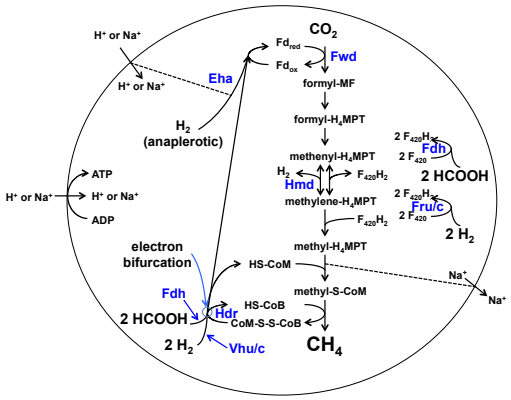
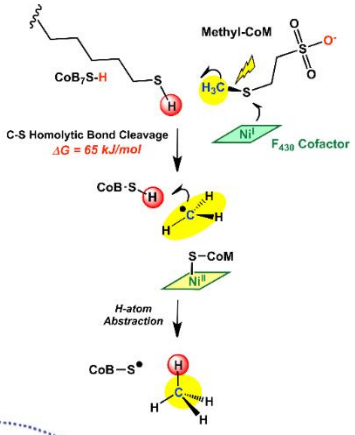
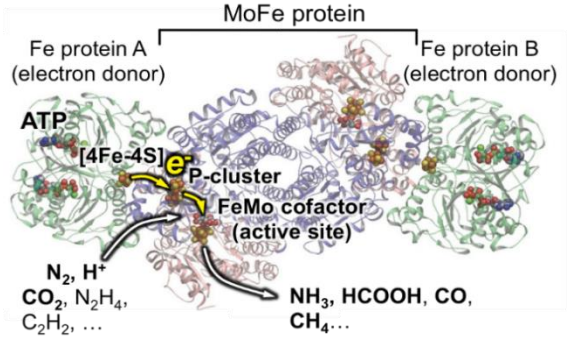
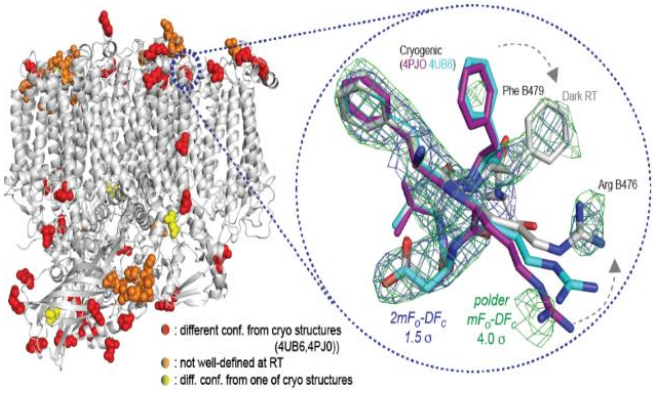
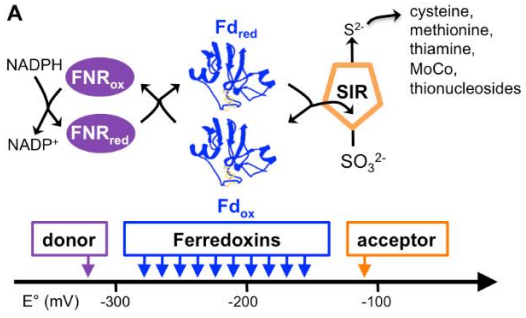
2016
Physical Biosciences Research Meeting











Marriott Washingtonian Hotel
Gaithersburg, MD
October 16-19, 2016

U.S. DEPARTMENT OF ENERGY | Office of Science

Office of Basic Energy Sciences
Chemical Sciences, Geosciences & Biosciences Division

2016 Physical Biosciences Research Meeting

Program and Abstracts

Washingtonian Marriott Hotel
Gaithersburg, MD
October 16-19, 2016

Chemical Sciences, Geosciences, and Biosciences Division
Office of Basic Energy Sciences
Office of Science
U.S. Department of Energy

The research grants and contracts described in this document are, unless specifically labeled otherwise, supported by the U.S. DOE Office of Science, Office of Basic Energy Sciences, Chemical Sciences, Geosciences, and Biosciences Division.

Foreword

This volume provides a record of the 5th biennial meeting of investigators funded by the "core" Physical Biosciences program, along with scientists in programmatically-relevant areas that are supported by the Office of Basic Energy Sciences (BES) through the Energy Frontier Research Center (EFRC) program. Physical Biosciences and Photosynthetic Systems are the two complimentary programs within DOE-BES that fund basic research in energy-relevant biological sciences. These two programs, along with Solar Photochemistry, comprise the Photochemistry and Biochemistry Team within the Chemical Sciences, Geosciences and Biosciences Division of DOE-BES.

We have an exciting meeting planned for you! We'll start off with the usual BES and program updates, as your Program Manager continues to adjust funding priorities in response to departmental initiatives, budgetary pressures, and other evolutionary drivers. Then we'll move on to the real science, with an exciting Keynote Lecture delivered by Tobias Erb from the Max Planck Institute on the best CO_2 fixation pathway that you've never heard of – because he and his colleagues just invented it! Later on Monday afternoon I think you'll be equally excited to hear about important new insights into the mechanism of water oxidation catalyzed by Photosystem II (PS II) being generated by Junko Yano and her colleagues at Lawrence Berkeley National Lab (LBNL). Junko is our distinguished Photosynthetic Systems Cross-Over Speaker, meaning this is work funded by the Photosynthetic Systems program that I think is especially worthy of bringing to your attention. Finally, at my request our last talk on Monday before dinner will be a short one from our very own W.E. Moerner from Stanford on "How My Life Has Changed Since I Won the Nobel Prize." It may be hard to believe, but it has already been two years since W.E. won the Nobel Prize in Chemistry!

Tuesday morning will kick off with our New Tools Drive New Discoveries Lecture, featuring George Maracas from DOE-BES who will tell you all about the BES Nanoscience Research Centers (NSRCs) – there are five of these user facilities with some amazing capabilities that are available to you without charge. In fact, looking back over time one of the most gratifying things to me as a PM has been to see so many of you follow up on these "Tools Talks" (my version of TED Talks) by either taking advantage of these opportunities and in some cases even instituting collaborations with previous speakers in this series that resulted in some incredible progress – see Session II for example! Of course in addition to the "special" talks highlighted above, this year's meeting will also feature some amazing oral and poster presentations from many of you that really showcases the quality of the science you do and its impact on a much larger community.

In closing this section, I want to express my appreciation to Diane Marceau at DOE-BES for her invaluable help in planning this meeting, along with Connie Lansdon and Tim Ledford from Oak Ridge Institute for Science and Education (ORISE) for their help ensuring its successful execution. Joshua Haines from DOE-BES put in an extraordinary effort to deliver the program book on a ridiculously short time line, and everyone above deserves credit for performing these functions with grace, humor, and wit. Finally, I am deeply indebted to my colleagues, Steve Herbert and Gail McLean, for making every day an exciting adventure as we do our best to enable you to deliver the foundational knowledge needed to achieve next generation energy solutions…

Robert J. Stack, Program Manager, Physical Biosciences, DOE-BES

Images on cover taken from abstracts submitted by Leigh, Ragsdale, Raugei, Yano, and Bennett/Silberg.

Agenda

AGENDA

5th Biennial Physical Biosciences Research Meeting
Marriott Washingtonian, Gaithersburg, MD
October 16-19, 2016

Sunday, October 16, 2016

3:00 – 6:00 p.m. Meeting Registration (Diane Marceau and Connie Lansdon)
6:00 – 9:00 Dinner On Your Own in Washingtonian Center, Gaithersburg, MD
8:00 – 11:00 Informal/Optional No-Host Reception at the Marriott Washingtonian Lounge

Monday, October 17, 2016

7:00 – 8:00 a.m. Breakfast and Ongoing Registration (Diane Marceau)

Session I: Welcome, Program Notes, and Keynote Lecture

8:00 – 8:15 a.m. Welcome, DOE-BES Updates
Gail McLean, Division Director (Acting), CSGB, DOE-BES

8:15 – 9:00 Physical Biosciences Program Notes
Robert Stack, Program Manager, Physical Biosciences, DOE-BES

9:00 – 10:00 ***Invited Keynote Lecture:***
We Do it Our (Path)Way: Bringing Inorganic Carbon into Life with Synthetic CO_2-Fixation
Tobias Erb, Max Planck Inst. for Terrestrial Microbiology, Marburg, Germany

10:00 – 10:30 Break

Session II: Novel Paths to CO_2 Reduction: Computational Tools Really Do Provide Key Insights

10:30 – 11:00 Novel Microbial Based Enzymatic CO_2 Fixation Mechanisms
John Peters, Montana State University

11:00 – 11:30 Enzymology of Methanogenesis: Mechanism of Methyl-Coenzyme M Reductase
Stephen Ragsdale, University of Michigan

11:30 – 12:00 Nitrogenase Reduction of CO_2 to Hydrocarbons
Dennis Dean, Virginia Polytechnic Institute and State University
Lance Seefeldt, Utah State University

12:00 – 12:30 Elucidating Enzymatic Mechanisms with Theory and Computation
Simone Raugei, Pacific Northwest National Laboratory

12:30 – 1:30 Lunch

1:30 – 3:30 Free/Discussion Time (Put up all posters)

3:30 – 4:00 Break/Munchies Served

Session III: *Let There Be Light – in the Form of Photons as Well as Knowledge*

4:00 – 5:00 ***Photosynthetic Systems Program Cross-over Lecture:***
X-ray Crystallography and Spectroscopy of Photosystem II Using a Femtosecond X-ray Laser
Junko Yano & Vittal Yachandra, Lawrence Berkeley National Lab

5:00 – 5:30 Investigation of Hydrocarbon Formation by Vanadium Nitrogenase
Markus Ribbe, University of California, Irvine

5:30 – 6:00 Fine-Tuning Redox Regulation in an Anaerobe
Carrie Harwood, University of Washington, Seattle

6:00 – 6:15 How My Life Has Changed Since I Became a Nobel Laureate
W.E. Moerner, Stanford University

6:15 – 7:30 Dinner at Marriott Washingtonian

Poster Session I

8:00 – 10:00 Poster Session, Odd Numbered Posters (No-Host)

Tuesday, October 18, 2016

7:15 – 8:00 a.m. Breakfast

Session IV: *New Tools from DOE-BES and the DOE-MSU Plant Research Laboratory*

8:00 – 9:00 ***New Tools Drive New Discoveries Lecture:***
DOE Nanoscience Research Centers (NSRCs): Resources for Collaborative Research
George Maracas, Program Mgr, Nanoscience Research Centers (DOE-BES)

9:00 – 9:30 Photosynthetic Energy Capture, Conversion and Storage: From Fundamental Mechanisms to Modular Engineering
Project A: Robust Photosynthesis in Dynamic Environments
David Kramer, Michigan State University/Plant Research Laboratory

9:30 – 10:00 Project B: Construction and Operation of the Biological Solar Panel
Danny Ducat, Michigan State University/Plant Research Laboratory

10:00 – 10:30 Project C: Characterizing and Engineering Subcellular and Cellular Modules for Photosynthetic Productivity
Beronda Montgomery, Michigan State University/Plant Research Laboratory

10:30 – 11:00 Break

Session V: *Addressing and Modeling the Sinks for Reduced Carbon in Plants at Brookhaven*

11:00 – 11:30 Modification of Plant Lipids
John Shanklin, Brookhaven National Laboratory

11:30 – 12:00 Quantitative Analysis of Central Metabolism and Seed Storage Synthesis
Jorg Schwender, Brookhaven National Laboratory

12:00 – 12:30 Post-translational Regulation and Macromolecular Organization of Lignin Biosynthesis
Chang-Jun Liu, Brookhaven National Laboratory

12:30 – 1:30 Lunch

1:30 – 3:30 Free/Discussion Time

3:30 – 4:00 Break/Munchies Served

Session VI: The Plant Cell Wall: Biosynthesis and New Concepts of Structure

4:00 – 4:30 Functional Analysis and Genetic Manipulation of Plant ABCB Organic Ion Transporters
Angus Murphy, University of Maryland

4:30 – 5:00 Secondary Wall Formation in Fibers
Zheng-Hua Ye, University of Georgia

5:00 – 5:30 Myosins XI are involved in cellulose deposition and CESA dynamics
Christopher Staiger, Purdue University

5:30 – 6:00 Structural Studies of Recombinant Plant CESA Domains of the Plant Cellulose Synthesis Complex
Hugh O'Neill, Oak Ridge National Laboratory

6:00 – 6:30 Multiscale Modeling of the Plant Cell Wall: Elucidating Chemical and Architectural Control Points
Peter N. Ciesielski & Michael F. Crowley, National Renewable Energy Laboratory

6:30 – 8:00 Dinner on Your Own

Poster Session II

8:00 – 10:00 Poster Session, Even Numbered Posters (No-Host)

Wednesday, October 19, 2016

7:15 – 8:00 a.m. Breakfast

Session VII: Hydrogenases

8:00 – 8:30 Role of HydF in Hydrogenase Maturation
Joan Broderick, Montana State University

8:30 – 9:00 Hyperthermophilic Multiprotein Complexes and Pathways for Energy Conservation and Catalysis
Michael Adams, University of Georgia

9:00 – 9:30 Utilization of Protein Film Electrochemistry to Characterize the Mechanisms Imparting Aerotolerance and Bidirectionality in Soluble, Multimeric [NiFe]-Hydrogenases
Anne Katherine Jones, Arizona State University

9:30 – 10:00 Mechanistic Studies on the Activation of Hydrogen by Algal [FeFe]-Hydrogenase
David W. Mulder, National Renewable Energy Laboratory

10:00 – 10:30 Break

Session VIII: Local and Global Challenges in Electron Management

10:30 – 11:00 Elucidating Biological Energy Transduction from Ammonia
Kyle M. Lancaster, Cornell University

11:00 – 11:30 Energy Conservation During Methanogenesis in Methanosarcina Species
William W. Metcalf, University of Illinois, Urbana-Champagne

11:30 – 12:00 Mechanism of Electron Bifurcation
Cara E. Lubner, National Renewable Energy Laboratory

12:00 – 1:15 Lunch **(Reminder: Make sure all posters are down**)

Session IX: Frontiers in Proton, Dinitrogen, and CO_2 Redox Biochemistry: A Forum to Discuss Key Basic Research Challenges and How to Translate What We Learn from Nature to Achieve DOE Mission

1:15 – 3:00 **All of you and…**
Robert Stack, Program Manager, Physical Biosciences, DOE-BES
Steve Herbert, Program Manager, Photosynthetic Systems, DOE-BES
Gail McLean, Lead, Photochemistry and Biochemistry, DOE-BES

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Session I

We do it our (path)way: Bringing inorganic carbon into life with synthetic CO2-fixation

Tobias J. Erb, Principal Investigator
Lennart Schada von Borzyskowski, Dominik Peter, Thomas Schwander, Raoul G. Rosenthal
Biochemistry and Synthetic Biology of Microbial Metabolism Group
Max Planck Institute for terrestrial Microbiology, D-35043 Marburg, Germany
Email: toerb@mpi-marburg.mpg.de; Website: http://www.mpi-marburg.mpg.de/erb

Overall research goals of our group:

Research in our group revolves around the interface of metabolism, biochemistry, and synthetic biology. One major goal of our research is to understand and reconstruct those principles that allow for the conversion of inorganic carbon into living organic matter. For that, we focus on (1) discovering novel enzymes and pathways for CO_2-fixation, (2) understanding the underlying biochemical and catalytic principles of these enzymes, and (3) constructing novel pathways for CO_2-fixation in vitro and in vivo with synthetic biology.

Keynote lecture abstract:

Carbon dioxide (CO_2) is a potent greenhouse gas that is a critical factor in global warming. At the same time atmospheric CO_2 is a cheap and readily carbon source. Yet, synthetic chemistry lacks suitable catalysts to functionalize the CO_2-molecule, emphasizing the need to understand and exploit the CO_2-mechanisms offered by Nature. In my talk I will (1) discuss the evolution and limitation of naturally existing CO_2-fixing enzymes and pathways. I will (2) present strategies for the engineering and design of artificial CO_2-fixation reactions and pathways (Fig. 1), and (3) outline how these artificial pathways can be realized in vitro and in vivo to create minimal CO_2-fixation modules and novel CO_2-fixing microorganisms.

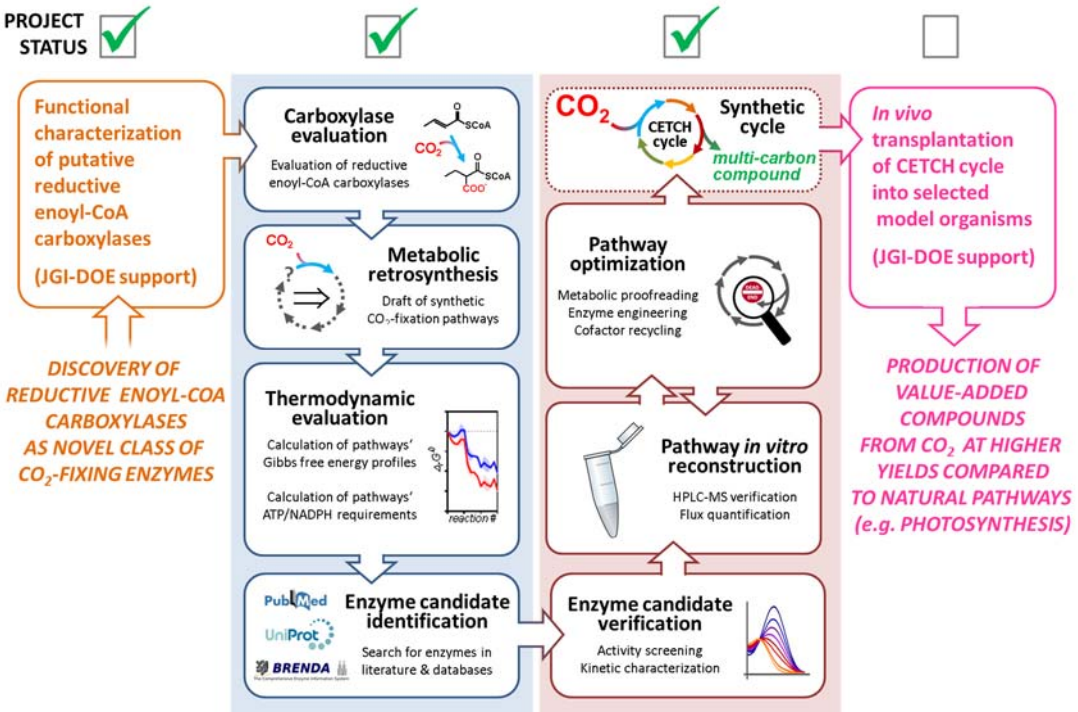


Fig. 1: Design and realization of the CETCH cycle, a synthetic CO_2-fixation pathway that is based on the principle of reductive enoyl-CoA carboxylation, a novel principle of CO2-fixation, discovered lately by us.

Significant achievements (2013-2015):

We lately discovered enoyl-CoA carboxylases/reductases (ECR), a novel class of highly efficient CO_2-fixing enzymes. In the last two years, we were able to resolve individual steps of the catalytic cycle of ECRs to follow CO_2-fixation in 'slow-motion' and could engineer these enzymes to catalyze novel carboxylation reactions. We also used ECRs to design and realize a synthetic cycle for the continuous fixation of CO_2 in vitro. The so-called crotonyl-CoA/ethylmalonyl-CoA/hydroxybutyryl-CoA (CETCH) cycle is a reaction network of 17 enzymes that converts CO_2 into organic molecules. The CETCH cycle was drafted by metabolic retrosynthesis and established with enzymes from nine different organisms of all three domains of life.

My scientific areas of expertise: biochemistry, synthetic biology, microbiology, metabolism & pathways

To take my project to the next level, my ideal collaborator would have expertise in: structural biology, reaction mechanisms, enzyme evolution, computational biology.

Selected recent publications:

1. R. G. Rosenthal, M.-O. Ebert, P. Kiefer, D. M. Peter, J. A. Vorholt, T. J. Erb "Direct evidence for a covalent ene adduct intermediate in NAD(P)H-dependent enzymes." Nature Chemical Biology **10**, 50-55 (2014)
2. D. M. Peter, L. Schada von Borzyskowski, P. Kiefer, P. Christen, J. A. Vorholt, T. J. Erb, "Screening and engineering the synthetic potential of carboxylating reductases from central metabolism and polyketide biosynthesis." Angewandte Chemie International Edition **45**, 13457-13461 (2015)
3. T. Schwander, L. Schada von Borzyskowski, S. Burgener, N. S. Cortina, T. J. Erb, „A synthetic patwhays for the fixation of carbon dioxide in vitro." Science, *in press*

Session II

Novel microbial based enzymatic CO_2 fixation mechanisms

John Peters, Principal Investigator
Montana State University, 224 Chemistry and Biochemistry Bldg., Bozeman, MT 59717
Email: john.peters@chemistry.montana.edu; Website: www.chemistry.montana.edu/~john.peters/

Overall research goals:

We are examining the catalytic mechanism of carboxylation enzymes in the microbial metabolism of propylene and acetone. Metabolic studies point to the key role of CO_2 as a substrate in these pathways. Two of the CO_2 fixing enzymes in these processes, acetone carboxylase (AC) and 2-ketopropyl-coenzymeM oxidoreductase/carboxylase (2-KPCC), are distinct mechanistically and have unique cofactor requirements. Our research plan involves kinetic studies and structure/function experiments including x-ray structure determination to dissect the molecular mechanism of these CO_2 fixing enzymes.

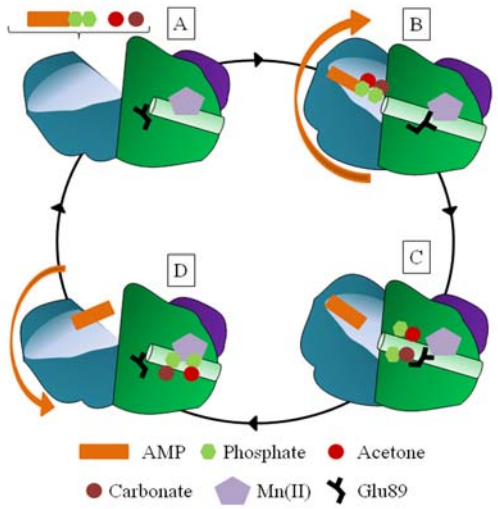
Significant achievements:

Substitution of a conserved catalytic dyad into 2-KPCC causes loss of carboxylation activity – 2-KPCC is a bifunctional enzyme coupling reductive C-S bond cleavage with carboxylation. The latter reactivity makes 2-KPCC a unique member of the DSOR family of enzymes. We have focused our efforts on unique elements of the active site environment of 2-KPCC compared with other members of the DSOR family, hypothesizing that these would be involved specifically in carboxylation. Probably the most interesting difference between the 2-KPCC reaction and other well-characterized DSOR enzymes like glutathione reductase (GR) or lipoamide dehydrogenase is that the active sites of these other DSOR-family enzymes facilitate the protonation of products. 2-KPCC exhibits a key structural difference relative to other members of the DSOR family: the absence of a His-Glu motif, termed a catalytic dyad, which has been shown to accelerate GR catalysis by protonating the reduced thiolate product. In 2-KPCC, the conserved His is replaced by Phe, and we have hypothesized that this difference is key for preventing the protonation of the enolacetone intermediate formed upon 2-KPC reductive cleavage leading to the formation of acetone instead of acetoacetate. We have shown that substitution of the Phe by His, essentially restoring the active element of the catalytic dyad found in other DSOR members, significantly compromises carboxylation activity but retains near wild-type levels of overall turnover. This favors the production of acetone even in the presence of CO_2. The results are quite profound and indicate that these differences in 2-KPCC are key to discriminating between CO_2 and protons as attacking electrophiles.

A new pathway for coenzyme M biosynthesis in proteobacterium Xanthobacter autotrophicus Py2 –In the methanogenic archaea, CoM is synthesized by either phosphoenolpyruvate (PEP)- or L-phosphoserine-dependent pathways. In *X. autotrophicus* Py2, a putative suite of five CoM biosynthetic genes has been identified through informatics and proteomic approaches. Analysis of their protein sequences and partial characterization of selected gene products allowed for a hypothetical scheme for CoM biosynthesis in *X. autotrophicus* Py2 to be proposed, starting with the phosphosulfolactate product of XcbB1. XcbC1, a member of the argininosuccinate lyase family, catalyzes a β-elimination on this substrate to yield sulfoacrylic acid and inorganic phosphate. XcbD1 then catalyzes a hypothetical addition reaction between sulfoacrylic acid and AMP, forming the AMP conjugate of sulfopropionate. For the final step of the pathway, XcbE1, a pyridoxal phosphate (PLP) dependent enzyme related to cysteine desulfhydrase, catalyzes concerted decarboxylation and thiolation with cysteine as a cosubstrate to yield the final product, coenzyme M. Of the four protein products described, B1, C1 and E1 have been expressed, purified, and partly characterized biochemically. XcbD1 has been recently expressed and purified, and awaits further studies. The hypothesized activities of B1 and C1 have been confirmed via biochemical studies while the activities of E1 and D1

are still under investigation. The proposed reactions represent new enzyme activities for three separate enzyme families, and they define a complete, plausible pathway for CoM biosynthesis for bacteria that is distinct from CoM biosynthesis in the archaea.

Mechanism of ATP-Dependent acetone carboxylation – We have determined the first structure of the $\alpha_2\beta_2\gamma_2$ heterohexameric acetone carboxylase in the native, ligand-free state and in the presence of bound AMP, providing significant insights into the AC mechanism. From our initial analysis of the structure, we can infer that ATP binding and subsequent acetone and bicarbonate activation is coupled to large scale protein conformational changes. The structure of AC in the presence of bound AMP and in the absence of phosphoenolacetone and carboxyphosphate we believe represents a trapped state with the product AMP but lacking intermediates to proceed further in the reaction. This trapped state reveals the channel that protects the semistable phosphorylated intermediates traveling from the nucleotide-binding site to the Mn site from the aqueous solvent. Once the intermediates are bound at the Mn site, the electron-rich double bond of the phosphoenol acetone could attack the highly reactive carboxyphosphate, forming acetoacetone and the first phosphate product. Attack of a potentially Mn-activated water/hydroxide on the carboxyphosphate could drive the release of the second of AMP and reciprocal conformational changes that initiate the start of another cycle of catalysis (left).



AC structural transition states. β subunit (blue) with nucleotide binding site and α subunit (green) go through conformational changes that assist catalysis of substrates at the nuclotide binding site and Mn active site.

Science objectives for 2016-2017:
Identify the steps of the 2-KPCC reaction, and determine which presents the energetic barrier that the enzyme lowers in order to achieve catalysis.
Elucidate the biochemistry of bacterial biosynthesis of CoM
Determine how structural transitions control the entry of substrates (acetone, HCO_3^-, and ATP) and movement of intermediates through AC

My scientific area(s) of expertise is/are: Mechanistic enzymology and structure determination using x-ray diffraction methods.

To take my project to the next level, my ideal collaborator would have expertise in: Organic synthesis/bioorganic mechanism

Publications supported by this project:
1. G.A. Prussia, G.H. Gauss, F. Mus, L. Conner, J.L. DuBois, J.W. Peters. "Substitution of a conserved catalytic dyad into 2-KPCC causes loss of carboxylation activity" *FEBS Lett.* doi: 10.1002/1873-3468.12325.[Epub ahead of print] (2016) – *Highlight by FEBS*
2. S.E. Partovi, A.E. Gutknecht, H.A. Martinez, J.L. DuBois, and J.W. Peters, A new pathway for coenzyme M biosynthesis in proteobacterium *Xanthobacter autotrophicus* Py2 In revision for *J. Bacteriol.* (2016)
3. F. Mus, B.J. Eilers, A.B. Alleman, B.V. Kabasakal, J.W. Murray, B.P. Nocek, J.L. DuBois, J.W. Peters, Mechanism of ATP-Dependent acetone carboxylation. In review, *Nat. Struct. & Mol. Biol.* (2016)

Enzymology of Methanogenesis: Mechanism of Methyl-Coenzyme M Reductase

Stephen W. Ragsdale, Principal Investigator
Anjali Padwarthan, Thanyaporn Wongnate, Johanna Mock
Department of Biological Chemistry, Univ. of Michigan Medical School, Ann Arbor, MI 48109-0606
Email: sragsdal@umich.edu, Web: http://www.biochem.med.umich.edu/?q=ragsdale

Overall research goals: Methyl-coenzyme M reductase (MCR) from methanogenic archaea catalyzes the terminal step in the biological synthesis of methane. Using coenzyme B (HSCoB) as the two-electron donor, MCR reduces methyl-coenzyme M to methane. The mechanism of methane synthesis is thought to involve either methyl-nickel(III) or methyl radical/Ni(II)-thiolate intermediates. We are testing our current working hypotheses that the MCR mechanism involves a Ni(II)-SCoM ($MCR_{ox1\text{-}silent}$ intermediate) and a methyl radical and that there is significant kinetic bias in the "methanogenic" and "methanotrophic" MCRs to optimize methane formation and methane oxidation, respectively, at their physiological levels of substrates.

Significant achievements 2014-2016: Our goals were (a) to isolate and characterize intermediates in the MCR mechanism using presteady-state kinetic and spectroscopic methods and (b) to perform and compare computational and experimental studies with wild-type and site-directed variants of the methanogenic and "methanotrophic/ANME" MCRs in forward and reverse directions.
We employed transient kinetic, spectroscopic and computational approaches to study the reaction between the active Ni(I) enzyme and substrates (Fig. 1). Consistent with the methyl radical–based mechanism, there was no evidence for a methyl-Ni(III) species; furthermore, magnetic circular dichroism spectroscopy identified the Ni(II)-thiolate intermediate. Temperature-dependent transient kinetics also closely matched density functional theory predictions of the methyl radical mechanism. This work was recently published (1).

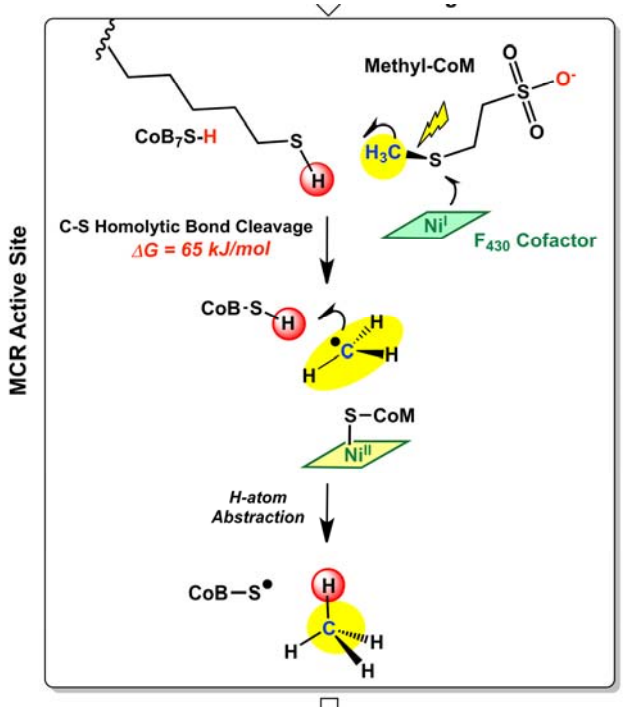


Fig. 1. Methanogenesis and the Initial step in the MCR mechanism.

We also studied the reaction of MCR with its native substrates using static binding, chemical

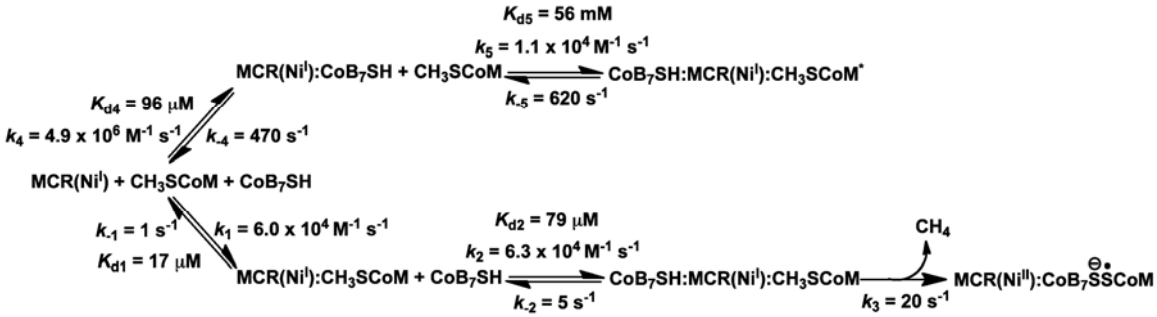


Fig. 2. Steady State MCR mechanism.

quench and stopped-flow techniques (2). We have characterized each of the steps in the MCR mechanism and measured all of the rate constants. We discovered that MCR utilizes an unusual mechanism by which it enforces a strictly ordered mechanism for methane synthesis (methyl-Coenzyme M first and coenzyme B second) (Figure 2). We find that MCR can bind either substrate; however, only the MCR:methyl-SCoM binary complex is productive while the other (MCR:CoB_7SH) is inhibitory. While methyl-SCoM binds extremely poorly to the inhibitory MCR:CoB_7SH complex (K_d = 56 mM), CoB_7SH has high affinity for the productive MCR:methyl-SCoM complex. This facilitates formation of the active ternary complex (CoB_7SH:MCR(Ni^I):CH_3SCoM) in which methyl-SCoM is bound near the Ni center and CoBSH bound in the channel above the methyl-SCoM. Only then can the chemical reaction occur (k_{obs} = 20 s^{-1} at 25 ^oC). Furthermore, it appears that substrate-induced conformational changes promote correct binding order and chemistry. The paper also has general impact to the area of protein chemistry and enzymology in that it describes a way that an enzyme can enforce a strictly ordered ternary complex mechanism even though it can bind either substrate with high affinity.

Science objectives for 2016-2018:

- Isolate and characterize intermediates in the MCR mechanism using presteady-state kinetic and spectroscopic methods
- Perform and compare computational and experimental studies with wild-type and site-directed variants of the "methanogenic" and "methanotrophic/ANME" MCRs in the forward and reverse directions.

References to work supported by this project 2014-2016: **(Also change this to 2014-2016 or N.A.)**

1. Wongnate, T., Sliwa, D., Ginovska, B., Smith, D., Wolf, M. W., Lehnert, N., Raugei, S., and Ragsdale, S. W. (2016) The Radical Mechanism of Biological Methane Synthesis by Methyl-Coenzyme M Reductase, *Science* **352**, 953-8
2. Wongnate, T., and Ragsdale, S. W. (2015) The reaction mechanism of methyl-coenzyme M reductase: how an enzyme enforces strict binding order, *J. Biol. Chem.* **290**, 9322-34.

And finally, to take my project to the next level my ideal collaborators would have expertise in: crystallography, XAS, spectroscopy, protein mutagenesis and expression in methanogens.

REFERENCES:

1. Wongnate, T., Sliwa, D., Ginovska, B., Smith, D., Wolf, M. W., Lehnert, N., Raugei, S., and Ragsdale, S. W. (2016) The Radical Mechanism of Biological Methane Synthesis by Methyl-Coenzyme M Reductase, *Science* **352**, 953-8
2. Wongnate, T., and Ragsdale, S. W. (2015) The reaction mechanism of methyl-coenzyme M reductase: how an enzyme enforces strict binding order, *J. Biol. Chem.* **290**, 9322-34

Nitrogenase Reduction of CO_2 to Hydrocarbons

Dennis R. Dean, Principal Investigator
Lance C. Seefeldt, Co-PI(s)
Department of Biochemistry, Virginia Tech, Blacksburg, Virginia 24060.
Email: deandr@vt.edu

Overall research goals:

The overall goal of this research is to gain a molecular level understanding of the activation and reduction of N_2 to NH_3 and CO_2 to hydrocarbons catalyzed by the bacterial enzyme nitrogenase. Nitrogenase catalyzes the multi-electron/proton reduction of dinitrogen (N_2) to two ammonia (NH_3).

$$N_2 + 8e^- + 8H^+ \rightarrow 2NH_3 + H_2 \qquad \text{eqn 1}$$

This is one of the most demanding reduction reactions achieved in nature. We have discovered that the protein surrounding the active site metal cluster, FeMo-cofactor, can be remodeled, allowing larger compounds to become substrates for nitrogenase. In one such remodeled nitrogenase, we were able to demonstrate that nitrogenase could reduce CO_2 by 8 electrons/protons all the way to methane (CH_4) in a catalytic reaction unprecedented in biology.

$$CO_2 + 8e^- + 8H^+ \rightarrow CH_4 + 2H_2O \qquad \text{eqn 2}$$

$$CO_2 + C_2H_2 + 8e^- + 8H^+ \rightarrow C_3H_6 + 2H_2O \qquad \text{eqn 3}$$

We have also demonstrated that it is possible to couple the reduction of two substrates at the same time. Acetylene (C_2H_2) and CO_2 could be activated, with observation of C3 hydrocarbon products. Our goals are to understand the molecular details of how nitrogenase catalyzes these challenging reduction reactions, thus providing foundational knowledge that can be applied to the design of robust catalysts for these reactions.

Significant achievements (2013-2016):

Our progress over the last three years has resulted in 10 publications (listed below). The key findings address how nitrogenase holds electrons and protons to achieve N_2 and CO_2 reduction. For N_2 reduction, we have published 7 papers (references 1, 2, 3, 5, 6, 9, 10 below) revealing that metal-hydrides ($M\text{-}H^-$) are a central feature of how nitrogenase reduces N_2. We show that for N_2 reduction, a central reaction is the formation of H_2 from two bound metal hydrides through reductive elimination (*re*). The *re* mechanism for N_2 reduction, that we have put forward with our collaborator Brian Hoffman (Northwestern University), provides a comprehensive description of the key step in N_2 reduction catalyzed by nitrogenase.

We have also examined the role of metal-hydride in CO_2 reduction catalyzed by nitrogenase. Here, we find that CO_2 reduction results from migratory insertion (*mi*) into the metal-hydride rather than reductive elimination (*re*) (reference 7 below). We further predicted and report for the first time that nitrogenase will reduce CO_2 to formate or CH_4 in competing reaction pathways. Molecular mechanic (MM) calculations with Simone Raugei (Pac. Northwest Natl. Lab) provided the energy profile for these competing reactions (Figure 1) and we show that it is possible to change the product profile by means of amino acid substitutions near the active site. These finding will be powerful in understanding how to control CO_2 reduction products and in allowing the rational design of changes to achieve predicted product profiles from CO_2 reduction.

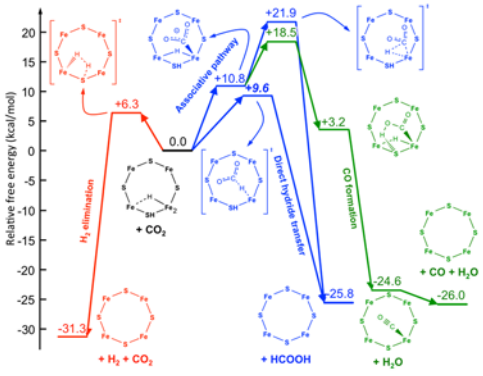


Figure 1: Reaction pathway from CO_2 to formate or CO. From reference 7.

Finally, we have made significant advances in figuring out how to deliver electrons to nitrogenase without the complications of the Fe protein cycle. In three publications (4, 6, 8 below), we show that it is possible to deliver electrons to the MoFe protein using small molecule reductants, solvated electrons (cyroreduction), or electrochemistry. These findings open new ways for gaining insights into the mechanism of N_2 and CO_2 reduction catalyzed by nitrogenase, and will be a central tool in the studies going forward.

Science objectives for 2016-2017:

- Exploit a system for delivery of electrons to nitrogenase MoFe protein using mediated and direct electrolysis, thus providing the unprecedented opportunity to examine mechanistic steps without the complications of the Fe protein cycle. We should be able to address key rate limiting chemical steps of N_2 and CO_2 reduction.
- Develop and apply new methods for rapid purification of fully active nitrogenase.
- Continue to attempt conversion of nitrogenase to a physiological nitrate reductase.

My scientific area(s) of expertise is/are: Microbial genetics, metallocluster assembly, enzymology. metalloenzymes.

To take my project to the next level, my ideal collaborators would have expertise in: X-ray crystallography of metalloenzymes and advanced spectroscopy of metalloenzymes.

Publications supported by this project 2013-2016:

1. Hoffman, B. M., Lukoyanov, D., Yang, Z.-Y., Dean, D. R., and Seefeldt, L. C. (2014) Mechanism of nitrogen fixation by nitrogenase: the next stage. *Chem. Rev. 114*, 4041–4062. DOI: 10.1021/cr400641x
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3. Smith, D., Danyal, K., Raugei, S., and Seefeldt, L. C. (2014) Substrate channel in nitrogenase revealed by a molecular dynamics approach. *Biochemistry 53*, 2278–2285. DOI: 10.1021/bi401313j
4. Danyal, K., Rasmussen, A. J., Keable, S. M., Inglet, B. S., Shaw, S., Zadvornyy, O. A., Duval, S., Dean, D. R., Raugei, S., Peters, J. W., and Seefeldt, L. C. (2015) Fe protein-independent substrate reduction by nitrogenase MoFe protein variants. *Biochemistry 54*, 2456–2462. DOI: 10.1021/acs.biochem.5b00140
5. Lukoyanov, D., Yang, Z.-Y., Khadka, N., Dean, D. R., Seefeldt, L. C., and Hoffman, B. M. (2015) Identification of a key catalytic intermediate demonstrates that nitrogenase is activated by the reversible exchange of N_2 for H_2. *J. Am. Chem. Soc. 137*, 3610–3615. DOI: 10.1021/jacs.5b00103
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8. Milton, R. D., Abdellaoui, S., Khadka, N., Dean, D. R., Leech, D., Seefeldt, L. C., and Minteer, S. D. (2016) Nitrogenase bioelectrocatalysis: heterogeneous ammonia and hydrogen production by MoFe protein. *Energy Environ. Sci. 9,* 2550-2554. DOI: 10.1039/C6EE01432A
9. Lukoyanov, D., Khadka, N., Yang, Z.-Y., Dean, D. R., Seefeldt, L. C., and Hoffman, B. M. (2016) Reversible photoinduced reductive elimination of H_2 from the nitrogenase dihydride state, the E4(4H) Janus intermediate. *J. Am. Chem. Soc. 138*, 1320–1327. DOI: 10.1021/jacs.5b11650
10. Lukoyanov, Khadka, Yang, Dean, Seefeldt, Hoffman (2016) Reductive elimination of H_2 activates nitrogenase to reduce the N≡N triple bond: characterization of the E_4(4H) Janus intermediate in wild-type enzyme. *J. Am. Chem. Soc.* (*in press*).

Elucidating Enzymatic Mechanisms with Theory and Computation

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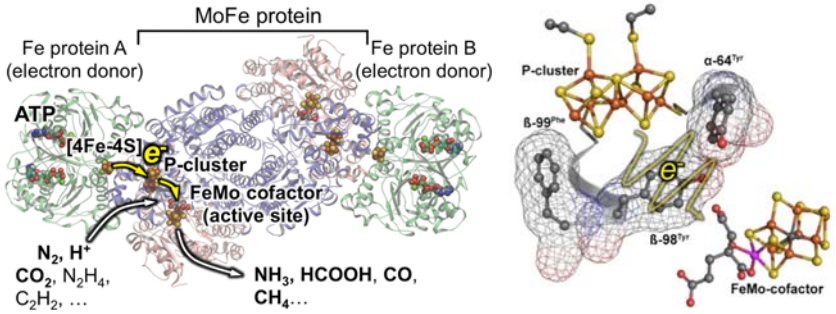
Overall research goals: Nitrogenase is an extremely versatile enzyme, which, in addition to the reduction of dinitrogen (N_2) to ammonia (NH_3), catalyzes a number of energy-relevant reactions, such as carbon dioxide (CO_2) reduction to formate ($HCOO^-$), carbon monoxide (CO) and methane (CH_4), at ambient condition using ATP, electrons and protons. Our goal is to gain basic structure-function information necessary to understand the mechanism of fast electron transfer and substrate activation in order to provide functional principles leading to new strategies for improved electro-chemical transformations.

