Solid-state NMR analysis of biomolecules (carbohydrate, lipid, protein, lignin) for understanding their structure, dynamics, and interactions.

The ideal collaborator for my project would have expertise in: plant biology, natural and synthetic polymers, microbiology, algae.

Publications supported by this project [2020-2025]:

1. Kirui, A.; Zhao, W.; Deligey, F.; Yang, H.; Kang, X.; Mentink-Vigier, F.; Wang, T. "Carbohydrate-aromatic interface and molecular architecture of lignocellulose." *Nat. Commun.* 13, 538 (2022). *Selected as the editors' highlight. Highlighted on BES's website.*
2. Poulhazan, A.; Dickwella Widanage, M.; Muszynski, A.; Arnold, A.; Warschawsi, D.; Parastoo, A.; Isabelle, M.; Wang, T. "Identification and quantification of glycans in whole cells: architecture of microalgal polysaccharides described by solid-state NMR." *J. Am. Chem. Soc.* 143, 46, 19374-19388 (2021). *Front cover article.*
3. Ling, Z.; Tang, W.; Su, Y.; Huang, C.; Kirui, A.; Wang, T.; French, A.D.; Yong, Q. "Stepwise allomorphic transformations by alkaline and ethlenediamine treatments on bamboo crystalline cellulose for enriched Enzymatic digestibility." *Ind. Crops. Prod.* 177, 114450 (2022).
4. Zhao, W.; Kirui, A.; Deligey, F.; Mentink-Vigeir, F.; Zhou, Y.; Zhang, B.; Wang, T. "Solid-state NMR of unlabeled plant cell walls: high-resolution structural analysis without isotopic enrichment." *Biotechnol. Biofuels* 14, 1-14 (2021).

Elucidating the Biochemical Mechanisms Controlling Secondary Wall Biosynthesis in Plants

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Email: yh@uga.edu; Website: https://www.plantbio.uga.edu/directory/people/zheng-hua-ye

Overall research goals:

The major goal of this project is to carry out biochemical characterization of enzymes involved in the biosynthesis of xylan, the second most abundant polysaccharide in secondary cell walls. Secondary walls in the form of wood and fibers are the most abundant stored energy in plant biomass. Understanding how secondary walls are synthesized will provide fundamental insight into how plants convert the fixed carbon through photosynthesis into a long-term stored energy. Xylan is composed of a linear backbone of β-1,4-linked xylosyl (Xyl) residues substituted with various side chains and often acetylated at *O*-2 or *O*-3. In grass xylans, the side chains include 2-*O*- and/or 3-*O*-linked arabinofuranose (Ara), 3-*O*-linked Ara substituted at *O*-2 with another Ara [Ara-(1->2)-Ara] or Xyl [Xyl-(1->2)-Ara], and 2-*O*-linked glucuronic acid (GlcA)/4-*O*-methylglucuronic acid (MeGlcA) residues. The biochemical mechanisms controlling the substitutions of xylan with these various side chains remain to be investigated. The specific aims of this proposed research are to carry out a comprehensive biochemical characterization of glycosyltransferases catalyzing the substitutions of xylan, the results of which will shed light on the biochemical mechanisms controlling secondary wall biosynthesis.

Significant achievements: [2022-2025]:

1. A rice GT61 glycosyltransferase is a xylan arabinosyl 2-*O*-xylosyltransferase (XAXT1) catalyzing the addition of Xyl onto *O*-2 of Ara*f* side chains of xylan to form the Xyl-Ara*f* disaccharide side chains

We have discovered that a novel GT61 glycosyltransferase, OsXAXT1, is able to mediate the addition of Xyl-Ara*f* disaccharide side chains onto xylan when co-expressed with a xylan α-1,3-arabinosyltransferase (OsXAT2) in the Arabidopsis *gux1/2/3* triple mutant that is devoid of sugar substitutions in xylan. 1D and 2D NMR analyses of xylan from the transgenic plants showed the presence of Xyl-Ara*f* disaccharide side chains in which Xyl is attached to *O*-2 of Ara*f* (Fig. 1). We further revealed that recombinant OsXAXT1 expressed in HEK293 cells was able to transfer Xyl onto the Ara*f* side chains of arabinoxylooligomers, establishing that it is a 2-*O*-xylosyltransferase catalyzing the addition of Xyl onto the Ara*f* side chains of xylan. Moreover, we have found that homologs of OsXAXT1 from other grass species, including maize, sorghum, *Brachypodium* and switchgrass, also possess the same xylosyltransferse activity as rice OsXAXT1, indicating their functional conservation in grass species. Our findings provide the first line of evidence demonstrating that grass XAXTs are xylosyltransferases catalyzing the addition of Xyl onto *O*-2 of Ara*f* side chains of xylan to form the Xyl-Ara*f* disaccharide side chains, which enriches our understanding of genes involved in xylan biosynthesis.