Significant achievements 2014-2016: We performed a series of combined computational and experimental investigations aimed at understanding how the association between the biological electron donor (the ATP-dependent Fe protein) and the MoFe protein allosterically regulates some aspects of catalysis, specifically, intramolecular electron transfer and/or substrate binding and reduction. Our studies added to earlier findings that substitution of amino acid residues buried inside the MoFe protein near to the FeMo-co and the P-cluster allow for reduction of a number of substrates without the Fe protein and ATP. Computational analysis showed that the Fe protein and this region of the MoFe protein are mechanically coupled, suggesting a possible mechanism for how the Fe protein communicates with the MoFe protein.

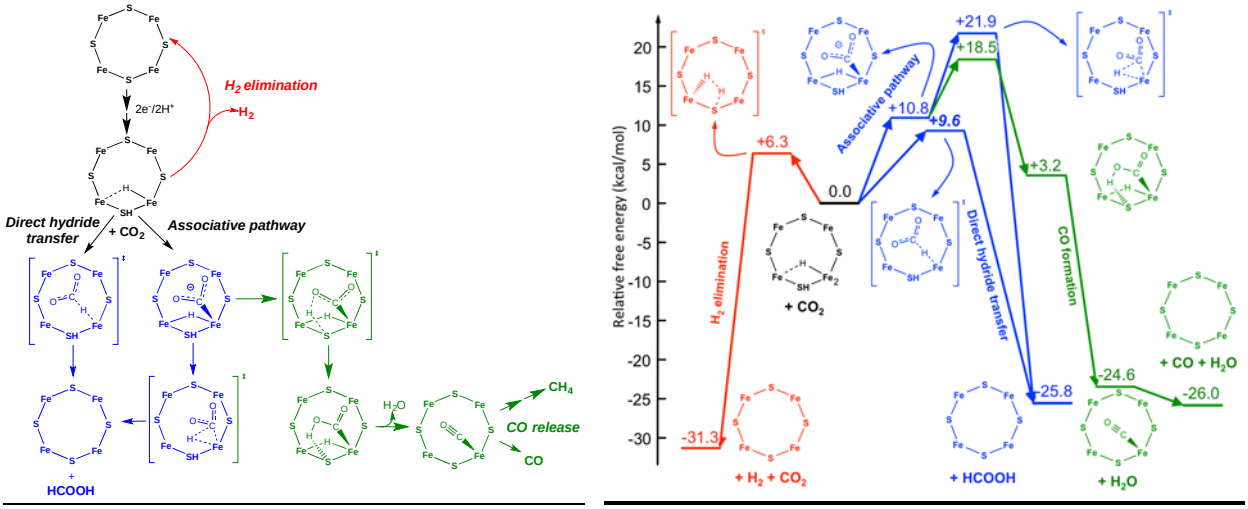


Nitrogenase and metal clusters. Left: Structure of the nitrogenase complex. Right: The three amino acid residues relevant to the Fe protein-independent reduction of the substrates (N_2H_4, N_3^- and H^+) by the MoFe protein are shown, along with the P cluster and FeMo-co.

Taking a step further, using kinetic measurements of electron transfer, ATP hydrolysis and Pi release, along with normal modes analysis of the nitrogenase complex motions, we also showed that the two halves of the nitrogenase complex do not undergo the electron transfer cycle independently. Rather the electron transfer within one half of the nitrogenase dimer induces conformational changes that allow for ATP hydrolysis and subsequent steps, while allosterically suppressing (partially) the electron transfer in the other half.

In a parallel effort, we investigated possible pathways for CO_2 reduction to $HCOO^-$, CO and CH_4. We demonstrated that nitrogenase preferentially reduces CO_2 by 2 electrons/protons to formate ($HCOO^-$) at rates more than 10 times higher than rates of CO_2 reduction to CO and CH_4. Quantum mechanical (QM) calculations on the doubly reduced FeMo-cofactor with a Fe-bound hydride and S-bound proton (E_2 state) favour a direct reaction of CO_2 with the hydride ('direct hydride transfer' reaction pathway), with facile hydride transfer to CO_2 yielding

formate. In contrast, a significant barrier is observed for reaction of Fe-bound CO_2 with the hydride ('associative' reaction pathway), which leads to CO and CH_4. Importantly, computations revealed that protein residues above the reactive face of the FeMo-co limit the CO_2 access to the hydridic hydrogen, introducing a barrier for the direct hydride transfer favouring the competitive elimination of H_2 over $HCOO^-$ formation. Consistent with this finding, MoFe proteins with amino acid substitutions near FeMo-cofactor (α-$70^{Val\rightarrow Ala}$, α-$195^{His\rightarrow Gln}$) are found to significantly alter the distribution of products between formate and CO/CH_4.



Possible pathways for CO_2 reduction (left): Reduction to formate (blue) can go by either a direct hydride transfer or an associative pathway; A pathway to formation of CO and CH_4 is shown in green; Six additional electrons and protons are added to the bottom structure to achieve reduction to CH_4; competitive formation of H_2 is shown in red. **Computed free energy diagram for CO_2 reduction and H_2 formation occurring at the E_2 state of FeMo-cofactor (right).**

Science objectives for 2016-2018:

- Characterization of the mechanism for the allosteric regulation of electron transfer.
- Atomistic study of reductive elimination mechanism of N_2 activation.
- Characterize the basic structural changes occurring within the nitrogenase complex during turnover utilizing an array of electron microscopy methods utilizing MoFe proteins that are modified to provide reporter molecules.

Our scientific areas of expertise are: Theoretical and computational biophysics (Simone Raugei) and biochemistry (Lance Seefeldt).

To take our project to the next level my ideal collaborator would have expertise: in EPR and related spectroscopy techniques. In this regard, we are already fruitully collarating with Brian Hoffman at Northwestern University.

References to work supported by this project 2014-2016:

1. K. Danyal, S. Shaw, T. R. Page, S. Duval, A. J. Fielding, M. Horitani, A. R. Marts, D. Lukoyanov, D. R. Dean, S. Raugei, B. M. Hoffman, L. C. Seefeldt, E. Antony (2016). Negative Cooperativity in the Nitrogenase Fe Protein Electron Delivery Cycle. *PNAS, in press.*
2. N. Khadka, D. R. Dean, D. Smith, B. M. Hoffman, S. Raugei, L. C. Seefeldt (2016). CO_2 Reduction Catalyzed by Nitrogenase: Pathways to Formate, Carbon Monoxide, and Methane. *Inorg. Chem. 55, 8321-8330.*
3. K. Danyal, A. J. Rasmussen, S. M. Keable, B. S. Inglet, S. Shaw, O. A. Zadvornyy, S. Duval, D. R. Dean, S. Raugei, J. W. Peters, L. C. Seefeldt (2015). Fe Protein-Independent Substrate Reduction by Nitrogenase MoFe Protein Variants. *Biochemistry 54, 2456-2462.*
4. D. M. A. Smith, K. Danyal, S. Raugei, and L. C. Seefeldt (2014). Substrate Channel in Nitrogenase Revealed by a Molecular Dynamics Approach. *Biochemistry* ***53,*** *2278-2285.*

Session III

X-ray Crystallography and Spectroscopy of Photosystem II Using a Femtosecond X-ray Laser

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Overall research goals: The objective of this proposal is to understand the mechanism by which H_2O is oxidized to O_2 in Photosystem II (PS II). We propose to develop and utilize new methodologies of X-ray spectroscopy and crystallography using synchrotron and X-ray free electron laser (XFEL) sources. The focus of our studies are: 1) What are the geometric and electronic structural changes of the catalytic Mn_4CaO_5 cluster of PS II? 2) How does the protein environment and the catalytic center interact with each other to carry out the sequential events during the four-electron water oxidation reaction, under ambient conditions? 3) Development of new X-ay spectroscopic techniques to study the natural and artificial photosynthetic systems.

Significant achievements 2014-2016:

1) We developed the method of simultaneous X-ray diffraction (XRD)/X-ray emission spectroscopy (XES) to collect time-resolved data at room temperature using XFELs. Using this method, we collected data from PS II from all the S-states, some of which with improved crystal resolution of up to 2.25 Å. We demonstrated that we can proceed through the entire S-state cycle, including time-points between the S_3 and S_0 states. The S-state intermediates of PS II were generated by multiple visible laser excitations, with 0.5 s intervals between flashes to achieve S-state turnover. We used a similar system to measure O_2 evolution yields using $H_2{}^{18}O$ labelled water and optimized the flow rate, laser power and time interval required for S-state turnover. The Kβ emission spectra show that the Mn cluster was undamaged and advanced through the S-states.

2) We collected the undamaged Mn L-edge X-ray absorption spectrum (XAS), using the XFEL, from the S_1 state of PS II. We used a specially designed 100-element zone-plate spectrometer, that discriminates the Mn Lα signal from the overwhelming signal from the O Kα from the aqueous solution, and demonstrated that the XAS data can be collected either with an XFEL SASE beam with monochromator or with a seeded beam without a monochromator. Theoretical simulations for understanding the spectrum and the electronic structure of the Mn cluster is underway.

3) We developed new computational methods and protocols for analyzing both XRD and XES data from the XFEL in real time. We also showed using the new protocols that the analysis of the XFEL data requires

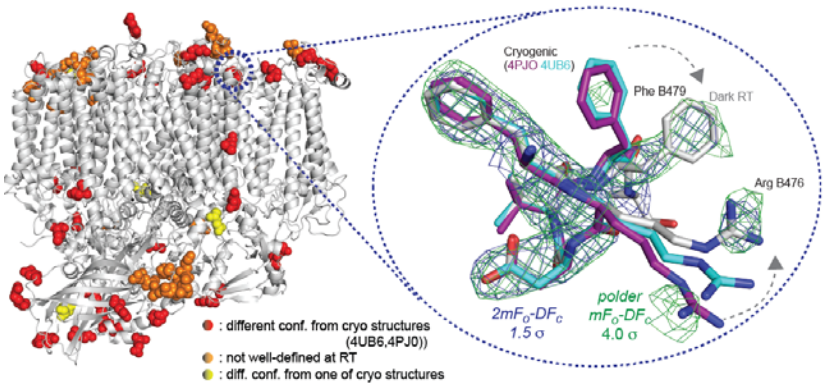

Fig. 1. Comparison of the room temperature (RT) and cryogenic structures in the dark state. Location of residues that show different side chain orientations at room temperature compared to the cryogenic structures. Examples of different side chain positions in the RT structure are shown in the zoomed view; the RT model (grey) together with the $2mF_o$-DF_c electron density map and the *polder* mF_o-DF_c map upon omitting residues B 475-479 and the cryogenic structures (cyan, purple).

fewer diffraction images than previously expected. We have developed a new drop on demand acoustic transducer-based sample delivery method, that improved the data collection efficiency significantly, and as a consequence improved the quality of both diffraction and spectroscopy data. Because of this drop-on-demand sample delivery method, we can more precisely control the time-intervals and the visible illumination protocols. This procedure also allows us to study larger crystals, that usually show diffraction to higher resolution. Using this method, we can collect the XES data from one time-point in less than 20 mins, making time-resolved spectroscopy a reality.

Science objectives for 2016-2017:

- We plan to conduct simultaneous XRD of all the S-states at room temperature to higher resolution, and follow the fast steps between the S_3 to S_0 transition in a time-resolved manner. The S_3 to S_0 transition, and the time-points between these transitions will be the focus of these studies.
- Mn L-edge spectroscopy of all the S-states and the intermediate states between the S_3 and S_0 states will be studied using the XFEL to determine the electronic structural changes that are involved in the O-O bond formation.
- We collected preliminary $K\beta_{2,5}$ XES data from Mn model complexes using the new acoustic droplet sample dispenser and we plan to collect data from PS II.
- We will continue our development of the stimulated emission spectroscopy for solution samples.

References to work supported by this project 2015-2016:

1. Young, I. D., *et al.* (2016) Structure of photosystem II and substrate binding at room temperature, *Nature* (*accepted*).
2. Kroll, T., *et al.* (2016) X-ray absorption spectroscopy using a self-seeded soft X-ray Free-Electron Laser. *Optics Express*, (*In press*).
3. Alonso-Mori, R., *et al.* (2016) Towards Characterization of Photo-Excited Electron Transfer and Catalysis in Natural and Artificial Systems Using XFELs. *Faraday Discuss.*, (*In press*).
4. Chatterjee, R., *et al.* (2016) Structural changes correlated with magnetic spin state isomorphism in the S-2 state of the Mn_4CaO_5 cluster in the oxygen-evolving complex of photosystem II, *Chem. Sci*. **7**, 5236-5248.
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7. Sauter, N. K., *et al.* (2015) No observable conformational changes in PSII, *Nature* **533**, E1-E2.
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9. Roessler, C. *et al.* (2015) Acoustic Injectors for Drop-on-demand Serial Femtosecond Crystallography. *Structure* **24**, 631-640.
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Investigation of hydrocarbon formation by vanadium nitrogenase

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Overall research goals:

The V-nitrogenase is considerably more active in CO reduction than its Mo-counterpart, making it an attractive template for investigations of the chemical and mechanistic details of this unique reaction. Using combined genetic (homologous recombination), biochemical (metal and activity analyses) and spectroscopic (EPR, XES and XAS spectroscopy) approaches, we propose to define the functional determinants of CO reduction by V-nitrogenase. Through our proposed studies, we hope to provide insights into the reaction of enzymatic CO reaction, which will enable engineering of nitrogenase-based, hydrocarbon-producing microbes and inform designs of novel CO- and CO_2-reducing catalysts in the long run.

Significant achievements (2015-2016):

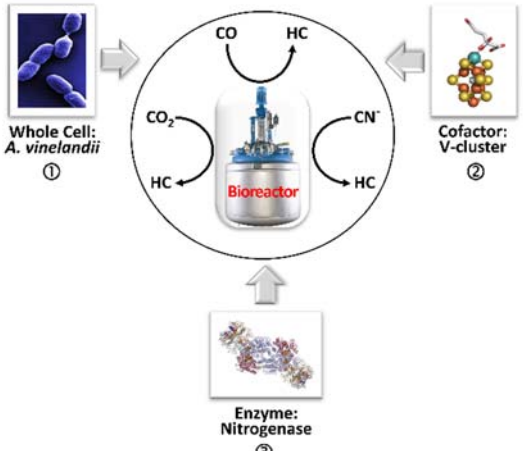

Figure 1. Schematic presentation of the long-term goal of our research.

During the initial 12 months of this proposal, we have made substantial progress in investigating the processes of hydrocarbon formation by (**1**) whole cells of *Azotobacter vinelandii* expressing the V-nitrogenase, (**2**) isolated nitrogenase cofactors and model compounds, and (**3**) purified nitrogenase enzymes, which provide a solid foundation for future designs of nitrogenase-based bioreactors for conversion of C1 substrates into hydrocarbon products (**Figure 1**).

Regarding **1**, we have shown that an *A. vinelandii* strain expressing the V-nitrogenase is capable of *in vivo* reduction of CO to ethylene (C_2H_4), ethane (C_2H_6) and propane (C_3H_8) (**Figure 2**). Moreover, we have demonstrated that CO is not used as a carbon source for cell growth; instead, it is reduced to hydrocarbons in a novel secondary metabolic pathway. These findings have broad implications in evolution-, environment- and energy-related areas, suggesting a possible role of the ancient nitrogenase as an evolutionary link between the carbon and nitrogen cycles on Earth while laying a solid foundation for biotechnological adaptation of a whole-cell approach to recycling carbon wastes into hydrocarbon products.

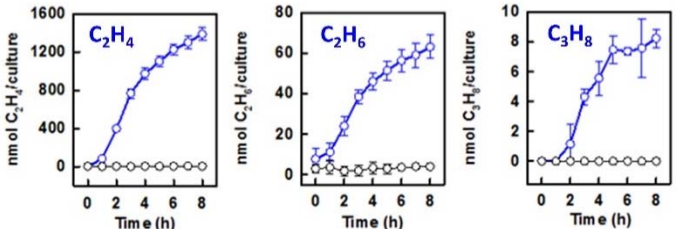

Figure 2. Formation of ethylene (C_2H_4), ethane (C_2H_6) and propane (C_3H_8) from CO by *A. vinelandii* expressing V-nitrogenase (*blue*) and Mo-nitrogenase (*black*).

Regarding **2**, we have established a new research focus on synthetic model chemistry in our lab and generated the first asymmetric synthetic mimic of the nitrogenase cofactor (designated the Mo-cluster) (**Figure 3**) in collaboration with Prof. Kazuyiuki Tatsumi and Prof. Yasuhiro Ohki at Nagoya University, Japan. In addition, we have synthesized an all-iron synthetic topolog of the nitrogenase cofactor (designated the Fe_6-cluster) based on a procedure described previously by Prof. Dick Holm's group at Harvard. Using a strong samarium (Sm)-based reductant, we have established ATP-independent CO-reducing activities not only for the isolated V- and M-clusters, but also for the two synthetic model compounds we synthesized recently. Both the biogenic V- and M-clusters and the

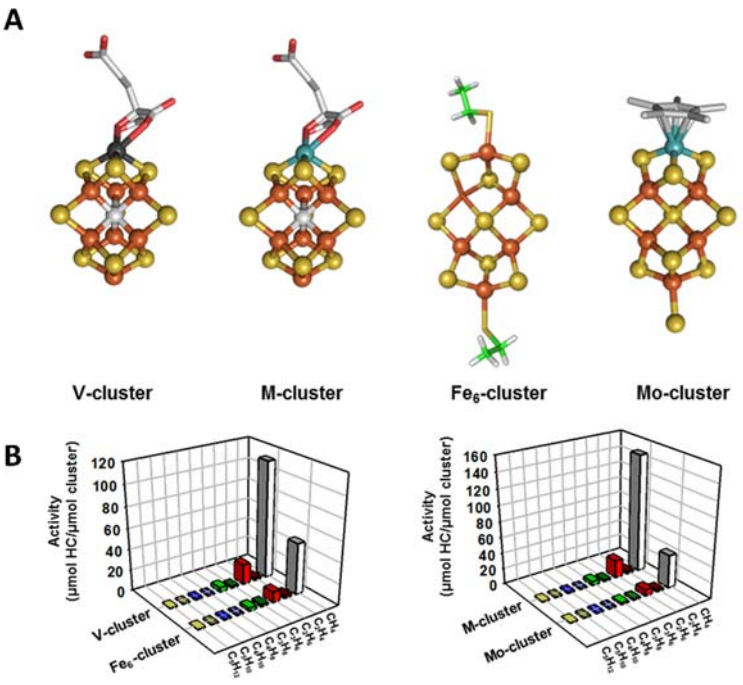

Figure 3. (**A**) Structural models of the V-, M-, Fe_6- and Mo-clusters. The atoms are colored as follows: Fe, orange; S, yellow; Mo, cyan; V, dark gray, O, red; C, light gray; C (in Fe_6-cluster), green; H (in Fe_6-cluster), gray. (**B**) Formation of hydrocarbons from CO-reduction by clusters in A. The turnover numbers are 205, 225, 92 and 73, respectively, for the V-, M-, Fe_6- and Mo-clusters.

synthetic Mo and Fe_6-clusters generated C1-C5 hydrocarbons as products of CO reduction and, remarkably, the synthetic clusters show turnover numbers as high as 30-50% of those of the biogenic clusters (**Figure 3**). Our capability to generate synthetic mimics of the nitrogenase cofactors expands our cluster library for further studies along this line, which could prove instrumental in establishing the criteria that are necessary for this type of clusters to perform the Fischer-Tropsch-like reaction of hydrocarbon formation.

Regarding **3**, our goal is to construct three P-cluster-replete but cofactor-deplete forms of variant apo-MoFe proteins, which carry (i) the domains of the cofactor site of VFe protein; (ii) the domains of the P-cluster site of VFe protein; and (iii) the domains of both cofactor and P-cluster sites of VFe protein. We have already constructed a parent strain on the basis of which the variants will be generated. In addition, we have also completed the mutagenic work and generated DNA fragments encoding the proposed apo-MoFe protein variants. Currently, we are in the process of transforming these DNA fragments into the parent strain and screening for *A. vinelandii* colonies carrying these fragments at the correct location.

Science objectives for 2016-2017:

- We plan to finish the construction of *A. vinelandii* strains expressing the apo-MoFe protein variants and characterize the V- and M-cluster-reconstituted proteins. In addition, we will include the available synthetic clusters in our proposed cofactor insertion studies based on our previous success of combining the Fe_6-cluster and the apo-MoFe protein into an artificial 'nitrogenase'. Our efforts along this line will not only facilitate identification of the key structural determinants of nitrogenase for its CO-reducing activity, but also enable generation of interesting artificial enzymes with altered/novel substrate-reducing activities.

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

To take my project to the next level, my ideal collaborator would have expertise in: ENDOR and FTIR spectroscopy.

Publications supported by this project 2015-2016:

1. Y. Hu, M. W. Ribbe, "Nitrogenases - A tale of carbon atom(s)." *Angew. Chem. Int. Ed. Engl.* **55**, 8216-8226 (2016). DOI: 10.1002/anie.201600010
2. J. G. Rebelein, Y. Hu, M. W. Ribbe, "The *in vivo* Hydrocarbon Formation by Vanadium Nitrogenase follows a Novel Secondary Metabolic Pathway. " *Nat. Commun.*, in final revision (2016)
3. K. Tanifuji, N. Sickerman, C. C. Lee, T. Nagasawa, K. Miyazaki, Y. Ohki, K. Tatsumi, Y. Hu, M. W. Ribbe, "Structure and Reactivity of an Asymmetric Synthetic Mimic of Nitrogenase Cofactor." *Angew. Chem. Int. Ed. Engl.*, in submission (2016)

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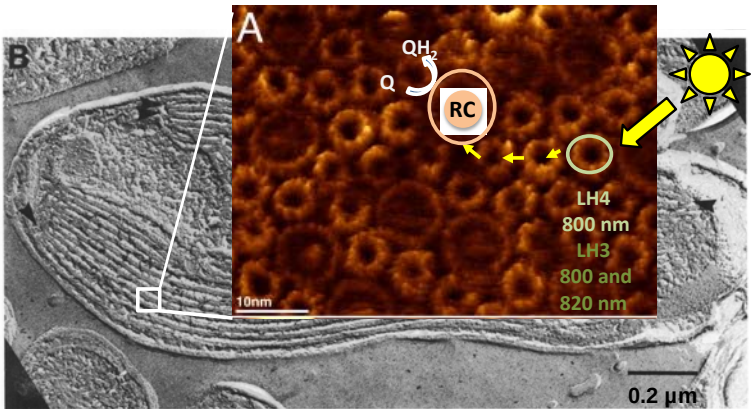
Overall research goals:

The development of devices that can capture sunlight and convert it to readily consumable forms of energy is of interest in the alternative energy arena. Ideally, effective light harvesting devices should be able to 1) capture light with maximum efficiency; 2) maintain this efficiency even when subjected to changes in light intensity caused by shading or the diurnal cycle; 3) be composed of abundant and inexpensive materials; and 4) self-repair or limit damage. Photosynthetic organisms meet these criteria and are ideal models for understanding/developing light harvesting devices and as a platform for biofuel production.

Our research goal is to understand how the synthesis of light harvesting (LH) complexes is regulated in response to low light in the purple non-sulfur photosynthetic bacterium *Rhodopseudomonas palustris* (Rpal). This species carries out cyclic photophosphorylation under anaerobic conditions and does not generate oxygen. Light harvesting complexes are circular pigment (bacteriochlorophyll/carotenoids)-protein complexes that absorb light and transfer it to a reaction center.



EM image of an Rpal cell showing its laminated photosynthetic membranes. Overlain on top (A) is an AFM image of light harvesting and reaction center complexes. Light absorption and the flow of energy are shown. A reduced quinone (QH_2) pool is used to generate a protonmotive force for ATP synthesis. [from: Scheuring *et al.*(2006). *J Mol Biol.* Varga and Staehelin. (1983). *J Bact.*

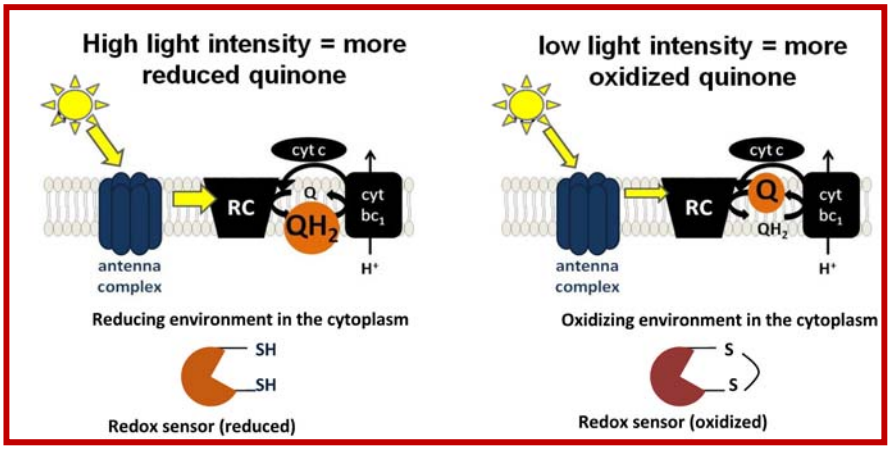
Significant achievements (2013-2016):

- Our most significant accomplishment is to show that Rpal senses low light intensity as a redox signal. Although this is known to be the case in cyanobacteria, our finding is important because it provides an opportunity to study redox regulation in an anaerobe. To date redox regulation has been studied almost exclusively in response to oxygen or, as with cyanobacteria, in the presence of oxygen.

- We defined a gene set that is required for low light LH4 expression. It encodes a signal transduction cascade that has two bacteriophytochromes and a redox sensing protein as components.

- The bacteriophytochromes (Bph2/Bph3) sense light quality to fine-tune LH4 expression and are part of a phosphorelay leading to LH4 gene expression, but they are not the major sensors of light intensity.

- We determined that redox, not light *per se* is the signal that controls LH4 gene expression in response to light intensity. A proposed mechanism for this in the context of cell physiology is shown in the following figure.



- We received a competitive user facility award from the Environmental Molecular Sciences Laboratory (EMSL) to augment this award and to work in collaboration with Aaron Wright, who uses activity based proteomics to identify redox responsive proteins. From an initial data set we have identified over 200 proteins that have redox responsive cysteines.

Science objectives for 2014-2015:

- Use microscopy and flow cytometry to provide a general probe-based evaluation of the overall cellular protein redox in Rpal under different light intensities and growth conditions.
- Identify characterize the specific cysteine residues undergoing redox regulation in proteins of interest in the context of bioenergy generation. A major target protein is nitrogenase.
- Start to work out the general mechanism of redox-responsive modification of proteins in response to low light. Determine what enzymes are involved.

My scientific area(s) of expertise is/are: Microbial physiology and biochemistry.

To take my project to the next level, my ideal collaborator would have expertise in: *In vitro* studies of nitrogenase. Our data indicate that nitrogenase is redox regulated – a new finding. This is an important enzyme and it is of interest to uncover the mechanism of redox regulation. Lance Seefeldt at Utah State University.

Publications supported by this project 2013-2016:

Fixen KR, A. W. Baker, E. A. Stojković, J. T Beatty, and C.S. Harwood. 2014. Apo-bacteriophytochromes modulate bacterial photosynthesis in response to low light. Proc. Natl. Acad. Sci. USA. 111: E237-44.

McKinlay JB, Y. Oda, M. Ruhl, AL Posto, U. Sauer and CS Harwood. 2014. Non-growing *Rhodopseudomonas palustris* increases the hydrogen gas yield from acetate by shifting from the gloxylate shunt to the tricarboxylic acid cycle. J Biol Chem. 289:1960-70.

Heiniger, E. K., and C. S. Harwood. 2015. Posttranslational modification of a vanadium nitrogenase. 2015. MicrobiologyOpen. Aug;4(4):597-603. doi: 10.1002/mbo3.265.

Fixen, KR, Oda Y and Harwood CS. 2015 Clades of photosynthetic bacteria belonging to the genus *Rhodopseudomonas* show marked diversity in light harvesting antenna complex gene composition and expression. **mSystems**. 1(1):e00006-15.doi:10.1128/mSystems.00006-15.

Fixen, KR and Harwood CS**.** 2016.A polymorphism in the oxygen-responsive repressor PpsR2 confers a growth advantage to *Rhodopseudomonas palustris* under low light. Photosynth. Res. 129: 199-20.

How My Life Has Changed Since I Became a Nobel Laureate

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Overall abstract goal:

The primary objective of this abstract, not submitted by W.E. Moerner but instead surreptitiously inserted into this book by his Program Manager, is to extend a hearty congratulations to W.E. from DOE-BES! And I am sure, all of you - his colleagues in the Physical Biosciences program – salute him as well!

The Nobel Prize in Chemistry 2014 was awarded jointly to Eric Betzig, Stefan W. Hell and William E. Moerner *"for the development of super-resolved fluorescence microscopy"*.

Life

W. E. Moerner was born in Pleasanton, California, but grew up in Texas. After studies at Washington University in St. Louis, Missouri and Cornell University in Ithaca, New York, in 1982 he received his doctorate from Cornell. He then worked at the IBM Almaden Research Center in San Jose, California until 1995. After three years at the University of California at San Diego, he moved to Stanford University in California. He has been a visiting professor at ETH Zurich and at Harvard University. W. E. Moerner is married and has one son.

Work

In normal microscopes the wavelength of light sets a limit to the level of detail possible. However this limitation can be circumvented by methods that make use of fluorescence, a phenomenon in which certain substances become luminous after having been exposed to light. Around 2000, Eric Betzig and William E. Moerner helped create a method in which fluorescence in individual molecules is steered by light. An image of very high resolution is achieved by combining images in which different molecules are activated. This makes it possible to track processes occurring inside living cells.

Moerner Nobel Interview

https://www.nobelprize.org/nobel_prizes/chemistry/laureates/2014/moerner-interview.html

Nice Photo Gallery Here

https://www.nobelprize.org/nobel_prizes/chemistry/laureates/2014/moerner-photo.html

After the Nobel Prize, What Do You Do for an Encore?

http://science.energy.gov/news/featured-articles/2016/10-05-16/

Poster Session I

Elucidating the Cellular Machinery for Lipid Storage in Plants

Kent D. Chapman, Principal Investigator
Robert T. Mullen (University of Guelph), John M. Dyer (USDA-ARS), Co-Principal Investigators
Department of Biological Sciences, BioDiscovery Institute, University of North Texas, Denton, TX 76203
Email: chapman@unt.edu Web: http://www.biol.unt.edu/~chapman/

Overall Research Goals:
A detailed understanding of the molecular mechanisms by which photosynthetic organisms capture, convert and store reduced carbon could provide important insights necessary to develop renewable, bio-based forms of usable energy. Of the cellular processes that participate in energy conversion and storage, perhaps the least well understood are the mechanisms for packaging reduced lipids into structures compatible with the aqueous environment of the cell. In fact, even the inventory of proteins that participate in the compartmentalization of storage lipids in plant tissues is far from complete. **Our overarching goal is to understand the biochemical and cellular processes important for compartmentalization of storage lipids in plant tissues, ultimately to manipulate the energy storage capacity of plants.** Toward this end, we will focus our near-term efforts on elucidating the composition and function of proteins involved in the biogenesis and stability of cytosolic lipid droplets in plant tissues through three specific aims: 1) Functionally characterize Lipid Droplet Associated Proteins (LDAPs) and their interacting proteins in the regulation of LD dynamics, 2) Functionally evaluate SEIPINs and their interacting proteins to produce lipid droplets at the ER, and 3) Assess the involvement of additional candidate lipodystrophy-related genes in lipid storage in plants.

Significant achievements 2014-2016:
This is a new award from the BES. However in support of our scientific aims, over the last few years our group has collaborated to identify several new players involved in the accumulation of plant lipid droplets, including Lipid Droplet Associated Proteins (LDAPs) and SEIPINs. Still, the information on how these proteins function to efficiently package and stabilize storage lipids remains to be elucidated.

Science objectives for 2016-2018:
Our efforts now will be directed toward identifying the protein networks that function to support the formation and stability of lipid droplets in plant tissues. Assessment of how this protein machinery is integrated to influence lipid droplet size, number, composition, and rate of lipid droplet formation in cell-free systems and in various plant tissues will be used to elucidate the mechanisms that regulate the biogenesis, dynamics and storage of hydrophobic, energy-dense lipids. We anticipate that our results will help define the mechanistic basis for variation and/or conservation of lipid storage across plant tissue types and in a wide range of organisms.

References to work supported by this project 2014-2016: Not Applicable, New award Sept 1, 2016.

My scientific area(s) of expertise is/are: Lipid Biochemistry, Cell Biology

And finally, to take my project to the next level my ideal collaborator would have expertise in:
Proteomics, Protein Structure, Time-lapse Imaging, Reconstitution of Cellular Systems *in vitro*

Molecular Mechanism of Energy Transduction by Plant Membrane Proteins
Prof. Michael R. Sussman, Principal Investigator
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Email: msussman@wisc.edu; Website:https://www.biotech.wisc.edu/sussmanlab/research/publications

Overall research goals. The plasma membrane is the point of contact between a cell and its external environment and plays an essential role in the growth and development of all organisms. In plants and fungi, the plasma membrane pump (H^+-ATPase) converts the chemical energy of ATP into a proton-motive force (PMF) comprised of both a chemical gradient of protons and an electrical potential. This PMF in turn is used to drive the transport of all other solutes (e.g. minerals, sugars, amino acids) and in addition plays a critical role in regulating the rate of cell expansion. My laboratory has been developing and implementing genomic and proteomic technologies that allow us to reveal the molecular details of how the plant plasma membrane proton pump converts chemical into electrical energy and also, how this process is regulated in response to environmental and developmental cues. This work has lately focused especially on the carboxy terminal 100 amino acids since it resides in the cytoplasm and appears to act as an inhibitory domain that presumably folds back onto, and inhibits, the catalytic domain. When the carboxy tail is phosphorylated (e.g., in response to auxin, light, pathogens) it's inhibitory function is diminished, resulting in enhanced pump activity and cell expansion.

Although the plant plasma membrane proton pump (called AHA, for **A**rabidopsis **H^+-A**TPase) M_r=100,000 dalton polypeptide has been crystallized and a low resolution X-ray diffraction structure determined, the carboxy terminal 100 amino acid regulatory domain was <u>not</u> observed in the diffraction pattern and thus, there is no clear understanding, at the molecular level, of how this domain operates. We have used a combination of genetic and chemical approaches to address this problem. Our genetic studies use a yeast heterologous expression system in which the fungal pump gene (called PMA1) is replaced by AHA2, one of the most highly expressed of the eleven genes encoding the plant enzyme. This provides a facile method of identifying key residues involved in regulating the plant pump expressed in yeast, but since the protein kinases and phosphatases involved in regulating the pump *in planta* are not present in yeast, we are also performing reverse genetic experiments with knockout plants. This work has identified an essential role of phosphorylation of the penultimate T947 in the plant's elongation response to auxin and blue and surprisingly, in collaboration with Prof. William Gray (U of Minneapolis), we have discovered that a protein phosphatase, rather than a protein kinase, is the key player in determining how phosphorylation of this specific residue is controlled by auxin. At the same time, recent work has indicated that regulation is considerably more complicated than we anticipated since there are a half dozen additional phosphorylated residues in both the C and amino terminal portions of the enzyme that may play opposing roles in regulating the pump. Our genetic studies with Arabidopsis knockout plants have also allowed us to fluorescently label the plant protein with various mutant GFP's, and use state of the art methods for localizing single pump molecules in living plant cells. These studies provide, for the first time, to measurents of the mobility and location of the pump protein within the two dimensional plane of the plasma membrane and will help us determine whether chemical (auxin, blue light e.g.) or genetic (e.g., phosphomimetic versions of T947) alter these properties *in vivo*.

More recently, we have ramped up our chemical approaches to understanding how the C terminus interacts with and regulates the catalytic portion of the protein, using two methods of monitoring solution structure via tandem mass spectrometry: (1) hydroxyl radical mediated footprinting of solvent accessible side chains in protein at microsecond scale, using our newly invented plasma-mediated device and (2) photoaffinity cross-linking via unnatural amino acid incorporation.

Significant achievements (past year):

- Using millisecond pulses of hydroxyl radicals produced by rapid mixing devices employing the Fenton reaction, 'footprinting' has been used for decades to study solvent accessibility in nucleic acids. Since proteins fold and unfold with a time scale much faster than nucleic acids, it is necessary to use methods that generate microsecond pulses of hydroxyl radicals. Prior to our work, there were only two methods available to achieve this: (1) high energy radiation produced via a synchrotron to generate hydroxyls from water or (2) a 248nm excimer laser to generate hydroxyls from hydrogen peroxide. For the past two years I have been collaborating with an engineering professor at UW-Madison to invent an inexpensive device that creates microsecond pulses of hydroxyl radicals from water, with an electrically generated plasma. We have now demonstrated that this device works very well for footprinting model proteins, and are now applying it in our studies on the C-terminus of AHA2.
- We have begun hydroxyl radical modifying experiments comparing wildtype and mutant AHA2 protein expressed in yeast, and our preliminary results demonstrate that we can identify a handful of discrete tryptic peptides in the N-terminal domains that show a large difference in solvent accessibility due to the presence or absence of the C terminal regulatory domain.
- We have successfully demonstrated the incorporation of the photoaffinity label unnatural amino acid, BPA, (3-(4-benzoylphenyl)alanine), into a half dozen different locations in AHA2 and purified sufficient protein to perform SDS-gel based analysis to determine the degree of UV induced intermolecular and intramolecular cross linking of monomers.
- We have begun applying appropriate software (e.g., Stavros) and synthesized deuterated BPA, to increase the ability of using tandem mass spectrometry for finding a cross-linked peptide.
- We have successfully applied state of the art single molecule visualization experiments with AHA2 labeled with suitable GFP translational fusions, to determine the location of this molecule within the plane of the plasma membrane, in living Arabidopsis cells.

Science objectives for 2016-2017:

- Identify specific amino acids within peptides whose degree of oxidation by hydroxyl radicals informs us which parts of AHA2 are involved in its regulation by the C terminus.
- Identify the sequence of UV cross-linked peptides using BPA with AHA2 expressed in yeast.
- Determine whether the location of individual AHA2 molecules in root cells *in planta* is affected by genetic and chemical agents known to affect the C-terminal regulation of activity.

My scientific areas of expertise are: tandem mass spectrometry, genomic profiling technologies.
To take my project to the next level, my ideal collaborator would have expertise in: use of X-ray crystallography and NMR to study protein structure dynamics.
Publications supported by this project:

1. Haruta M, Sabat, G, Stecker, K, Minkoff, BB and Sussman, MR 2014 A Peptide Hormone And Its Receptor Protein Kinase Regulate Cell Expansion in *A. thaliana*. Science 343:408-411.
2. Spartz AK, Ren H, Park MY, Grandt KN, Lee SH, Murphy AS, Sussman MR, Overvoorde PJ, Gray WM. 2014 SAUR Inhibition of PP2C-D Phosphatases Activates Plasma Membrane H^+-ATPases to Promote Cell Expansion in Arabidopsis. Plant Cell. 26:2129-2142.
3. Rodrigues RB, Sabat G, Minkoff BB, Burch HL, Nguyen TT, Sussman MR. 2014 Expression of a translationally fused TAP-tagged plasma membrane proton pump in *Arabidopsis thaliana*. Biochemistry. 53:566-578
4. Faraz A. Choudhury, Grzegorz Sabat, Michael R. Sussman, Yoshio Nishi, and J. Leon Shohet 2015 Fluorophore-based sensor for oxygen radicals in processing plasmas, Journal of Vacuum Science & Technology A33, 061305; doi: 10.1116/1.4930315
5. Haruta M, Gray WM, Sussman MR. 2015 Regulation of the plasma membrane proton pump (H^+-ATPase) by phosphorylation. Curr Opin Plant Biol 28:68-75

Molecular Mechanisms of Plant Cell Wall Loosening: Expansin Action

Daniel J. Cosgrove, Principal Investigator
Yuning Chen, Postdoctoral Research Associate
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Overall research goals:

We are studying the biophysical and molecular basis of plant cell wall loosening. This process is essential for plant cell growth, the unfurling of the photosynthetic leaf canopy and the storage of carbon and energy in plant cell walls, which constitute the biomass for the next generation of renewable biofuels. Our focus is on expansins. These proteins have the remarkable ability to induce wall stress relaxation, polymer creep, and cell wall enlargement. They act on selective sites within cell walls, loosening the cell walls, but without detectable enzymatic activity. Our goal is to understand how expansins loosen cell walls at the molecular scale. This has implications for cell wall structure and for biotechnological approaches to enhancing plant growth for food, fiber and energy needs of humanity. Moreover, expansins are important in plant adaption to environmental stresses such as water deficits and oxidative damage, so understanding their mechanisms of action may provide novel approaches for increasing resilience of plant crops to climate-related stresses.

We divide the universe of expansins into three groups:

1. **α-expansins (EXPAs)**; they mediate acid-induced extension of plant cell walls;
2. **β-expansins (EXPBs)**; we have studied an EXPB that is expressed abundantly in grass pollen and that selectively loosens grass cell walls. The major group of EXPBs ('vegetative homologs') are implicated plant growth, but active protein has not been available for study;
3. **Microbial expansins (EXLXs)**; they facilitate plant-microbe interactions. Recently we leveraged the facile expression of bacterial expansins in *E. coli* for study of these proteins by crystallography, site-directed mutagenesis and advanced solid-state NMR (ssNMR).

Current and ongoing work studies all three classes of expansins, which differ in the biophysical effects on cell walls. This research will yield critical insights into the molecular biophysics of plant cell walls and wall enlargement. Insights could inspire new approaches for improving the use of plant cell walls for the energy and material needs of society, e.g. to improve the efficiency of biofuel production from cellulosic biomass or to develop novel materials that mimic the versatile cell wall.

Significant achievements (2014-2016):

- Used phylogenetic analysis to conclude that multiple horizontal gene transfers from plants to microbes, followed by horizontal gene transfers among microbes, accounts for the diversity and scattered appearance of expansins in the microbial world. We also expressed a variety of these microbial expansins in *E. coli* and characterized their wall-loosening activity.
- The pronounced selectivity of the beta-expansin (ZmEXPB1) from maize pollen for grass cell walls, compared with cell walls in the evolutionary lineage leading to grasses, indicates that a substantial restructuring of the cell wall occurred early in the evolution of the grass family, most likely related to new structural functions of arabinoxylans, the major matrix polysaccharide found in grass walls.
- Combining binding isotherms with ssNMR with ZmEXPB1 linked to Mn^{2+} as a paramagnetic cell wall probe, we showed that ZmEXPB1 binds predominantly to glucuronoarabinoxylan (GAX) in complex grass cell walls and that this beta-expansin alters GAX mobility in a complex manner: rigid GAX becomes more rigid, mobile GAX becomes more mobile. This study provides the first molecular evidence of β-expansin's target in grass CWs and demonstrates a new strategy for investigating ligand binding for proteins that are difficult to express heterologously.

Science objectives for 2016-2018:

- Assess expansin binding/activity relations by directed protein evolution in vitro. This work will use phage display for a program of directed evolution to screen for mutants with increased binding affinity of a bacterial expansin to dissect the relationship between cellulose binding and wall-loosening activity.
- Analyze expansin action by FESEM imaging and AFM nanomechanical mapping. This will leverage our recent progress in imaging and nanomechanical assessment of cell walls to understand expansin binding and activity at the scale of individual cellulose microfibrils.
- Express plant expansins in mammalian HEK293 cells for structure/function analyses. This is a collaboration with Dr. Breeanna Urbanowicz (CCRC) and builds on her recent successes in expressing cell wall related enzymes in HEK293 cells. With this goal we hope to produce active EXPAs and vegetative EXPBs for studies of their activity. Recombinant proteins will be studied by site-directed mutagenesis, crystallography, extensometry of cell walls, and biochemical analyses.
- Test whether acid growth results from pH-dependent relocation of expansin within cell walls. We will test the novel hypothesis that expansin-induced wall loosening is the result of pH-dependent re-partitioning of expansin between pectin (non-productive) and the cellulose 'hotspots' where wall extensibility is controlled. Three approaches will be used, including ssNMR in collaboration with Prof. Mei Hong (MIT).

My scientific area(s) of expertise is/are: plant cell growth; cell wall biophysics and structure; biomechanics; plant molecular biology; recombinant protein expression; structural biology; modeling.

To take my project to the next level, my ideal collaborator would have expertise in: novel methods for assessing conformation and interactions of complex polysaccharide; coarse-grain modeling of polymers; polymer biophysics;

Publications supported by this project 2014-2016:

1. Georgelis, N., Nikolaidis, N., & Cosgrove, D. J. 2014. Biochemical analysis of expansin-like proteins from microbes. Carbohydrate Polymers 100: 17-23
2. Nikolaidis N., Doran N., & Cosgrove D.J. 2014. Plant expansins in bacteria and fungi: evolution by horizontal gene transfer and independent domain fusion. Mol Biol Evol 31: 376-386.
3. White, P. B.; Wang, T.; Park, Y. B.; Cosgrove, D. J.; Hong, M. 2014 Water – polysaccharide interactions in the primary cell wall of *Arabidopsis thaliana* from polarization transfer solid-state NMR. Journal of the American Chemical Society 136: 103990-10409
4. Sampedro J., Guttman M., Li L.C., Cosgrove D.J. 2015 Evolutionary divergence of beta-expansin structure and function in grasses parallels emergence of distinctive primary cell wall traits. Plant J 81(1):108-20 doi:10.1111/tpj.12715
5. Georgelis N, Nikolaidis N, Cosgrove DJ (2015) Bacterial expansins and related proteins from the world of microbes. Applied Microbiology and Biotechnology 99: 3807-3823
6. Offermann LR, Giangrieco I, Perdue ML, Zuzzi S, Santoro M, Tamburrini M, Cosgrove DJ, Mari A, Ciardiello MA, Chruszcz M. 2015. Elusive Structural, Functional, and Immunological Features of Act d 5, the Green Kiwifruit Kiwellin. Journal of Agricultural and Food Chemistry 63: 6567-76 doi:10.1021 /acs.jafc.5b02159
7. Cosgrove DJ. 2015. Plant expansins: diversity and interactions with plant cell walls. Curr. Opin. Plant Biol., 25, 162-172.
8. Cosgrove DJ. 2016. Catalysts of plant cell wall loosening. F1000 Reviews 5:119-ff doi 10.12688/f1000research.7180.1

EFRC: CENTER FOR LIGNOCELLULOSE STRUCTURE AND FORMATION (CLSF)
PI: DANIEL COSGROVE

WEBSITES: http://science.energy.gov/bes/efrc/centers/clsf/; http://www.lignocellulose.org/

TEAM: **Penn State University (Lead)**: Daniel J. Cosgrove (Director), Charles Anderson, Ying Gu, Seong H. Kim, Manish Kumar, Janna Maranas, Karl Mueller, B. Tracy Nixon, Ming Tien; **Massachusetts Institute of Technology**: Mei Hong; **North Carolina State Univ.**: Candace Haigler, Yaroslava Yingling; **Oak Ridge National Laboratory**: Hugh O'Neill; **Stony Brook Univ.:** Chang-Jun Liu; **Univ. of Rhode Island**: Alison Roberts; **Univ. of Texas at El Paso:** James Kubicki; **Univ. of Virginia**: Jochen Zimmer

SCIENTIFIC MISSION AND APPROACH

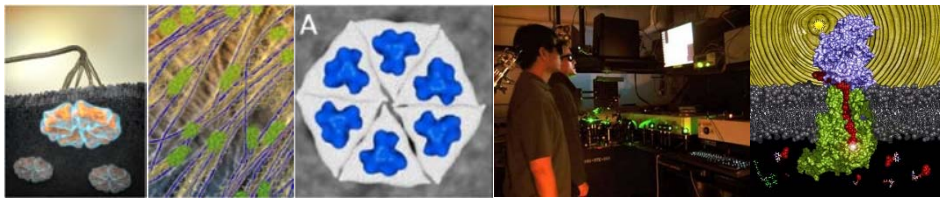
The goals of CLSF are to develop a detailed nano- to meso-scale understanding of plant cell walls, from cellulose microfibril (CMF) formation to the assembly of CMFs with other wall components to form the versatile plant cell wall. Cellulosic biomass (lignocellulose) holds great promise as a large-scale, renewable and sustainable source of liquid biofuels for transportation, if technical obstacles stemming from its complex, hierarchical structure could be overcome. CLSF's research will form the scientific foundation for designing methods to deconstruct cell walls and for coaxing plants into making modified walls for significant advances in sustainable energy and materials. The research is organized around two interconnected themes that lie at the interface of biology and physics:

1) **Cellulose Synthesis and Crystallization:** Elucidate how plants synthesize CMFs, including how cellulose synthases (CESAs) polymerize and secrete individual glucan chains and how multimeric cellulose synthase complexes (CSCs) assemble multiple chains into 3-nm-wide CMFs. CMFs are long, crystalline arrays of glucans that form the major load-bearing component of the plant cell wall.

2) **Structure and Assembly of Cell Walls:** Elucidate the rules of assembly by which CMFs interact with diverse matrix polymers to form complex, multilamellate structures, ranging from highly extensible primary cell walls in growing tissues to rigid lignified secondary cell walls in xylem and other cells.

SELECTED SCIENTIFIC ACCOMPLISHMENTS

- Determined the 3D molecular structure for the catalytic domain of a plant CESA. The structure was first proposed based on computational modeling, then confirmed experimentally in a bacterial CESA.
- For the first time, characterized *in-vitro* cellulose biosynthesis in a bacterial cellulose synthesis complex reconstituted from purified components.
- Computationally predicted a deleterious CESA mutation that was then verified through genetic testing and live cell imaging of cellulose synthesis complexes, opening up the possibility of rationally designing CESAs to engineer the properties of cellulose, biomass and biomaterials.
- Discovered that CSC is made from three types of CESAs in equal proportion. Additional experiments and modeling indicate that CSC contains 18 CESAs (3 in each lobe of 6), contrary to the long held assumption that CSC contains 36 CESAs.
- Elucidated the connection between CMF orientation and the cytoskeleton: a protein, CSI1, binds to CESA and microtubules, providing the long-hypothesized link between these two components.
- Developed Sum Frequency Generation Spectroscopy to quantify the amount, orientation, and mesoscale packing of crystalline CMFs in intact plant cell walls. This tool revealed CMFs in secondary cell walls are often aligned in anti-parallel directions.
- Used atomic force microscopy and solid-state NMR to discern direct cellulose-cellulose junctions and cellulose-pectin interactions that dominate primary cell wall structure and its mechanical properties.



CLSF research, from left: Model of cellulose synthesis complex; Model of a primary cell wall: CMF (blue), xyloglucan (green), and pectins (yellow); Experimental evidence for 18 CESAs within the CSC rosette; Yong Bum Park and Chris Lee, CLSF members, utilize sum frequency generation vibration spectroscopy to detect crystalline cellulose in lignocellulosic materials; Afterlife of a Photon – Winner of the 2015 EFRC Poetry of Science Contest (http://www.energyfrontier.us/content/afterlife-photon)

IMPACT

- When the CLSF was initiated, a vast communication gap existed between the physical chemists who study cellulose structure and the biologists who are discovering how this material is synthesized by plants. CLSF helped to bridge this gap by organizing and sponsoring two symposia at the American Chemical Society meeting in 2011 and 2012 and a 3-day international symposium at Penn State in 2013 with about 150 attendees.
- Overthrew the "tethered network" model of primary cells walls that dominated the field for the last 25 years. Xyloglucans were postulated to form load-bearing tethers linking dispersed CMFs into a cohesive network, but this was refuted when enzymatic cutting of xyloglucans did not loosen the cell wall. Computational modeling, NMR experiments and atomic force microscopy indicate a cell wall model where strength is controlled at limited, direct junctions formed between CMFs.
- CLSF researchers are recognized as leaders in the cell wall community: they garnered more invitations to speak at the last two international cell wall conferences than any other group.

PUBLICATIONS AND INTELLECTUAL PROPERTY

As of May 2016, CLSF had published 132 peer-reviewed publications cited over 1,200 times and filed one US patent application. The most highly cited papers are:

- Li, S., Lei, L., Somerville, C. & Gu, Y. Cellulose synthase interactive protein 1 (CSI1) links microtubules and cellulose synthase complexes. *PNAS* **109**, 185-190, doi:10.1073/pnas.1118560109 (2012). [90 citations]
- Park, Y. & Cosgrove, D. A Revised Architecture of Primary Cell Walls Based on Biomechanical Changes Induced by Substrate-Specific Endoglucanases. *Plant Physiology* **158**, 1933-1943, doi:10.1104/pp.111.192880 (2012). [85 citations]
- Wang, T. *et al.* Sensitivity-enhanced solid-state NMR detection of expansin's target in plant cell walls. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 16444-16449, doi:10.1073/pnas.1316290110 (2013). [60 citations]
- Park, Y. & Cosgrove, D. Changes in Cell Wall Biomechanical Properties in the Xyloglucan-Deficient xxt1/xxt2 Mutant of Arabidopsis. *Plant Physiology* **158**, 465-475, doi:10.1104/pp.111.189779 (2012). [55 citations]
- Sethaphong, L. *et al.* Tertiary model of a plant cellulose synthase. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 7512-7517, doi:10.1073/pnas.1301027110 (2013). [49 citations]
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A Tale of Two Enzymes: CO Dehydrogenase and Formate Dehydrogenase

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Overall research goals: The overall goals of this research program are to probe the relationship of structure to function for molybdenum-containing enzymes catalyzing energy-relevant reaction, and to determine their detailed chemical mechanism of action. This project focuses on two air-stable enzymes involved in the biotransformation of energy-relevant one-carbon compounds, the Mo- and Cu-containing CO dehydrogenase (CODH) from *Oligotropha carboxidovorans* and the Mo-containing formate dehydrogenase from *Ralstonia eutropha* (also known as *Cupriavidus necator*). The first of these enzymes catalyzes the oxidation of CO to CO_2, with reducing equivalents thus obtained being transferred to the quinone pool; the second catalyzes the reversible oxidation of formate to CO_2 by NAD^+. This latter enzyme, FdsABG, is a member of the NADH dehydrogenase superfamily of enzymes that play crucial roles in the bioenergetics nearly all organisms. The active site of CODH is a unique binuclear cluster with the structure $LMo^{VI}O_2$-μS-$Cu^{I}(H_2O)$(S-Cys), while the active site of FdsABG has an L_2MoS(S-Cys) structure; in both cases, L is a bidentate pyranopterin cofactor coordinated to the molybdenum via an enedithiolate side chain. In addition to the intrinsic interest in the reactions catalyzed by these enzymes, their active sites constitute attractive synthetic targets for inorganic chemists seeking to mimic the structural and functional characteristics of biological catalysts.

Significant achievements 2014-2016: We have undertaken an ENDOR analysis of the EPR-active form of CO-reduced CD dehydrogenase that we had previously characterized. This work has definitively established the signal-giving species to be a copper carbonyl complex of the partially reduced binuclear center, an assignment that substantively supports our working hypothesis regarding the reaction mechanism of the enzyme, that back-bonding of CO bound to the copper substantially weakens the C-O bond and renders it more susceptible to nucleophilic attack.

We have also examined the rapid-reaction kinetics of CO dehydrogenase reduction by H_2, determining a limiting k_{red} at high [H_2] of 5 s^{-1} and a K_d of 525 μM; both are largely independent of pH. From a temperature-dependence study at pH 7.2, we find $\Delta H^{‡}$ = 28 kJ/mol, $\Delta S^{‡}$ = -146 J/K•mol and $\Delta G^{‡}$ = 72 kJ/mol for reduction by H_2, which compare with $\Delta H^{‡}$ = 45 kJ/mol, $\Delta S^{‡}$ = -60 J/K•mol and $\Delta G^{‡}$ = 63 kJ/mol for reduction of the native enzyme by CO, and $\Delta H^{‡}$ = 40 kJ/mol, $\Delta S^{‡}$ = -94 J/K•mol and $\Delta G^{‡}$ = 68 kJ/mol for reduction of the silver-substituted enzyme by CO. Reduction of native enzyme by CO has a substantial entropic component that is even larger with H_2.

We have also characterized two new EPR signals attributable to the Mo/Cu binuclear cluster of CO dehydrogenase, seen upon reduction of enzyme with H_2 and with dithionite-reduced enzyme complexed with bicarbonate. By contrast to the signal seen with CO, the signal seen with H_2-reduced enzyme shows coupling to two inequivalent protons, both of which are solvent exchangeable. This signal most likely arises from either an η_2-Cu (I)•H_2 complex or a Cu (I) hydride. Unlike the EPR signal exhibited by enzyme partially reduced with sodium dithionite, which exhibits coupling to two inequivalent protons, the EPR signal seen in the presence of bicarbonate complex is devoid of proton coupling. The bicarbonate signal is also interesting in that it more closely resembles that of the H_2-reduced rather than CO-reduced enzyme in its g values and copper hyperfine. The bicarbonate complex is mechanistically relevant given the recent suggestion that the immediate product of the enzyme-catalyzed reaction is bicarbonate rather than CO_2. Our work strongly suggests that the observed EPR signal does not arise from an E_{red}•P complex.

We have also examined the FAD site of CO dehydrogenase, characterizing the semiquinone oxidation state by EPR and identifying it to be of the blue neutral from with a linewidth of 20 G. The signature long-wavelength absorbance of FADH• is also observed in the absorption spectrum of partially reduced enzyme at low pH. The enzyme exhibits a pH-dependent absorption spectrum in the oxidized state that is lost upon covalent modification of the enzyme by the flavin-specific agent diphenyliodonium cation. The pH dependence is attributed to Tyr 193 of the FAD-containing CoxM subunit, which sits atop the *re* face of the isoalloxazine ring in van der Waals contact with it. Electron equilibration among the enzyme's four redox-active centers (including two [2Fe-2S] clusters in addition to the binuclear center and FAD) is found to be pH-dependent, but too fast to be followed using a stopped-flow pH jump protocol. Electron transfer from the iron-sulfur clusters to the FAD is thus much faster than in other members of the xanthine oxidase family of molybdenum-containing enzymes to which CO dehydrogenase belongs.

In work with the FdsABG formate dehydrogenase from *Ralstonia eutropha*, we have developed an efficient purification protocol for the enzyme and characterized the kinetics of enzyme reduction by formate. We find biphasic kinetics accounting for >90% of the total absorbance change. For the fast phase of the reaction, we find a limiting k_{red}^{fast} of 140 s^{-1} and a K_d^{fast} of 82 μM, and for the slow phase k_{red}^{slow} = 19 s^{-1} and K_d^{slow} = 230 μM. Given the capacity of the enzyme to take up a total of 11 reducing equivalents in its molybdenum center, FMN and seven iron-sulfur clusters, we attribute the fast phase to the reaction of more oxidized enzyme forms and the slower phase to more reduced forms in which the molybdenum center in a portion of the enzyme population possesses a partially reduced molybdenum center and is therefore unreactive toward formate.

We have also examined the EPR signal of the molybdenum center of formate-reduced FdsABG and find a signal with $g_{1,2,3}$ = 2.009, 2.001, 1.992 and strong coupling to one solvent-exchangeable proton with $A_{1,2,3}$ = 138, 82,45 Mhz. Importantly, we have shown that the strongly coupled proton is derived from the C_α of substrate, constituting strong evidence in support of a hydride transfer mechanism. We have also begun characterizing the several iron-sulfur clusters of the enzyme by EPR, and by varying temperature, level of enzyme reduction and microwave power have identified four well-defined EPR signals, with evidence of two other, less well-characterized signals. The signals themselves are unexceptional but, significantly, one is magnetically coupled to the molybdenum center below 20 K. We are thus able to assign this signal, with $g_{1,2,3}$ = 2.044, 1.937, 1,898, to the [4Fe-4S] cluster that lies nearest (~14 Å) to the molybdenum center of the enzyme.

Science objectives for 2016-2018

CO Dehydrogenase

- Examine the ENDOR of H_2-reduced CODH and of the enzyme in complex with bicarbonate.
- Perform enzyme-monitored turnover experiments to identify spectral intermediates.
- Examine electron transfer in CODH using a stopped-flow pH-jump method and pulse radiolysis.

FdsABG

- Investigate the structure of the enzyme by EXAFS and SAXS synchrotron methods.
- Examine electron transfer within FdsABG by pH-jump and pulse radiolysis.
- Determine the rapid-reaction kinetic parameters for the oxidative and reductive half-reactions of the enzyme.
- Attempt to substitute the molybdenum-coordinate cysteine with selenocysteine and characterize the resulting variant.

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And finally, to take my project to the next level my ideal collaborator would have expertise in: Your choice!

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

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Project Goals: The Complex Carbohydrate Research Center (CCRC) of the University of Georgia is a national resource for the study of complex carbohydrates. The DOE Center enables the CCRC to provide collaborations, services and training to academic, government, and industrial researchers who study the complex carbohydrates of plants and microbes by providing support of experts who operate, maintain, and assist in interpreting the data obtained from a variety of scientific instrumentation. The expanding need for expertise in studies of the structure/function of complex carbohydrates is rapidly growing as the importance of carbohydrate research in areas such as biomass conversion to biofuels, biomedical glycobiology and vaccine development is being recognized.

Analytical Services and Collaboration Studies: The DOE Center enables several types of collaboration and services to be offered to researchers. Scientists who request analytical services (see below) receive a written report containing a description of (i) the analytical procedures used, (ii) publishable quality results (data) of the analyses of their samples, and (iii) an authoritative interpretation of the results. CCRC personnel also provide collaborative service by becoming involved in "in depth" scientific research projects with individuals from other laboratories. One hallmark of the collaboration and services offered is the continued addition of new technologies that are originally developed in the CCRC research laboratories and are disseminated to outside investigators. Over the last two years, the DOE Center at the CCRC has processed over **191** projects from **174** outside investigators in analytical or collaborative services. The service and collaborative activities of the Center has resulted in **70** peer-reviewed publications where the DOE Center Grant has been cited either through co-authorship with the DOE Center scientists or the work that has been carried out by the DOE Center. Center personnel consult with external scientists via e-mail and telephone, helping the scientists address specific analytical problems, interpretation data and provide protocols. The CCRC's website (www.ccrc.uga.edu) provides freely accessible, internet-searchable databases in aspects of carbohydrate science, a scheduler for shared use of instrumentation, and descriptions of the CCRC's various research projects, service and training.

The CCRC provides several "high demand" collaboration and analytical services to the scientific community as a result of the funding by the DOE Center grant. The major areas of collaboration and analytical services that we currently provide include:

1. Purification and analysis of plant and microbial polysaccharides
2. Purification and characterization of plant and microbial glycoproteins
3. Molecular weight determination: SEC, MALDI-MS, or ESI-MS
4. Glycosyl composition analysis: GC-MS and HPAEC
5. Glycosyl linkage analysis: GC-MS
6. Determination of absolute configuration
7. Structural characterization by mass spectrometry
    a. ESI-MS and ESI-MS/MS

b. MALDI-MS and MALDI-MS/MS

c. Online liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS/MS)

8. 1-D and 2-D NMR spectroscopy for detailed structural elucidation and sequencing
9. Lignin analysis using Py-MBMS and Py-GC-MS
10. CarboSource Services: Production and distribution of rare nucleotide-sugars, acceptors required for polysaccharide and glycoconjugate biosynthesis, and of monoclonal antibodies reactive against plant cell wall epitopes
11. Glycome profile analysis: Cell wall-directed monoclonal antibodies/ELISA to probe detailed structural analysis of plant cell walls

Training: Training students and scientists in various fields of carbohydrate science is a very important part of the CCRC's mission. Training occurs when undergraduate students, graduate students, postdoctoral fellows, and visiting scientists undertake research projects with or take formal courses from CCRC faculty and staff. In addition, several annual one-week training courses and two- or four-day specialized courses are offered for individuals from academic institutions, government laboratories, and private industry. In the last two years **123** scientists have taken part in our hands-on training courses in carbohydrate analysis from **43** different national and international institutions.

Training courses currently offered at the CCRC include:

Course 1. *Techniques for Characterization of Carbohydrate Structure of Polysaccharides*

Course 2. *Separation and Characterization of Glycoprotein and Glycolipid Oligosaccharides*

Course 3. *Analytical Techniques for Structural Analysis of Glycosaminoglycans (GAGs)*

Course 4. *Mass Spectrometry of Glycoproteins*

Electron Flow and Energy Conservation in a Hydrogenotrophic Methanogen

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Overall research goals:

Methanococcus maripaludis is a model species of hydrogenotrophic methanogen. Hydrogen or formate serves as electron donor to reduce CO_2 to methane. No cytochromes are present and a central component of energy coupling is electron bifurcation at the heterodisulfide reductase (Hdr) step (Fig. 1). Our overall goal is to exploit the favorable growth characteristics and genetic tools of *M. maripaludis* to test hypotheses regarding electron flow and energy conservation in hydrogenotrophic methanogens.

Significant achievements (2012-2016):

Electron bifurcation, a circular pathway, and an anaplerotic role of an energy-converting hydrogenase. We constructed mutants of *M. maripaludis* that were deficient in multiple hydrogenases. A mutant deficient in Fru/c, Vhu/c, and Hmd (Fig. 1) required both formate and H_2. Sub-stoichiometric amounts of H_2 were required, suggesting that the energy-converting Eha hydrogenase functions only to provide anaplerotic electrons to the formyl-methanofuran dehydrogenase (Fwd) step of the pathway. The results were consistent with a circular pathway (named the Wolfe cycle) in which electron bifurcation couples the heterodisulfide reductase (Hdr) step to the formylmethanofuran dehydrogenase (Fwd) step. At a stoichiometric level, either H_2 or formate directly provides electrons to Hdr for electron bifurcation, and either hydrogenase (Vhu/c) or formate dehydrogenase (Fdh) directly associates with Hdr.

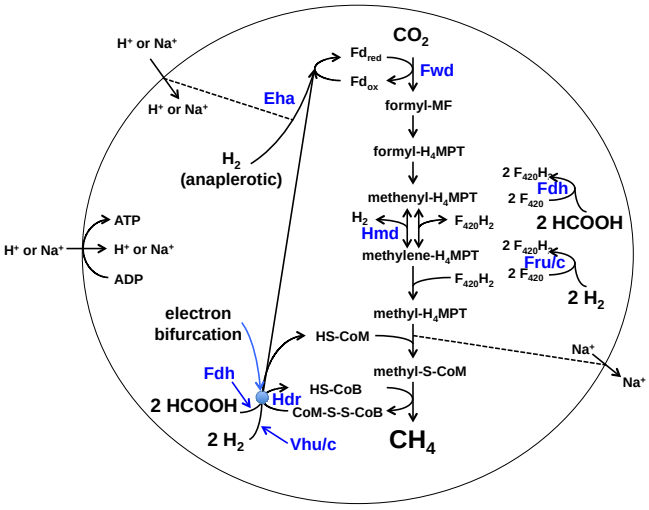

Fig. 1. Pathway of hydrogenotrophic methanogenesis

Regulation of formate dehydrogenase gene expression. Another hydrogenase mutant, deficient in Vhu/c, grew robustly on formate and extremely poorly on H_2. Curiously, growth was intermediate in the presence of both formate and H_2 (Fig 2A), suggesting that H_2 inhibits growth on formate. We had previously shown that several key genes of methanogenesis were repressed by high H_2, including *fdh* encoding formate dehydrogenase. We attributed H_2-inhibition of growth of the *vhu/c* mutant to H_2-inhibition of *fdh* gene expression. This hypothesis led us to search for factors affecting *fdh* expression by selecting for suppressor mutants where H_2 no longer inhibited growth on formate. Re-sequencing the genomes of the suppressor mutants revealed three classes of spontaneous mutations, affecting the gene for the methenyltetrahydromethanopterin-reducing hydrogenase Hmd and two uncharacterized genes, Mmp0267 and Mmp1381. We constructed deletion mutations in each gene and confirmed that the mutants grow well in the presence of both formate and H_2 (Fig.

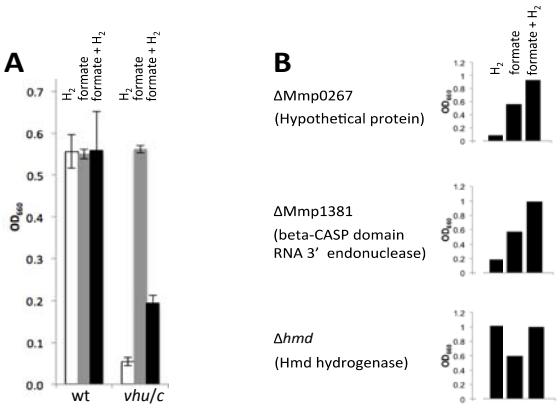

Fig. 2. Growth of (A) wild type (wt), *vhu/c*, and (B) *vhu/c* with deletions in three genes.

2B) and have increased levels of *fdh* mRNA.

Science objectives for 2016-2017:

- Identify ferredoxins that deliver stoichiometric electrons from Hdr to Fwd and anaplerotic electrons from Eha to Fwd.
- Determine the role of Hmd in the regulation of *fdh* expression.

My scientific area(s) of expertise is/are: Genetics of hydrogenotrophic methanogenesis.

To take my project to the next level, my ideal collaborator would have expertise in: Biochemistry of hydrogenotrophic methanogenesis.

Publications supported by this project 2012-2016:

1. Lie, T. J., K. C. Costa, B. Lupa, S. Korpole, W. B. Whitman, and J. A. Leigh. 2012. Essential anaplerotic role for the energy-converting hydrogenase Eha in hydrogenotrophic methanogenesis. Proc. Natl. Acad. Sci. USA 109:15473-15478. (A "from the cover" article) DOI: 10.1073/pnas.1208779109
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Understanding redox proportioning through ferredoxins, low potential iron-sulfur proteins acting as electrical hubs to control metabolism

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Overall research goals:

Ferredoxins (Fd) are thought to function as central energy-conserving redox hubs, serving as conduits between multiple redox donors and acceptors. Our goal is to elucidate the physical parameters that underlie Fd sequence-function relationships (redox potential, surface charge, structure, binding affinity) sufficiently so that we can anticipate what proportion of Fd redox is delivered to various natural redox partners within cells containing multiple Fd homologs and Fd-partner proteins. Our specific aims are to: (1) develop selections for Fd function and survey how the energy transduction activities of Fd homologs from different lineages perform in isolated redox pathways, (2) dissect Fd sequence elements that control their partner specificity by analyzing the redox activities of Fd chimeras created by recombining homologs that differ in their partner specificity, and (3) evaluate how Fd homologs vary in their control over redox delivery to different Fd-acceptor proteins within the same cell.

Significant achievements (2015-2016):

We built a cellular assay for comparing Fd activities in *Escherichia coli* through expression of a three-component redox pathway that rescues the growth of a sulfide auxotroph that cannot use sulfite as the only sulfur source. We showed that auxotroph growth is rescued when it expresses an e- donor protein (Fd-NADP reductase, FNR), a ferredoxin, and an e- acceptor (Fd-dependent sulfite reductase, SIR). We have also shown that three different FNR donors can be used as donors by *Mastigocladus laminosus* Fd, which can donate the e- to corn SIR. We have created an expression system that places Fd expression under control of the tetracycline repressor (TetR). In this system, Fd expression depends upon the addition of anhydrotetracycline (aTc), while FNR and SIR are constitutively expressed. Because expression is dependent upon aTc, selections can be performed over a range of expression conditions to establish the concentration of aTc required to elicit half maximal complementation.

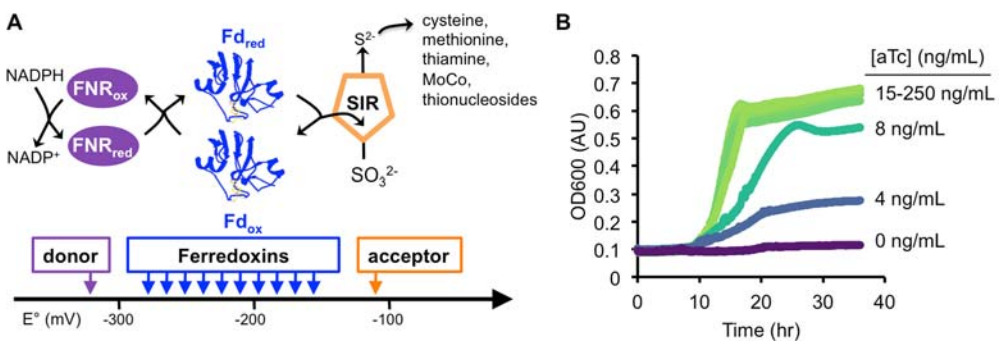

Fig. 1. (A) Redox pathway used to complement the growth a sulfide auxotroph. (B) Effect of aTc induction of *Mastigocladus laminosus* Fd on the complementation of the auxotroph.

We have also visualized sequence and structure differences across the Fd family by analyzing the prevalence of Fd in 92 organisms from 18 phyla (74 bacteria, 10 archaea, 8 eukaryotes). We found that 58% of these organisms have five or more proteins with Fd-like Fe-S binding motifs. We created a sequence similarity network, which shows clear demarcations between several of the Fd categories. However, some Fd do not clearly cluster with the classical Fd groups, including many unclassified Fd with 2Fe2S- or 4Fe4S- cluster motifs (in gray). We also generated a structure-anchored maximum parsimony phylogenetic tree, which reveals Fd that do not fall into the classical categories. Many of

these unclassified Fd are located at the junction between different Fd categories and have small confidence value, for example, between the nitrogenase-protecting and hydrogenosomal Fd.

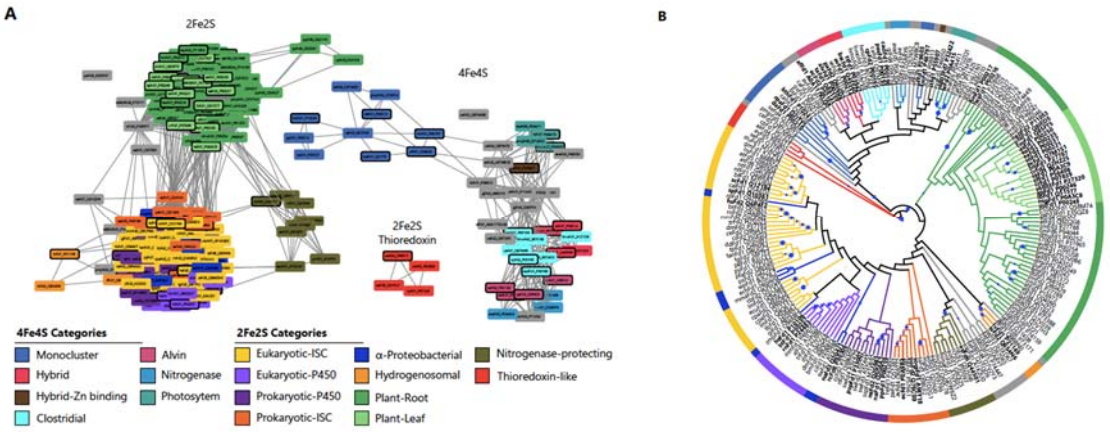

Fig. 2. (A) Sequence similarity network and (B) phylogenetic tree illustrating the relationships of 201 Fd. Fd are colored by categories, with unclassified family members that do not cluster with previously described categories in gray. Fd with known structures are outlined in black.

We have also evaluated how the control of redox in *Clostridium acetobutylicum* affects the pattern of metabolites. In the acid phase, reducing power goes towards H_2 production, while later in the solvent-associated stationary phase reducing power is used to generate butanol and ethanol. Reduced Fd produced during formation of acetyl-CoA is utilized directly to form H_2. While the formation of acetate from pyruvate does not require reducing power, formation of longer chain length butyric acid and alcohol products requires NAD(P)H derived in part from reduced Fd. A synthetic version of *Chlorobium tepidium* Fd:NAD(P)H oxidoreductase (FNR) was expressed in *C. acetobutylicum* ATCC824, the degenerate non-solventogenic counterpart M5, and a high solvent producing *buk*- strain using vector pJIR750 with FNR expressed from the phospotransbutyrylase (*ptb*) promoter. Addition of FNR increased relative butyrate and butanol production: the butyrate/acetate ratio in M5 expressing FNR was 3.8, vs 1.8 in the vector control. In wild type 824 with FNR, the butanol/acetone ratio was 2.9, vs 1.6 for the control. In the high solvent buk- mutant, the butanol/acetone ratio was 4.3 vs 2.4 in the control. Growth, OD and total alcohol level were similar between FNR-expressing strains and corresponding controls.

Science objectives for 2016-2017:

- Use cellular assays to compare Fd activities (and chimeras), and determine Fd cellular cycling efficiencies. Determine how varying the donor/acceptor partners affect Fd efficiencies.
- Examine the effects of constructs expressing various levels of FNR with or without different Fd in clostridial hosts.

Our scientific area(s) of expertise is/are: Clostridial microbiology, redox homeostasis, combinatorial biophysics, iron-sulfur proteins.

To take my project to the next level, my ideal collaborator would have expertise in: (i) computational docking of Fd and partner proteins, (ii) high-throughput biophysical analysis and (iii) quantitave MS for determining intracellular protein concentrations.

Publications supported by this project (2015-2016):

1. Atkinson, J.T, Campbell, I.J., Bennett, G.N. Silberg, J.J., *Biochem.*, Cellular assays for Fd: a strategy to understand electron flow through protein carriers that link metabolic pathways, *in review* (2016).

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

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Overall research goals:

The primary objective of this research is to study and understand the behavior of individual photosynthetic proteins and other biomolecules involved in energy capture and processing redox in a physiologically relevant solution environment. Single-molecule measurements have been firmly established as a cutting-edge technique for elucidating mechanistic details of numerous biological processes. These measurements are most powerful when they are applied over a long enough time to watch a process undergo multiple, statistically meaningful state transitions. At the same time, because such measurements should not be perturbed by surface attachment or encapsulation, we make use of the ABEL trap (Anti-Brownian ELectrokinetic trap) to suppress the usual Brownian motion of single biomolecules. By measuring multiple observables for each single molecule, such as emission brightness, excited state lifetime, emission spectrum, and emission anisotropy, we aim to infer mechanisms for observed photodynamics of both antenna proteins and energy-related enzymes. Using the newest incarnation of the ABEL trap, which senses the diffusion coefficient and electrokinetic mobility of the trapped molecule, we infer the size and charge state of the biomolecule, enabling detection of protein-protein interactions and oligomerization state of multimeric proteins.

Significant achievements (2014-2016):

- **Single-molecule identification of quenched and unquenched states of the green plant light-harvesting pigment-protein complex LHCII.** The molecular mechanisms that balance efficient light harvesting and photoprotection in photosynthesis are not fully understood. To determine how LHCII adapts to fluctuating sunlight intensity, we used the ABEL trap to investigate the fluorescence from individual copies of LHCII in a solution-phase environment without perturbations from surface attachment or encapsulation. We completed the first simultaneous measurements of fluorescence intensity (brightness), excited-state lifetime, and emission spectrum of single LHCIIs, and discovered previously unknown unquenched and quenched forms in normal conditions and under conditions mimicking high light. The relative population of one of the quenched states increases under conditions of nonphotochemical quenching. Instead of responding directly to light intensity (as in the case of bacterial antenna protein LH2), the conformation and the degree of quenching in LHCII are controlled through changes in the immediate environment of the pigments.
- **Dissecting pigment organization principles of Allophycocyanin, a major antenna complex in cyanobacteria**. Oligomerization plays a critical role in shaping the light-harvesting properties of many photosynthetic pigment-protein complexes, but a detailed understanding of this process at the level of individual pigments is still lacking. To study the effects of oligomerization, we used the ABEL trap on APC monomers and trimers to record step-wise photobleaching and multi-parameter state measurements, and extracted pigment-specific spectroscopic information with unprecedented detail. Our data reveals that the two chemically-identical pigments in APC have different roles. One (α) is the functional pigment that red-shifts its spectral properties upon trimer formation, while the other (β) is a "protective" pigment that persistently quenches the excited state of α in the pre-functional, monomer state of the protein. These results on the monomer and additional detailed studies on the trimer show how subtleties in pigment organization give rise to functionally important aspects of energy transfer and photoprotection in antenna complexes.
- **Exploration of the oligomerization states of the Rubisco activase enzyme (Rca)**. Regulation of Rubisco activity is of critical importance to energy assimilation on Earth and is carried out

by the enzyme Rubisco activase (Rca). How Rca works to regulate Rubisco is poorly understood, and the lack of a clear mechanism is partially due to the complication that the enzyme forms a range of oligomerization states in vitro (and likely in vivo as well). Using material from Prof. Rebekka Wachter's group at Arizona State University, we have completed preliminary experiments to utilize the ABEL trap's unique size and charge-sensing capability to quantify the oligomerization pathway of Rca *in vitro*, at the single-molecule level. We directly observe the formation of hexamers and other smaller oligomers, and find that the formation equilibrium depends upon the nucleotide provided to the enzyme.

Science objectives for 2016-2017:

- We will complete the study of the oligomerization states of the Rubisco activase protein in collaboration with Prof. Rebekka Wachter's group at Arizona State University.
- We have begun exploration of the three-pigment cyanobacterial phycobiliprotein, C-PC, which assembles via trimers and hexamers into rod-like antennae around an APC core to form the phycobilisome, and will fully characterize its photodynamics and quenching mechanisms.
- We have trapped single copies of phycocyanin PC645, the major antenna complex of Chroomonas, and will complete single-molecule fluorescence measurement of this cryptophyte antenna complex.
- We will study the photoactivated quenching of orange carotenoid protein (OCP) acting on APC monomers and trimers, in collaboration with Prof. Robert Blankenship's group at Washington University in St. Louis. OCP is known to interact with APC in its photoactivated form, but the exact quenching mechanism and site are not currently known.
- We are developing a method to measure the anisotropy decay of single molecules in the ABEL trap, which will provide a direct and sensitive measure of their rotational mobility. This technique is a complementary method to our D/μ measurements to detect changes in particle conformation and size.

My scientific area(s) of expertise is/are: physical chemistry and chemical physics of single molecules, single-molecule biophysics, photodynamics of photosynthetic protein and redox enzymes, super-resolution imaging and tracking beyond the diffraction limit in cells, and trapping of single molecules in solution.

To take my project to the next level, my ideal collaborator would have expertise in: expression and purification of photosynthetic antenna pigment-protein complexes or reaction centers, biochemistry of oligomeric protein complexes, site-specific mutation and fluorescent reporter protein labeling and validation.

Publications supported by this project 2014-2016:

1. Samuel D. Bockenhauer, Thomas M. Duncan, W. E. Moerner and Michael Börsch, "The regulatory switch of F1-ATPase studied by single-molecule FRET in the ABEL Trap, " *Proc. SPIE* **8950**, 89500H 1-14 (2014).
2. Quan Wang and W. E. Moerner, "Single-molecule motions enable direct visualization of biomolecular interactions in solution," *Nature Methods* **111**, 555-558 (2014).
3. Gabriela S. Schlau-Cohen, Samuel Bockenhauer, Quan Wang, and W. E. Moerner, "Single-molecule spectroscopy of photosynthetic proteins in solution: exploration of structure–function relationships," Minireview, *Chem. Sci.* **5**, 2933-2939 (2014).
4. Gabriela S. Schlau-Cohen, Hsiang-Yu Yang, Tjaart P. J. Krüger, Pengqi Xu, Michal Gwizdala, Rienk van Grondelle, Roberta Croce, and W. E. Moerner, "Single-Molecule Identification of Quenched and Unquenched States of LHCII," *J. Phys. Chem. Lett*. **6**, 860-867 (2015).
5. Quan Wang and W. E. Moerner, "Dissecting pigment architecture of individual photosynthetic antenna complexes in solution," *Proc. Nat. Acad. Sci. (USA)* **112**, 13880-13885 (2015).
6. W. E. Moerner, Yoav Shechtman, and Quan Wang, "Single-molecule spectroscopy and imaging over the decades," Introductory Review, *Faraday Discuss.* **184**, 9-36 (2015)

Intracellular Lipid Transfer in the Biosynthesis of Photosynthetic Membrane Lipids and Storage Triacylglycerol

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Overall research goals: Our long-term goal is to understand the process of lipid movement between the two major compartments for glycerolipid assembly, namely, the endoplasmic reticulum (ER) and the plastid at the molecular genetic level and to understand the factors that regulate the flux of fatty acids between membrane lipids and storage triacylglycerol (TAG). The specific objectives are to: (1) identify the genes affected in lipid-trafficking mutants isolated in a forward genetic screen; (2) determine the functional role of the encoded proteins at the molecular, biochemical, and physiological levels; (3) investigate the factors regulating carbon partitioning into fatty acids and the flux of fatty acids between membrane lipids and storage TAG in vegetative tissues of plants. The results from this study should create the knowledge base to enhance our ability to manipulate the processes that regulate photosynthetic membrane biogenesis and carbon partitioning and storage in plants, and lay the intellectual foundation for future engineering endeavors aimed at the development of novel biomass crops for the production of nutrition-rich feed and renewable fuels.

Significant achievements (2014-2016):
We carried out the in-depth biochemical and genetic analysis of *tgd5* mutants identified in a forward genetic screen for lipid trafficking mutants. Similar to *tgd1* mutants, both the rate of fatty acid synthesis and TAG level are significantly increased in *tgd5* mutants. *TGD5* encodes a glycine-rich protein that is localized in envelope membranes of the chloroplast. TGD5 physically and functionally interacts with several other TGD components of the plastid lipid import pathway. Our current hypothesis is that TGD5 facilitates lipid transfer from the outer to the inner plastid envelope by bridging between TGD4 and the TGD1,2,3 transporter complex.

We also performed the initial studies of two lipid trafficking mutants designated *reduced lipid transport 3* (*rtl3*) and *tgd4-4*. The *rtl3* mutant exhibits *tgd* mutant-like lipid phenotypes except that oligogalactolipids, which is diagnostic for all *tgd* mutants is absent in leaf lipid extracts of *rtl3*. Sequencing of *tgd4-4* identified a G to A mutation at base pair of 39 relative to ATG. The mutation is predicted to convert a Trp residue (TGG) to a stop codon (TGA), which would lead to the production of a truncated protein of 13 amino acids instead of the full-length 479 amino acid TGD4. Unlike other *tgd4* alleles (88), *tgd4-4* exhibits a pale-green appearance, likely because of substantial decreases in levels of galactolipids, and is completely fertile, providing the first opportunity to carry out additional genetic analyses to isolate new components in ER-to-plastid lipid trafficking.

Additional work related to this project focused on analyzing the pathway of TAG metabolic pathways and the physiological significance of TAG metabolism in vegetative tissues of plants. Taking advantage of Arabidopsis *tgd* mutants that accumulate TAG in vegetative tissues, we found that there is an intricate interplay between TAG metabolism, fatty acid oxidation and membrane lipid homeostasis. In the *tgd1* mutant, blocking the release of fatty acids from TAG or blocking fatty acid peroxisomal oxidation severely impairs fatty acid turnover and boosts leaf

TAG content by approximately 150-fold relative to wild-type plants. These biochemical genetic results support the role of TAG as an intermediate in the fatty acid degradation pathway. Analysis of *tgd1* mutants also indicates that the increased fatty acid synthesis is attributable to the posttranslational activation of heteromeric acetyl-CoA carboxylase (hetACCase).