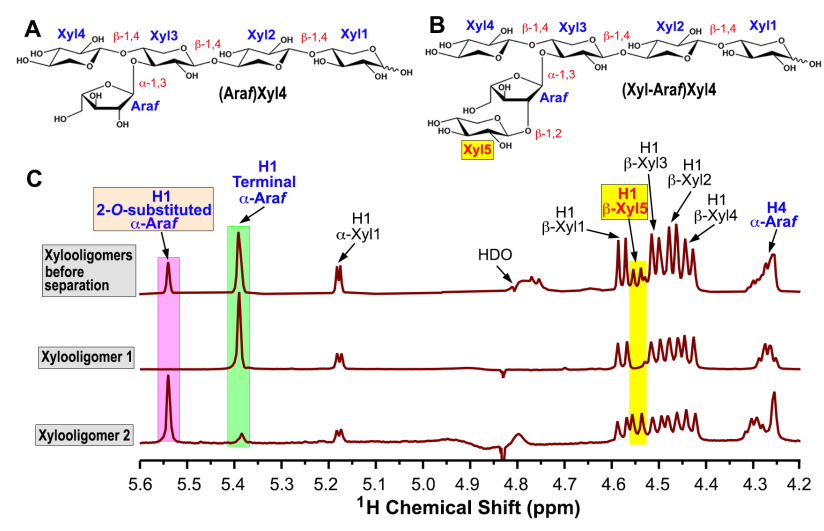

Fig. 1 Structural analysis of xylooligomers from transgenic *gux1/2/3* plants co-expressing OsXAXT1 and OsXAT2.

(A) Diagram of 3-*O*-Araf substituted xylotetraose [(Ara*f*)Xyl_4].

(B) Diagram of Xyl-Araf disaccharide substituted xylotetraose [(Xyl-Ara*f*)Xyl_4].

(C) ^{1}H NMR spectra of xylooligomers from *gux1/2/3* co-expressing OsXAXT1 and OsXAT2 before and after their separation. Xylooligomer 1 had the structure of (Ara*f*)Xyl_4 with resonances at 5.39 ppm corresponding to H1 of 3-*O*-linked terminal α-Ara*f*. Xylooligomer 2 had the structure of (Xyl-Ara*f*)Xyl_4 with resonances at 5.54 ppm corresponding to H1 of 2-*O*-substituted α-Ara*f* and at 4.55 ppm attributed to H1 of Xyl_5 attached to *O*-2 of α-Ara*f*.

2. Discovery of arabinosyltransferases and xylosyltransferases catalyzing the transfer of 2-*O*-Ara*f*, 3-*O*-Ara*f* and 2-*O*-Xyl side chains onto xylan in pine

We have uncovered four pine wood-associated GT61 members that exhibit three distinct glycosyltransferase activities involved in xylan substitutions. Two of them catalyzed the addition of 2-*O*-α-Ara*f* or 3-*O*-α-Ara*f* side chains onto xylooligomer acceptors and thus were named *Pinus taeda* xylan 2-*O*-arabinosyltransferase 1 (PtX2AT1) and 3-*O*-arabinosyltransferase 1 (PtX3AT1), respectively. Two other pine GT61 members were found to be xylan 2-*O*-xylosyltransferases adding 2-*O*-β-Xyl side chains onto xylooligomer acceptors. Our findings have established several pine GT61 members as xylan 2-*O*- and 3-*O*-arabinosyltransferases and 2-*O*-xylosyltransferases, which provides a knowledge basis for genetic improvement of plant biomass composition tailored for diverse end uses.

Science objectives for 2022-2023:

We have found that mutation of a secondary wall-associated Arabidopsis gene causes a severe defect in secondary wall thickening. We hypothesize that it is a new glycosyltransferase involved in xylan biosynthesis and will test this hypothesis by performing structural analysis of xylan from the mutant and biochemical characterization of its function. We will also continue our efforts on identification and biochemical characterization of additional glycosyltransferases involved in xylan substitutions. These research objectives will further our understanding of glycosyltransferases responsible for the synthesis of xylan, the second most abundant polysaccharide in plant biomass.