Science objectives for 2016-2018:

- Potential mechanisms underlying hetACCase regulation include protein phosphorylation, redox modulation, feedback inhibition and regulation by PII and biotin/lipoyl attachment domain containing proteins. Their roles in regulating hetACCase will be systematically test to determine the mechanistic basis for the increased fatty acid synthesis in leaves of *tgd* mutants.

- The newly isolated mutant *rtl3* exhibits a major increase in the rate of fatty acid synthesis, a substantial decrease in ER-to-plastid lipid trafficking activity, an alteration in fatty acid and lipid composition and an accumulation of TAG in leaves. Detailed genetic and biochemical experiments will be performed to identify the gene, characterize the mutant and establish the function of the protein.

- TAG synthesis shares common carbon precursors and energy substrates with the synthesis of transient starch in leaves. Studying the interrelationship between fatty acid and starch metabolism in *tgd* mutants will help to understand how carbon partitioning into major metabolic pathways is regulated in vegetative tissues of plants.

My primary expertise is in: molecular genetics and biochemistry of plant lipids

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

Publications supported by this project 2014-2016:

1. Xu C, Shankin J. Triacylglycerol metabolism, function and accumulation in plant vegetative tissues. Annu Rev Plant Biol 67: 179-206 (2016).
2. Xu C, Andre C, Fan L, Shanklin J. Cellular organization of triacylglycerol biosynthesis in microalgae. Subcell Biochem 86:207-21 (2016).
3. Li N, Xu C, Li-Beisson Y, Philippar K. Fatty acid and lipid transport in plant cells. Trends Plant Sci 21:145-58 (2016).
4. Fan J, Zhai Z, Yan C, Xu C. Arabidopsis TRIGALACTOSYLDIACYLGLYCEROL5 interacts with TGD1, TGD2, and TGD4 to facilitate lipid transfer from the endoplasmic reticulum to plastids. Plant Cell 27:2941-55 (2015).
5. Xu, C. and Shanklin, J. Insights from recent plant studies: Triacylglycerol metabolism, fatty acid β-oxidation and lipid homeostasis. Invited communication for ASBMB Today "Lipid News" 14(4): 11-13 (2015).
6. Fan J, Yan C, Boston R, Shanklin J, Xu C. Arabidopsis lipins, PDAT1 acyltransferase and SDP1 triacylglycerol lipase synergistically direct fatty acids toward ß-oxidation thereby maintaining membrane lipid homeostasis. Plant Cell 26: 4119-4134 (2014).

Cell-type specific pectins in plant cell wall structure, interaction and function

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Overall research goals:

Borate cross-linking of the pectic polysaccharide rhamnogalacturonan II (RG-II, see Fig 1) is a key event in controlling the assembly and functions of the primary wall surrounding growing plant cells.

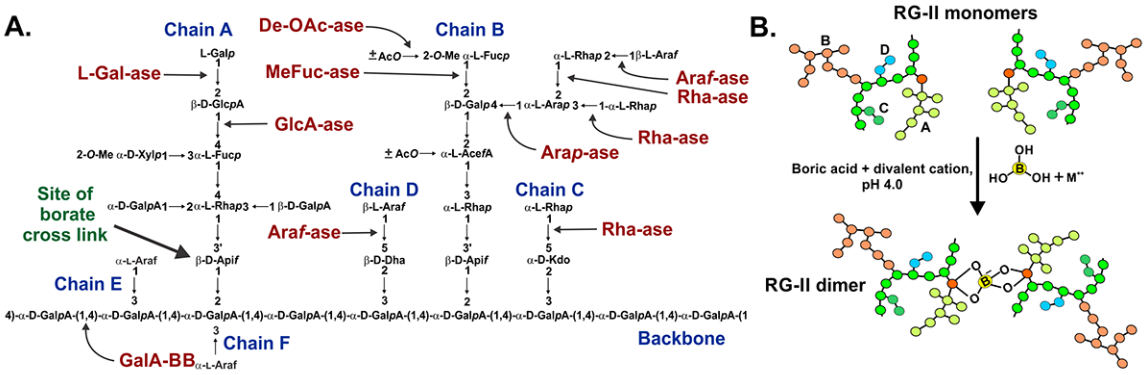

Fig. 1. A, The glycosyl sequence of RG-II and selected *Bacteroides thetaiotaomicron* enzymes used to fragment it. B, RG-II dimer formation *in vitro*. Two RG-II molecules are linked to one another by a borate di-ester.

Our research is focused on defining the structural features of RG-II that regulate its interaction with borate and on elucidating the molecular and cellular mechanisms involved in RG-II synthesis. Such information is required to understand the underlying principles leading to the assembly of a functional plant cell wall.

Because of the structural complexity of RG-II, tools to specifically modify RG-II are required to determine the relationship between its structure and function. Until now, virtually no enzymes that fragment RG-II have been identified. However, our collaborator Harry Gilbert (Newcastle Univ, UK) has recently shown that *Bacteroides thetaiotaomicron*, a human gut bacterium, produces a suite of enzymes that specifically hydrolyze all but one of the glycosidic bonds of RG-II.

Our main goal is determine how RG-II structure and cofactors contribute to dimer formation and the dynamics of the borate cross-link. To this end we are developing a toolbox of recombinant *B. thetaiotaomicron* enzymes to modify RG-II structure *in vitro*. We are also using these enzymes to validate the primary structures and positions of the four sidechains on the backbone and to generate libraries of well-characterized RG-II fragments. These fragments will be used as acceptor substrates to identify and functionally characterize candidate enzymes involved in RG-II sidechain synthesis.

Significant achievements (2014-2016):

To date we have expressed and used 11 recombinant *B. thetaiotaomicron* enzymes (see Fig. 1A). Our studies show that:

1. The sidechains of the monomer are more susceptible to the hydrolases than the dimer, which suggests that there are extensive interactions between sidechains in the dimer.
2. Removal of L-Gal and GlcA from chain A substantially reduces the rate of dimer formation.
3. Removal of 2-*O*-Me Fuc from chain B and trimming of the GalA backbone substantially increases the rate of dimer formation.

4. Modifying chain D has a small but discernible influence on dimer formation.

Our real time ^{1}H-NMR analysis has shown that during dimer formation the line-widths and chemical shifts of resonances diagnostic of many residues change. Notably, the resonances of methyl-ether protons of the *O*-methylated sugars in the monomer become broader and their chemical shifts move up-field during the transition from the monomer to the dimer. Such data is consistent with our enzymatic fragmentation studies indicating that the 2-*O*-Me Fuc of chain B has a role in controlling dimer formation (see Poster).

We have shown that the ability to form apiose, a branched monosaccharide present in RG-II, apiogalacturonan (ApiGalA) and plant secondary metabolites, existed prior to the appearance of these glycans in vascular plants (Smith et al 2016). We have also shown that even though bryophytes and algae form UDP-Api, they lack the glycosyltransferase machinery required to synthesize Api-containing cell wall glycans.

Our studies of the cell walls from 16 of the 37 species of duckweed have shown that RG-II structure and cross-linking is conserved in these aquatic plants even though the structures of other pectins, xyloglucan and glucuronoxylan changed considerably as duckweeds diversified. This again emphasizes how crucial maintaining RG-II structure and cross-linking is for vascular plants to grow.

Science objectives for 2016-2018:

- We will continue studies to validate the primary structure of RG-II. In particular the conformation of the Rha-3'Api linkage has been questioned by the Gilbert lab. Their data obtained using the *B. thetaiotaomicron* enzymes indicate that the Rha-3'Api linkage is α rather than β as in previous models of RG-II (see Fig. 1) and suggest that the Api is 3-linked rather than 3'-linked. Since this will have considerable effects on molecular models of RG-II, the Rha-Api disaccharide will be generated in amounts sufficient for high-resolution NMR analyses to establish with certainty the anomeric form and position of the glycosidic linkage.
- To determine the functional role of RG-II sidechains, we generated a fragment of RG-II containing chains A and D linked to the GalA backbone. The fragment will be fully characterized to establish the glycosyl sequence of RG-II. We will also determine if the fragment self-assembles into a dimer in the presence of borate.
- Arabidopsis genes encoding putative apiosyl and Kdo transferases have been identified. The genes have been expressed in HEK cells and the recombinant proteins isolated. We will perform *in vitro* assays to determine if the proteins are functional GTs. The sugar donors UDP-Api and CMP-Kdo are themselves unstable so they will have to be generated *in situ* during the GT assays. We will also continue to use bioinformatics to identify other potential enzymes involved in RG-II synthesis
- Oligosaccharides containing apiose linked to *O*-2 of a 4-linked GalA backbone are required for use as acceptors to identify the RhaT that catalyzes the formation of the Rha-Api linkage. We have found that a commercial enzyme (Viscozyme) contains endo-glycanases that generate oligosaccharides composed of Api and GalA from duckweed apiogalacturonan. These fragments will be isolated, characterized and used as potential substrates in the RhaT and KdoT characterization studies

My scientific area(s) of expertise are: Plant cell walls. Polysaccharide structure and function.

To take my project to the next level, my ideal collaborator would have expertise in: Solution conformation/properties of polysaccharides. Imaging (e.g. AFM) of polysaccharides.

Publications supported by this project:

1. Smith, JA, Yang, Y, Levy, S, Adelusi, OO, Hahn, MG, O'Neill, MA, Bar-Peled, M (2016). Functional characterization of UDP-apiose synthases from bryophytes and green algae provides insight into the appearance of apiose-containing glycans during plant evolution. *J. Biol. Chem. In press. doi:10.1074/jbc.M116.749069.*

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

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Overall research goals:

This project focused on understanding the assembly, biophysical charge transport mechanism(s), and energetic consequences of extracellular redox chains that mediate long-distance extracellular electron transport (EET) between microbial biofilms and inorganic surfaces ranging from natural minerals to electrodes. A physics-based understanding will enable the conversion of energy at hybrid biomolecular/synthetic interfaces. The 2013-2016 project's objectives were to:

(1) Test whether electron transport measurements in bacterial nanowires and individual multiheme cytochromes agree with the theoretical predictions of a multistep redox hopping mechanism.

(2) Quantify interfacial microbe-surface electron transfer at the level of single *Shewanella oneidensis* MR-1 cells, and discover how this fundamental single-cell respiration rate is impacted by surface redox potential and selected mutations.

(3) Monitor, *in vivo*, the assembly of bacterial nanowires from individual cells, thereby identifying their composition, formation mechanism, and respiratory impact.

Significant achievements (2014-2016):

Goal 1. Single-molecule tunneling spectroscopy and Kinetic Monte Carlo (KMC) simulations of electron transport in the multiheme cytochrome (MtrF) from *S. oneidensis MR-1* demonstrated reasonable agreement between a multi-step hopping model and measured transport rates [1-3]. In collaboration with colleagues at the Naval Research Laboratory, the temperature dependence of electron transport through *Geobacter sulfurreducens* biofilms was also measured [4]. The results demonstrated thermally activated transport, a telltale feature of multistep redox hopping, with activation energies matching those of bacterial multiheme cytochromes. On another front, to shed light on the previously proposed role for flavins as electron 'shuttles' between cytochromes and external surfaces, we performed electrochemical measurements that revealed both flavin-dependent and flavin-independent EET pathways from *S. oneidensis* MR-1 [5]. Our results indicate that flavins accelerate EET from *Shewanella* as cytochrome-bound cofactors, rather than soluble shuttles, supporting one of the two intensely debated models of how flavins enhance EET.

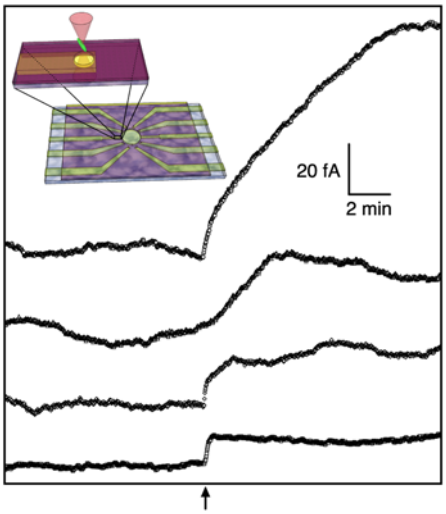

Fig. 1. Measurements of extracellular electron transport from single *Shewanella oneidensis* MR-1 cells manipulated using optical tweezers above microelectrodes (inset schematic). Arrow marks the time of contact for four individual cells. From [6].

Goal 2. To achieve the goal of single-cell EET measurements, individual bacteria were manipulated using optical tweezers, placed on microelectrodes, and a potentiostat was used to detect the respiration current correlated with cell-electrode contact. Our publication [6] details single cell measurements of *S. oneidensis* MR-1,

which resulted in respiration rates up to 6.2×10^5 s^{-1} under our measurement conditions. At this rate, and comparing to the single molecule rates above (10^4 s^{-1}), only 1-10% of the experimentally estimated average density of 10^3–10^4 outer membrane cytochromes per cell can satisfy the entire cellular respiration rate.

Goal 3. This task was complete in project year 1 (2013-2014), when we made the first *in vivo* observations of the formation and respiratory impact of bacterial nanowires [7], using a platform developed with AFOSR funding. We found that *S. oneidensis* MR-1 nanowires are extensions of the outer membrane and periplasm that include the multiheme cytochromes (MtrC and OmcA) responsible for electron transport. Atomic force microscopy suggested that nanowires originate from fusion of membrane vesicles into membrane 'tubes'. This was confirmed in 2015, when we developed protocols for correlative fluorescence and electron microscopy of nanowires, resolving the lipid bilayers and even capturing connections of individual vesicles under native conditions (manuscript in preparation). Motivated by this success, we have initiated a separate project with Grant Jensen (Caltech) to resolve the 3D ultrastructure of the *Shewanella* nanowires using electron cryo-tomography, including the spacing and nanoscale localization of MtrC and OmcA, for which X-ray structures already exist.

Science objectives for 2016-2017:

Our renewal project activities will build on the achievements outlined above by

- Measuring electron transport in cytochrome networks *in situ* through electrochemical gating and bipotentiostatic conductance measurements (in solution) of living *Shewanella* biofilms and cell-free synthetic systems where multiheme cytochromes are reconstituted in artificial lipid bilayers
- High performance computations to extend KMC simulations from individual cytochromes to the entire redox networks of nanowires, cells, and even biofilms. Parallel simulations will examine the effect of cytochrome density, orientation and network size on overall electron transport rates.
- Electrochemical characterization of new bacterial isolates that acquire energy by electron *uptake* from electrodes. An understanding of these cathodic microbes will impact future applications that drive reductive metabolisms for synthesis of desirable end products.

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

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

Publications supported by this project 2014-2016:

1. H.S. Byun, S. Pirbadian, A. Nakano, L. Shi, M.Y. El-Naggar. *Kinetic Monte Carlo simulations and molecular conductance measurements of the bacterial decaheme cytochrome MtrF*, ChemElectroChem, 1, 1932-1939, 2014.
2. C. Masato Nakano, H.S. Byun, H. Ma, T. Wei, M.Y. El-Naggar. *A framework for stochastic simulations and visualization of biological electron-transfer dynamics*, Computer Physics Communications 193, 1-9, 2015.
3. C. Masato Nakano, E. Moen, H.S. Byun, H. Ma, B. Newman, A. McDowell, T. Wei, M.Y. El-Naggar, *iBET: Immersive visualization of biological electron-transfer dynamics*, Journal of Molecular Graphics and Modelling, 65, 94-99, 2016
4. M.D. Yates, J. Golden, J. Roy, S.M. Strycharz-Glaven, S. Tsoi, J. Erickson, M.Y. El-Naggar, S. Calabrese Barton and L. Tender, *Thermally activated long range electron transport in living biofilms*, Physical Chemistry Chemical Physics, 17, 32564-32570, 2015.
5. S. Xu, Y. Jangir, M.Y. El-Naggar. *Disentangling the roles of free and cytochrome-bound flavins in extracellular electron transport from Shewanella oneidensis MR-1*, Electrochimica Acta, 198, 49-55, 2016
6. B.J. Gross and M.Y. El-Naggar. *A combined electrochemical and optical trapping platform for measuring single cell respiration rates at electrode interfaces*, Review of Scientific Instruments, 86, 064301, 2015.
7. S. Pirbadian, S. E. Barchinger, K.M. Leung, H.S. Byun, Y. Jangir, R.A. Bouhenni, S.B. Reed, M.F. Romine, D.A. Saffarini, L. Shi, Y.A. Gorby, J. H. Golbeck, M.Y. El-Naggar. *Shewanella oneidensis MR-1 nanowires are outer membrane and periplasmic extensions of the extracellular electron transport components*, PNAS, 111, 12883-12888, 2014.

Multidimensional Spectroscopies for Probing Coherence and Charge Separation in Photosystem II

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Overall research goals:

Photosystem II (PSII) is the only known natural enzyme that uses solar energy to split water, making the elucidation of its design principles critical for our fundamental understanding of photosynthesis and for our ability to mimic PSII's remarkable properties. This project focuses on key deficits in our current understanding of the PSII reaction center (PSII RC). The project involves the development of new spectroscopic methods, and their application to address the following open questions:

1) What is the electronic structure of the PSII RC?
2) What are the charge separation pathways in the PSII RC?
3) Do key pigment and/or protein dynamics enhance energy transfer and charge separation in the PSII RC?

Significant achievements (2015-2016):

In the past year we have performed several different types of spectroscopic measurements aimed at addressing the key open questions about the PSII RC. These include 2D electronic spectroscopy (2DES) experiments in different spectral regions, and two variations on 2DES, one that enables sensitive measures of coherent dynamics[1] and another, 2D electronic Stark spectroscopy, that reveals charge transfer states[2]. We also recently reviewed experimental implementations of 2D electronic spectroscopy[3].

Our previous 2DES experiments have been performed on the D1D2 cyt-b559 complex. Concerns that this complex may have a perturbed excitonic structure motivated us to study PSII core complexes that consist of the intact PSII RC complex (with quinones) and the neighboring antenna complexes CP43 and CP47. We have performed polarization-dependent 2DES studies of the PSII core preparation at 77K, shown in Figure 1. Despite the predicted small electronic coupling in this system, we see extremely rapid energy transfer within the first 100 fs, as shown by the cross-peak features in the parallel and perpendicular 2D spectra at the earliest waiting time delays. We have simulated the early energy transfer events using two different approaches. The first approach considers evolution of only the electronic energy in the system and fails to reproduce the ultrafast energy transfer dynamics. A second approach that considers the combined dynamics of both electronic and vibrational degrees of freedom reproduces the observed sub-100 fs evolution. This implies that vibrational degrees of freedom not only induce population transfer between the excitonic states in the PSII CC, but also shape the energy landscape of the system[4].

Following our observation of vibronic coherence in the PSII RC, we have continued experiments to probe the functional relevance of the coherences. We developed a two-color coherence spectroscopy method to provide high sensitivity measurements of coherent dynamics[1]. We have applied this approach to study the ~820nm anion band of the PSII RC, and have observed coherent dynamics, consistent with our previous work. We are currently performing control experiments to rule out possible contributions from excited state chlorophyll a and plan to examine other anion signatures to determine whether we are seeing coherent formation of the charge separated state. We have also performed a careful comparison between the frequencies and 2D spectral signatures of coherence in chlorophyll a and the PSII RC. We see clear evidence of vibronic coupling and are currently writing this up for publication.

To uncover the charge-transfer states involved in the charge-separation in the PSII RC we developed 2D electronic Stark spectroscopy (2DESS) and transient-grating Stark spectroscopy (TGSS) and have recently submitted a methods paper on this work[2]. We expect these methods to be widely applicable to studies of charge separation in other systems. We have collected high quality 2DESS data on the PSII RC and are currently working on modelling the data. The modelling will build on our previous excitonic model, but will include explicit charge transfer states and will allow us to test different charge separation mechanisms.

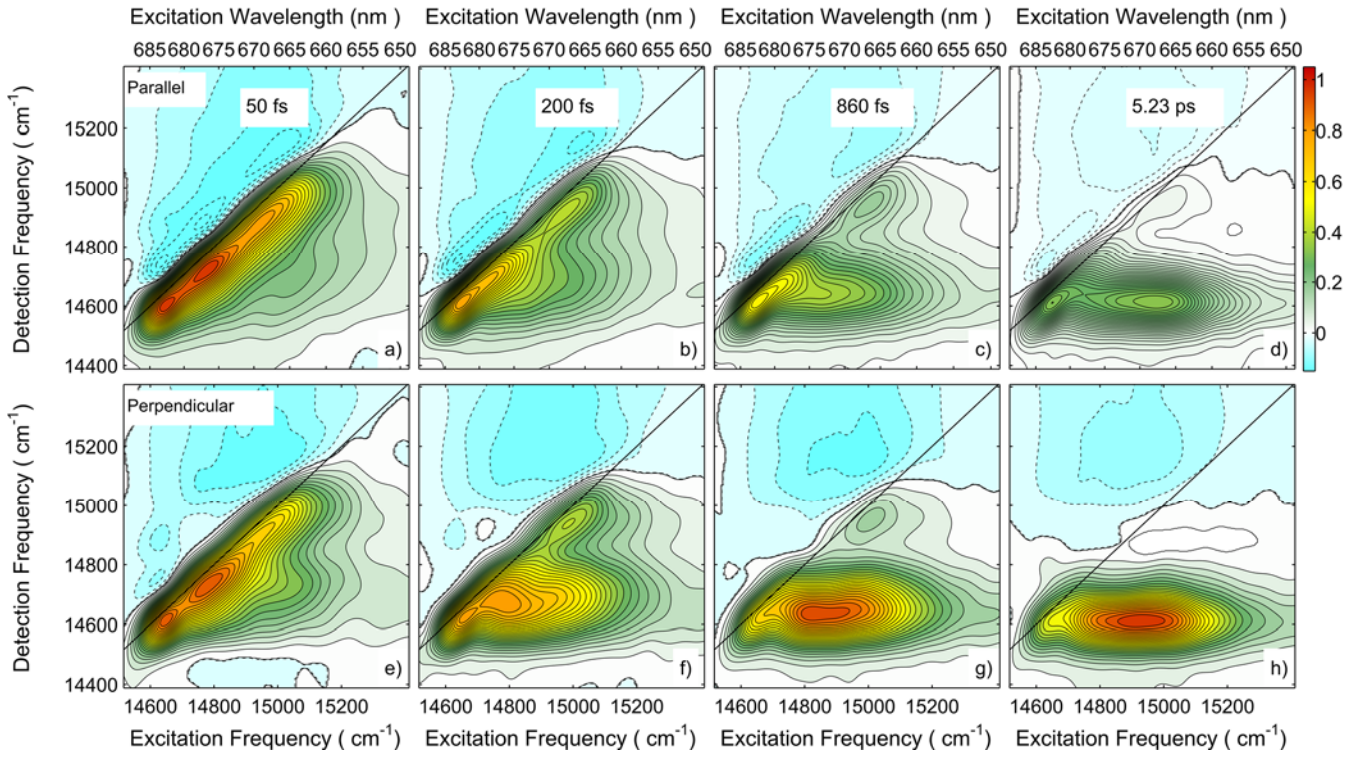

Figure 2: 2D Electronic Spectra of the PSII core complexes at 77K recorded at different t_2 waiting times as labeled. Upper panels, all pump and probe pulses are parallel (a-d); Bottom panels, the polarization of two pump pulses was perpendicular to that of the probe pulse (e-h). In both polarization conditions, spectral amplitudes are normalized to the maximum of the earliest t_2 time spectrum after pulse overlap coherent effects are largely complete. Positive amplitude is shown in solid contour lines, and negative amplitude in dashed lines.

Science objectives for 2016-2017:

- Make additional Stark 2DES measurements of the PSII RC and PSII cores and perform simulations to test and refine excitonic and charge separation models of the PSII RC.
- Further experiments to probe the importance of vibronic coherence to charge separation in the PSII RC. These include studies of site-directed mutants and anion band experiments.
- Perform our first 2D electronic-vibrational spectroscopy to help elucidate the charge separation mechanism of the PSII RC.

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

To take my project to the next level, my ideal collaborator 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 : (2015-2016)

1. Senlik, S.; Policht, V.; Ogilvie, J. P. Two-Color Nonlinear Spectroscopy for the Rapid Acquisition of Coherent Dynamics. *Journal of Physical Chemistry Letters* **2015,** *6*, 2413-2420.
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Regulated reductive flow through archaeal respiratory and energy production systems

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Overall research goals:

The metabolism and energy production strategies supporting growth of hyperthermophilic archaea push the known limits of energy conversation mechanisms. The central metabolism of *T. kodakarensis* utilizes a modified Embden-Meyerhof (EM) pathway wherein the early steps of glycolysis do not result in immediate substrate-level phosphorylation, but rather result in the reduction of ferredoxin proteins. The reduced ferredoxins (Fd_{red}) act as temporary carriers of high-energy cargo, and typically deliver electrons to membrane-bound multi-subunit complexes that couple the exergonic transfer of electrons to protons or elemental sulfur with the simultaneous pumping or translocation of ions across the cellular membrane. Transfer of the electrons to a terminal acceptor oxidizes and thus recycles the ferredoxin proteins (Fd_{ox}) and the resulting ion-gradient is then used to drive ATP synthesis via an A_1A_o-ATP synthase.

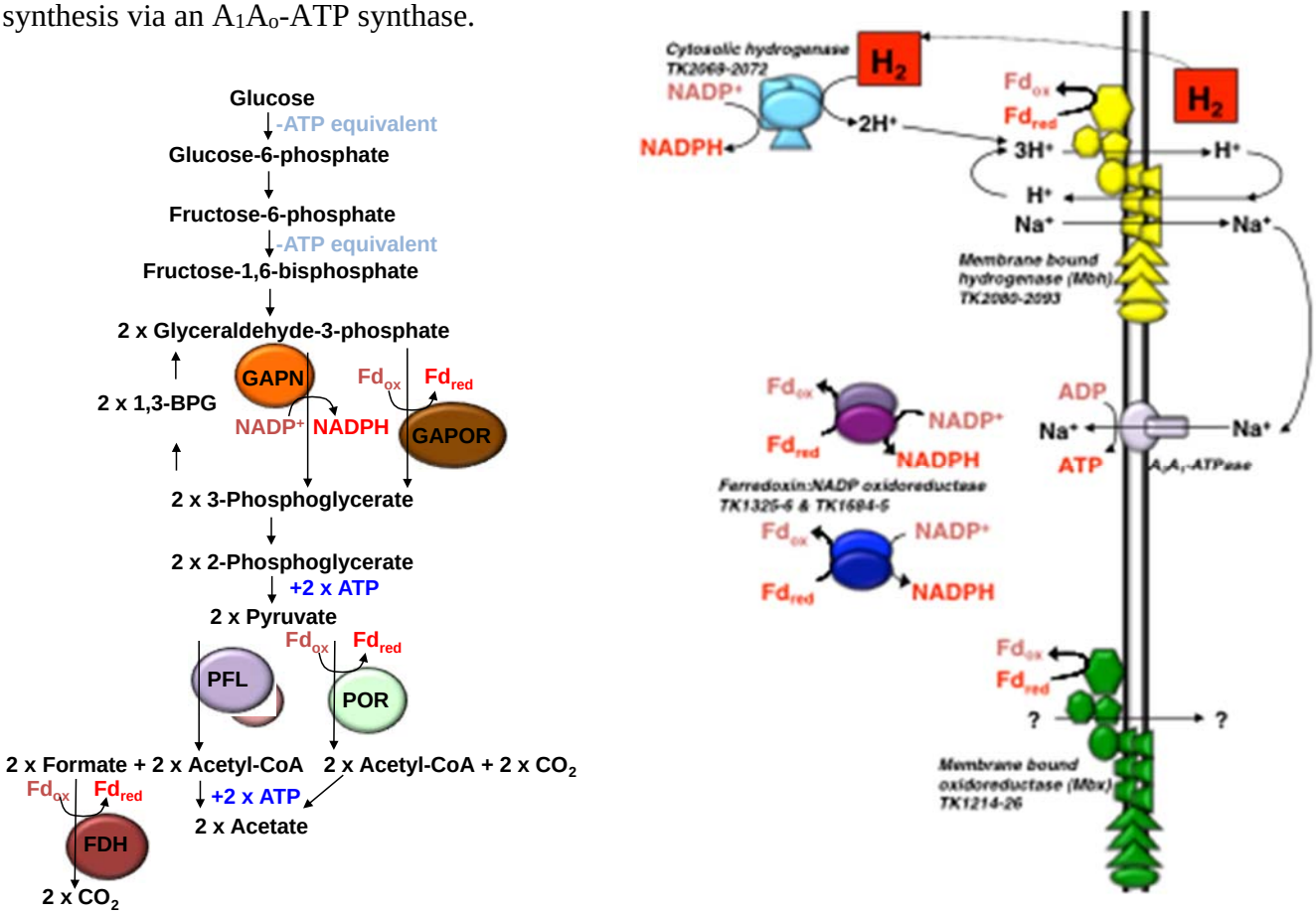

Fig 1. The ferredoxin redox cycle in *T. kodakarensis.* The modified EM pathway (left) yields 4 Fd_{red} that can be reoxidized by a variety of complexes (right), some of which provide routes to ATP synthesis.

Although many studies have focused on the individual enzymes involved in the EM pathway that transfer electrons to Fd proteins, as well as the soluble hydrogenase, the membrane-bound hydrogenase (Mbh), and the membrane-bound oxidoreductase (Mbx) that are known to except electrons from the Fd proteins, very little is known about the regulation and roles of the distinct ferredoxin proteins that are critical to the flux of electrons from glycolysis to energy conserving systems. We are leveraging our expertise in archaeal genetics, transcription, and physiology to establish the interplay, reactivity, and distinct role(s) of the three genetically encoded ferredoxin proteins in *T. kodakarensis*.

Our specific objectives are i) establishing the essentiality of each ferredoxin under different growth conditions, ii) ascertaining the mechanisms underlying the regulation of their expression, iii) determining the *in vivo* protein interactions made by each ferredoxin, and iv) detailing the overlap and unique functions of each ferredoxin with respect to energy transfer to the Mbh, Mbx, and soluble hydrogenases known to be critical to energy transactions in *T. kodakarensis.*

Significant achievements (Year 2016):

- Via total transcriptomics under different growth conditions we have established that the three loci encoding known ferredoxin proteins in *T. kodakarensis* (TK1087, TK1694, and TK2012, encoding ferredoxin-2, -1, and -3, respectively) are subject to distinct regulatory mechanisms that imply specific ferredoxins are utilized to shuttle electrons to distinct respiratory complexes during different physiological states.
- By *in vivo* tagging, purification, and mass-spectrometry analyses, the *in vivo* protein interaction partners of each ferredoxin have been established.
- TK2012 (Fd_3) was shown to be non-essential. Deletion strains are viable but show significantly decreased H_2 production – this result is congruent with the established interation of Fd_3 with Mbh.

Science objectives for 2016-2017:

- Alter, at the transcription level, the expression of each Fd to alter the cellular pool of Fds and shift the flux of electron flow to specific Fd oxidation complexes.
- Remove and alter the antisense, redox-mediated transcription of Fd_3 to establish the regulatory potential of this antisense transcription.
- Establish, through purification and biochemical techniques, direct interactions between each Fd and the redox complexes identified by mass spectrometry analyses.

My scientific area(s) of expertise is/are: genetics in archaeal systems; genome modifications; metabolic engineering of extremophiles; archaeal replication and transcription mechanics.

To take my project to the next level, my ideal collaborator would have expertise in: metabolic modeling; whole-cell flux analyses.

Publications supported by this project (2016):

Not applicable.

Transcriptional feedback mechanisms that impact phenylpropanoid metabolism in Arabidopsis

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Overall research goals: Understanding the molecular mechanisms by which carbon flux is partitioned in plants, and how flux through the phenylpropanoid pathway in particular is regulated and allocated to different branches of the pathway is essential for the eventual rational manipulation of phenylpropanoid flux for bioenergy applications. We previously reported an allelic series of three Arabidopsis mutants with a *reduced epidermal fluorescence* phenotype, *ref4-1*, *ref4-2*, and *ref4-3*, which exhibit global decreases in phenylpropanoids. These mutations, all of which exhibit semi-dominant inheritance, map to At2g48110, a gene now known to encode the MED5 subunit of Mediator, a complex that bridges gene-specific activators and repressors with basal transcription factors and the rest of the core transcriptional machinery. Our overall scientific goals are to expand our understanding of the function of Mediator, and elucidate the mechanisms by which Mediator influences phenylpropanoid biosynthesis and how metabolite accumulation within the pathway and between metabolic pathways is essential for feedback regulatory control. These experiments will inform future bioengineering efforts aimed at altering carbon allocation and cell wall engineering by contributing to our understanding of the role of Mediator in regulating lignin biosynthesis, a pathway of plant metabolism that is important on a global scale.

Significant achievements 2014-2016:

Over the past funding period, we have identified three separate phenomena that suggest that Mediator plays a critical role in metabolic homeostasis. First, we studied the *ref8* mutant of Arabidopsis, which is defective in the early phenylpropanoid pathway enzyme *p*-coumaroyl shikimate 3′-hydroxylase. To test whether MED5a and MED5b could be involved in the repression of lignin biosynthesis in *ref8-1* mutants, we generated *med5a med5b ref8-1* triple mutants. Surprisingly, disruption of both *MED5a* and *MED5b* in the *ref8-1* mutant background led to almost complete rescue of the *ref8-1* growth phenotype, with the morphology of *med5a med5b ref8-1* plants virtually indistinguishable from that of *med5a med5b* plants at multiple stages of growth (Fig. 1; Bonawitz et al., 2014).

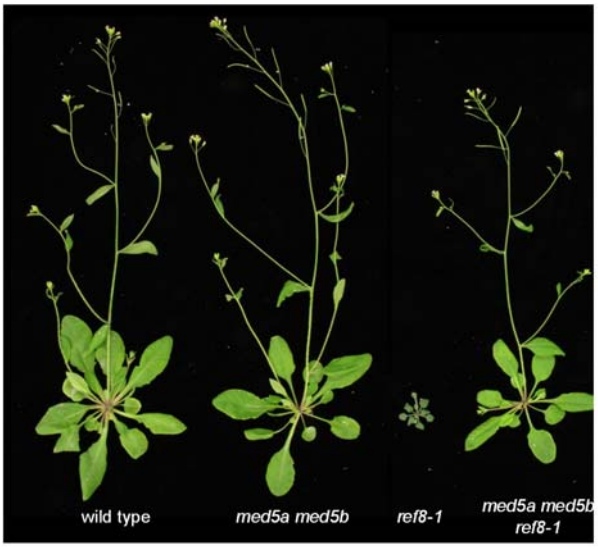

Fig. 1. Disruption of Mediator rescues growth and fertility of Arabidopsis *ref8* mutants. Three-week-old wild-type, *ref8-1*, *med5a med5b*, and *med5a med5b ref8-1* plants photographed at four weeks of age.

Second, we studied the sinapoylmalate-deficient *ref5-1* mutant. Positional cloning was used to map the *REF5* locus which was ultimately shown to encode the cytochrome P450-dependent monooxygenase CYP83B1, an enzyme involved not in phenylpropanoid biosynthesis but in glucosinolate metabolism (Kim et al., 2015). Our results indicates that the perturbation of phenylpropanoids in *ref5* mutants is not due to a deficiency in indole glucosinolates but is instead the result of the

accumulation of the CYP83B1 substrate indole acetaldoxime or a derivative. To identify genes required for the crosstalk between glucosinolate biosynthesis and phenylpropanoid metabolism, we performed a *ref5* suppressor screen and isolated three independent lines which had a restored fluorescence phenotype when observed under UV light. (Kim et al., 2015). Whole genome sequencing analyses revealed that all three of the suppressors have mutations in the same gene, At3g23590, which encodes the Arabidopsis Mediator subunit MED5b, providing more evidence for the role of Mediator in metabolic homeostasis.

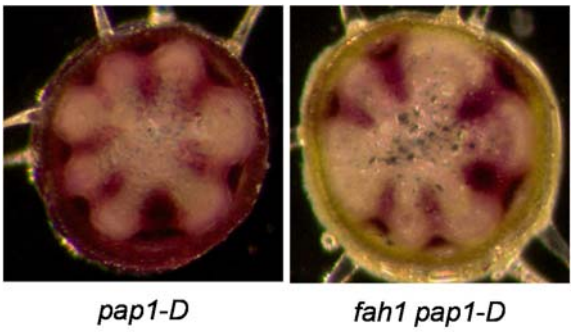

Fig. 2. Anthocyanin accumulation is inhibited in the cortex of *fah1 pap1-D* inflorescence stems, but is unaffected in vascular bundles. Semi-thin sections of *pap1-D* and *fah1 pap1-D* inflorescence stems taken from the bottom of the second internode as viewed without staining using a light microscope.

Finally, although the lignin biosynthetic enzyme ferulate 5-hydroxylase (F5H) is required for the formation of the sinapate moiety that decorates the most abundant anthocyanin in Arabidopsis leaves, this addition is not required for anthocyanin accumulation. Nevertheless, we repeatedly noticed a clear reduction in anthocyanin accumulation in F5H-deficient *fah1* plants. This effect can be even more easily seen in *pap1-d* plants, which overexpress the transcription factor MYB75 (Fig. 2). Analysis of *med5a med5b fah1* seedlings showed that the inhibition of anthocyanin accumulation in *fah1* is Mediator-dependent (Anderson et al., 2015), suggesting that a metabolite that accumulates upstream of F5H may trigger down-regulation of anthocyanin accumulation.

Science objectives for 2016-2018:

- explore the mechanism(s) involved in phenylpropanoid suppression in Arabidopsis mutants,
  - Mediator dependent down-regulation of phenylpropanoid metabolism occurs at multiple levels.
- characterize *ref4-3* suppressor mutants,
  - Comparative transcriptomic and phenotypic analyses will identify additional genes involved in phenylpropanoid homeostasis and phenylpropanoid-deficiency phenotypes.
- identify additional genes involved in phenylpropanoid homeostasis.
  - Loss of multiple tail subunits will phenocopy the *ref8* growth suppression exhibited in *ref8 med5a med5b*.

References to work supported by this project 2014-2016:

1. Anderson NA, Bonawitz ND, Nyffeler KE, Chapple C. (2015) Loss of FERULATE 5-HYDROXYLASE leads to Mediator-dependent inhibition of soluble phenylpropanoid biosynthesis in Arabidopsis. *Plant Physiol*, 169: 1557-1567
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And finally, to take my project to the next level my ideal collaborator would have expertise in: cryo-EM

Technical Advances in Mass Spectrometric Imaging of Plant Metabolites

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Overall research goals: The overarching goal of our research is to understand plant metabolic biology at the level of a single cell, and eventually at the subcellular and possibly the sub-organelle levels using mass spectrometric (MS) imaging techniques. Such information provides unprecedented details about the distribution of metabolites from cell to cell, and enables the visualization of cooperative and antagonistic effects among the metabolites that are programmed by the genetics of the organisms and modified by environmental influences. These details, integrated with functional genomics, will ultimately lead to a predictive understanding of the mechanisms that multicellular organisms use to regulate metabolic processes.

Significant achievements in 2014-2016: First, we have achieved 5 µm high-spatial resolution in MS imaging, which is almost an ultimate limit in typical matrix-assisted laser desorption ionization (MALDI)-MS. This ultrahigh resolution capability was applied to maize leaf cross-sections and demonstrated cellular and subcellular features of different metabolites. Fatty acyl chain dependent heterogeneous distribution of phosphatidylglycerol (PG) was observed between chloroplasts of bundle sheath cells and mesophyll cells. This non-uniform distribution of PG is further studied along the developmental gradient of leaf and influenced by genetics anong B73 and Mo17 inbreds and their hybrids. More recently, we have further improved the imaging technology to reach 3~4 µm laser spot size allowing routine 5 µm imaging. Most importantly, this modification allows easy change of laser spot size with simple exchange of beam expanders in less than 5 min. The left panel of Figure 1 illustrates the application of this capability to a single section of maize root section with 50, 10, and 5 µm spatial-resolution obtained with 45, 7, and 4 µm laser spot size.

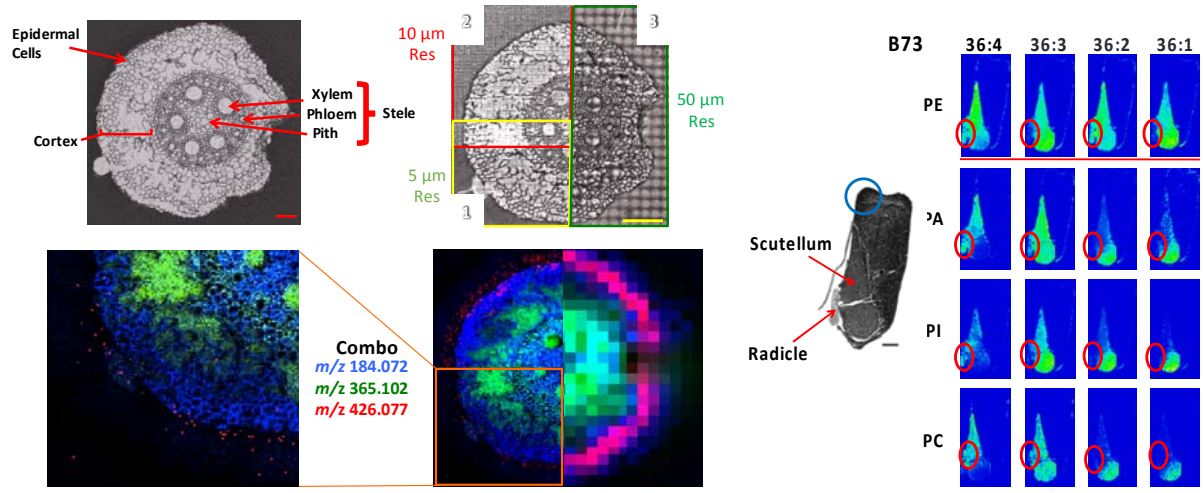

Figure 1. (Left) Multi-resolution MS imaging on a single root cross-section, obtaining 50, 10, and 5 µm resolution imaging comparing sensitivity vs imaging quality (Ref 1). (Right) Application of MS imaging to maize seed germination. Phosphatidylethanolamine shows unique heterogeneity for even number of saturation at radicle, while other phospholipids show gradual decrease of the abundance as saturation (manuscript to be submitted).

Second, we improved the lack of useful matrices in MALDI-MSI by adopting nanoparticles (NPs) as matrices. Nanoparticles can be uniformly applied to tissue samples and generate minimal backgrounds at low *m/z* values. However, their analyte selectivity is largely unknown. We have

performed a large scale screening for 13 NPs against two dozen of metabolites and established empirical correlation between different NPs and metabolite classes. Fe_3O_4 NPs were found to be most effective for MALDI-MS of triacylglycerols (TAGs), overcoming the ion suppression by phosphatidylcholines (PCs), as commonly occurs with organic matrixes such as dihydroxybenzoic acid (DHB); unfortunately, it displays ion suppression of PCs instead. We have shown, however, that the binary matrix of Fe_3O_4 NPs and DHB resulted in effective detection of both TAGs and PCs, allowing more effective study of TAG biosynthesis from PC. Additionally, this binary matrix has a synergy effect efficiently ionizing large oligosaccharides, not possible with either of the two matrices. Finally, we have demonstrated MS imaging can be used for large scale metabolomics studies, by combining the use of multiple matrices on consecutive tissue sections and adopting multiplex MS imaging. We have applied this technology to the germination of maize seeds, comparing B73 and Mo17 inbreds at various germination stages. One interesting behavior is shown in the Right-panel of Figure 1, which shows a correlation between the unsaturation of phospholipids and their abundances in the radicle, with phosphatidylethanolamine (PE) as exception being abundant in radicle for even number of unsaturation.