My project addresses BES cross-cutting priority areas by:

Because the bulk of fixed carbon through photosynthesis by land plants is stored in the form of secondary walls, my project on the study of secondary wall biosynthesis addresses the broad research area set by the Physical Biosciences program, which is to seek "a fundamental understanding of the complex processes that convert and store energy in living systems", and more specifically, addresses one of the supported studies by the program, which is "the underlying biochemical and biophysical principles determining the architecture of biopolymers and the plant cell wall". Because the bulk of plant biomass targeted for lignocellulosic biofuel production is from secondary walls, the knowledge generated from this research fits into the program's goals to "impact numerous DOE interests, including improved biochemical pathways for biofuel production".

My scientific area(s) of expertise is/are: Biochemistry and molecular genetics.

The ideal collaborator for my project would have expertise in: Protein and carbohydrate structures.

Publications supported by this project [2022-2025]:

1. Zhong, R., Lee, C., Cui, D., Phillips, D.R., Adams, ER, Jeong, H.-Y., Jung, K.-H. and Ye, Z.-H. (2022) Identification of xylan arabinosyl 2-*O*-xylosyltransferases catalyzing the addition of 2-*O*-xylosyl residue onto arabinosyl side chains of xylan in grass species. *Plant J.* doi:10.1111/tpj.15939.
2. Zhong, R., Phillips, D.R. and Ye, Z.-H. (2022) Independent recruitment of glycosyltransferase family 61 members for xylan substitutions in conifers. *Planta* 256, 70.
3. Ye, Z.-H. and Zhong, R. (2022) Outstanding questions on xylan biosynthesis. *Plant Sci.* 325, 111476.
4. Ye, Z.-H. and Zhong, R. (2022) Cell wall biology of the moss *P. patens*. *J. Exp. Bot.* 73, 4440-4453.
5. Zhong R, Cui D, Phillips DR, Sims NT, Ye Z-H. (2021) Functional analysis of GT61 glycosyltransferases from grass species in xylan substitutions. *Planta* 254, 131.
6. Zhong R, Phillips DR, Ye Z-H. (2021) A single xyloglucan xylosyltransferase is sufficient for generation of the XXXG xylosylation pattern of xyloglucan. *Plant Cell Physiol.* 62, 1589-1602.
7. Zhong R, Lee C, Haghighat M, Ye Z-H. (2021) Xylem vessel-specific SND5 regulates secondary wall biosynthesis through activating secondary wall NAC binding elements. *New Phytol.* 231, 1496-1509.
8. Zhong R, Kandasamy MK, Ye Z-H. (2021) XND1 regulates secondary wall deposition in xylem vessels through inhibition of VND functions. *Plant Cell Physiol.* 62, 53-65.

Exploring the Role of TOR Kinase in the Regulation of Central Metabolism and Lipid Synthesis

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John Shanklin, Co-PI(s)
Dora Farago, Postdoctoral Research Associate
Biology Department, Brookhaven National Laboratory, Building 463, Upton NY11973
Email: zzhai@bnl.gov; Website: None

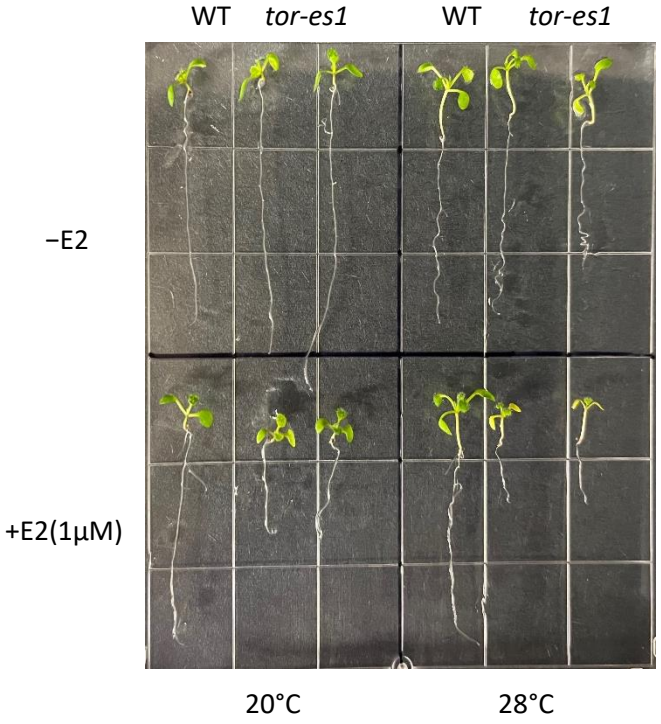
Overall research goals:

Our overall research goal is to explore the role of TOR kinase in the regulation of central metabolism, particularly, to define regulatory role of TOR signaling in plant lipid metabolism.