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2. A. D. Feenstra, K. C. O'Neill, G. B. Yagnik, Y. J. Lee, "Organic-Inorganic Binary Mixture Matrix for Comprehensive Laser-desorption Ionization Mass Spectrometric Analysis and Imaging of Medium-size Molecules including Phospholipids, Glycerolipids, and Oligosaccharides", RSC Advances, in revision.
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Session IV

5th Biennial Physical Biosciences Research Meeting
Marriott Washingtonian, Gaithersburg, MD
October 16-19, 2016
Session IV: New Tools from DOE-BES and the DOE-MSU Plant Research Laboratory

New Tools Drive New Discoveries Lecture:
DOE Nanoscale Science Research Centers (NSRCs): Resources for Collaborative Research

George Maracas, Program Manager, Nanoscience Research Centers (DOE-BES)

Abstract

The Nanoscale Science Research Centers (NSRCs) are DOE's premier user centers for interdisciplinary research at the nanoscale, serving as the basis for a national program that encompasses new science, new tools, and new computing capabilities. Each center has particular expertise and capabilities in selected theme areas, such as synthesis and characterization of nanomaterials; catalysis; theory, modeling and simulation; electronic materials; nanoscale photonics; soft and biological materials; imaging and spectroscopy; and nanoscale integration. The centers are housed in custom designed laboratory buildings near one or more other major BES facilities for x-ray, neutron, or electron scattering, which complement and leverage the capabilities of the NSRCs. These laboratories contain clean rooms, nanofabrication resources, one-of-a-kind signature instruments, and other instruments not generally available or co-located except at major user facilities. These facilities are routinely made available on a scientific merit basis to the broad research community. There are five NSRCs:

• Center for Functional Nanomaterials (CFN) at Brookhaven National Laboratory
• Center for Integrated Nanotechnologies (CINT) at Los Alamos National Laboratory and Sandia National Laboratories
• Center for Nanophase Materials Sciences (CNMS) at Oak Ridge National Laboratory
• Center for Nanoscale Materials (CNM) at Argonne National Laboratory
• The Molecular Foundry (TMF) at Lawrence Berkeley National Laboratory

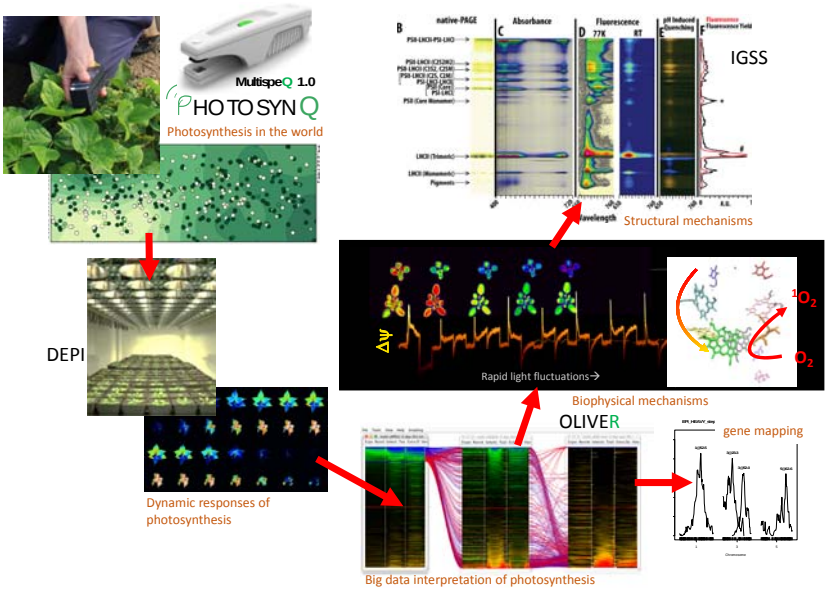
This talk will provide an overview of the NSRC Program and their capabilities for synthesis, fabrication, characterization and theory. Emphasis will be put on capabilities of interest to the Physical Biosciences community. The intent is to show that the NSRCs are places where researchers can access unique and state-of-the-art instruments and also able to collaborate in an interdisciplinary culture to accelerate their research.

Photosynthetic Energy Capture, Conversion and Storage:
From Fundamental Mechanisms to Modular Engineering
Project A: Robust Photosynthesis in Dynamic Environments

Christoph Benning, Principal Investigator
D.M. Kramer (subproject A lead), C. Benning, F. Brandizzi, J. Chen, D. Ducat, G. Howe, J. Hu, D, B. Montgomery, T. Sharkey, M. Thomashow
MSU-DOE Plant Research Lab, Michigan State University

Overall research goals: Project A addresses one of the grand challenges in basic energy science, understanding how the components of natural photosynthesis are integrated into living organisms, to operate as self-organizing molecular assemblies that respond to diverse, highly dynamic and unpredictable challenges of natural environments.

Research accomplishments: To understand (and thus improve) natural photosynthesis, we need to probe, in high throughput specific biophysical, biochemical and metabolic reactions of photosynthesis not only in isolated complexes, but as they occur *in vivo*, under field conditions. To meet these challenges, we developed several new phenotyping platforms (see Figure), including: 1) PhotosynQ (www.photoynq.org) [1] an open platform for photosynthetic phenotyping that includes a sophisticated yet inexpensive, field-deployable photosynthesis sensor, MultispeQ, that allows us to capture in detail the responses of photosynthesis in the real world. 2) Dynamic Environmental Photosynthetic Imaging (DEPI) [2], a high throughput phenotyping platform that images photosynthesis, growth and other parameters under simulated environmental conditions, allowed us to identify genes and functions critical for photosynthetic responses; and 3) In Gel Spectral Scanning (IGSS), a novel, high-resolution, high throughput approach to characterizing how the organization of photosynthetic supercomplexes affects function.



Analyses of more than 300,000 biophysical photosynthesis measurements around the world from the PhotosynQ platform suggest that under permissive conditions, photosynthesis is nearly "optimally" regulated, i.e. decreases in photochemical efficiency (ϕ_{II}) is balanced by equal increases in the efficiency of nonphotochemical quenching (ϕ_{NPQ}). Intriguingly, imbalances in this ratio either with increased or decreased ϕ_{NPQ}, strongly correlate with stresses (especially when imposed rapidly.), photodamage and loss of yield, suggesting that this regulatory balance is of paramount importance for robust photosynthesis. Using our controlled lab-based spectroscopic tools, we showed that this critical balancing depends in large part on the interaction of electron transfer components with the thylakoid proton motive force (*pmf*). On one hand, treatments or mutations that prevent the buildup of *pmf*, prevent the regulation of light capture and electron transfer, and lead to "over-reduction" and subsequent photodamage to photosystem I [3]. On the other hand, our work shows that rapid changes in light intensity results in large spikes in the electric field ($\Delta\psi$) component of *pmf*, accelerating recombination reactions in photosystem II, leading to the production of highly reactive singlet oxygen ($^1O_2$) [4]. These $\Delta\psi$ spikes are observed under even mild field-like conditions, implying that $\Delta\psi$-induced photodamage is a major limiting factor for plant productivity, but also suggests specific ways in which the effect may be avoided, potentially allowing for targeted improvement, including activation of specific thylakoid ion transporters [5, 6].

Large-scale screening of mutants and ecotypes in our DEPI system led to the discovery of new, emergent photosynthetic processes and genes that are only seen under non-laboratory conditions, including previously unreported phenomenon reflecting the remodeling of the photosynthetic apparatus [2]. This screen also showed that a large fraction of the chloroplast-targeted genes likely code for "ancillary functions" to cope with the effects of fluctuating environmental conditions. Phylogenetic analysis suggests that these components evolved more recently and diverged more rapidly than the core complexes of photosynthesis, rather than modify the ancient, core-components of photosynthesis, nature evolved add-on modifiers tune the regulatory systems of photosynthesis. Because these components are transferable, they make promising targets for improvement of photosynthesis.

Key research objectives for 2016-2017: 1) We have initiated a collaborative exploration of the ancillary roles of specialized chloroplast lipids, phycobilisome components [7, 8] and peroxisome proteins [9]. We will continue to examine the roles of ancillary processes identified in our large screens, by a) identify specific classes of genes that affect the interactions among light capture, the *pmf* and metabolism; and b) explore a broader set of environmental conditions, including temperatures, humidity, light quality and biotic factors [10, 11]; 2) Test hypothetical models for the mechanism of regulation of the thylakoid *pmf* and its effects on photodamage, both in the lab and in the real world; 3) Complete the first use of quantitative trait loci mapping in algae, and its application to understanding the biophysical, biochemical and genetic basic processes that allow some algae to cope with hyperoxic conditions; 4) Understand the connections between feedback limitations and the light reactions in plants and cyanobacteria; 5) Use a newly-developed method to image assimilation, to identify the processes and genes that limit CO_2 diffusion through leaves; 6) Develop our photosynthetic phenotyping platforms, both to address specific research questions, and to make both tools and data public resources.

Highlighted publications supported by this project:

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

Project B: Construction and Operation of the Biological Solar Panel

Christoph Benning, Principal Investigator
G.A. Howe (Subproject Lead), F. Brandizzi, **D. Ducat,** S.Y. He, J. Hu, C. Kerfeld, D.M. Kramer, B. Montgomery, T. Sharkey, M. Thomashow, C.P. Wolk, Co-PI(s)

The overarching goal of Project B is evolving from prior research efforts, with objectives to further our understanding of integrated processes in the biological solar panel across multiple temporal and spatial scales, and a focus on the conversion of sunlight into energy-dense organic compounds. The project involves collaborative, cross-disciplinary research with the long-term goal of developing multiscale models to describe photosynthesis as an integrated system, thus allowing improvement in photosynthetic efficiency through redesign of component parts. The objectives and approaches of Project B encompass multiple disciplines, including biophysics, biochemistry, physiology, photobiology, genetics, and cell biology. Moving forward, Project B is comprised of the following four aims: 1. Analysis of photosynthetic membrane assembly within the chloroplast, and the influence of metabolite exchange between chloroplasts and other organelles on photosynthetic processes; 2. Examination of the influence of structural features of the biological solar panel on CO_2 accessibility to photosynthetic compartments and regulatory links between photorespiration and the Calvin-Benson cycle; 3. Determination of how carbon outputs from the Calvin-Benson are coordinated with changing light intensity and matched with the availability of ATP/NADPH to meet the metabolic demands of the cell, including specialized cell types that use photosynthesis for non-canonical purposes; and 4. Determination of cellular processes that sense shifts in carbon partitioning within engineered plant and cyanobacterial systems towards the discovery of regulatory links between alterations in sink demand and the photosynthetic apparatus. Collectively, the research in Project B aims to elucidate pathways and mechanisms that act to integrate the flow of energy in photosynthetic arrays from the mesoscale down to the nanoscale.

Achievement Highlights during the period of 2014-2016:

- Within the funding period, Project B-associated research has resulted in the publication of 6 original research articles and 5 related review articles.
- Developed a comprehensive plant model relating plant specific leaf area growth and biomass accumulation as a function of integrated, area-based photosynthetic parameters. Application of this model to a number of mutant lines indicated that leaf architecture is critical in determining the relationship between photosynthesis and net deposition of energy; thin leaves maximize light interception and minimize respiration, whereas dense leaves suffer high resistance to CO_2 diffusion.
- Examined the tradeoffs between growth and defense in Arabidopsis, discovering that activation of jasmonate (JA) signaling halts leaf area growth, while maintaining normal efficiencies of photosynthesis. Analysis of JA mutants revealed, among other discoveries, that the growth-defense tradeoff can be uncoupled through genetic derepression of phytochrome-mediated (PIF transcription) pathways. Plants mutated for both JA and PIF signaling exhibited increased photosynthetic activity and increased mass, while maintaining production of a robust defense response.
- Developed a cyanobacterial model for the study of source/sink dynamics of photosynthesis. Demonstrated that activation of heterologous metabolic sinks (i.e. sucrose export) is coupled to increases in photosynthetic efficiency through relief of photosynthetic sink limitation under standard laboratory growth conditions. These data suggest a novel approach to engineer cyanobacteria for higher photosynthetic efficiencies while also achieving photobiological production of key metabolites.
- Uncovered a role for the ubiquitin conjugating enzyme 22 (UBC22) in regulating the partitioning of energy between growth and reproduction. UBC22 activity promotes reproductive development at the expense of plant growth, leading to decreased total photosynthesis and total mass.
- Uncovered a relationship between the architecture of the extracellular matrix of plant leaf tissue and the photosynthetic efficiency of the contained cells. Identified methyltransferases (CGR2 and CGR3) that

modulate pectin rigidity and demonstrated that the activity of these factors can be tuned to optimize leaf architecture for increased total plant growth.

- Developed a novel gnotobiotic plant growth system, the FlowPot system, that facilitates the study of genetic and mechanistic bases of plant growth-promoting properties of bacterial species, (e.g. *Burkholderia caribensis* XV). The FlowPot system permits the study of Arabidopsis in the absence of any endogenous plant microbiome. This system permitted mutational analysis of *B. caribensis*, revealing potential roles for specific bacterial pathways in promoting plant growth.
- Uncovered unexpected functions for the CAMTA family of transcription factors traditionally associated with plant bacterial defense in the regulation of photosynthesis, carbon metabolism, and growth. Analysis of CAMTA mutants indicated roles in plant responses to low sugar status.

Key research objectives for 2016-2017:

- Study the synthesis and turnover of thylakoid membrane lipids. Examine the involvement of contact sites between the endoplasmic reticulum and the chloroplast outer membrane in facilitating inter-organelle exchange of lipid precursors and the function of syntaxin-like proteins in these junctions.
- Use a forward genetic screen to identify components that reside at documented peroxisome-chloroplast contact sites and which may promote efficient transfer of photorespiratory intermediates.
- Initiate a novel chlorophyll-fluorescence-based, high-throughput screen for mutants with reduced CO_2 mesophyll conductance. Identify genetic regulators of cell wall thickness affecting CO_2 mesophyll conductance, which impacts photosynthic efficiency and photorespiration rates.
- Explore the impact of cell shape on photosynthetic efficiency through analysis of engineered cyanobacterial strains that can be tuned across a broad range of lengths and shapes.
- Examine regulatory links between photorespiration and carbon input/recycling by identifying mutants with reduced chloroplast uptake of the end-products of the photorespiratory pathway.
- Investigate carbon partitioning to a novel glucose-6-phosphate shunt, that may account for ~10% of flux through the Calvin-Benson Cycle (CBC) by examination of carbon flux and post-translational regulation of key enzymes (e.g. glucose-6 phosphate dehydrogenase) under varied environmental conditions.
- Systematically investigate photosynthetic processes in plant tissues where photosynthesis is used for specialized purposes, such as trichomes, through analysis of photosynthetic parameters, thylakoid architecture and composition, and flux of carbon towards tissue-specialized secondary metabolites.
- Identify carbon sensing mechanisms and changes in carbon flux through core metabolism of Arabidopsis mutants that constitutively partition cellular resources to defense compounds (i.e. have a high defense "sink"). Similarly examine suppressor mutants of these lines that display accelerated growth and enhanced photosynthesis, and determine the mechanistic basis of these phenotypes.
- Identify changes in the flux of photosynthetically fixed carbon through the CBC and central carbon intermediates that potentially sense alterations in sink strength in engineered cyanobacterial strains that permit inducible and tunable regulation of source/sink dynamics.

Highlighted publications supported by this project 2014-2016 (of 11 total publications):

1. Kim, S. J., Held, M. A., Zemelis, S., Wilkerson, C., & Brandizzi, F. (2015). CGR2 and CGR3 have critical overlapping roles in pectin methylesterification and plant growth in Arabidopsis thaliana. *The Plant Journal*, **82** (2), 208-220.
2. Weraduwage, S. M., Chen, J., Anozie, F. C., Morales, A., Weise, S. E., and Sharkey, T. D. (2015) The relationship between leaf area growth and biomass accumulation in Arabidopsis thaliana. *Front Plant Sci* **6**, 167
3. Campos, M. L., Yoshida, Y., Major, I. T., de Oliveira Ferreira, D., Weraduwage, S. M., Froehlich, J. E., Johnson, B. F., Kramer, D. M., Jander, G., Sharkey, T. D., and Howe, G. A. (2016) Rewiring of jasmonate and phytochrome B signaling uncouples plant growth-defense tradeoffs. *Nature Communications* **7**, 12570
4. Abramson, B. A., Kachel, B., Kramer, D. M., and Ducat, D. C. (2016) Increased photochemical efficiency in cyanobacteria via an engineered sucrose sink. *Plant and Cell Physiology*, In press
5. Havko, N. E., Major, I. T., Jewell, J. B., Attaran, E., Browse, J., and Howe, G. A. (2016) Control of carbon assimilation and partitioning by jasmonate: An accounting of growth-defense balance. *Plants* **5**, 7

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

Project C: Characterizing and engineering subcellular and cellular modules for photosynthetic productivity

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The overarching goal of the group of projects that comprise Project C is to gain a fundamental mechanistic understanding of cyanobacterial photosynthesis that can be applied to guide strategies for engineering improvements in primary productivity. Conceptually, we are addressing key questions in photosynthetic energy capture and storage using concepts borrowed from engineering, such as modularity. Modules are recognized as semi-autonomous functional units, with strong intramodule connectivity among components and weaker, often transient, inter-module connections. Modules can exist on multiple scales, including protein domains, co-regulated genes and operons, pathways, and compartments.

Photosynthesis in cyanobacteria is readily dissociated into functional modules, beginning with the distinction between electron transport and carbon reactions. These can be further subdivided to finer levels of resolution. For example, carbon metabolism involves different types of modules, including both structural and functional: the inorganic carbon uptake systems in the cell membrane, the carboxysome, and the remainder of the Calvin-Benson cycle. These modules are connected through metabolites such as the bicarbonate that connects the transporters to the carboxysome, and the phosphoglycerate and ribulose bisphosphate that connect the carboxysome to the Calvin-Benson cycle. All of these structural and functional modules also can be further deconstructed.

Project C places special emphasis on the features and interconnectivity of two cyanobacterial modules, light harvesting and the carboxysome, and also on a modular photoactive protein, the Orange Carotenoid Protein (OCP). These modules are prominent components of photosynthesis and possess structural features broadly useful for bioengineering applications. As a structural and functional module at the core of cyanobacterial carbon fixation, the carboxysome's importance to photosynthetic efficiency is difficult to overstate. Yet, the regulatory connections between the carboxysome and other photosynthetic modules, such as those comprising the light reactions have been largely neglected. The architectural features inherent to the carboxysome also make it a particularly attractive structural and functional module to be repurposed in other contexts. Likewise, the OCP is a modular protein that acts to control photosynthetic efficiency by attaching at the interface of the light-harvesting antenna and photosystem II (PSII). Regulation of the OCP is directly related to photosynthetic efficiency in cyanobacteria, and the particular mechanism by which OCP senses and conformationally responds to changes in light quality and intensity makes it useful as an engineering module in other contexts.

Project C applies "modular thinking" across the biological continuum, from the protein domain to the concept of the cell as a module in a community in the environment. Projects included in this group are aimed at gaining a fundamental mechanistic understanding of the structure and function of photosynthetic modules and the requisite means of communication between them, and using this knowledge to engineer and recombine modules to improve capture and conversion of light energy. One subproject which will be described in detail is devoted to understanding the relationship between light perception and carbon fixation in cyanobacteria, with particular attention on the carboxysome. The second subproject focuses on structure and function of the carboxysome as a bacterial microcompartment, knowledge which can be used to design and construct new types of compartments and scaffolds for engineering metabolism. The third subproject is centered on using structural and mechanistic insights about the OCP to repurpose it as a light-responsive redox-carrier that can be used to connect natural and engineered photosynthetic modules.

Significant achievements (2014-2017):

The overarching theme of Project C, a modular approach to understanding and engineering photosynthesis, has been articulated in three publications, and a fourth is in preparation.

- Gonzalez-Esquer, C.R., Newnham, S.E. and Kerfeld, C.A. Bacterial Microcompartments as Metabolic Modules for Plant Synthetic Biology. Plant Journal 87: 66-75, 2016.
- Kerfeld, C.A. Plug and Play for Improving Primary Productivity. American Journal of Botany 102: 1949-50, 2015.
- Montgomery, B.L., Lechno-Yossef, S. and Kerfeld, C.A. Interrelated Modules in Cyanobacterial Photosynthesis: The Carbon Concentrating Mechanism, Photorespiration and Light Perception. Journal of Experimental Botany 67: 2931-2940, 2016.
- Young, E.J., Burton, R.L., Fuentes-Cabrera, M.A., Mahalik, J.P., Kerfeld, C.A. and Ducat, D.C. Designing Molecular Scaffolds from Bacterial Microcompartment Protein Modules. In preparation

Science objectives for 2016-2017:

- Testing environmental regulation of carboxysome structure and function, as well as potential encapsulation of key components such as Rubisco activase
- Building Synthetic Compartments and Scaffolds for Cellular Engineering
- Light-Controlled Delivery of Electron Carriers for Synthetic Microcompartments and Scaffolds

Publications supported by this project 2014-2017 [10 out of 19 total]:

1. Aussignargues, C., B. C. Paasch, R. Gonzalez-Esquer, O. Erbilgin, and C. A. Kerfeld. 2015. Bacterial microcompartment assembly: The key role of encapsulation peptides. Commun Integr Biol 8:e1039755.
2. Aussignargues, C., M.-E. Pandelia, M. Sutter, J. Zarzycki, A. Turmo, J. Huang, J. Plegaria, D. Ducat, E. Hegg, B. Gibney, and C. Kerfeld. 2016. Structure and function of a bacterial microcompartment shell protein engineered to bind a [4Fe-4S] cluster. JACS 138 (16): 5262–5270.
3. Gonzalez-Esquer, C. R., T. B. Shubitowski, and C. A. Kerfeld. 2015. Streamlined Construction of the Cyanobacterial CO2-Fixing Organelle via Protein Domain Fusions for Use in Plant Synthetic Biology. Plant Cell 27:2637-2644.
4. Gupta, S., M. Guttman, R. L. Leverenz, K. Zhumadilova, E. G. Pawlowski, C. J. Petzold, K. K. Lee, C. Y. Ralston, and C. A. Kerfeld. 2015. Local and global structural drivers for the photoactivation of the orange carotenoid protein. Proc Natl Acad Sci U S A 112:E5567-5574.
5. Leverenz, R. L., M. Sutter, A. Wilson, S. Gupta, A. Thurotte, C. Bourcier de Carbon, C. J. Petzold, C. Ralston, F. Perreau, D. Kirilovsky, and C. A. Kerfeld. 2015. A 12 A carotenoid translocation in a photoswitch associated with cyanobacterial photoprotection. Science 348:1463-1466.

   Noël, C., F. Cai, and C. Kerfeld. 2015. Purification and characterization of protein nanotubes assembled from a single bacterial microcompartment shell subunit. Adv Mater Interfaces:10.1002/admi.201500295.
6. Gonzalez-Esquer, C.R., Newnham, S.E. and C.A. Kerfeld. 2016. Bacterial Microcompartments as Metabolic Modules for Plant Synthetic Biology. Plant J 87: 66-75.
7. Kerfeld, C.A. 2015. Plug and Play for Improving Primary Productivity. Am J Bot 102: 1949-50.
8. Kerfeld, C.A. and M.R. Melnicki. 2016. Assembly, Function and Evolution of Cyanobacterial Carboxysomes. Curr Opin Plant Biol 31:66-75.
9. Zarzycki; J., Erbilgin, O., and C.A.Kerfeld. 2015.Bioinformatic Characterization of a Major Family of Bacterial Organelles: Glycyl Radical Enzyme-Associated Bacterial Microcompartments. Appl Environ Microbiol 81: 8315-8329.
10. Sutter, M., Roberts, E.W., Gonzalez-Esquer, R.C., Bates, C., Dawoud, S., Landry, K., Cannon, G.C., Heinhorst, S and C.A. Kerfeld. 2015. Structural characterization of a newly identified component of alpha-carboxysomes: The AAA+ domain Protein cso-CbbQ. Sci Rep 5: 16243.

Session V

Modification of Plant Lipids

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Overall research goals:

The program seeks to provide fundamental understanding of the molecular mechanisms involved in conversion of photosynthetically derived carbon into desired lipid storage forms that will contribute to United States energy and fossil feedstock independence. Lipids and oils are energy-dense compounds that occur in a wide variety of forms, including the storage lipids of higher plants. The molecular mechanisms involved in chiral lipid-modification reactions using fatty acid desaturation as a model are being studied. X-ray crystallography, spectroscopy, molecular genetics, and biochemistry are used to probe structure-function relationships within these enzymes. Understanding the factors that control the selectivity and specificity of these processes is allowing us to redesign lipid-modification enzymes with improved function. The ultimate goal of this program is to be able to optimize the transformations necessary for creating improved accumulation of desired chiral lipids in non-food crops. In the current funding period we have initiated investigation into the posttranscriptional regulation of the WRINKLED1 transcription factor so that we can design mechanism-based interventions to increase its abundance and increase the accumulation of triacylglycerol (TAG).

Progress 2014-2016:

- Characterized *in vivo* interaction between the FAD family of desaturases and performed metabolic flux analysis to demonstrate a metabolic channel between 18:1 and 18:3 exists by FAD2 and FAD3 forming a functional heterodimer in which the FAD2 18:2 product is directly converted to 18:3 by FAD3 without release of the 18:2 intermediate[1].
- With co-PI Xu, discovered factors key to TAG accumulation in vegetative tissues[2], and coauthored in-depth Annual Review on the topic[3].
- In collaboration with Ed. Cahoon (Nebraska) we used our Arabidopsis proof-of-principle metabolic engineering strategy using an engineered 16:0-Δ^9-desaturase enzyme (Plant Physiol. 154:1897-904) to create a camelina seed crop with 70% ω-7 fatty acids[4].
- Mechanistic studies demonstrated a half-of-the-sites reactivity of the castor Δ9-18:0-ACP desaturase that buffers against oxidative damage to a single subunit[5].
- Reconstructed the evolutionary intermediates on the path between Δ^9-18:0-ACP desaturase and a Δ9-16:0-ACP desaturase that gave rise to downstream chemical attractants allowing speciation to occur in orchids[6].

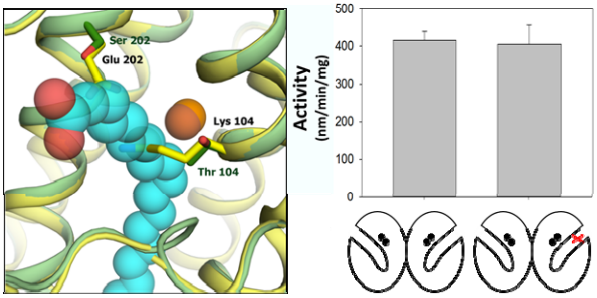

***Fig. 1**. Left panel, Structures of WT (green) and inactive mutant with occluded substrate binding channel (yellow) and blocking of fatty acid substrate (cyan). Right panel, activities of homodimer with two active subunits vs. that of heterodimer comprising one active and one inactive subunit.*

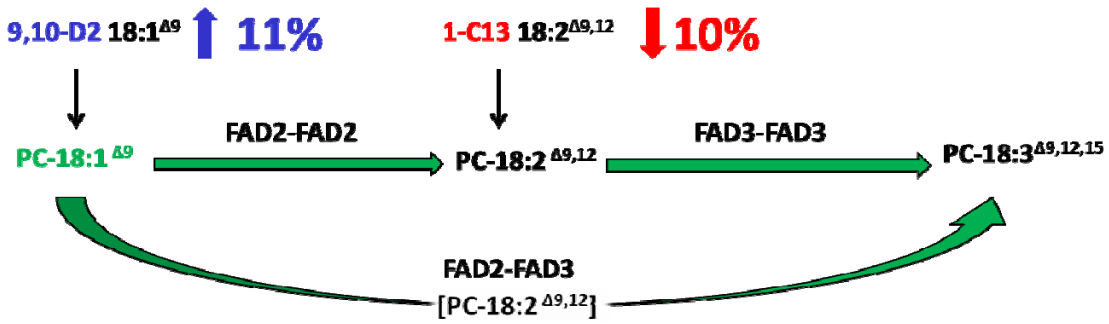

Fig. 2. *Metabolic flux analysis of competing routes between 18:1 and 18:3 by feeding mass labelled 18:1 and 18:2 to yeast expressing plant FAD2 and FAD3 enzymes shows that FAD2-FAD3 form a channel in which 18:2 formed by FAD2 is preferentially converted to 18:3 over fed 18:2).*

Science objectives for 2016-2018:

- Discover the mechanism of FAD2 and FAD3 selectivity by identifying the specificity determining residues in the FAD2 Δ^{12} and FAD3 ω-3 desaturases, using a loss of function assay for the fungal bifunctional Δ^{12}/ω-3 Fm1 as the model test system.
- Explore the basis for irreversible acetyl Co-A carboxylase inhibition using Tween-80 feeding to simulate oversupply of oleic acid.
- Identify the mechanism of sugar status-dependent posttranslational regulation of WRINKLED1 and use that knowledge to reduce its turnover as a strategy to upregulate oil accumulation in camelina. Use this information to overcome the negative regulation and increase triacylglycerol accumulation.

References to work supported by this project 2014-2016:

1. Lou, Y., J. Schwender, and J. Shanklin. 2014. FAD2 and FAD3 desaturases form hetero-dimers that facilitate metabolic channeling in vivo. J. Biol. Chem. 289(26):17996-18007.

2. Fan, J., C. Yan, R. Roston, J. Shanklin, and C. Xu. 2014. Arabidopsis lipins, PDAT1 acyltransferase, and SDP1 triacylglycerol lipase synergistically direct fatty acids toward β-oxidation, thereby maintaining membrane lipid homeostasis. Plant Cell 26(10): 4119-4134.

3. Xu, C., and J. Shanklin. 2016. Triacylglycerol Metabolism, Function and Accumulation in Plant Vegetative Tissues. Annu. Rev. Plant Biol. 2016. 67:179–206.

4. Nguyen, H. T., H. Park, K. L. Koster, R. E. Cahoon, H. T. Nguyen, J. Shanklin, T. E. Clemente, and E. B. Cahoon. 2015. Redirection of metabolic flux for high levels of omega-7 monounsaturated fatty acid accumulation in camelina seeds. Plant Biotech. J. 13(1): 38-50.

5. Liu, Q., J. Chai, M. Moche, J. Guy, Y. Lindqvist, and J. Shanklin. 2015. Half-of-the-sites reactivity of the castor Δ9-18:0-ACP desaturase. Plant Physiol., 169:432-441.

6. Sedeek, K. E. M., E. Whittle, D. Guthör, U. Grossniklaus, J Shanklin, and P. M. Schlüter. 2016. Amino acid change in an orchid desaturase enables mimicry of the pollinator's sex pheromone. Current Biology 26:1505-11.

To take my project to the next level my ideal collaborator would have expertise in: Protien kinase biochemistry.

Quantitative Analysis of Central Metabolism and Seed Storage Synthesis

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Overall research goals: Plant biomass is of increasing importance as renewable resource for the production of fuels and for chemical feedstocks that replace petroleum based materials. Rational engineering of plant biomass production is still hampered by the lack of understanding of the very complex biochemical processes. The long term goal of this project is to increase the basic understanding of control and regulation of central metabolism in plants, an evolutionary conserved metabolic network that fuels all cellular activities with building blocks and energy cofactors. We combine experimental and computational approaches to increase understanding of the functioning of synthesis of storage reserve compounds as a basis for rational engineering of seeds and other plant storage organs. Different genotypes and transgenics of oilseed rape (*Brassica napus*) and related crucifer oilseed crop plants field pennycress (*Thlaspi arvense*) and false flax (*Camelina sativa*) will be studied. Seeds/embryos in the stage of seed development and storage synthesis are dissected and cultured in vitro. Embryo tissue is characterized by quantitation of metabolic flux, enzyme activities, metabolites and transcripts. Based on modeling approaches like flux balance analysis and enzyme kinetic models, a detailed view of the particular metabolic state is obtained. The plasticity of the central metabolic network is revealed and new hypotheses on regulation and function of central metabolism can be derived and tested by additional genetic and physiological perturbations. Overall this approach will increase understanding of the biochemical processes involved in partitioning carbon and nitrogen into seed storage compounds. It is aimed at mechanistic understanding of regulatory processes at the metabolic level as well as to test the effect of transgenic alterations with regards to carbon partitioning and storage synthesis.

Significant achievements 2014-2016: We further extended our large scale stoichiometric model (*bna572*$^{+}$) by adding metabolic pathways relevant for minor seed storage compounds. In order to make use of transcriptome sequencing we referenced the model to the genome of *Brassica napus*. We compared two *B. napus* genotypes that differ in seed oil content by transcriptome profiling, analysis of metabolite levels and metabolic fluxes. We found that flux predictions made with our large scale stoichiometric model (*bna572*$^{+}$) are more realistic if flux information from ^{13}C-tracer based flux analysis is added as model constraints. Comparing the two genotypes differing in oil content we also found that changes in metabolic flux in central metabolism of *Brassica napus* developing seeds do not correlate well with changes in transcript abundance of enzyme encoding genes. This suggests that transcript profiling has only limited value as an indicator of metabolic fluxes in central metabolism and that regulatory circuits at the level of allosteric enzyme control have a significant share of control in central metabolism.

Changes in gene expression, posttranslational covalent modifications of enzymes and allosteric metabolic effects tend to have overlapping control over metabolism. In order to untangle different levels of regulation we compared 9 *B. napus* genotypes which produce triacylglycerol and other seed storage compounds in varying proportions. We characterized metabolic flux, metabolite levels and enzyme activities in central metabolism in parallel. Within the genotype spectrum, increasing flux through glycolysis and lipid synthesis is accompanied by decreasing levels of most glycolysis pathway intermediates. This is consistent with a bottom-up control mechanism of glycolysis that has been described to be characteristic for plants. Overall, the results are consistent with partitioning of flux between lipid and starch to be dominantly controlled by pyruvate kinase enzyme levels and by metabolic (allosteric) effects.

Science objectives for 2016-2018: We will explore specific features of the allosteric feedback regulation in central metabolism in more detail and in independent ways. For this purpose we will employ transgenic approaches at the levels of key enzyme steps. Enzymes will be expressed under control of chemical inducible promoters in order to measure key metabolic responses (flux, metabolite level) in dependence of a graded titration of gene expression. We will also characterize allosteric properties of pyruvate kinase based on in-vitro assays with the recombinant expressed and purified enzyme from *Arabidopsis thaliana*.

References to work supported by this project 2014-2016:

1. Schwender J, Hebbelmann I, Heinzel N, Hildebrandt T, Rogers A, Naik D, Klapperstuck M, Braun HP, Schreiber F, Denolf P, Borisjuk L, Rolletschek H (2015) Quantitative Multilevel Analysis of Central Metabolism in Developing Oilseeds of Brassica napus during in vitro culture. Plant Physiol. 168: 828-848

2. Hay J, Shi H, Heinzel N, Hebbelmann I, Rolletschek H, Schwender J (2014) Integration of a constraint-based metabolic model of Brassica napus developing seeds with 13C-Metabolic Flux Analysis. Frontiers in Plant Science 5:724

3. Hay JO, Schwender J (2014) Flux variability analysis: application to developing oilseed rape embryos using toolboxes for constraint-based modeling. Methods in molecular biology 1090: 301-316

4. Schwender J, König C, Klapperstück M, Heinzel N, Munz E, Hebbelmann I, Hay JO, Denolf P, Bodt SD, Redestig H, Caestecker E, Jakob PM, Borisjuk L, Rolletschek H (2014) Transcript abundance on its own cannot be used to infer fluxes in central metabolism. Frontiers in Plant Science 5:668

5. Lou Y, Schwender J, Shanklin J (2014) FAD2 and FAD3 Desaturases Form Heterodimers That Facilitate Metabolic Channeling in Vivo. J Biol Chem 289: 17996-18007

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

Post-translational Regulation and Macromolecular Organization of Lignin Biosynthesis

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Xuebin Zhang, Mingyue Gou, Postdoctoral Research Associate
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Overall research goals:

As one of the major cell wall structural components, lignin represents a substantial metabolic sink of reduced carbon, accounting for up to 30% photoassimilates. As an irreversible biological process, cell wall lignification is under tight regulatory control. The metabolic regulation occurs both at transcriptional and post-translational levels, which coordinately controls carbon skeleton and energy channeled into phenylpropanoid-lignin metabolism. One of our long-term research goals is to discover the biochemical and molecular mechanisms governing carbon flux channeled to the phenylpropanoid pathway during plant lignification. Specifically our researches are 1) to define the proteolytic regulators and the related regulatory networks controlling carbon allocation into phenylpropanoid metabolism; 2) to determine the biochemical and biological functions of redox partners associated with cytochrome P450 monooxygenases for phenylpropanoid-lignin biosynthesis; and 3) to continually engineer and evaluate the effects of the functionally specialized monolignol 4-*O*-methyltransferases on plant lignification and cell wall ultrastructure.

Significant achievements 2014-2016:

- **Novel proteolytic regulators negatively controlling phenylpropanoid biosynthesis in response to sugar signal.** With previous characterization of three E3 ligase components KFB^{PALs}, we further discovered two additional KFB homologs that mediate ubiquitination and degradation of PAL, the first rate limiting enzyme of phenylpropanoid pathway, and chalcone synthase (CHS), a committed enzyme directing metabolic flux from general phenylpropanoid pathway to a variety of flavonoid metabolites, respectively. Examining the expression of At*KFB*^{PALs} and At*KFB*^{CHS}, we found that their transcript abundances were severely suppressed in the seedlings grown on the medium containing high concentration of sugars. Consequently the cellular concentration of PAL (and CHS) enzymes and the phenylpropanoid metabolite content in those seedlings were substantially increased (**Fig. 1**). These data imply that the KFB^{PALs} (and KFB^{CHS}) may function as the key integrators of the transcriptional and proteolytic regulation network in response to sugar signals to control carbon allocation into phenylpropanoid metabolism.

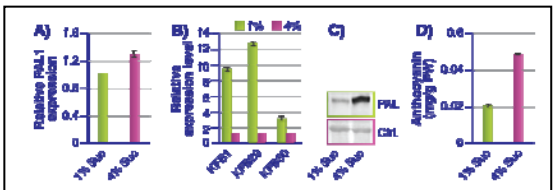

Fig. 1 *Relative gene expression levels of PAL1 (A), and KFB^{PALs} (B) by qRT-PRC, and PAL protein concentration (C) by western blot, as well as anthocyanin accumulation level (D) in seedlings grown on the medium containing 1% and 4% sucrose (Suc), respectively.*

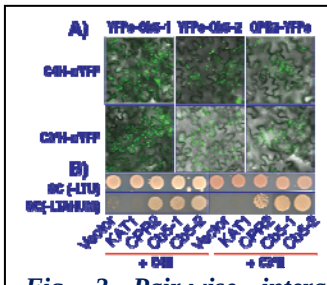

Fig. 2 Pair-wise interaction verification. *(A) BiFC assays of Cb5 isoforms (1 and 2) and CPR2 with C4H or C3'H. (B) Membrane Y2H assay. Sc(-LTU): growth medium; Sc(-LTAHUM): selective medium. KAT1: control protein.*

- **The interactions of cytochrome P450 monooxygenases with redox partners cytochrome P450 reductase and cytochrome b5.** In phenylpropanoid-monolignol biosynthesis, NADPH: Cytochrome P450 Reductases (CPRs) commonly serve as the redox partner of P450 monooxygenases (C4H, C3'H and F5'H) to deliver electrons from reducing agents for hydroxylation of phenolic substrates. Our immunoprecipitation

coupled with mass spectrometry analyses discovered that cytochrome b5 (Cb5) physically associates with monolignol P450 enzymes. To validate their interactions, we conducted Bimolecular Fluorescence Complementation (BiFC) and split-ubiquitin membrane Y2H assays. Both experiments revealed that C4H and C3'H indeed directly interact with CPR or Cb5 (**Fig. 2**). These data suggest that Cb5 might act as an alternative electron carrier protein to stimulate P450 activity.

- **Engineering a set of monolignol 4-*O*-methyltransferases with high selectivity for particular lignin precursors.** Lignin composition dictates the degree of lignin condensation and thus the degradability of plant cell walls. Preferentially modifying the *para*-hydroxyl of lignin precursor to deprive its dehydrogenation propensity would disturb the formation of particular lignin subunits. Combining crystal structure determination with combinatorial active site saturation mutagenesis, we remodelled the substrate binding pocket of a previously engineered substrate promiscuous enzyme, yielding a set of mutant enzymes capable of preferentially modifying G- or S-lignin precursors, or producing novel monomers. Furthermore, an engineered enzyme via expressing it in poplar was demonstrated significant effects on enhancing digestibility and fermentative ethanol yield of transgenic wood.

Science objectives for 2016-2018:

- Availability of carbon source in plant fluctuates significantly over the course of a day. We will evaluate the regulatory roles of KFB^{PALs} in controlling phenylpropanoid-lignin biosynthesis in response to the fluctuation of intrinsic sugar signals. As KFB^{PALs} is transcriptionally in response to the sugar signal, we will discover the molecular players and the potential regulatory network controlling KFB^{PAL} gene expression.
- Cb5 may function as electron carrier protein or allosteric regulator stimulating P450 enzyme activity. We will constitute the redox components in yeast system to examine the effects of those redox partners on the activities of monolignol P450 enzymes. We will also adopt molecular genetic approaches to define and differentiate Cb5 and CPR's biological functions in monolignol biosynthesis.
- The functionally specialized MOMTs will be expressed in the plants with tissue specific promoters. Comprehensive analyses on lignin content, composition/structure of transgenic plants will be conducted to evaluate their *in vivo* functions.

References to work supported by this project 2014-2016:

1. Liu, C.-J*., Cai, Y., Zhang, X., Gou, M., and Yang, H. (2014) Tailoring lignin biosynthesis for efficient and sustainable biofuel production. ***Plant Biotech. J.*** 12:1154-1162.
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4. Cai, Y., Bhuiya, M.-W., Shanklin, J., and Liu, C.-J* (2015). Engineering a monolignol 4-*O*-methyltransferase with high selectivity for the condensed lignin precursor coniferyl alchohol. ***J. Biol. Chem.*** 290: 26715-26724
5. Cai, Y., Zhang, K., Kim, H., Hou, G., Zhang, X., Yang, H., Feng, H., Miller, L., Ralph, J., and Liu, C.-J.* (2016). Enhancing digestibility and ethanol yield of *Populus* wood *via* expression of an engineered monolignol 4-*O*-methyltransferase. ***Nature Communications*** 7:11989 doi: 10.1038/ncomms11989

And finally, to take my project to the next level my ideal collaborator would have expertise in: Cell wall/lignin structure determination, Redox chemistry.

Session VI

Abstract

Functional Analysis and Genetic Manipulation of Plant ABCB Organic Ion Transporters

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Department of Energy, Basic Energy Sciences, CSGB Division, grant no. DE-FG02-13ER16405

The objective of this BES-funded project is the identification and characterization of structural characteristics of plant ABCB/PGP transporters that determine overall functionality, regulate membrane stability, and specificity for aromatic organic acid substrates. Plant ABC transporters are primary transporters of hydrophobic and phenylpropanoid compounds that are resource materials for biofuel conversion. Overall functionality of plasma membrane ABC transporters is regulated by pairing of "half-transporter" subunits and conserved association with HSP90 and/or FKBP506-type foldases (FKBPs). Membrane stability is conferred by association with sphingolipid / sterol-enriched ordered membrane domains as well as by a novel protease-dependent endocytotic mechanism associated with the EXO70a turnover mechanism. Specificity for hydrophilic substrates is regulated by charges of amino acid residues within the transmembrane helix core. However, transport of more hydrophobic aromatic organic acid substrates appears to be additionally regulated by lipid- protein interactions in the inner leaflet of the plasma membrane. Here we report the following:

1. Simple modification of amino acid residues associated with substrate transport in ABCB auxin transporters reduces activity, but does change substrate specificity as is seen for hydrophilic substrates. However, alteration of the lipid environment for these transporters does reduce substrate specificity. Further, addition of plant-like membrane components to plant transporters in *Lactococcus* increases ABCB activity in isolated membranes.
2. Phenotypic plasticity observed in *abcb* auxin transport mutants of Arabidopsis that has been attributed to differential function of the transporters is more likely an effect of differential background auxin levels.
3. Half transporter forms of some ABCB transporters exhibit partial transport functionality, suggesting that dimerization to form full transporters can partially substitute for full-length activity.
4. The aspartyl protease dependent endocytosis and turnover of the ABCB4 transporter can be regulated by manipulation of protease activity and trafficking events within the *trans* Golgi Network.
5. Enhanced production of stable ABCB proteins in plant, *Lactococcus*, *S. pombe,* and *Pichia pastoris* expression systems can be achieved by manipulation of the factors described above – thus making fermenter-scale expression at levels sufficient for structural determination possible.

Secondary Wall Formation in Fibers

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Overall research goals:

The overall goal of the DOE-funded project is to study functional roles of genes involved in secondary wall biosynthesis in plants. Secondary walls are mainly composed of cellulose, xylan and lignin, biosynthesis of which requires a coordinate expression of all the biosynthetic pathway genes. Xylan in dicots (typically called glucuronoxylan) is composed of a linear backbone of β-1,4-linked xylosyl residues, about 10% of which are substituted with α-1,2-linked glucuronic acid and/or 4-*O*-methylglucuronic acid residues. In addition, about 60% of xylosyl residues are acetylated at *O*-2 and/or *O*-3. Although a number of genes have been implicated in xylan backbone elongation and substitution of glucuronic acid residues, genes and their encoded enzymes involved in the acetylation of xylosyl residues and those involved in the synthesis and transport of UDP-xylose, the xylosyl donor for xylan biosynthesis, are not well studied. Because the presence of xylan in cellulosic biomass has been shown to hinder the efficiency of conversion of biomass into bioethanol, further understanding of how xylan is made will not only contribute to our knowledge of cell wall biosynthesis in general but also have important economic and agronomic implications, such as providing genetic tools for custom-designing cell wall composition tailored for biofuel production.

Significant achievements (2014-2016):

Xylan, a major polysaccharide in plant lignocellulosic biomass, is acetylated at *O*-2 and/or *O*-3 and its acetylation impedes the use of biomass for biofuel production. In Arabidopsis, about 60% of xylosyl residues in xylan are acetylated and the biochemical mechanisms controlling xylan acetylation are largely unknown. We have found essential roles of 7 DUF231 domain-containing proteins, in xylan acetylation in Arabidopsis. These DUF231 proteins are involved in position-specific acetylation of xylosyl residues, i.e., 2-*O*-and 3-*O*-monoacetylation, 2,3-di-*O*-acetylation, and 3-*O*-acetylation of xylosyl residues substituted at *O*-2 with glucuronic acid. While mutation of one of these DUF231 gene, *ESK1*, causes a specific reduction in 2-*O*-and 3-*O*-monoacetylation, simultaneous mutations of two other DUF231 genes, TBL32 and TBL33 result in a complete loss of 3-*O*-acetylated 2-*O*-GlcA-substituted xylosyl residues (Fig. 1). Our finding indicates the complexity of the biochemical mechanisms underlying xylan acetylation

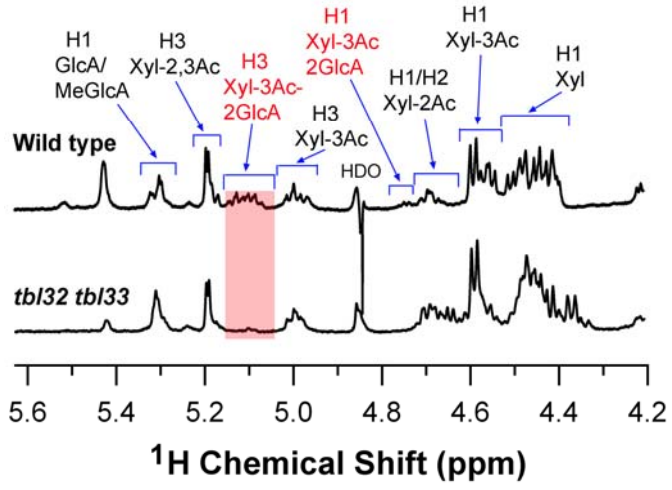

Fig. 1 NMR spectroscopy of distribution patterns of xylan acetyl substitutions in the wild type and the *tbl32* and *tbl33* double mutant defective in DUF231 genes. The resonances for non-acetylated (Xyl), 2-*O*-acetylated (Xyl-2Ac), 3-*O*-acetylated (Xyl-3Ac), 2,3-di-*O*-acetylated (Xyl-2,3Ac), 3-*O*-acetylated 2-*O*-GlcA-substituted xylosyl residues (Xyl-3Ac-2GlcA) and GlcA/MeGlcA are labeled. Note that the *tbl32 tbl33* mutation caused a loss of the resonances corresponding to 3-*O*-acetylated 2-*O*-GlcA-substituted xylosyl residues (Xyl-3Ac-2GlcA) at 5.1 p.p.m., indicating a specific role of TBL32 and TBL33 in 3-*O*-acetylation of xylosyl residues substituted at *O*-2 with glucuronic acid.

We have demonstrated that xylans from *Selaginella moellendorffii* and *Physcomitrella patens* are acetylated at *O*-2 and *O*-3 and that genes involved in xylan backbone elongation and glucuronic acid methylation are evolutionarily conserved in plants.

We have found a number of NAC and MYB transcription factors that are master switches controlling secondary wall biosynthesis in switchgrass, a promising biofuel crop, which provides molecular tools for genetic manipulation of biomass production in switchgrass.

We have characterized five Arabidopsis NAC transcription factors for their specific roles in regulating secondary wall biosynthesis in vessels, and three Arabidopsis DUF579 genes and four poplar DUF579 genes for their roles in glucuronic acid methylation in xylan.

Science objectives for 2016-2018:

The main objective for the following year will be to investigate several genes of unknown functions for their roles in secondary wall biosynthesis. We have recently identified several genes that are expressed during secondary wall biosynthesis and we propose that they are involved in xylan biosynthesis. We will generate mutants mutated in these genes, and examine effects of the mutations on xylan structure. The study of xylan biosynthesis will provide knowledge foundation for genetic modification of biomass with altered wall composition.

Publications supported by this project [2014-2016]:

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To take my project to the next level my ideal collaborator would have expertise in: protein and carbohydrate structures

Myosins XI are involved in cellulose deposition and CESA dynamics

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Overall research goals:

In the current proposal, we will test the ***central hypothesis*** that actin and myosin control the rate of delivery of cellulose-synthase complexes (CSCs) to the plasma membrane and thereby regulate cellulose biosynthesis. The ***specific aims*** include:

1) determining the role of *Arabidopsis* myosins XI in CESA trafficking and dynamics;

2) testing whether actin filament length and lifetime alter exo- and endocytosis of CSCs

The Staiger laboratory aims to understand dynamic control of the cortical actin cytoskeleton and how it is used to deliver materials to and from the PM and cell wall. Although it is commonly accepted that actin filaments serve as tracks for exocytosis, there is little direct evidence that secreted cargo traffics along single filaments or bundles. Moreover, which dynamic properties of the cortical cytoskeleton regulate vesicle trafficking remain poorly understood. Recently, we described a model for the regulation of axial cell expansion and cell wall deposition based on specific properties of single actin filaments. Fortunately, with genetic control over the availability of actin filament ends, and thus the regulation of individual filament length and lifetime, we are uniquely positioned to test this model. We have also demonstrated that class XI myosins from *Arabidopsis* participate in cellulose deposition during primary cell wall formation. Capitalizing on the identification of a new small molecule inhibitor, we have implicated myosin in trafficking of CSCs and CESA dynamics. We will pursue further analysis of myosin activity and its role in cell wall assembly by combining high spatiotemporal live-cell imaging with molecular genetic analysis of class XI myosins.

Significant achievements (2015-2016):

- Identified and characterized a new, small molecule inhibitor of plant myosins; pentabromopseudilin (PBP).
- Isolated a *myosin xi* triple-knockout mutant (*xi-1 xi-2 xi-k*), expressing CESA6-YFP in *prc1-1* background for live-cell imaging of CSC trafficking and membrane dynamics.
- Showed that *myosin xi 3KO* mutants have reduced total and crystalline cellulose content in primary cell walls.
- Demonstrated the involvement of myosins, using both pharmacological and genetic approaches, in maintaining steady-state levels of CSC particles at the plasma membrane (PM).
- Discovered a population of SmaCC/MASCs that accumulate in the cortical cytoplasm just below the PM when myosin is inhibited.
- Used fluorescence recovery after photobleaching (FRAP) experiments to quantitatively analyze the rate of delivery of CSC particles to the PM. Acute and constitutive inhibition of myosin activity results in up to 50% reduction in exocytosis of CESA.
-

Science objectives for 2016-2017:

- Characterize the tracks that myosin XI uses to deliver CESA to the PM, single actin filaments *versus* bundles by two-color fluorescence, timelapse microscopy.

- Genetically manipulate the abundance of single actin filaments in the cortical cytoplasm using Capping Protein mutants (e.g. *cpb-1* and *CP OX*) to test the hypothesis that a dense array of actin filaments serves as a meshwork that hinders exocytosis of CESA.
- Demonstrate that PBP inhibits plant myosins XI *in vitro* by purification of native Myosin XIK-YFP from plant cells and establishing actin filament gliding assays.
- Characterize the secretion of noncellulosic polysaccharides and PM proteins in the *myosin xi 3KO* mutant.

My scientific area(s) of expertise is/are: live-cell imaging, high-performance fluorescence microscopy, quantitative cell biology, cytoskeletal dynamics.

To take my project to the next level, my ideal collaborator would have expertise in: carbohydrate chemistry and cell wall composition analyses; fluorescence bioreporters.

Publications supported by this project (2014-16):

1. Li J, Staiger BH, Henty-Ridilla JL, Abu-Abied M, Sadot E, Blanchoin L, **Staiger CJ**. 2014. The availability of filament ends modulates actin stochastic dynamics in live plant cells. *Mol. Biol. Cell* 25:1263-1275.
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6. Li J, Blanchoin L, **Staiger CJ**. 2015b. Signaling to actin stochastic dynamics. *Annu. Rev. Plant Biol.* 66:415-440.

Sub-project title: Structural Studies of Recombinant Plant CESA N-terminal Domains of the Plant Cellulose Synthesis Complex

Presenter: Hugh O'Neill, Oak Ridge National Laboratory, Email: oneillhm@ornl.gov
Hugh O'Neill, B. Tracy Nixon, Loukas Petridis, Yaroslava G. Yingling (Co PIs)
Venu Gopal Vandavasi, Juan Du, Abhishek Singh, Joel Lapin, (Postdoctoral Associates)

Project Award DE-SC0001090: Center for Lignocellulose Structure and Formation

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Overall Research Goals

Cellulose synthesis in plants is carried out by the synergistic action of a large number of membrane bound cellulose synthase (CESA) proteins that form the cellulose synthesis complex (CSC). There are many outstanding questions regarding the roles of different CESA isoforms in the CSC and how they assemble to form a functioning complex. Knowledge of the structure of individual CESA proteins that form the CSC would provide critical information about the molecular mechanism of cellulose synthesis. Recent structural studies of *Arabidopsis thaliana* CESA3 catalytic domain using small-angle scattering (SAS), and freeze-fracture and negative-stain transmission electron microscopy (FF-TEM, TEM) analysis of *Physcomitrella patens* CSC supported by computation, provided strong evidence that there are 18 CESA proteins in the plant CSC [1,2]. These studies contradict previous reports that proposed a 36 subunit CSC, and in turn, implicate an 18 chain cellulose microfibril as the fundamental product of cellulose synthesis in plants.

Significant Achievements

Here, we report on our progress in investigating the structure of the N-terminal domain of CESA proteins to gain insight into the role of this domain in cellulose synthesis. Recombinant N-terminal domains of *A. thaliana* CESA1 and CESA3, and *Gossypium hirsutum* CESA1, were cloned, expressed and purified from *Escherichia coli* extracts. Physicochemical characterization of the recombinant proteins indicate that they are purified as homodimers, bind up to 2 mol zinc per monomer, and are flexible disordered proteins. Small-angle scattering analysis showed that the proteins have an extended and flexible conformation (Figure 1). Fusion proteins comprised of the N-terminal domains linked to maltose binding protein (MBP) were made to get more in-depth information about location of the dimerization interface. Small-angle scattering analysis supported by negative-stain transmission electron microscopy revealed that the dimerization interface is likely to be towards the C-terminal end of the proteins (Figure 1). Molecular dynamics simulations of the *A. thaliana* and *G. hirsutum* N-terminal domains provide structural details about the interface region and point to a well conserved alpha-helix as the interface. The effect of amphiphiles on the conformation of the dimers was also studied and revealed that in the presence of the detergents such as sodium dodecyl sulfate the secondary structure of the proteins is increased and the conformation of the protein transitions from an extended to a compact state. The experimental and computational results will be discussed in the context of previously published work that proposed a redox dependent N-terminal domain dimerization mechanism and more recent work on in vitro synthesis of cellulose by heterologously expressed *Populus*

CESA proteins [3,4]. Finally, we will present a model for the role of the N-terminal domain in the assembly of a functional CSC.

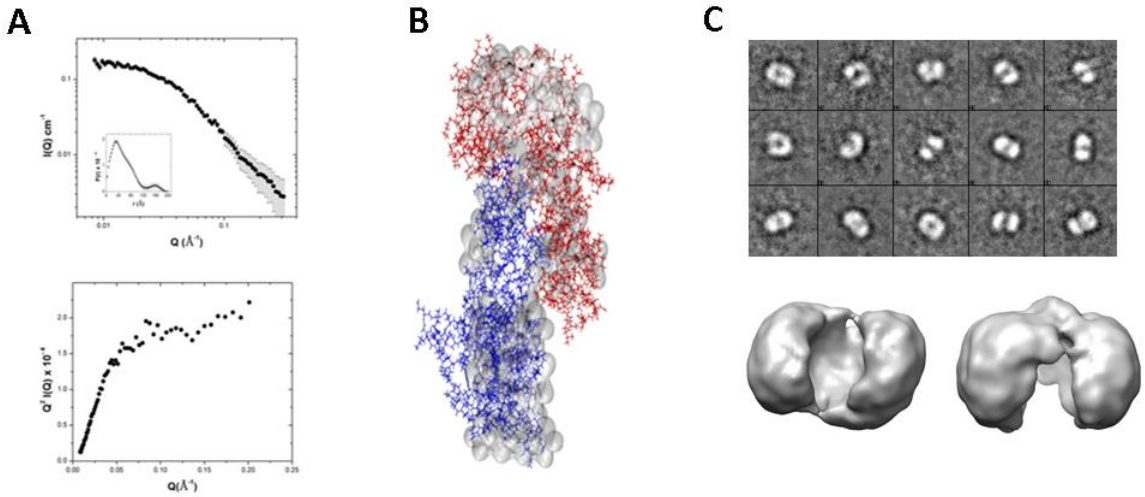

Figure 1. **A**. Small-angle neutron scattering profile (top) and Kratky plot (bottom) of *A. thaliana* CESA3 N-terminal domain (Inset: P(r) curve). **B**. *Ab initio* shape reconstruction from SANS data (grey spheres) overlaid with dimer structures from MD simulations. **C**. Negative stain TEM images of CESA3 N-terminal domain- MBP fusion protein (top). Representative class average images of the CESA3 N-terminal domain- MBP fusion protein

Science objectives for 2015 – 2016

- Structural analysis of *A. thaliana* catalytic domain trimer using cryo-electron microscopy
- Identification of the interface region of the catalytic domain trimer using mass spectrometry
- Production of recombinant *A. thaliana* CESA transmembrane domain and structural studies using small-angle scattering

My scientific area of expertise: Biochemistry, molecular biology, small angle scattering

To take my project to the next level, my ideal collaborator would have expertise in: synthetic biology, cell-free protein expression, cryo-electron microscopy

References

1. Vandavasi VG, Putnam DK, Zhang Q, Petridis L, Heller WT, Nixon BT, Haigler CH, Kalluri U, Coates L, Langan P, O'Neill, H A structural study of CESA1 catalytic domain of arabidopsis cellulose synthesis complex: evidence for CESA trimers. Plant physiology. 2016;170(1):123-35.
2. Nixon BT, Mansouri K, Singh A, Du J, Davis JK, Lee J-G, Slabaugh E, Vandavasi VG, O'Neill H, Roberts EM, Haigler CH Comparative structural and computational analysis supports eighteen cellulose synthases in the plant cellulose synthesis complex. Scientific Reports. 2016;6:28696
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The Center for Direct Catalytic Conversion of Biomass to Biofuels (C3Bio)

Multiscale Modeling of the Plant Cell Wall: Elucidating Chemical and Architectural Control Points

Peter N. Ciesielski and Michael F. Crowley, Principal Investigators (co-presenters)
Bryon S. Donohoe, Micheal E. Himmel, Co-PI(s)
Vivek Bharadwaj, Postdoctoral Research Associate
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Email: peter.ciesielski@nrel.gov; michael.crowley@nrel.gov
Website: http://www.nrel.gov/bioenergy/computational-modeling.html

Overall research goals:

Biomass holds great potential as a renewable source of fuels, chemicals, and materials that can displace large portions of fossil fuel-derived products. The development of cost-competitive processes for the conversion of raw biomass into these desirable products will required significant advances in the development of conversion strategies that specifically address the inherent chemical and biological resiliency and multiscale complexity of biomass. Such advances are informed by improving our fundamental understanding of the chemical, physical, and biological characteristics that govern the properties and functionality of the biopolymer composite that constitutes plant cell walls.

We are leveraging the high-performance computing resources of the DOE, in tandem with experimentation and advanced imaging, to identify and quantify "control points" that can be leveraged to affect the outcomes of biomass conversion processes. Due to its hierarchical structure, such control points manifest at length scales that span many orders of magnitude: at the molecular and macromolecular scales, the biosynthesis, composition, and decoration of biopolymers dictate their assembly into nanostructures; at mesoscales, the association of cellulose nanofibrils with hemicelluloses and lignins forms an impressive polymer composite termed lignocellulose that contributes the majority of biomass feedstocks by weight; and at the tissue scale, the arrangement of cell walls forms a macroporous matrix that impacts the transport of heat and mass throughout biomass particles during conversion processes. We are developing interconnected modeling tools to probe the structure and functionality of biomass at each of these length scales to better understand how these features dictate the behavior of biomass in conversion process with the goal of leveraging this new knowledge toward targeted process design and optimization.

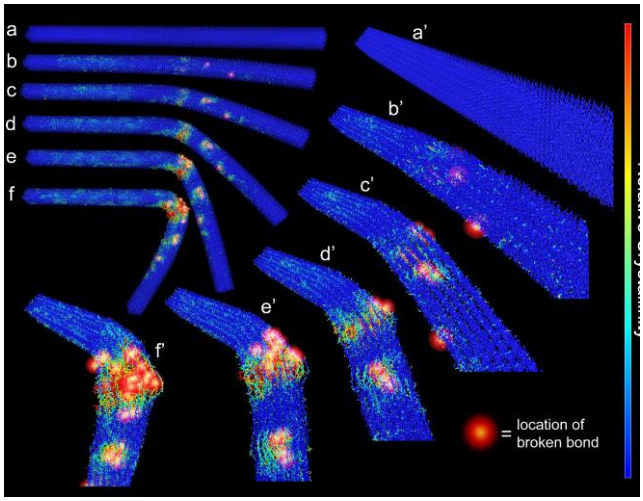

Figure 1. Molecular modeling of stress-induced defects provides insight into the effectiveness of conversion processes that combine mechanical and chemical deconstruction.

Significant achievements (2014-2017):

- Simulated structural mechanics of cellulose nanofibrils to relate macromolecular stresses to crystallinity and molecular defects
- Simulated aggregation of hemicellulose polymers and cellulose nanofibrils to understand favorable interactions and decoration patterns
- Developed methods to construct mesoscale models of lignocellulose based on quantitative structural parameters for use in finite element simulations of transport phenomena coupled to conversion kinetics

- Constructed species-specific biomass particle models for use in simulations of various conversion processes including fast pyrolysis and acid hydrolysis.

Science objectives for 2016-2017:

- Relate nanoscale geometry of cellulose nanofibrils to deviations in crystallinity as a function of fibril size and shape
- Streamline molecular dynamics simulations to provide quantitative characteristics to parameterize mesoscale of models lignocellulose
- Develop mesoscale lignocellulose models that capture the effects of specific genetic variants and thermochemical treatment technologies developed throughout the center (e.g., high S lignin, steam explosion pretreatment)
- Leverage quantitative image analysis to further inform and validate structural models across multiple length scales

My scientific area(s) of expertise is/are: Materials science, molecular dynamics simulation, finite element simulation, multimodal imaging and image analysis, structural geometry, enzymology.

To take my project to the next level, my ideal collaborator would have expertise in: High speed, in-situ chemical and/or spectroscopic imaging.

Publications supported by this project 2014-2016:

1. Ciesielski, P. N.; Crowley, M. F.; Nimlos, M. R.; Sanders, A. W.; Wiggins, G. M.; Robichaud, D.; Donohoe, B. S.; Foust, T. D., Biomass Particle Models with Realistic Morphology and Resolved Microstructure for Simulations of Intraparticle Transport Phenomena. *Energy & Fuels* **2015,** *29* (1), 242-254.
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5. Wei, H.; Yang, H.; Ciesielski, P. N.; Donohoe, B. S.; McCann, M. C.; Murphy, A. S.; Peer, W. A.; Ding, S.-Y.; Himmel, M. E.; Tucker, M. P., Transgenic ferritin overproduction enhances thermochemical pretreatments in Arabidopsis. *Biomass and Bioenergy* **2015,** *72*, 55-64.
6. Ciesielski, P. N.; Wang, W.; Chen, X.; Vinzant, T. B.; Tucker, M. P.; Decker, S. R.; Himmel, M. E.; Johnson, D. K.; Donohoe, B. S., Effect of mechanical disruption on the effectiveness of three reactors used for dilute acid pretreatment of corn stover Part 2: morphological and structural substrate analysis. *Biotechnol Biofuels* **2014,** *7* (1), 1.
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Poster Session II

EFRC: BIOLOGICAL ELECTRON TRANSFER AND CATALYSIS (BETCY)
PI: JOHN PETERS

AWARDS: $10M (August 2014 – July 2018)
WEBSITES: **http://science.energy.gov/bes/efrc/centers/betcy/;** http://betcy-efrc.org/default.htm
TEAM: **Montana State University (Lead):** John W. Peters (Director), Brian Bothner, Eric Boyd, Ross Carlson; **University of Georgia**: Michael W.W. Adams; **National Renewable Energy Laboratory**: Pin-Ching Maness, Paul King; **University of Washington**: Caroline Harwood; **Arizona State University**: Anne Jones; **Utah State University**: Lance Seefeldt; **University of Kentucky**: Anne-Frances Miller

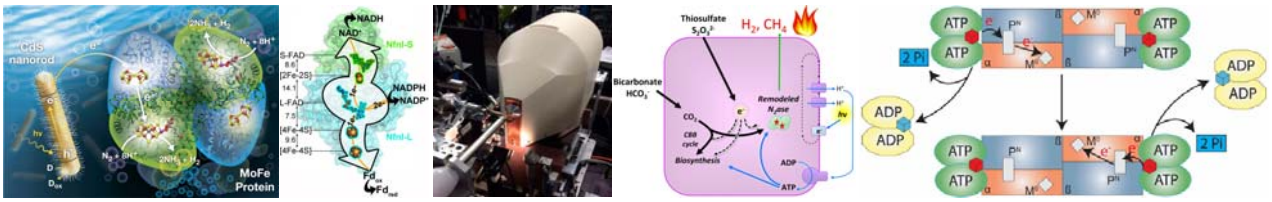
SCIENTIFIC MISSION AND APPROACH

The focus of the Biological Electron Transfer and Catalysis (*BETCy*) EFRC research is elucidating mechanisms of conversion of electrochemical potential into chemical bond energy and is organized into three integrated Thrusts.

- **Thrust 1** – Mechanism of coupling exergonic and endergonic electron transfer reactions by electron.
- **Thrust 2** – Mechanism of coupling chemical bond energy and electrochemical potential in electron transfer to achieve very low potential reductions.
- **Thrust 3** – Atomic level determinants of enzymatic redox properties and their relationship to catalytic bias.

SELECTED SCIENTIFIC ACCOMPLISHMENTS

- Demonstrated the first example of a light-driven N_2 reduction reaction to ammonia catalyzed by nitrogenase in a paper published in the journal *Science.*
- Key contributors to the commissioning x-ray free electron laser experiments on protein crystals published in the *Proceedings of the National Academy of Sciences USA*.
- First complete mechanistic description of a model bifurcating enzyme, provided insights on fundamental protein design and thermodynamic principles these enzymes have acquired in order to accomplish energy conserving biochemical reactions. Provides a template by which to evaluate how broader structural context of bifurcating enzymes accomplish this catalytically demanding reaction.
- Demonstrated the first enzyme catalyzed light driven methane formation in work in press in the *Proceedings of the National Academy of Sciences*.
- Redefined the nitrogenase electron delivery cycle by showing that the rate-limiting step is phosphate release, not component protein dissociation in work published in *Biochemistry*.
- Demonstrated negative cooperativity in nitrogenase during the electron delivery cycle. Article is in press at the *Proceedings of the National Academy of Sciences USA.*



BETCy research, from left: Light driven N_2 reduction by nitrogenase coupled to light-harvesting cadmium sulfide nanorods; X-ray structure of NfnI enzyme used to develop a model for describing the mechanism of electron bifurcation; Goniometer-based femtosecond crystallography at Stanford LCLS-XPP; Light driven methane production by remodeled nitrogenase. Conformational control of electron transfer events in the nitrogenase enzyme complex.

IMPACT

- BETCy and CME PIs teamed to organize Telluride workshop on chemistry at metal active sites of enzymes emphasizing fundamental of catalytic bias and proton coupled electron transfer reactions.
- Invited talks on electron bifurcation mechanism at Gordon Research Conferences on 1) The Molecular Basis of Carbon One Metabolism, 2) Electron Donor-Acceptor Interactions, and 3) Metals in Biology and the American Society for Microbiology General Meeting.
- Results of light driven nitrogen reduction published in *Science* highlighted in numerous science popular press forums including *Chemical and Engineering News*.
- Invited talks on light driven nitrogen reduction at Gordon Research Conferences on 1) Metallocofactors, and 2) Metals in Biology.
- BETCy PIs commissioned by Springer Publishing Company to edit the first book on Electron Bifurcation

PUBLICATIONS AND INTELLECTUAL PROPERTY

As of August 2016, BETCy has 10 peer-reviewed publications.

- Cohen, A. E. *et al*. Goniometer-based femtosecond crystallography with X-ray free electron lasers. *Proceedings of the National Academy of Sciences of the United States of America* **111**, 17122-17127, doi:10.1073/pnas.1418733111 (2015). [31 citations]
- Peters, J. W. *et al*. [FeFe]- and [NiFe]-hydrogenase diversity, mechanism, and maturation. *Biochimica et Biophysica Acta-Molecular Cell Research* **1853**, 1350-1369, doi:10.1016/j.bbamcr.2014.11.021 (2015). [16 citations]
- Brown, K. A. *et al*. Light-driven dinitrogen reduction catalyzed by a CdS:nitrogenase MoFe protein biohybrid. *Science* **352,** 448-450, doi:10.1126/science.aaf2091 (2016). [2 citations]
- Peters, J. W. *et al*. Electron bifurcation. *Current Opinion In Chemical Biology* **31**, 146-152, doi:10.1016/j.cbpa.2016.03.007 (2016). [1 citation]
- Yang, Z-Y. *et al*. Evidence that the P-i release event is the rate-limiting step in the nitrogenase catalytic cycle. *Biochemistry* **55**, 3625-3635, doi:10.1021/acs.biochem.6b00421 (2016). [0 citations]
- Schut, G. J. *et al.* The role of geochemistry and energetics in the evolution of modern respiratory complexes from a proton-reducing ancestor. *Biochimica et Biophysica Acta-Bioenergetics* **1857**, 958-970, doi:10.1016/j.bbabio.2016.01.010 (2016). [1 citation]
- Schut, G. J. *et al.* Heterologous production of an energy-conserving carbon monoxide dehydrogenase complex in the hyperthermophile *Pyrococcus furiosus*. *Frontiers in Microbiology* **7**, 1-9, doi:10.3389/fmicb.2016.00029 (2016). [0 citations]
- Poudel, S. *et al*. Unification of [FeFe]-hydrogenases into three structural and functional groups. *Biochemica Biophysica Acta* **1860**, 1910-1921, doi:10.1016/j.bbagen.2016.05.034 (2016). [0 citations]
- Fixen, K. R. *et al*. Light-driven carbon dioxide reduction to methane by nitrogenase in a photosynthetic bacterium. *Proceedings of the National Academy of Sciences of the United States of America* (in press)
- Danyal, K. *et al.* Negative Cooperativity in the Nitrogenase Fe Protein Electron Delivery Cycle. *Proceedings of the National Academy of Sciences of the United States of America* (in press).

Wendy J. Shaw

Controlling Catalytic Reactivity with Scaffolds that Range in Size and Flexibility

Wendy J. Shaw, Nilusha Borolugodage, Molly O'Hagan, Abhi Karkamkar, Simone Raugei, Thomas Ward[†], Sascha Keller[†]
Pacific Northwest National Laboratory
[†]University of Basel, Switzerland

Enzymes display incredible efficiency, selectivity, and specificity, much of which is a result of the protein scaffold which surrounds the active site. Despite knowing that the scaffold plays a role, identifying specific features and then mimicking those features in simpler or more robust catalysts remains a challenge. Our program is focused on learning from enzymes and applying that knowledge to capture the features of the scaffold into molecular and heterogeneous catalysts. As a successful example of this approach, we have attained enzymatic-like electrochemical reversibility for interconverting H_2 and protons by introducing two scaffold features around the well understood $[Ni(P^{R}_2N^{R'}_2)_2]^{2+}$ active site, those of proton transport and controlling the active site structure (Figure 1, left). We are also attempting to apply the principles learned here to heterogeneous catalysts and artificial enzymes to utilize the scaffold environment to control catalytic reactivity and develop a mechanistic understanding of the many roles of the scaffold. We will show preliminary results demonstrating initial success in generating site controlled heterogeneous catalysts in which we can control both the number of metal sites as well as the functional groups and features of the scaffold. We will also show preliminary success using a redesigned protein to create a complex scaffold around a well understood molecular complex.

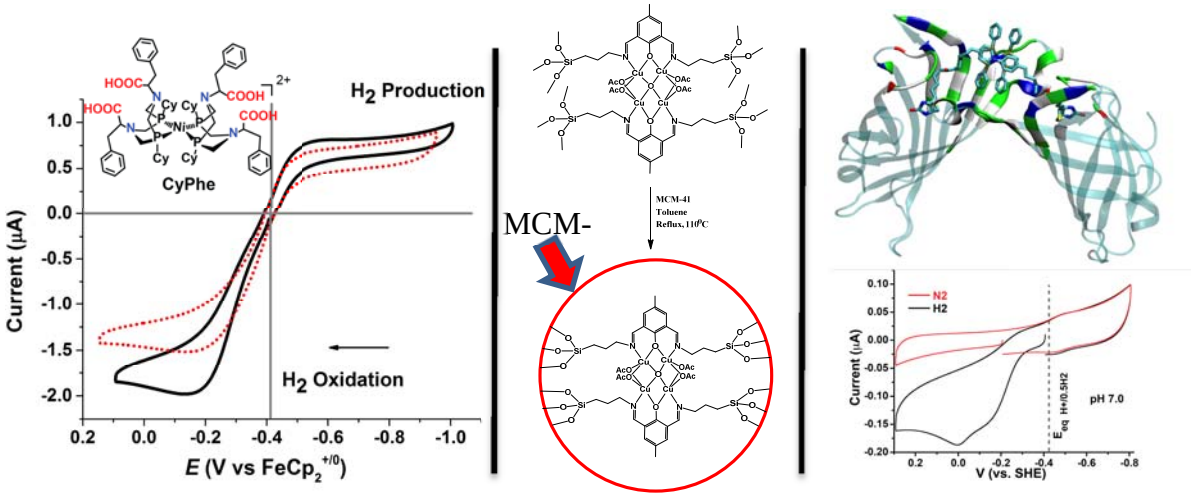


Figure 1. (Left) Electrochemical reversibility was achieved at room temperature for $[Ni(P^{Cy}_2N^{Phe}_2)_2]^{2+}$ (Cy=cyclohexyl, Phe=phenylalanine), as a result of features in the scaffold. **(Middle)** Heterogeneous scaffolds can be prepared from well-defined molecular catalysts allowing site-specific synthetic control. **(Right)** Redesigning proteins can provide a scaffold from which to learn, as shown here by attaching a molecular complex to streptavidin, which still shows electrocatalysis.

Mechanisms of self-assembly and catalysis in protein microcompartments

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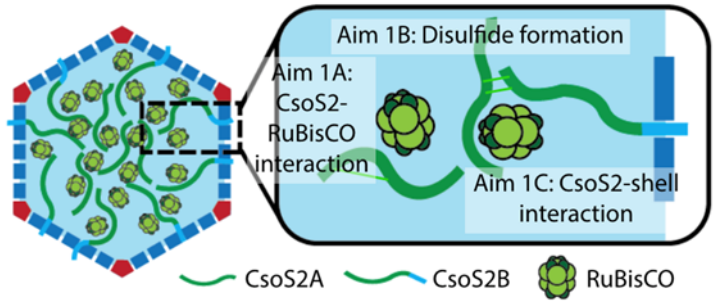
Overall research goals:

The compartmentalization of biochemical reactions is a fundamental principle employed by the cell to segregate and facilitate metabolism. Understanding and engineering compartmentalization has the potential to dramatically improve the rate, yield, and toxicity of biocatalysts metabolically engineered for energy capture and conversion. Despite obvious applications, however, the predictable engineering of compartmentalized biochemical reactions within the cell remains challenging. Many bacteria use protein microcompartments to specifically encapsulate and facilitate multi-step metabolic pathways. For example, the carboxysome is a ~300 MDa capsid-like structure that compartmentalizes carbonic anhydrase and the Calvin Cycle enzyme RuBisCO in order to optimize CO_2 fixation. These modular, protein-based complexes could provide a unique scaffold for the development of novel biocatalysts, but, generally, their mechanism of assembly and function remains ambiguous. The goal of our work is to elucidate the molecular mechanisms which govern the self-assembly and function of the carboxysome. Ultimately, we hope to leverage this understanding in order to develop protein-based microcompartments with enhanced catalytic properties.

Significant achievements (2011-2016):

- Developed fluorescence microscopy-based assays to track the assembly of carboxysomes in living cells.
- Established a heterologous system for expressing carboxysomes in *E. coli*. This enables both biochemical assays to elucidate critical protein-protein interactions for assembly and also the system to produce engineered microcompartments. Further work revealed that one of these proteins, CsoS2, is highly regulated and essential for carboxysome assembly.
- Constructed a reaction-diffusion model of the carboxysome's function in cells to quantify and explore its function.
- Developed a simplified protein compartment, encapsulin, as new class of model compartment.

Figure 1. Model of the carboxysome and the critical role of the protein CsoS2 in making a number of critical interactions to modulate assembly.



Science objectives for 2016-2017:

- In initial work we have discovered a critical interaction in alpha-carboxysome formation between RuBisCO and the poorly characterized protein CsoS2. We are characterizing the specific molecular nature of this interaction and functional consequences using a variety of techniques including biochemistry and physiological analysis.
- We have additional evidence that CsoS2 makes a series disulfides bonds during the carboxysome maturation process. We are identifying these interactions and determining their significance.
- We have established a new type of compartment, encapsulin, as a model system for protein encapsulation. While doing so, we have observed this compartment binds a flavin molecule and are now determining the function of the flavin on the native encapsulin's function mediating iron redox chemistry.

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

To take my project to the next level, my ideal collaborator would have expertise in: cryo-electron microscopy.

Publications supported by this project 2011-2016:

1. N. M. Mangan, A. Flamholz, R. D. Hood, R. Milo, D. F. Savage, pH determines the energetic efficiency of the cyanobacterial CO2 concentrating mechanism. *Proc. Natl. Acad. Sci. U.S.A.* **113**, E5354–62 (2016).
2. R. D. Hood, S. A. Higgins, A. Flamholz, R. J. Nichols, D. F. Savage, The stringent response regulates adaptation to darkness in the cyanobacterium Synechococcus elongatus. *Proc. Natl. Acad. Sci. U.S.A.* (2016), doi:10.1073/pnas.1524915113.
3. C. Cassidy-Amstutz *et al.*, Identification of a Minimal Peptide Tag for in Vivo and in Vitro Loading of Encapsulin. *Biochemistry*. **55**, 3461–3468 (2016).
4. T. Chaijarasphong *et al.*, Programmed Ribosomal Frameshifting Mediates Expression of the α-Carboxysome. *J. Mol. Biol.* **428**, 153–164 (2016).
5. R. Yokoo, R. D. Hood, D. F. Savage, Live-cell imaging of cyanobacteria. *Photosynthesis Research*. **126**, 33–46 (2015).
6. A. H. Chen, A. Robinson-Mosher, D. F. Savage, P. A. Silver, J. K. Polka, The bacterial carbon-fixing organelle is formed by shell envelopment of preassembled cargo. *PLoS ONE*. **8**, e76127 (2013).
7. W. Bonacci *et al.*, Modularity of a carbon-fixing protein organelle. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 478–483 (2012).
8. A. H. Chen, B. Afonso, P. A. Silver, D. F. Savage, Spatial and temporal organization of chromosome duplication and segregation in the cyanobacterium Synechococcus elongatus PCC 7942. *PLoS ONE*. **7**, e47837 (2012).

Nanotube-Supported Phospholipid Bilayers

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Overall research goals: The overarching goal of this project is to attain a detailed understanding of, and to gain control of molecular biologically-inspired self-assembly at bio-nano interfaces and, particularly, at lipid bilayer surfaces. Using the latest advances in fabrication of ceramic nanoporous substrates, the chemistry of paramagnetic EPR-active tags responding to local electric fields, and parallel developments in EPR, NMR, and double-resonance methods, we propose to infer the role of electrostatic interactions in the interfacial self-assembly and assess the oligomerization and structure of membrane proteins including bacterial photoreceptors. We will also expand the arsenal of self-assembled soft nanostructures under conditions of nanoscale confinement from lipids to peptide tubules and/or hybrid lipid/electron transfer enzymes with the ultimate goals of building hybrid biologically-inspired nanodevices for energy capture and biological energy transduction.

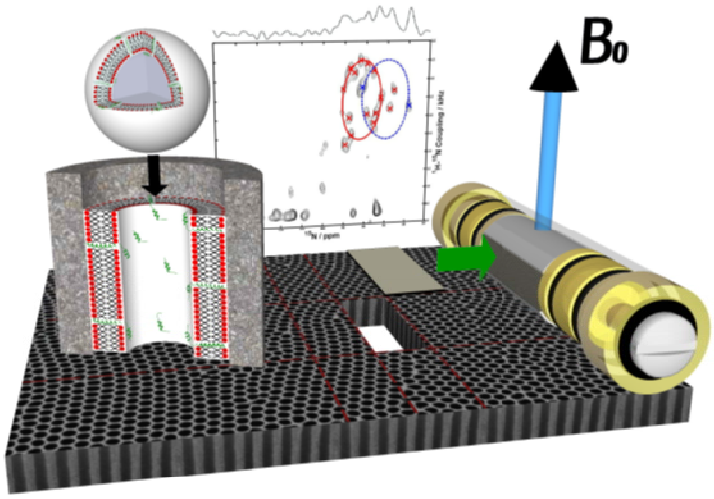

Figure 1. SEM image captures well-organized structure of anodic aluminum oxide (AAO) nanopores of *ca*. 80 nm in diameter employed for macroscopic alignment of fully hydrated bilayers of different lipid composition containing uniformly ^{15}N-labeled Pf1 coat protein. AAO was cut and packed into a 4×5 mm NMR tube to obtain 2D NMR spectra to reveal changes in the protein conformation caused by the lipid bilayer composition. The helix tilt was entirely governed by the membrane thickness. The AAO method allows for studying the role played by the lipids in shaping membrane proteins.

Significant achievements 2015-2016:

- Demonstrated high-resolution oriented sample solid-state (OS ss) NMR spectroscopy of the uniformly ^{15}N-labeled Pf1 coat protein reconstituted in nanoporous AAO substrate-confined lipid bilayers. Two different lipid compositions have revealed some marked deviations in the positions of the spectral peaks attributed to structural changes. This is consistent with the hypothesis that folding of the membrane-spanning proteins is lipid-dependent. Thus, the largely improved AAO alignment technique provides a general method for studying lipid-induced structural conformations of membrane proteins under physiologically relevant conditions by OS ssNMR.[1,2]

- By using molecular probes and associated spin-labeling EPR methods we developed,[3,4] it was shown that the acid−base equilibrium inside the nanopores pores is affected by the pore diameter below d ≈ 29 nm but is independent of the ionic strength. Specifically, for the 29 and 18 nm nanopores the values of pH_{int} were found to be 0.5−0.8 pH unit lower than the bulk pH_{ext}. This local but a large in magnitude effect can attributed to a distortion of the water structure in the immediate vicinity of the pore wall and an influence of the surface charge near the double layer formed inside the pores. As pH_{int} and electrical surface potential are essential characteristics in heterogenic catalysis and for forming substrate-supported and nanopore confined lipid bilayers, our results are

expected to assist in studying different catalytic reactions and serve as a basis for a rational design of hybrid catalytic self-assembled catalytic systems based on nanoporous AAO membranes.[7,8]

- A synthesis and characterization of a novel biradical that can be site-specifically, covalently attached to exposed cysteines, either natively present in a protein or introduced by site-directed mutagenesis has been reported.[3,9] Structurally, this biradical originates from a known DNP NMR polarizing agent TOTAPOL, but contains an essential new feature - a flexible side chain terminated with thiol-specific methanethiosulfonate functionality. To the best of the authors' knowledge, this is the first demonstration of the use of stable biradicals that can be covalently attached to a protein as polarization agents for DNP to achieve dramatic (*i.e.*, ×10-15 fold or higher) enhancement of NMR signals.[9]

Science objectives for 2016-2018:

- Develop atomic layer deposition (ALD) and consequent chemical modification methods of the inner nanopore surface for covalent attachment and macroscopic alignment of photosensitizers//bio-catalysts and consequent characterization by a combination of EPR, NMR, X-ray, optical, IR techniques (in collaboration with group of Dr. David Tiede (ANL).
- Employ AAO nanopore technology to build hybrid self-assembled and macroscopically aligned structures containing transmembrane peptides and integral and membrane associated proteins involved in transduction of biological energy (*e.g.*, rhodopsins, cytochromes) for consequent characterization with magnetic resonance and optical methods.