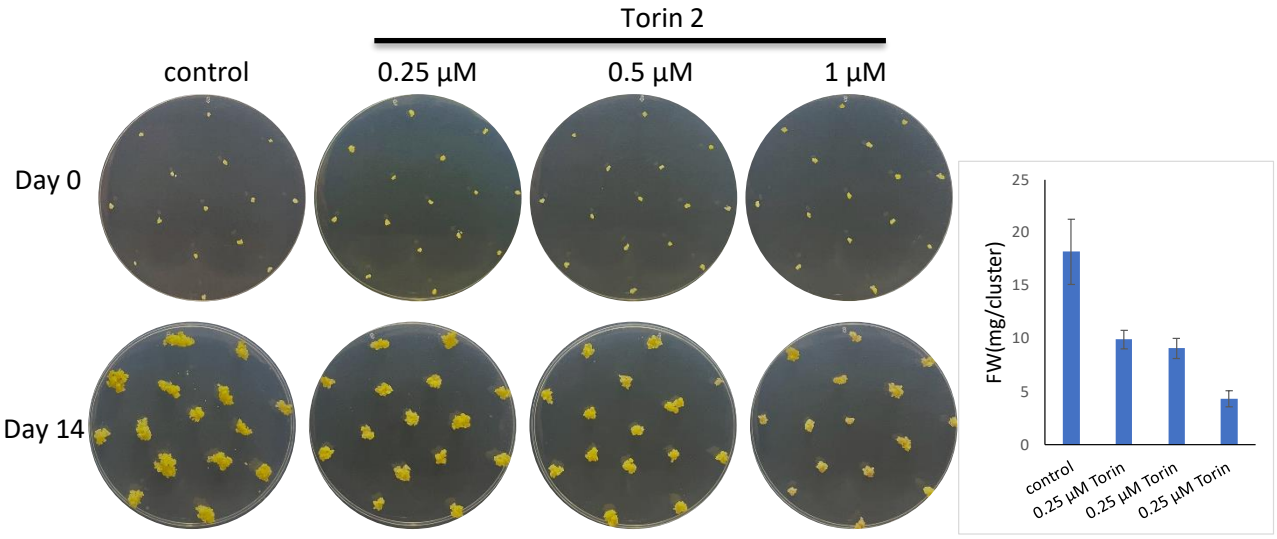
Significant achievements: 2022:

1. We have already established heat-induced morphogenesis of Arabidopsis plant and heat-induced cell growth of Brassica napus suspension cell line as plant model systems to study TOR carbon sensing mechanism.

2. We have either generated or attained Arabidopsis mutants and transgenic lines that have silenced or enhanced TOR activity.



TOR is necessary for heat-induced morphogenesis of Arabidopsis. WT and β-estradiol (E2) induced tor silencing transgenic lines (*tor-es1* and *tor-es2*) were germinated and grown on ½ MS+1% Sucrose for 3 days, then transferred to ½ MS with or without 1μM of E2 and grown under 20°C or 28°C for 7D.



TOR is necessary for heat-induced cell growth of Brassica napus. Effects of TOR inhibitor (torin2) on growth of Brassica napus suspension cells at the high temperature (28°C)

Science objectives for 2021-2022:

1. How carbon signals interact with and modulate the activity of plant TOR.
   1.1 To establish robust plant systems to study TOR carbon sensing mechanism.
   1.2 To test some potential potent TOR activators in heat-induced plant cell growth.
2. Define the mechanism(s) of TOR signaling regulation of plant lipid metabolism through short- and long-term suppression or enhancement of TOR protein kinase activity.
   2.1 To quantitate the role of TOR signaling in regulating lipid synthesis by suppression of TOR.
   2.2 To determine the effects of enhanced TOR signaling on lipid synthesis and accumulation.

My project addresses BES cross-cutting priority areas by:
Discovering underlying principles that govern how plants convert and store energy and developing knowledge that underpins the development of renewable sources of reduced carbon.

My scientific area(s) of expertise is/are: Plant metabolism, Plant genetics, protein biochemistry.

The ideal collaborator for my project would have expertise in: Proteomics, Cryo-EM.

Publications supported by this project 2022-2027:
None