References to work supported by this project 2015-2016:

1. A. Marek, W. Tang, S. Milikisiyants, A. A. Nevzorov, A. I. Smirnov, "Nanotube Array Method for Studying Lipid-Induced Conformational Changes of a Membrane Protein by Solid-State NMR", *Biophys. J*., 108: 5-9 (2015) DOI: 10.1016/j.bpj.2014.11.011.
2. A. A. Nevzorov and A. I. Smirnov, "Orientational and Motional Narrowing in Solid-State NMR of Membrane Proteins Macroscopically Aligned by Nanopores", *Biol. Magn. Reson*. v. 32, pp. 159-185 (2015). DOI: 10.1007/978-1-4899-7621-5_5.
3. M. A. Voinov, D. B. Good, M. E. Ward, S. Milikisiyants, A. Marek, M. A. Caporini, M. Rosay, R. A. Munro, M. Ljumovic, L. S. Brown, V. Ladizhansky, and A. I. Smirnov, "Cysteine-Specific Labeling of Proteins with a Nitroxide Biradical for Dynamic Nuclear Polarization NMR", *J. Phys. Chem. B*, 119: 10180-10190 (2015) DOI: 10.1021/acs.jpcb.5b05230.
4. M. A. Voinov and A. I. Smirnov, "Ionizable Nitroxides for Studying Local Electrostatic Properties of Lipid Bilayers and Protein Systems by EPR", *Methods Enzymology*, (2015) 564:191-217; doi:10.1016/bs.mie.2015.08.007
5. T. I. Smirnova and A. I. Smirnov, "Peptide-membrane Interactions by Spin-labeling EPR", *Methods Enzymology* (2015) 564: 219-58. doi: 10.1016/bs.mie.2015.08.018.
6. Z. Liu, D. Leininger, A. Koolivand, A. I. Smirnov, O. Shenderova, D. W. Brenner, J. Krim, "Tribological properties of nanodiamonds in aqueous suspensions: effect of the surface charge", RSC Advances, 5: 78933-78940 (2015), *RSC Adv*., 2015,5, 78933-78940.
7. E. G. Kovaleva, L. S. Molochnikov, U. Venkatesan, A. Marek, D. P. Stepanova, K. V. Kozhikhova, M. A. Mironov, and A. I Smirnov, "Acid-base Properties of Nanoconfined Volumes of Anodic Aluminum Oxide Pores by EPR of pH-sensitive Spin Probes", *J. Phys. Chem. C* (2016) 120 (5), pp 2703-2711, DOI: 10.1021/acs.jpcc.5b10241
8. E. G. Kovaleva, L. S. Molochnikov, D. P. Stepanova, A. V. Pestov, D. G. Trofimov, I. A. Kirilyuk, and A. I. Smirnov, "Interfacial Electrostatic Properties of Hydrated Mesoporous and Nanostructured Alumina Powders by Spin Labeling EPR", *Cell Biochem. Biophys*., provisionally accepted. (2016).
9. M. A. Voinov and A. I. Smirnov, "Site-Specific Dynamic Nuclear Polarization NMR Agents, US Patent Application, No. 15/222,428, filed July 28, 2016.

To take my project to the next level my ideal collaborator would have expertise in: membrane protein biochemistry.

The Importance of Pyrophosphate in the Bioenergetics of the Syntrophic Benzoate Degraders

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Overall research goals:

Syntrophy is essential for the efficient conversion of organic wastes and crop residues into the energy rich fuel, methane. Syntrophic metabolism operates close to thermodynamic equilibrium. We are interested in how syntrophic bacteria conserve energy using catabolic growth reactions with low free energy changes. The objectives of this proposal are: 1) to determine the mechanism of ATP formation from acetyl-CoA in *Syntrophus aciditrophicus*, a syntrophic fatty and aromatic acid-degrading bacterium, and 2) to understand the thermodynamic regimes of key catabolic and energy conserving reactions involved in syntrophic metabolism by quantifying metabolites including pyrophosphate, acyl-CoA intermediates, adenylates, and NAD^+/NADH.

Significant achievements (2014-2016):

- We used transcriptomic, proteomic, metabolite, and enzymatic approaches to show that *S. aciditrophicus* uses a unique approach to make ATP from acetyl-CoA metabolism, an AMP-forming, acetyl-CoA synthetase (Acs1) (Fig. 1). High pyrophosphate levels and a high AMP to ATP ratio (5.9 ± 1.4) in *S. aciditrophicus* cells favor the operation of Acs1 in the acetate-forming direction. Pyrophosphate cycling using Acs1, provides an efficient mechanism for energy conservation for *S. aciditrophicus*, which is critical for microorganisms that operate close to thermodynamic equilibrium.

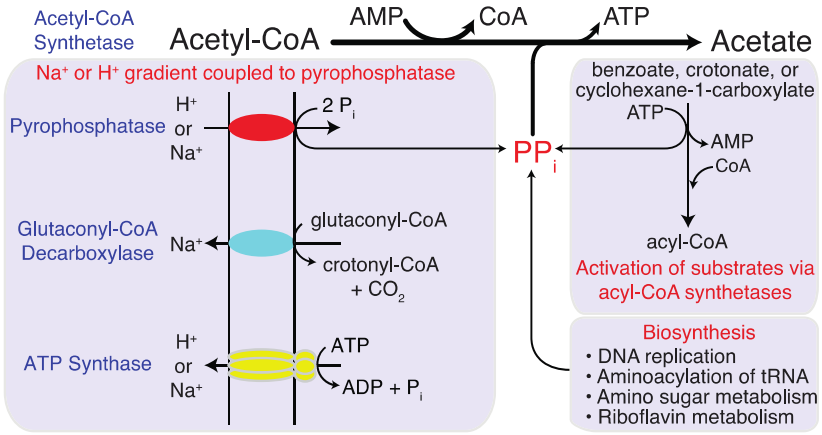

Fig. 1: Importance of pyrophosphate cycling and Acs1 in the bioenergetics of *S. aciditrophicus*. Pyrophosphate formed during substrate activation or during biosynthesis is used to make ATP by Acs1. Additional pyrophosphate needed for the Acs1 reaction can be made by membrane-bound pyrophosphatases (red) using ion gradients formed by glutaconyl-CoA decarboxylase (blue) or ATP synthase (yellow).

- *S. aciditrophicus* maintains a very low energy charge (0.24 ± 0.04) compared to other bacteria such as *Escherichia coli*, which has an energy charge of 0.93 ± 0.005. The low energy charge suggests that the free energy for ATP formation (free energy of phosphorylation) may also be low, which would allow syntrophic bacteria to use reactions with low free energy changes such as Acs1.
- We identified and characterized one of the main enzymes involved in NADH cycling in *Syntrophomonas wolfei*, the Fe-Fe hydrogenase, Hyd1 (encoded by Swol_1017-1019). *S. wolfei* is a fatty acid-degrading, syntrophic bacterium found in many methanogenic environments. *hyd1* was cloned and heterologously expressed, and its gene product was characterized. Hyd1 oxidized NADH and reduced NAD^+ without the presence of ferredoxin. The presence of oxidized ferredoxin did not accelerate the rate of NAD^+ reduction nor did the presence of reduced ferredoxin increase the rate of hydrogen production from NADH as is reported for confurcating Fe-Fe hydrogenases. These results suggest that Hyd1 is a NADH-dependent Fe-Fe hydrogenase but not a confurcating hydrogenase, i.e., one that uses reduced ferredoxin to drive the unfavorable production of hydrogen from NADH.

- The isotopomer profiling and ^{13}C-NMR spectroscopy showed that *S. aciditrophicus* synthesizes glutamate by two pathways. The minor route involved the use of *Re*-citrate synthase (30-40%), whereas the majority of glutamate was synthesized via the reductive carboxylation of succinate. *S. aciditrophicus* is the second example of a microbial species to employ two pathways for glutamate synthesis.

Science objectives for 2016-2017:

- *S. aciditrophicus* maintains a much different energy charge (higher levels of AMP than ATP) than other microorganisms, which suggests that the free energy needed for ATP synthesis (free energy of phosphorylation) is also low. We will determine the metabolite pools of various syntrophic bacteria to determine if their free energy of phosphorylation is low.
- The NADH-linked hydrogenase in *S. wolfei* does not appear to use reduced ferredoxin to drive the unfavorable production of hydrogen from NADH. Thus, *S. wolfei* and other syntrophic bacteria must maintain high levels of NADH compared to NAD^+ to allow hydrogen production from NADH. We will test this hypothesis by measuring NADH/NAD^+ levels in various syntrophic bacteria.

My scientific area(s) of expertise is/are: Microbial physiology.

To take my project to the next level, my ideal collaborator would have expertise in: Protein chemistry/structural biology; metabolomics; metabolic modeling.

Publications supported by this project (2014-2016):

1. James, K L., L. A. Ríos-Hernández, N. Q. Wofford, H. Mouttaki, J. R. Sieber, C. S. Sheik, H. N. Nguyen, Y. Yang, Y. Xie, J. Erde, L. Rohlin, E. A. Karr, J. A. Loo, R. R. Ogorzalek Loo, G. B. Hurst, R. P. Gunsalus, L. I. Szweda, and M. J. McInerney. 2016. Pyrophosphate-dependent ATP formation from acetyl coenzyme A in *Syntrophus aciditrophicus*, a new twist on ATP formation. mBio 7(4): e01208-16. doi: 10.1128/mBio.01208-16.
2. R. P. Gunsalus, L. Cook, B. Crable, L. Rohlin, E. McDonald, H. Mouttaki, J. R. Sieber, N. Poweleit, H. Zhou, A. L. Lapidus, H. E. Daligault, M. Land, P. Gilna, N. Ivanova, N. Kyrpides, D. E. Culley, and M. J. McInerney. 2016. Complete genome of *Methanospirillum hungatei* type strain JF1. Stand. Genomic Sci. 11:2 DOI 10.1186/s40793-015-0124-8.
3. M. Kim, H. M. Le, X. Xie, X. Feng, Y. J. Tang, H. Mouttaki, M. J. McInerney, W. Buckel. 2015. Two pathways for glutamate biosynthesis in the syntrophic bacterium, *Syntrophus aciditrophicus*. Appl. Environ. Microbiol. 81:8434-8344.
4. J. R. Sieber, B. R. Crable, C. S. Sheik, G. B. Hurst, L. Rohlin, R. P. Gunsalus and M. J. McInerney. 2015. Proteomic analysis reveals metabolic and regulatory systems involved the syntrophic and axenic lifestyle of *Syntrophomonas wolfei*. Front. Microbiol. **6**:115. doi: 10.3389/fmicb.2015.00115.
5. Wang, L., P. Bradsock, C. Li M. J. McInerney, and L. R. Krumholz. 2016. The role of Rnf in ion gradient formation in *Desulfovibrio alaskensis*. Peer J. 4:31919; DOI 10.7717/peerj.1919.
6. Guoqiang Li and M. J. McInerney. 2016. Use of biosurfactants for oil recovery. In: Sang Yup Lee and Kenneth N. Timmis (eds.) Handbook of Hydrocarbon and Lipid Microbiology Series. Consequences of Microbial Interactions with Hydrocarbons, Oils and Lipids: Production of Fuels and Chemicals. Springer-Verlag Berlin Heidelberg, Germany, in press.

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

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Markus W. Ribbe, Co-PI(s)
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Overall research goals:

Nitrogenase catalyzes the conversion of inert dinitrogen to bioavailable ammonia. Despite major efforts in the past decades, the catalytic mechanism of nitrogenase has not been fully deciphered. Previous studies have shown that NifEN (a scaffold protein that hosts the biosynthesis of nitrogenase cofactor) is a functional homolog of the MoFe protein (the catalytic component of nitrogenase). Compared to the MoFe protein, NifEN has a lower enzymatic activity and a narrower substrate profile, making it a perfect mutational platform for (re)constructing a functional MoFe protein. This project aims to systematically reconstruct an equivalent or variant of the MoFe protein via genetic approaches, and monitor and analyze the (re)construction process by combined biochemical and spectroscopic methods. Success in generating an active nitrogenase equivalent on a simplified template will enable identification of the functional determinants for the catalytic activity of nitrogenase and, in the same time, provide proof-of-concept for minimizing the essential *nif* gene set and engineering a metabolic pathway of nitrogenase assembly via a synthetic biology approach; whereas success in generating a functional variant(s) of nitrogenase will facilitate capture of substrates/intermediates or permit identification of candidates with desired product profile, both of which contribute to a better understanding of the mechanism of ammonia synthesis by nitrogenase.

Significant achievements (up to 2016):

The fundamental significance and inherent complexity of nitrogenase have prompted extensive studies to obtain a molecular description of this enzyme. However, despite major efforts in the past decades, the mechanism of nitrogenase is yet to be fully deciphered.

One aspect yet to be explored is whether enzymatic systems homologous to nitrogenase can be compared with nitrogenase to map out features key to the catalytic activity of this enzyme. NifEN is as an ideal candidate for effort along this line. Better known as a scaffold protein in M-cluster assembly, NifEN is an $\alpha_2\beta_2$-heterotetramer that shares considerable sequence homology with the MoFe protein. Better known as a scaffold protein in M-cluster assembly, NifEN is an $\alpha_2\beta_2$-heterotetramer that shares considerable sequence homology with the MoFe protein. Additionally, it contains cluster-binding sites that are homologous to those found in the MoFe protein: a "P-cluster site" at the interface of each αβ-subunit dimer and an "M-cluster site" within each α-subunit. Consistent with sequence-based predictions, biochemical, spectroscopic and structural analyses have identified (*i*) a $[Fe_4S_4]$ cluster (designated the O-cluster) as an analog to the P-cluster at the α/β-subunit interface and (*ii*) a $[Fe_8S_9C]$ cluster (designated the L-cluster) as both a precursor and a structural homolog to the M-cluster in the α-subunit of NifEN (*1*).

Excitingly, we have shown in a recent study that upon maturation of the L-cluster on NifEN, the cluster species extracted from NifEN is spectroscopically equivalent and functionally interchangeable with the native M-cluster extracted from the MoFe protein (*2*). Both extracted clusters display nearly indistinguishable EPR features, XAS/EXAFS spectra and reconstitution activities, firmly establishing the M-cluster-bound NifEN as the second protein scaffold (other than the MoFe protein) to house the unique nitrogenase cofactor. Importantly, the M-cluster-bound NifEN displays comparable ATP-independent, substrate-reducing profiles to those of the MoFe protein, providing the first direct piece of evidence for the structural-functional homology between the two proteins and the feasibility of probing the key catalytic features of MoFe protein via reconstruction of a MoFe protein equivalent on the basis of a simplified template, NifEN (*2*).

(1) Hu Y, Ribbe MW, "Biosynthesis of the Metalloclusters of Nitrogenases." *Annu. Rev. Biochem.* **85**, 455-483 (2016). DOI: 10.1146/annurev-biochem-060614-034108.
(2) Fay AW, Blank MA, Rebelein JG, Lee CC, Ribbe MW, Hedman B, Hodgson KO, Hu Y, "Assembly scaffold NifEN: A structural and functional homolog of the nitrogenase catalytic component." *Proc. Natl. Acad. Sci. USA* **113**, 9504-9508 (2016). DOI: 10.1073/pnas.1609574113.

Science objectives for 2016-2019:

- We plan to use the NifEN protein of *Azotobacter vinelandii* as a mutational platform to (re)construct functional MoFe protein equivalent(s) or variant(s) for mechanistic investigations of ammonia synthesis by nitrogenase (Fig. 1). Specifically, we propose to generate a functional equivalent of nitrogenase by sequentially reconstructing a P-cluster site (Aim 1), duplicating an M-cluster site (Aim 2) and re-establishing proton gating residues (Aim 3) in NifEN; additionally, we propose to engineer functional variants of nitrogenase with altered activity and/or product profile by mixing-and-matching the reconstructions of the key catalytic features in NifEN (Aim 4). Genetic methods (mutagenesis and homologous recombination) will be used to systematically reconstruct an equivalent (or variants) of the 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. Through our proposed research, we hope to identify the functional determinants of nitrogenase and provide insights into the mechanism of this complex metalloenzyme. Further, we hope to further minimize the essential *nif* gene set and provide a template for engineering a simplified pathway of nitrogenase assembly via synthetic biology approach in the future. Together, success along this line of research could contribute to a better mechanistic understanding of nitrogenase while informing future designs of effective approaches to sustainable ammonia synthesis.

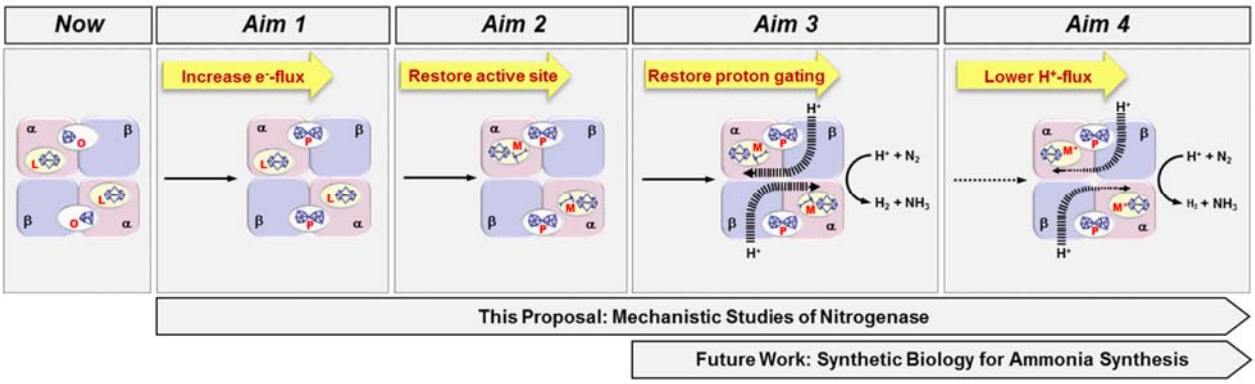

Figure 1. Proposed aims of this project.

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

To take my project to the next level, my ideal collaborator would have expertise in: ENDOR spectroscopy and X-ray crystallography

Trafficking to the Vacuoles in Plants/DE-FG02-11ER15295

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Overall research goals:

Our goals are to understand the mechanisms that control the ability of plant cells to accumulate energy reserve molecules such as proteins in specialized organelles known as vacuoles or to secrete molecules such cell wall precursors. In the current award our objectives are:
1. Understand the role of the exocyst in regulating vacuole trafficking. *We hypothesize that the exocyst functions as part of a regulatory mechanism controlling the dynamics of vacuole trafficking and exocytosis.*
2. Determine the structure of Arabidopsis EXO70A1 and map the ES2 binding sites and functional domains. *We hypothesize that identification of the binding site for ES2 will reveal the functional regions ofEXO70A that are responsible for regulation of vacuole trafficking and exocytosis.*

Vacuoles are essential and plants and serve roles in both protein and organelle degradation for reutilization and storage especially in seeds of high energy molecules such as storage proteins which are critical for seed germination under normal and stress conditions which impacts biomass production and nutrition. In addition, plants secrete many different molecules such as cell wall components, lipids, waxes and others that are important for plant protection and biomass. Vacuole accumulation and secretion are closely linked and highly regulated pathways within the endomembrane trafficking network. We have discovered many drug-like synthetic small molecules that can suppress vacuole trafficking and enhance secretion; other chemicals or enhance vacuole trafficking and suppress secretion. The ability of our compounds to modulate these pathways indicated that these pathways are tightly coupled and highly regulated. Thus, to achieve the goals of enhanced energy reserves or enhanced secretion it is essential to understand how this dynamic is controlled. Bioactive chemicals has provided us unique insight into natural mechanisms controlling the dynamics of vacuole trafficking and secretion that was not possible previously using past approaches. One such compound, ES2, negatively modulates secretion while enhancing vacuole trafficking. This is the first compound discovered that targets an essential secretory complex known as the exocyst and will reveal important regulatory domains controlling secretion and vacuole trafficking. We have achieved the objectives of determining the structure of the EXO70A1 in plants and mapping its identifying its binding site. To extend past these objectives and using sophisticated image analysis we have identified another novel compound that enhances secretion while negatively modulating vacuole trafficking. We are currently identifying its target. The combination of understanding the mechanisms controlling the dynamics of secretion vs vacuole accumulation and the use of synthetic modulators will permit us to both understand and regulate trafficking in order to enhance secretion cell wall biosynthesis or the accumulation of biological energy reserves for increased biomass, nutritional value, stress resistance, and the secretion of specialized plant products.

Significant achievements (2014-2017):

- To dissect the regulation of vacuole trafficking we discovered novel chemicals that modulate many pathways within endomembrane trafficking network focusing on those that perturb the dynamics of vacuole trafficking vs secretion of proteins to the plasma membrane
- One compound, ES2, enhanced vacuole trafficking and suppressed secretion. Using a combination of chemical proteomics, cell biology, synthetic chemistry, and synthetic chemistry the target of ES2 was

found to be the EXO70A1 subunit of the exocyst complex that is essential for secretion. This is first compound know to have this activity and has potential in many fields including medicine.
- The target was confirmed using a combination of mass spectroscopy, microscale thermophoreis and protease protection assays.
- The crystal structure of the plant EXO70A1 was determined and used to define the ES2 binding site. This may uncover regulatory sites important for controlling secretion/vacuole dynamics
- Beyond the proposed research we have discovered other novel modulators including ES17, which enhances secretion and suppresses vacuole trafficking and are working to identify its target.

Science objectives for 2016-2017:

- Complete ES2 EXO70A1 binding site mapping.
- Identify the target of ES17 using chemical proteomics approaches that were successful for ES2.
- Initially focusing on vacuole trafficking we will look for proteins interacting with the target of ES17.
- Use the combination of targets to investigate mechanisms leading to enhanced vacuole trafficking or enhanced secretion by using RNASeq to identify proteins or genes that are co-regulated by ES2 and ES17 that may be points of coordinate regulation between the secretory and vacuole trafficking pathways.

My scientific area(s) of expertise is/are: cell biology, chemical biology, genetics, microscopy/imaging.

To take my project to the next level, my ideal collaborator would have expertise in: Image analysis and quantification of vesicle dynamics, expert in transmission electron microscopy/tomography, larger scale synthetic chemistry.

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Redox Control of Ubiquitin-Like Protein Modification in Archaea

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Overall research goals:

Redox reactions are important for bioenergetic systems, yet damaging to biomolecules, particularly proteins with metal cofactors or sulfur-containing methionine and cysteine residues. While numerous studies demonstrate the pivotal role ubiquitin proteasome systems play in the selective recognition and destruction of oxidized proteins, the molecular mechanisms underlying how these systems are regulated by oxidative stress remains poorly understood. MsrA-type methionine sulfoxide (MSO) reductases are ancient enzymes that are important in reducing MSO residues in the control of signal transduction and in the repair of proteins damaged by oxidative stress. Here, we discovered that MsrA is required for the ubiquitin-like (Ubl) protein modifications that form during mild oxidative stress in archaea. Our research goal is to establish the molecular mechanism of how MsrA functions in Ubl protein modification; to achieve this, we will (1) determine whether MsrA is needed to guide the formation of the ubiquitin-like bonds that are generated under mild oxidizing conditions through MSO reductase dependent signal transduction or by a moonlighting function in direct catalysis, (2) identify the proteins (partners and substrates) that are found associated with MsrA, and (3) assess the impact small molecule ligands, such as DMSO, may have on the conformation and function of MsrA. Extending study of MsrA to its role in triggering Ubl modifications that form during mild oxidative stress should reveal novel insights into redox controlled post-translational protein modification. This project has implications in improving the viability and recovery of cells from disturbances in redox balance and maintaining the function of biocatalysts in extreme conditions.

Significant achievements (2015-2016):

MsrA-dependent Ubl modifications were reconstituted *in vitro* and found to require protein substrate, E1-like UbaA, ATP, MsrA active site residues (C13 and E56), and mild oxidant (DMSO). The modifications had biochemical features characteristic of Ubl isopeptide linkages (cleaved by a deubiquitinase-like enzyme). The recycling cysteines of MsrA (C16, C48 and C162) were not required for this activity. Reductant (DTT) was not needed for MsrA to reconstitute the Ubl modifications but was essential for its MSO reductase activity; by contrast, the mild oxidant DMSO (a small molecule ligand of MsrA-type enzymes) was required for the Ubl modifications but inhibited the MSO reductase activity. **MsrA was purified in high yield** from recombinant *Escherichia coli* (5-10 mg per liter culture) for the assays. This achievement will facilitate our definition of the MsrA structural features responsible for its moonlighting function in Ubl-protein modification.

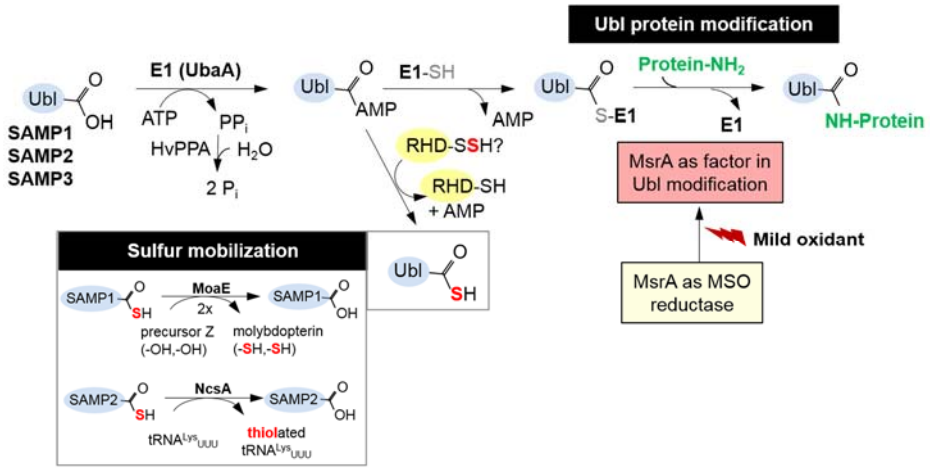

Fig. 1. Schematic representation of the MsrA as having a moonlighting function in ubiquitin-like (Ubl) protein modification that is distinct from its methionine sulfoxide reductase activity. In this pathway, the E1-like UbaA adenylates and forms a thioester intermediate with the Ubl SAMPs. HvPPA hydrolyzes inorganic pyrophosphate. MsrA undergoes a mild oxidant (DMSO/MSO) induced switch from an MSO reductase to a protein factor required for Ubl protein modification during mild oxidative stress.

Science objectives for 2016-2019:

Identify the proteins targets of the MsrA-dependent Ubl modification system. The MsrA-dependent Ubl conjugates will be purified by immunoprecipitation and/or affinity chromatography (from *in vitro* and *in vivo* assays with appropriate controls) and will be identified by LC-MS/MS analysis. The Fe-S cluster assembly system homolog SufB has been identified as a tentative target through initial screens, cloned and partially purified for use as a substrate in the *in vitro* reconstitution assays.

Determine the structure and function of MsrA as a moonlighting protein. We will characterize the molecular mechanism of how DMSO, a mild oxidant and small molecule ligand of the MsrA-type MSO reductases, switches the archaeal MsrA from a MSO reductase to a protein factor of the Ubl modification system.

My scientific area(s) of expertise is/are: protein biochemistry, proteomics, genetics, molecular biology, post-translational modification, proteases and microbiology (with emphasis on extremophiles).

To take my project to the next level, my ideal collaborator would have expertise in: methods to monitor the dynamics and atomic structure of enzymes (*e.g.,* MsrA) in the presence and absence of organic compounds (DMSO, MSO) and peptide ligands.

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Resolving protein-semiquinone interactions by advanced EPR spectroscopy.

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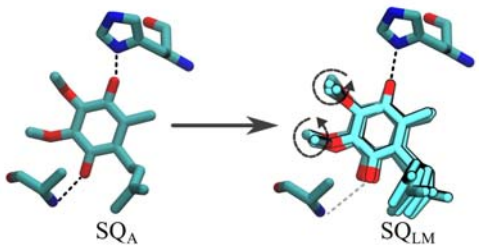
Overall research goals: Our focus is on use of modern, high-resolution EPR spectroscopy to explore the catalytic domains trapped in states with semiquinone (SQ) as an intermediate. The catalytic sites we propose to study, - the Q_A and Q_B sites of the bacterial reaction center (RC) from *Rhodobacter sphaeroides*, the Q_H site of the bo_3 quinol oxidase, and the Q_i site of the bc_1 complex, - all operate using ubiquinone (UQ), but have different electron transfer partners, and different operating potentials. EPR probes interactions between the electron spin of SQ and local magnetic nuclei, which provide direct information about *spatial* and *electronic* structure of the SQ and the immediate protein and solvent environment. The main question to be addressed is that of how the protein environment modifies the spatial and electronic structure of the SQ in different sites to fit the physiological function.

Significant achievements 2014-2016:

Hydrogen bond network around the Q_B site semiquinone. By utilizing a combined pulsed EPR and DFT approach, the hydrogen bond network around the Q_B site semiquinone (SQ_B) was determined. The development of such a technique is crucial toward an understanding of protein-bound SQs on the structural level, as (i) membrane protein crystallography typically results in low resolution structures, and (ii) obtaining protein crystals in the SQ form is rarely feasible. The SQ_B H-bond network was investigated with ^{1}H Q-band (~34 GHz) ENDOR and X-band (~9.7 GHz) HYSCORE on fully deuterated RCs from *Rb. sphaeroides* in an H_2O solvent. Three protons were detected, one with an anisotropic hyperfine (hfi) tensor component, T = 4.6 MHz, and two others with T = 3.2 and 3.0 MHz assigned to the $N_\delta H$ of His-L190 and to the peptide N_pH of Gly-L225 and Ile-L224, respectively. The principal values of the ^{1}H hfi tensors and their orientations with respect to the SQ_B g-tensor reference frame are obtained by the simulations of the orientation selective Q-band ^{1}H ENDOR spectra. The Euler angles describing the hfi tensors into the g-tensor reference frame define the locations of the H-bonded protons with respect to the SQ_B. These Euler angles are found to be in agreement with our geometry optimized DFT model of SQ_B providing the foundation for future joint pulsed EPR and DFT SQ structural determinations in other proteins.

Redox potential tuning through differential quinone binding in the RC of Rb. sphaeroides. Prior experimental results have shown that the RC from *Rb. spaheroides* is only fully functional with a limited set of methoxy-bearing UQs, suggesting that specific interactions with this substituent are required to drive electron transport and the formation of quinol. Molecular dynamics simulations of both the quinone-bound RC at equilibrium as well as thermodynamic integration calculations utilizing UQ and two monomethoxy UQ derivatives, we have investigated the protein interactions with the Q_A and Q_B quinones. In particular, we identify a specific interaction between the 2-methoxy (MeO) group of UQ in the Q_B site and the N_p of GlyL225 that we implicate in locking the orientation of the 2-MeO group, thereby tuning the redox potential difference between the Q_A and Q_B quinones. Disruption of this interaction leads to weaker binding in a UQ analog that lacks a 2-MeO group, a finding supported by reverse electron transfer EPR experiments of the $Q_A^-Q_B^-$ biradical.

Regulation of the primary quinone binding conformation by the H Subunit in RC from Rb. sphaeroides. Unlike photosystem II (PSII) in higher plants, RCs from *Proteobacteria* have an additional peripheral membrane subunit "H". The H subunit is necessary for photosynthetic growth, but can be removed chemically *in vitro*. The LM dimer retains the ability to form and stabilize the SQ_A like the RC, but is essentially incapable of the subsequent inter-quinone ET to Q_B. We investigated the influence of the H subunit on interactions between the primary SQ_{LM} and the protein matrix, using a combination of site-specific isotope labelling, pulsed EPR, and DFT calculations. Pulsed EPR data have revealed the structural features



of the SQ_{LM} in the LM dimer. The His-M219 N_δ – SQ_{LM} H-bond is elongated, but with a preserved binding conformation between the imidazole ring and SQ. The Ala-M260 N_p – SQ_{LM} H-bond is elongated even further and probably possesses a distribution of bond lengths and bond angles. The ^{13}C isotope labelling experiment suggests a greater degree of rotational freedom for the MeO groups of the SQ_{LM}, consistent with the overall weakening of SQ binding in the LM dimer. Assuming a similar influence of the H subunit removal on the quinone-protein interactions in the Q_B site the weaker H-bonding and disordered orientation of the 2-MeO group likely alters the quinone redox potential difference required for ET between the Q_A and Q_B sites.

Science objectives for 2016-2018:

- ^{13}C couplings provide insight into the SQ binding to the protein. We will apply a biochemical approach for selective ^{13}C labeling of ring carbons in the SQ_H. The quinones biochemically labeled in bo_3 enzyme will also be used in studies of bacterial reaction center and bc_1 complex.
- We will continue studying the influence of mutations on SQ_H in cyt bo_3. We will focus on two non-polar residues that are part of the Q_H binding pocket, Ile102 and Met78.

My scientific area(s) of expertise is/are: Continuous-wave and pulsed Electron Paramagnetic Resonance; magnetic resonance as a structural tool and its application in inorganic biochemistry, photosynthesis, structure-function relations in metalloproteins and quinone processing sites studies.

To take my project to the next level, my ideal collaborator would have expertise in: Pulsed EPR spectroscopy and methods of selective isotope labeling employed in this work will be beneficial for structural studies of metal cofactors and radical species in any other areas of bioenergetics, photosynthesis, and bioinorganic chemistry. The approach might also contribute to the development of strategies for design and engineering of new metalloenzymes and devices for biotechnological applications.

References to work supported by this project 2014-2016:

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The Center for Direct Catalytic Conversion of Biomass to Biofuels (C3Bio)

"Cell Wall Modeling: Synthesis, Components, Structure, Diffraction, and Multiscale Modeling"

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Overall research goals:

Cell walls are the source of all bioenergy and represent a promising raw material for many renewable materials and chemicals. Our understanding of the structure and knowledge of methods to manipulate cell walls and their components is a barrier to realizing the full potential of this incredible source of renewable carbon-based substances. Our goal is to understand cell walls from the molecular to macroscopic scale and all the interactions and contributions to structure and properties at these scales. We are aiming at understanding the major components at the molecular and meso scale: cellulose, hemicellulose, and lignin, including their interactions as they form the nanoscopic, microscopic, mesoscopic, and macroscopic scale structures. From these basic scientific understandings and characterizations, we will use modeling and simulation to predict methods to harness the potential uses of biomass for energy, chemicals, and materials. Our goals include a tight coupling of experiment, theory, and modeling to validate predictive capabilities and to create an environment of hypothesis-driven research and discovery where hypotheses and testing are done both in theory and experiment.

Significant achievements (2014-2017):

In the Modeling and Simulation portion of C3Bio, we have succeeded in the following:

- Developed models for mesoscale modeling of cell walls that include lignin, hemicellulose, and lignin for use in finite-element modeling of diffusion, heat transport, and chemical and enzymatic reaction.
- Characterized the interactions of glucuronyl xylan with cellulose at the molecular scale. We pushed the frontier of understanding of these interactions to show that this hemicellulose does not bind strongly to cellulose, but does have preferences for certain crystallographic faces of cellulose. Further, we showed that the side chain decoration of hemicellulose, in particular the pattern of decoration, is not a significant contributor to binding, in contrast to previously published hypotheses.
- Discovered the molecular source of twist in cellulose-I nanofibrils as coming from specific hydrogen bonds found only in cellulose-I.
- Determined the structural properties of cellulose bending and kinking in collaboration with researchers at NIST including bending modulus, force threshold for kinking and crystalline disruption and bond breaking within glucan chains under stress.
- Developed software for ultra-fast generation of X-ray diffraction patterns of cellulose nanofibrils.
- Characterized experimental patterns as composites of patterns from molecular structures.

Science objectives for 2016-2017:

- Construct a comprehensive library of diffraction patterns of molecular structures including several components of cell walls to more accurately interpret experimentally determined patterns.
- Construct a database of structures for use in developing interpretation software for measurements from NMR, neutron scattering, IR and other methods.
- Binding studies of other hemicellulose and lignin to cellulose.
- Determine thermodynamic stability of cellulose nanofibril shapes and cross sectional sizes.

My scientific area(s) of expertise is/are: Molecular Modeling and Simulation, Statistical Mechanics, HPC computing,

To take my project to the next level, my ideal collaborator would have expertise in: Diffraction techniques (WAXS, SAXS, SANS, WANS), NMR, SSNMR.

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

1. Yan Zhang, Hideyo Inouye, Michael Crowley, Leiming Yu, David Kaeli and Lee Makowski, Diffraction pattern simulation of cellulose fibrils using distributed and quantized pair-distances, accepted for publication in Journal of Applied Crystallography.
2. Ciesielski, P. N.; Crowley, M. F.; Nimlos, M. R.; Sanders, A. W.; Wiggins, G. M.; Robichaud, D.; Donohoe, B. S.; Foust, T. D., Biomass Particle Models with Realistic Morphology and Resolved Microstructure for Simulations of Intraparticle Transport Phenomena. Energy & Fuels 2015, 29 (1), 242-254.
3. Hinkle, J. D.; Ciesielski, P. N.; Gruchalla, K.; Munch, K. R.; Donohoe, B. S., Biomass accessibility analysis using electron tomography. Biotechnol Biofuels 2015, 8 (1), 1-16.
4. Bu,L.;Himmel,M.E.;Crowley,M.F.,ThemolecularoriginsoftwistincellulоseI-beta.Carbohydrate Polymers 2015, 125, 146-152.
5. Amore,A.;Ciesielski,P.N.;Lin,C.-Y.;Salvachúa,D.;iNogué,V.S.,DevelopmentofLignocellulosic Biorefinery Technologies: Recent Advances and Current Challenges. Australian Journal of Chemistry 2016.
6. Wei, H.; Yang, H.; Ciesielski, P. N.; Donohoe, B. S.; McCann, M. C.; Murphy, A. S.; Peer, W. A.; Ding, S.-Y.; Himmel, M. E.; Tucker, M. P., Transgenic ferritin overproduction enhances thermochemical pretreatments in Arabidopsis. Biomass and Bioenergy 2015, 72, 55-64.
7. Ciesielski, P. N.; Wang, W.; Chen, X.; Vinzant, T. B.; Tucker, M. P.; Decker, S. R.; Himmel, M. E.; Johnson, D. K.; Donohoe, B. S., Effect of mechanical disruption on the effectiveness of three reactors used for dilute acid pretreatment of corn stover Part 2: morphological and structural substrate analysis. Biotechnol Biofuels 2014, 7 (1), 1.
8. Inouye,H.;Zhang,Y.;Yang,L.;Venugopalan,N.;Fischetti,R.F.;Gleber,S.C.;Vogt,S.;Fowle,W.; Makowski, B.; Tucker, M., Multiscale deconstruction of molecular architecture in corn stover. Scientific reports 2014, 4.
9. Ciesielski,P.N.;Resch,M.G.;Hewetson,B.;Killgore,J.P.;Curtin,A.;Anderson,N.;Chiaramonti,A. N.; Hurley, D. C.; Sanders, A.; Himmel, M. E., Engineering plant cell walls: tuning lignin monomer composition for deconstructable biofuel feedstocks or resilient biomaterials. Green Chemistry 2014, 16 (5), 2627-2635.

Photosynthetic Energy Transduction

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Overall research goals:

Photosynthetic microorganisms have evolved complex metabolic networks consisting of multiple biochemical pathways that balance energy generation with utilization. Photosynthetic electron transport (PET) reactions establish the appropriate cellular ATP/NADPH ratio, which represents the collective contributions of cyclic electron flow (CEF), linear electron flow (LEF), and alternative electron flow (AEF). One hypothesis for the function of electron flow pathways peripheral to the core PET reactions is for adaptation to differential changes in metabolic and environmental conditions. These pathways cycle photosynthate and afford enormous flexibility in cellular energy homeostasis for acclimation to fluctuations in ambient conditions and nutrient availability. Remodeling of photosynthetic complexes, and the differential regulation of biochemical pathways, redox enzymes and electron carriers implies coordinated responses among adaptive pathways, but how this is controlled is not well understood. The broad goal of this project is to determine how molecular networks and enzyme mechanisms coordinate electron transfer reactions for energy balancing in model photosynthetic microbial systems.

Significant achievements (2015-2016):

Photosynthetic energy utilization in mutants with altered carbon allocation pathways.

- A prolyl-4-hydroxylase mutant (P4H) of *Chlamydomonas reinhardtii* is being studied as a biochemical regulatory model of how carbon storage affects balancing of photosynthate generation and utilization in green algae. The P4H mutant shows lower H_2 photo- and photofermentative production, lower accumulation of starch, and changes in the product profile of starch turnover.

Biochemical studies of photosynthetic flavodiiron (flv) enzymes.

- We obtained flv1-knockout clones (Δ*flv1*) in both wild-type and Δ*glgC*, and fully segregated *flv3*-knockout clones (Δ*flv3*) in wild-type and Δ*glgC* backgrounds. Construction of Δ*flv1*/Δ*flv3* strains is in progress. We have developed Flv3-overexpressing clones in wild-type strains that will be validated, and Flv1-overexpressing vectors are currently being constructed. Flv3 from *Synechocystis* has purified and showed pyridine-dependent O_2 reduction activity.

Site-differentiated [4Fe-4S] clusters in [FeFe]-hydrogenases.

- To investigate the properties of the unique site-differentiated His1Cys3 iron-sulfur cluster observed in [FeFe]-hydrogenases and NDH complexes, a domain containing this cluster and an adjacent [2Fe-2S] cluster from the [FeFe]-hydrogenase CaI has been expressed and purified. EPR redox titrations were performed showing a low reduction potential for the His1Cys3 [4Fe-4S] cluster with both $S = 1/2$ and 3/2 signals. Detailed assignment of the EPR spectrum is currently being completed.

Mechanism of H_2 activation by photosynthetic [FeFe]-hydrogenases.

- Mössbauer spectroscopy (collaboration with Dr. Y. Guo, Carnegie-Mellon University) was used to directly inform on the iron oxidation state and ligand geometries of the hypothesized terminal

hydride intermediate (H_{hyd}). H_{hyd} state consists of a diferrous diiron subcluster coupled to a paramagnetic $[4Fe\text{-}4S]^{+}$ subcluster. The isomer shifts of the diiron subcluster in H_{hyd} are consistent with a terminal hydride at the distal Fe atom in the Fe^{II} oxidation state.

Solar energy conversion and catalysis in photobiohybrid complexes.

- Cadmium sulfide (CdS) nanocrystals were used to photosensitize the nitrogenase molybdenum-iron (MoFe) protein. Light harvesting replaced ATP hydrolysis to drive the enzymatic reduction of N_2 into NH_3. The turnover rate was 75 per minute, 63% of the adenosine 5′-triphosphate-coupled reaction rate for the nitrogenase complex under optimal conditions. Inhibitors of nitrogenase (i.e., acetylene, carbon monoxide, and dihydrogen) suppressed N_2 reduction.
- Nanoporous "black" silicon (b-Si) photocathode with adsorbed [FeFe]-hydrogenase displayed a lower onset potential for H_2 generation than bare b-Si, and similar to that observed for a b-Si/Pt photoelectrode at the same light intensity. The b-Si/[FeFe]-hydrogenase electrode exhibited a turnover frequency of $\geq$1300 s^{-1}, a turnover number $>10^7$, and sustained current densities of 1 mA cm^{-2}, orders of magnitude greater than observed for previous enzyme-catalyzed electrodes.

Science objectives for 2016-2017:

- Determine the biochemical mechanism of P4H in the regulation of carbon metabolism of *Chlamydomonas reinhardtii* including photosynthetic electron transport; (ii) analyze the photosynthetic parameters (e.g., P700 re-reduction/oxidation kinetics, PQ pool reduction state) of various *Synechocystis* flv knockout and overexpression strains for changes in photosynthetic O_2 cycling.
- Purify *Synechocystis* Flv1 and Flv3 to homogeneity for biochemical and biophysical (EPR and Raman) analysis; (ii) complete the reduction potential/pH dependence, and interfacial electron transfer rate measurements of the His1Cys3 and Cys4 variant of the CaI [4Fe-4S] cluster; and (iii) conduct pulse EPR analysis on reduced CrHydA1 for properties of exchangeable protons of the reduced H-cluster.

My scientific area(s) of expertise is/are: Redox biochemistry; physical biochemistry; photocatalysis.

To take my project to the next level, my ideal collaborator would be: Lance Seefeldt's and his knowledge of chemically driven electron transfer, and multi-electron catalysis.

Publications supported by this project 2015–2016:

1. Boehm, M., M. Alahuhta, D.W. Mulder, E.A. Peden, H. Long, R. Brunecky, V.V. Lunin, P.W. King, M.L. Ghirardi and A. Dubini. 2015. "Crystal structure and biochemical characterization of *Chlamydomonas* FDX2 reveal two residues that, when mutated, partially confer FDX2 the redox potential and catalytic properties of FDX1." *Photosyn. Res.* **128**:45-57. DOI: 10.1007/s11120-015-0198-6.
2. Xiong, W., T.-C. Lee, S. Rommelfanger, E. Gjersing, M. Cano, P.-C. Maness, M. Ghirardi and J. Yu. 2015. **"**Phosphoketolase pathway contributes to carbon metabolism in cyanobacteria." *Nature Plants.* **2**:15187. DOI: 10.1038/nplants.2015.187.
3. Morra, S., S. Maurelli, M. Chiesa, D.W. Mulder, M.W. Ratzloff, E. Giamello, P.W. King, G. Gilardi, and F. Valetti. 2016. "Spectroscopic investigation by EPR and FTIR of CaHydA [FeFe]-hydrogenase and the effect of the C298D mutation: insights into the protein-metal cluster interaction." *BBA-Bioenergetics*. **1857**(1):98-106**.** DOI:10.1016/j.bbabio.2015.10.005.
4. Brown, K.A., P.W. King, and P. Ciesielski. 2016. "Photobiohybrid solar conversion with metalloenzymes and photosynthetic reaction centers." In *Biotechnology for Biofuel Production and Optimization*, edited by Carrie A. Eckert and Cong T. Trinh, Elsevier, Amsterdam, 2016, Pages 473-495. DOI: 10.1016/B978-0-444-63475-7.00018-2.
5. Brown, K.A., D. Harris, M.B. Wilker, A. Rasmussen, N. Khadka, H. Hamby, S. Keable, G. Dukovic, J.W. Peters, L.C. Seefeldt, and P.W. King. 2016. "Light-driven dinitrogen reduction catalyzed by a CdS:Nitrogenase MoFe protein biohybrid." *Science.* **352**:448-450. DOI: 10.1126/science.aaf2091.
6. Zhao, Y., N. Anderson, M.W. Ratzloff, D.W. Mulder, K. Zhu, J. Turner, N. Neale, P.W. King and H. Branz. 2016. "Overall water splitting using a hydrogenase-catalyzed nanoporous silicon photoelectrode." *ACS Appl. Mater. Interfaces*. DOI: 10.1021/acsami.6b00189.

Asparagine synthetase gene regulatory networks and plant nitrogen metabolism

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Overall research goals: The goal of this DOE project is to model and alter gene regulatory networks involved affecting nitrogen (N) and carbon (C) use efficiency. Specifically, we focus on the mechanisms that regulate N-assimilation into asparagine (Asn), a C/N-efficient amino acid that is used to transport/store N under conditions of N-excess and/or C-limitation. Using a combined genetic, genomic and systems biology approach, we uncovered molecular components of this C/N energy sensing mechanism that regulates the expression of the asparagine synthetase (ASN1) gene regulatory network. Through a positive genetic selection in Arabidopsis, we discovered chromatin modification and transcriptional mechanisms that control genome-wide responses to C, Light (L) and N signals. A major player in this C/N sensing system is a histone methyltransferase - SDG8 - which regulates the chromatin status and expression of genes involved in photosynthesis and energy metabolism in response to C, L and N sensing. Our goal is to further explore the plant responses to these nutritional and environmental stimuli, using a combination of epigenetic (Aim1), transcriptional (Aim2) and metabolic approaches (Aim3). With the identification of genes that integrate nitrogen, carbon and energy signaling at the genome-wide level, we now aim to characterize how these epigenetic and transcriptional regulators integrate these signals to regulate the major energy consuming (nitrogen assimilation) and energy producing (photosynthesis) pathways in plants.

Significant achievements (2014-2016):

AIM 1. CHROMATIN: Role of histone methylation in the ASN1 energy metabolism network. To uncover molecular components involved in C and L signaling, we used a positive genetic selection to identify an Arabidopsis mutant, *cli186,* impaired in repression of the ASN1 gene by C and L signals. We showed that the *cli186* mutant (now renamed *sdg8-5*) carries a complete deletion of SDG8, a gene encoding a Histone 3 lysine 36 (H3K36) methyltransferase. We identified 728 genome-wide targets of SDG8 based on SDG8-DNA binding and mis-methylation of H3K4 in the *sdg8-5* mutant. Remarkably, >50% of the 728 targets of SDG8 are light-regulated genes involved in energy generation and utilization. Moreover, H3K36me3 marks on SDG8 target genes correlate with their elevated gene expression level, and this is disrupted in the *sdg8-5* mutant. ***We thus propose a model*** in which SDG8 targets a specific subset of light- and energy-related genes for permissive H3K36me3 histone marks in the gene body, to allow high-level expression of its target genes under these conditions. Now, we aim to further test the hypothesis that the unique zf-CW domain of SDG8 serves to "read" H3K4 methylation (at promoters), which directs SDG8 to "write" H3K36 methylation of the gene bodies. Our results, and the H3K36me3 profile of mutants in the zf-CW "reading domain" of SDG8, support our hypothesis that the "reading" domain is important for correct targeting of H3K36me3 marks. **AIM 2: TRANSCRIPTION: Transcription control of the ASN1 energy metabolism network.** Recently, we have shown that SDG8 maintains high level of mRNA of its target genes in energy metabolism, by mediating H3K36 methylation in the gene body. Here, ***we tested the hypothesis*** that histone modification by SDG8 alters mRNA splicing for its energy-related gene targets – genome-wide. To test this, we characterized the transcriptome change between wild-type and the deletion mutant *sdg8-5* using RNA-seq. Interestingly, we found that exposure to a N-signal causes a significant amount of alternative splicing events in the *sdg8-5* background (compared to wild-type), specifically enriched in genes involved in transmembrane transport. This result supports that the gene body histone methylation by SDG8 affects mRNA splicing of its specific target genes. **AIM 3: METABOLISM: A gene regulatory network (GRN) controlling energy, C/N metabolism and nitrogen use efficiency.** To understand the regulation of energy and nitrogen use efficiency, we used network analysis to identify a gene regulatory subnetwork of ASN1, its key epigenetic regulator and transcription factors (TFs). We discovered that one such TF hub, GLK1 - a known master regulator of photosynthesis - affects nitrogen use efficiency according to our mutant analysis. In addition, we as the *sdg8* deletion mutant affected in histone methylation –and show that energy production (photosynthesis) and energy consumption (nitrogen assimilation) is disrupted in *sdg8* mutant. Collectively, our findings support the hypothesis that SDG8 and its downstream TFs (e.g. GLK1) coordinate the expression of genes that integrate photosynthesis, carbon and nitrogen metabolism.

Science objectives for 2016-2017:

- Our objectives are to further elucidate the regulatory mechanisms coordinating N-assimilation and C-metabolism/energy production at the level of chromatin regulation (Aim 1), transcriptional regulation (Aim 2), and in metabolites (Aim 3). This integrated approach enables us to identify regulatory factors that enable plants to coordinate N assimilation and storage with related processes including photosynthesis, energy and C-metabolism. Modifying the regulatory factors that mediate this integration should have implications for modifying N-use efficiency in crop plants at a systems-wide level as opposed to a single enzyme level.

My scientific area(s) of expertise is/are: Plant systems biology

To take my project to the next level, my ideal collaborator would have expertise in: photosynthesis & energy metabolism.

Publications and patents supported by this project 2004-2016:

1. Joan Doidy, Tim Jeffers, Molly Edwards, Alessia Para & Gloria Coruzzi. "Golden-like 1 (GLK1) is a transcription factor hub connecting gene regulatory networks of the chloroplast development with nitrogen signaling" (2016). Manuscript in preparation
2. Li Y, Yeoh-Wang J, Rock T, Varala K, McCombie WR, Coruzzi GM "The histone methyltransferase SDG8 mediates RNA splicing in response to a nutrient signal in Arabidopsis "(2016) Manuscript in preparation
3. Li Y, Varala K, Coruzzi GM (2015). "From milliseconds to lifetimes: tracking the dynamic behavior of transcription factors in gene networks." Trends in Genetics. 2015 June; 31(9):509-515.
4. Li Y*, Mukherjee I*, Thum KE, Tanurdzic M, Katari MS, Obertello M, Edwards MB, McCombie WR, Martienssen RA, Coruzzi GM (2015). "The histone methyltransferase SDG8 mediates the epigenetic modification of light and carbon responsive genes in plants." Genome Biology. 2015 Apr; 16(1):79.
5. Krouk G, Ruffel S, Gutiérrez RA, Gojon A, Crawford NM, Coruzzi GM and Lacombe B. (2011). A framework integrating plant growth with hormones and nutrients. ***Trends in Plant Sci***.16(4):178-182.
6. Obertello M, Krouk G, Katari M, Runko S, and Coruzzi G (2010). Modeling the global effect of the basic-leucine zipper transcription factor 1 (bZIP1) on nitrogen and light regulation in Arabidopsis ***BMC Systems Biology*** 4:111
7. Krouk G, Crawford NM, Coruzzi GM, Tsay YF (2010). Nitrate signaling: adaptation to fluctuating environments. ***Curr Opin Plant Biol***. 13.266-273.
8. Ruffel S, Krouk G, Coruzzi GM (2010). A Systems View of Responses to Nutritional Cues in Arabidopsis: Towards a Paradigm Shift for Predictive Network Modeling. ***Plant Physiol***. 152:445-52.
9. Coruzzi GM, Burga A, Katari MS, and Gutierrez RA (2009). Systems Biology: Principles and Applications in Plant Research. **In *Plant Systems Biology***, Annual Plant Reviews; Blackwell Publishing: Oxford, UK. Vol. 35. Pgs 3-31.
10. Thum KE, Shin, MJ Gutierrez RA, Mukherjee I, Katari MS, Nero D, Shasha D, and Coruzzi GM (2008). An integrated genetic, genomic and systems approach defines gene networks regulated by interaction of light and carbon signaling pathways in Arabidopsis. ***BMC Syst Biol*** 2, 31.
11. Gutierrez R, Stokes T, Thum K, Xu X, Obertello M, Katari MS, Tanurdzic M, Dean A, Nero D, McClung CR, and Coruzzi GM (2008). Systems approach identifies an organic nitrogen-responsive gene network that is regulated by the master clock control gene CCA1. ***Proc Natl Acad Sci USA*** 105, 4939-4944.
12. Gutierrez R, Shasha S, and Coruzzi G. (2005) Systems Biology for the Virtual Plant. ***Plant Phys.*** 138: 550-554.
13. Thum K, Shin M, Palenchar P, Kouranov A, Coruzzi G (2004) Genome-wide investigation of light & carbon signaling interactions in Arabidopsis. ***Genome Biology*** 5:R10.1- R10.20. (Faculty of 1000 & Review by Reyes & Chua (2004) *Genome Biology* 5:213)
14. Wong HK, Chan HK, Coruzzi GM, Lam HM (2004) Correlation of ASN2 gene expression with ammonium metabolism in Arabidopsis. ***Plant Physiology*** 134: 332-338.

Patents : The technology covered by these patents has been commercially licensed by two major US agricultural companies for crops including trees and corn, both major biofuel crops.

1. Patent #60/919,818 "Methods of affecting nitrogen assimilation in plants". Inventors: Gloria Coruzzi, Damion Nero and Rodrigo Gutierrez.
2. Patent #5,955,651 "Transgenic plants that exhibit enhanced nitrogen assimilation". Inventors: Coruzzi and Brears.
3. Patent #5,256,558 "Genes encoding plant asparagine synthetase". Inventors: Coruzzi and Tsai.

Unraveling the Mystery of how Dirigent Proteins/Proteins Harboring Dirigent Domains Control Lignin Formation: A New Frontier

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Overall research goals:

Lignin formation in the Casparian Strip (CS) membrane is proposed to be orchestrated via a supramolecular protein complex (Hosmani *et al.* (2013) PNAS **110**:14498, **Fig. 1**) involving dirigent proteins (DPs), Casparian Strip Membrane Domain Proteins (CASP) scaffold, peroxidase (PER), laccase (LAC), superoxide dismutase (SOD) and NADPH oxidase (NOX). Our objectives are: (i) Dissect lignin-forming complex, and establish precise nature of interacting proteins with DPs, particularly those providing $1e^-$ oxidation, cofactors and potential scaffolds; (ii) Apply DP X-ray crystallography, EPR, IMS/MS and computational modeling to establish biochemical mechanisms of CS DP substrate binding and phenoxy radical-radical coupling; and (iii) Establish DP role(s) in how macromolecular lignin formation is orchestrated.

Figure 1. Cartoon of possible interactions of lignin polymerization proteins and enzymes organized into a defined supramolecular proteinaceous complex.

Significant achievements: Advances in DP research include (i) their expanding metabolic roles in plant biochemical pathways, (ii) how redox reactions are biochemically controlled at the molecular level, stipulating phenoxy radical-radical coupling outcomes, and (iii) evolutionary ramifications.

(i) ***DP expanding metabolic roles in plant biochemical pathways***: Chronologically (see Fig. 2), this includes. 1) DP discovery and kinetic behavior, 2) hypothesis that proteins harboring dirigent domains control outcome of monolignol coupling in lignin formation, 4) establishing regions controlling different stereoselectivities (e.g. to different coupling product antipodes, such as in pinoresinol), 3) first DP crystal structure, i.e. of (+)-pinoresinol forming DP at 1.9 Å and DPs controlling 4) terpenoid stereoselective coupling to (+)-gossypol, and 5) enabling uniform lignin deposition in CS. Roles in other metabolic processes (e.g. stilbene, allyl/propenyl phenol biosynthesis) are also under investigation.

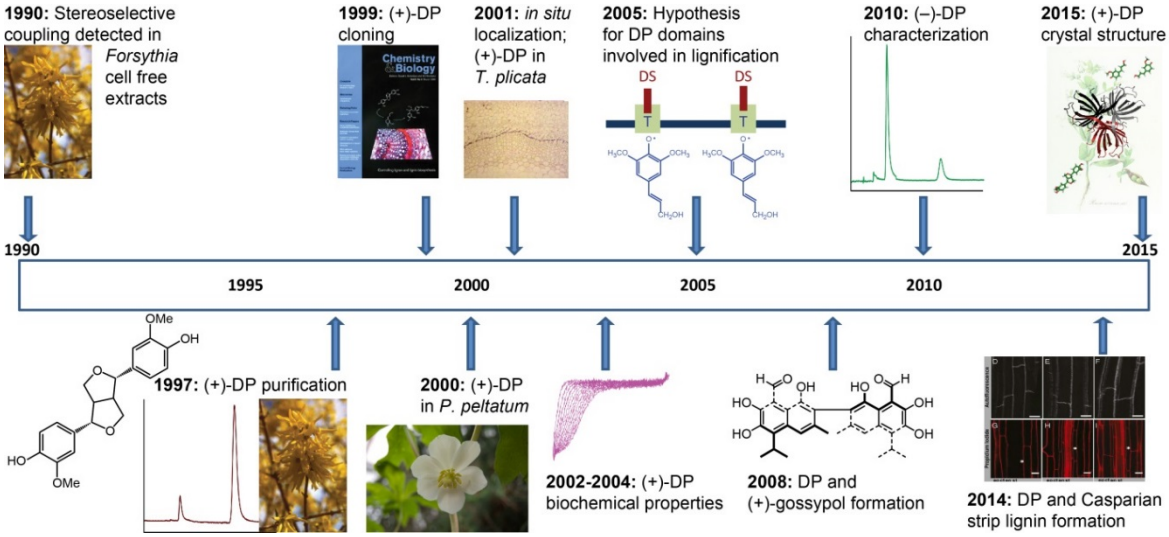

Figure 2. Chronology of dirigent protein (DP) discovery and related research.

(ii) ***DP structure determination and stipulating phenoxy radical-radical redox reaction outcomes.*** The pea 3D (+)-pinoresinol forming DP structure was determined at 1.95 Å resolution. From domain swapping experiments, ultimately giving different coupling stereoselectivities, we provisionally identified regions in substrate binding and coupling in the putative 'active site'. **Fig. 3A** shows amino

acid sequence alignments of (+)- and (−)-pinoresinol forming DPs (DRR206 and AtDIR6), as well as those implicated in lignification (AtDIR10) and (+)-gossypol formation (GhDIR4) in *Gossypium hirsutum*. **Fig. 3B** depicts DRR206 3D structure and our homology models of AtDIR10 and GhDIR4. We hypothesize that these loops hold important roles, possibly helping confer substrate specificity and, with AtDIR10, to (for example) orient DP active sites with respect to lignin macromolecular assembly

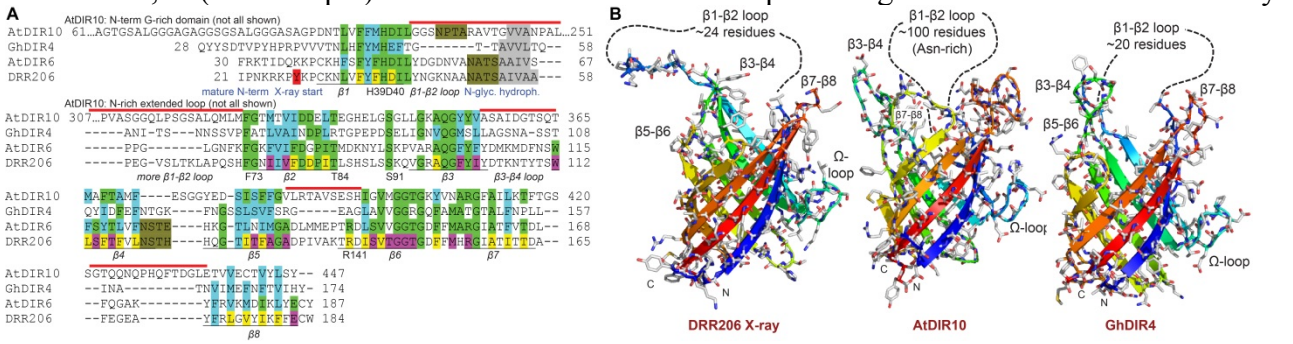

Figure 3. A. Multiple sequence alignment of AtDIR10 (a putative CS lignin forming DP), GhDIR4 (gossypol forming DP), AtDIR6 [(–)-pinoresinol (forming DP], and DRR206 [(+)-pinoresinol forming DP]. For DRR206, yellow indicates core residues and pink residues at oligomer interface. Green and blue highlight residues identical or conserved (respectively) with respect to DRR206. Brown highlights known glycosylation sites, and grey highlights a small hydrophobic region present in the large β1-β2 loop from many sequences. **B.** DRR206 3D structure and homology models of AtDIR10 and GhDIR4.

(iii) ***Evolutionary ramifications*****:** Our bioinformatics analysis of reported DP gene and DP-like gene sequences across the plant kingdom resulted in an unrooted phylogenetic tree (manuscript in preparation) which confirmed/extended both chemotaxonomy observations and our knowledge of lignin/lignan/ stilbenoid/terpenoid chemotaxonomy. This tree, generated from multiple sequence alignment of hundreds of DP sequences from numerous plant genomes, clustered into several large subfamilies: Dir b/d, Dir-g1, Dir-g2, Dir-c, Dir-a1, Dir-a2 (and/or Dir-f), Dir-h, Dir-I, Dir-e1, Dir-e2. One striking feature was in mapping distinct land plant family classes, namely Lycophytes, Bryophytes, Gymnosperms, and Angiosperms (Monocots and Eudicots)]. For extant "primitive" land plant genomes, there are reports of DPs only in *Selaginella,* which contain 8-8′ linked lignans and S-lignins. Here, 2 DP subfamilies, including those clustered in the dir-e1 subfamily with pinoresinol-forming DPs, and in the dir-a family ***may*** be associated with lignification. Interestingly, the bryophyte (*Physcomitrella*) has DP-like sequences, although it is the only non-vascular plant reported to do so. In short, there remain a large number of biochemical functions in DP sub-families that await discovery, including that of the precise role of AtDIR10 (and its homologs) in the CS.

Science objectives for 2016-2017: As above

Publications supported by this project 2013-2016:

Höhner, R., Marques, J.V., Ito, T., Amakura, Y., Budgeon, A D., Davin, L.B., Kirchhoff, H. and Lewis, N. G. (2016) Reduced arogenate dehydratase expression in *Arabidopsis*: photosynthesis and metabolism ramifications. (*Manuscript submitted*).

Kim, K.W., Smith, C.A., Daily, M.D., Cort, J.R., Davin, L.B., and Lewis, N.G. (2015) Trimeric structure of (+)-pinoresinol forming dirigent protein at 1.95 Å resolution with three isolated active sites. *J. Biol. Chem.* **290**, 1308–1318.

Dalisay, D.S., Kim, K.W., Lee, C., Yang, H., Rübel, O., Bowen, B.P., Davin, L.B., and Lewis, N.G. (2015) Dirigent protein-mediated lignan and cyanogenic glucoside formation in flax seed: Integrated omics and MALDI mass spectrometry imaging. *J. Nat. Prod.* **78**, 1231-1242.

Seneviratne, H.K., Dalisay, D.S., Kim, K.W., Moinuddin, S.G.A., Yang, H., Hartshorn, C.M., Davin, L.B., and Lewis, N G. (2015) Non-host disease resistance response in pea (*Pisum sativum*) pods: Biochemical function of DRR-206 and phytoalexin pathway localization. *Phytochemistry* **113**, 140-148.

Marques, J.V., Dalisay, D.S., Yang, H., Lee, C., Davin, L.B., and Lewis, N.G. (2014) A multi-omics strategy resolves the elusive nature of alkaloids in *Podophyllum* species. *Molecular BioSystems* **10**, 2838-2849.

Komvongsa, J., Luang, S., Marques, J.V., Phasai, K., Davin, L.B., Lewis, N.G., and Ketudat Cairns, J.R. (2014) Active site cleft mutants of Os9BGlu31 transglucosidase modify acceptor substrate specificity and allow production of multiple kaempferol glycosides *Biochim. Biophys. Acta* **1850**, 1405-1414.

Lee, C., Bedgar, D.L., Davin, L.B., and Lewis, N.G. (2013) Assessment of a putative proton relay in *Arabidopsis* cinnamyl alcohol dehydrogenase catalysis. *Org. Biomol. Chem.* **11**, 1127-1134.

Molecular Movies of Metalloenzyme Catalysis Using Ultrafast X-ray Pulses

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Overall research goals:

The fundamental questions in the field of enzyme mediated/catalyzed redox reactions in metalloenzymes are related to the reaction mechanisms and the interplay between the protein matrix and the metal cofactor. A detailed picture of the geometric as well as the electronic structure of the metalloenzyme active site at different points along the reaction coordinate is a prerequisite for understanding the reaction mechanism. The fs-X-ray pulses available from X-ray free electron laser (XFEL) sources allows one to perform X-ray studies under native conditions without the radiation damage issues encountered at conventional X-ray sources. The goal of this project is to develop tools to conduct combined X-ray diffraction and X-ray spectroscopic measurements of metalloenzymes under working conditions and couple these with other in-situ analytical tools to probe the geometric and electronic structure of intermediates involved in their reaction cycles.

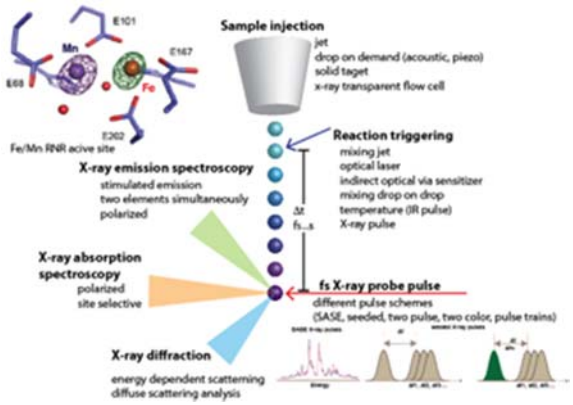

Fig. 1: Techniques to be developed/combined in the frame of this project.

Significant achievements:

- We developed an approach of parallel measurement of X-ray diffraction and X-ray spectroscopic data on crystals of metalloenzymes using XFEL fs pulses.
- Efficient sample injection strategies for XFEL measurements on protein solutions and crystal suspensions were developed and tested.
- In-situ light triggering and mixing schemes to activate photoactive or redox active enzymes and populate transient states were developed.
- We demonstrated this approach on photosystem II, populating different intermediate states and probing them by diffraction and X-ray emission.
- This approach was extended to other light inducible enzymes (phytochrome) as well as to other metalloenzymes (ribonucleotide reductase, O_2 tolerant hydrogenase, peroxidase) and to inorganic model systems.

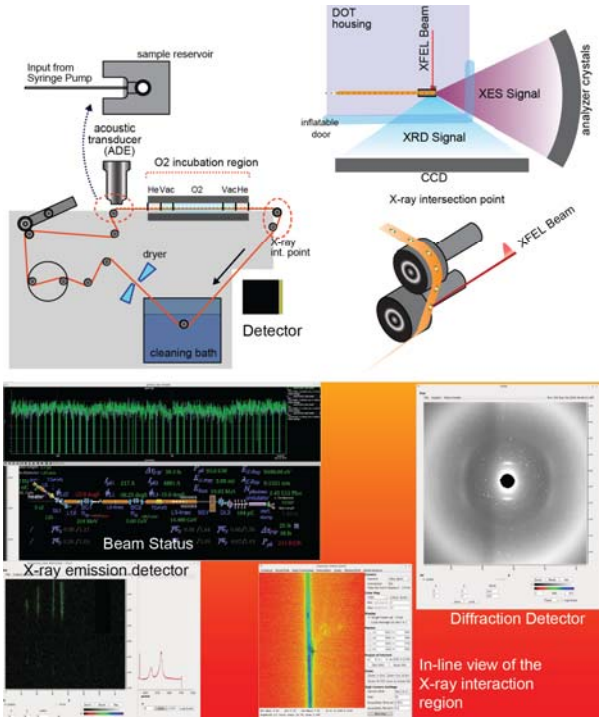

Fig. 2: Schematic of Droplet on Tape (DOT) setup with in-situ O_2 incubation to measure time resolved XRD and XES (top) and screen shot from a recent experiment at LCLS with RNR and hydrogenase, showing a view of the interaction region, the X-ray diffraction and the emission spectra as they are recorded (bottom).

- First XRD/XES data from in-situ O_2 activated redox active Fe and Mn containing enzymes were collected.

Science objectives for the coming years:

- Further develop the in-situ mixing techniques and the combined experimental setup that allows one to conduct spectroscopic (X-ray, UV-Vis, Raman) and structural biology methods on the same sample concomitantly.
- Elucidate radiation damage free structures of metalloenzymes under working conditions (room temperature) for various intermediate states in their reaction cycle. Targeted systems include oxygen tolerant hydrogenases (MBH), Fe/Mn ribonucleotide reductase and potentially other enzymes that can be activated by mixing for example with oxygen or other substrates.
- Correlate the structural information with parallel X-ray and other spectroscopic measurements to provide information on the evolution of the geometric and electronic structure of the active site over the reaction cycle, especially trying to target high-valent metal intermediates involved in these reactions. Detecting the transient ferryl (Fe^{IV}) intermediate that has been proposed in several systems is a high-priority goal.

My scientific area(s) of expertise is/are: X-ray spectroscopy, protein crystallography, metalloenzymes, photosynthesis.

To take my project to the next level, my ideal collaborator would have expertise in:
- Molecular biology and enzymology of other redox enzymes that can be activated by e.g. oxygen incubation or mixing with other substrates.
- Fast mixing and associated spectroscopic probes to study reaction intermediates in metalloenzymes.

Publications related to this project:

1. J. Kern, et al., Taking snapshots of photosynthetic water oxidation using femtosecond X-ray diffraction and spectroscopy, *Nat. Comm.* **5** (2014) 4371 **(**DOI: 10.1038/ncomms5371)
2. J. Kern, V.K. Yachandra, J. Yano, Metalloprotein structures at ambient conditions and in real-time: biological crystallography and spectroscopy using X-ray free electron lasers, *Curr Op Struct Biol* **34** (2015) 87–98 (DOI: 10.1016/j.sbi.2015.07.014)
3. S. Gul et al., Simultaneous detection of electronic structure changes from two elements of a bifunctional catalyst using wavelength-dispersive X-ray emission spectroscopy and in situ electrochemistry, *Phys. Chem. Chem. Phys.* **17** (2015) 8901-8912 **(**DOI: 10.1039/C5CP01023C)
4. R.G. Sierra, et al., Concentric-Flow Electrokinetic Injector Enables Serial Crystallography of Ribosome and Photosystem II, *Nat. Methods* **13** (2016) 59-62 (DOI:10.1038/nmeth.3667)
5. C.G. Roessler, et al,, Acoustic injectors for drop-on-demand serial femtosecond crystallography, *Structure* **24** (2016) 631–640 (DOI: 10.1016/j.str.2016.02.007)
6. R. Alonso-Mori, et al., Towards Characterization of Photo-Excited Electron Transfer and Catalysis in Natural and Artificial Systems Using XFELs, *Faraday Discuss.* **in press** (2016) (DOI: 10.1039/C6FD00084C)

Unraveling the regulation of terpenoid oil and resin biosynthesis for the development of biocrude feedstocks

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Narayanan Srividya, Senior Research Associate; Sean Johnson, Graduate Student
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Overall research goals:

Terpenoid essential oils and oleoresins are characterized by a high volumetric energy density and high degree of reduction, and are thus viable biocrude feedstocks for sustainably produced specialty chemicals. This project is aimed at developing single cell approaches to investigate plant cell types that are specialized for oil and resin biosynthesis. We are using essential oil biosynthesis in peppermint glandular trichomes as a model system for specialized secretory structures (during the previous funding period we also worked on *Citrus* and pine, which is reflected in the publication list).

Significant achievements (2015-2016):

- **Genome-scale stoichiometric model of peppermint glandular trichome metabolism.** We built a genome-scale stoichiometric model of secretory phase metabolism in peppermint glandular trichomes based on extensive biomass output measurements and RNA-seq transcriptome data. Simulated reaction deletions revealed two potential branch-points in trichome energy metabolism meriting further experimental investigation: (1) partitioning of ATP regeneration between oxidative phosphorylation and ethanolic fermentation and (2) non-photosynthetic regeneration of reduced ferredoxin in non-green plastids to support two electrons transfers that provide terpenoid precursors.

- **Assessing the relative contributions of oxidative phosphorylation and ethanolic fermentation to power metabolism in non-green glandular trichomes.** We demonstrated a small effect of the ATP synthase inhibitor oligomycin on the ability of isolated trichomes to produce terpenes from sucrose, indicating a minor role for oxidative phosphorylation in generating ATP. A much higher activity was determined for alcohol dehydrogenase at secretory (bot not post-secretory phase), indicating that the primary energy source for peppermint glandular trichomes is ethanolic fermentation (Fig. 1).

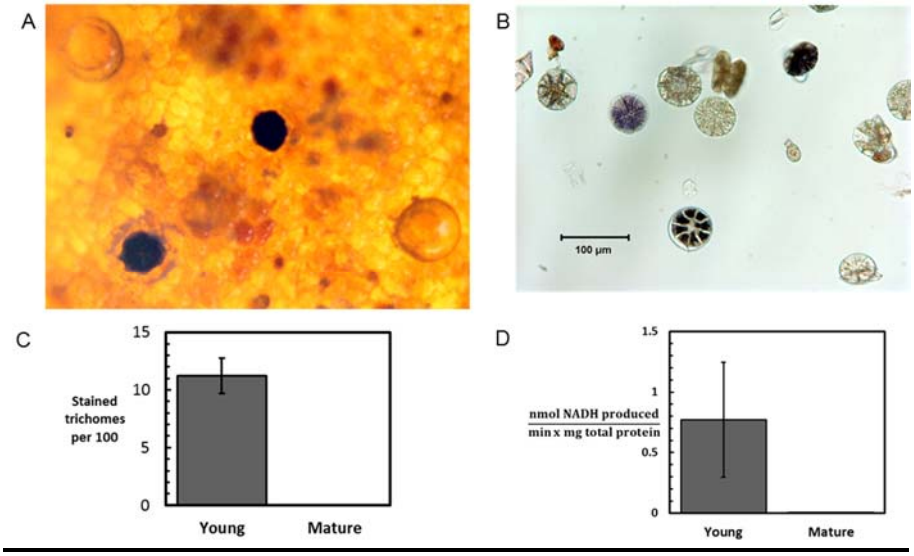

Fig. 1. Exceptionally high ethanol dehydrogenase (ADH) activity in secretory phase glandular trichomes of peppermint. (A) Stained leaf, image from near base of leaf. (B) Staining of glandular trichomes isolated from young leaves. (C) Counts of ADH activity-stained glandular trichomes in young and mature leaves. (D) Specific ADH activity in soluble protein extracted from isolated trichomes of young and mature leaves. Values are mean ± SD, n=3.

- **Evaluating ferredoxin (Fd) and ferredoxin-NADP$^+$ reductase (FNR) to facilitate reductive metabolism in non-green glandular trichomes**. Our biochemical assays with purified glandular trichome FNR indicated that it functions similar to FNR isoforms characterized previously in roots. Surprisingly, members of the genus *Mentha* appear to express glandular trichome-specific isofomrs of Fd. We hypothesize that these isoforms evolved to enable very high fluxes through reductive reactions involved in terpenoid biosynthesis.
- **Monoterpene synthase structure-function**. 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 sustainable specialty chemicals. Our previous work established which residues likely form the catalytically important residues in the active site of spearmint (-)-limonene synthase. We now demonstrate that we can use this enzyme as a protein engineering scaffold that can produce almost any type of target monoterpene in a predictable fashion.

Science objectives for 2016-2017:

- **Genome-scale modeling of glandular trichome metabolism and biochemical characterization of Fd/FNR.** Develop techniques to evaluate the interaction of glandular trichome-specific Fds with enzymes involved in terpenoid biosynthesis.
- **Terpene synthase structure-function.** Generate and test more monoterpene synthase mutants that are predicted to produce novel monoterpenes.
- **Manuscripts** are at the pre-submission or planning stage to report on the following topics:
  - Srvividya N., Lange B.M. (2017) Engineering a monoterpene synthase scaffold to predictably produce target monoterpenes, submission to *Science* or *Nature* planned for Q1, 2017.
  - Turner G.W., Parrish A.N., Fischedick J.T., Lange B.M. (2017) Developmental distribution of oleoresin formation in loblolly pine (*Pinus taeda* L.), submission to *Plant Physiol.* planned for Q4, 2016.
  - Johnson S.R., Lange B.M. (2017) Mathematical modeling and experimental testing of the processes that power monoterpenoid essential oil biosynthesis in peppermint glandular trichomes, submission to *Plant Cell* planned for Q4, 2016.

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

- Synthetic biology – development of microbial platform strains for generating diverse terpenoids.
- Terpene synthase structure-function – quantum mechanical modeling of catalysis involving carbocationic intermediates.

Publications supported by this project (published March 2015 - August 2016):

1. Srividya N., Lange I., Lange B.M. (2016) Generation and functional evaluation of designer monoterpene synthases. *Methods Enzymol.* **576**, 147-165.
2. Turner G.W., Lange B.M. (2015) Ultrastructure of grapefruit secretory cavities and immunocytochemical localization of (+)-limonene synthase. *Int. J. Plant Sci.*, **176**, 643-661.
3. Lange B.M. (2015) The evolution of plant secretory structures and emergence of terpenoid chemical diversity. *Annu. Rev. Plant Biol.* **66**, 139-159.
4. Srividya N., Davis E.E., Croteau R.B., Lange B.M. (2015) Functional analysis of spearmint (-)-limonene synthase mutants reveals determinants of catalytic outcome in a model monoterpene synthase. *Proc. Natl. Acad. Sci. USA* **112**, 3332-3337.

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

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Overall research goals: The oxo-acid:ferredoxin oxidoreductease (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.

Significant achievements 2014-2016: In the first two-year period of our new award, we were initially focused upon the OFORs from *Hydrogenobacter thermophilum*, an organism that uses the reverse TCA cycle, as a model system. We demonstrated the specificity of the reactivity of the *Ht* Fd1 protein with the native enzyme KorAB, an oxo-glutatrate:ferredoxin oxidoreductase. Through a comparison with a canonical PFOR that we also developed an over-expression system for in *E. coli*, the PFOR from *Desulfovibrio africanus*, we examined the impact of redox potentials of the native redox partner upon catalysis. Using either conventional steady-state assays, or a newly developed electrochemical assay, we demonstrated that the redox potential of Fd was sufficient to bias electrocatalysis for CO_2 reduction.

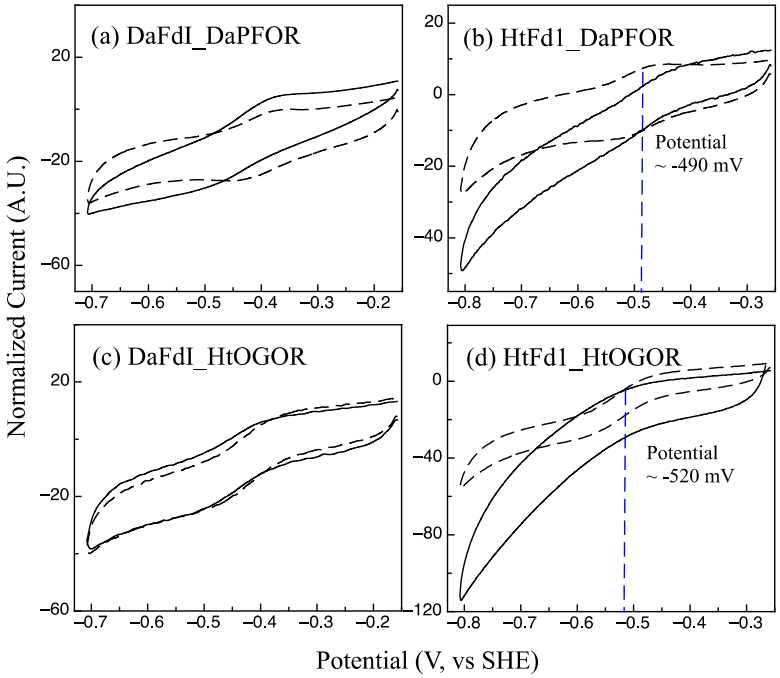

Figure 1. Electrochemical detection of CO_2 reduction for OFORs paired with either DaFd1 (a and c) or HtFd1 (b and d).

We have also investigated the impact of potential of Fds upon the reactivity exhibited by OFORs of different physiological role. The *Ht* Fd1 protein was used as a primary chassis for comparisons with *Da* Fd1, with respect to their native enzymes. While *Da* Fd has been crystallographically characterized previously, the *Ht* ferredoxin has been crystallographically characterized by us in the past granting period (Figure 2) . The protein forms a dimer *in crystallo*, which we have now shown to persist in solution as well. Using this protein, we have produced a small library of 20 mutants,

targeting single, double or triple mutations that render *Ht* Fd1 to be more similar *Da* Fd1. Through this strategy, we have assembled a series of proteins that now have a span of potential of approximately 100 mV (-560 mV to -440 mV) with the *Ht* Fd1 variants. We have corroborated that within the pairing of an OFOR and it's native redox partner, by altering the potential of the redox relay alone, we can tune the relative rates of oxidative and reductive chemistry.

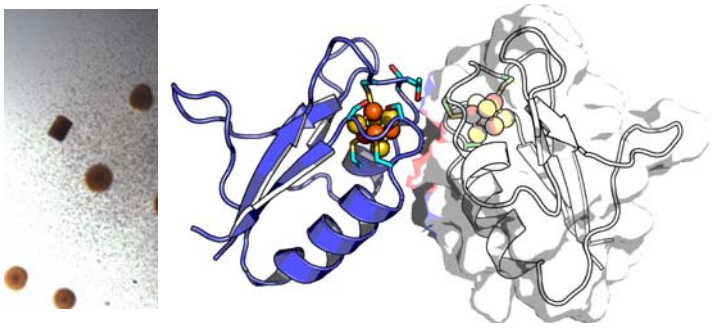

Figure 2. Crystals and final structure of HtFd1, at 2.0Å resolution.

We pivoted in the course of the past two years, to working enzymes from a mesophilic sources that are known to engage in the reverse TCA cycle. We have found that these (from *Magnetococcus* and *Chlorobium*), that have greater reactivity in our hands. We have developed a recombinant expression system for the two-subunit version of an OFOR, such as KorAB. We have purified the enzyme from *Magnetococcus marinus*, and have demonstrated that it possesses a single [4Fe-4S] cluster of low potential. We have demonstrated that this enzyme is active in oxidative and reductive directions, that a similar TPP• can be produced, similar to other PFORs that have been well-studied (Figure 3). In parallel we have developed an expression system for the *Chlorobium* PFOR, a single subunit PFOR bearing all three of the canonical subunits. The *Chlorobium* enzyme has been investigated by direct electrochemistry, allowing us to directly assess the redox potentials of all PFOR FeS clusters.

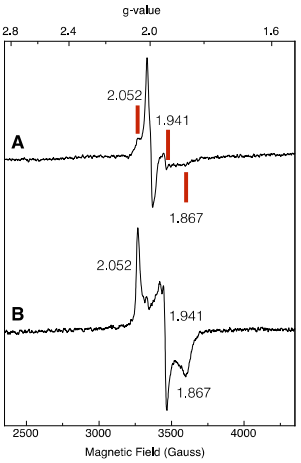

Figure 3. X-band EPR of *Mm* KorAB radical (A) and reduced KorAB (B).

Science objectives for 2016-2018:

- Pre-steady-state kinetic analyses of the *Mm* KorAB enzyme, as the difference in FeS cluster content implies a distinct difference in how timing of ET steps much occur in mechanistic chemistry.
- Determine the rate constants of Fd binding to KorAB, to ascertain of Fds provide a [4Fe-4S]x2 module to replace the apparently missing Fd domain found in most OFOR enzymes to date.

References to work supported by this project 2014-2016:

1. Li B and Elliott SJ. (2016) The catalytic bias of 2-oxoacid:ferredoxin oxidoreductase in CO_2 evolution and reduction through a ferredoxin-mediated electrochemical assay," *Electrochimica Acta, 199*, 349-356. DOI: 10.1016/j.electacta.2016.02.119

And finally, to take my project to the next level my ideal collaborator would have expertise in: Genetics of organisms engaging in the reverse TCA cycle, and the ability to make homologous expression systems (mutants) in cases where recombinant expression in *E. coli* fails.

Session VII

Role of HydF 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 H-cluster assembly through the use of physical biochemical approaches to elucidate the reactions catalyzed by the radical SAM maturases HydE and HydG and to explicitly identify the products of these enzymes and to determine how these products come together on the scaffold protein HydF. We made major strides in the past funding period towards these goals, by providing new insights into each of the three maturation enzymes while also laying the foundation needed to continue to pursue the complex biological process of H-cluster maturation. It is clear that these three enzymes interact with one another: two of them synthesize species used by the third in assembling an H-cluster precursor, and numerous lines of evidence point to the requirement for direct protein-protein interactions. Our approach involves first developing an understanding of each of the three proteins in isolation, and then incorporating studies of their interactions until a thorough understanding of H-cluster maturation is achieved.

Significant achievements (2016-2019):

- We have carried out a careful and detailed EPR study of the iron-sulfur cluster states on HydF. Through temperature- and power-saturation studies of HydF and the well-characterized proteins HydE and PFL-AE, we were able to demonstrate that HydF harbors a redox-active [2Fe-2S] cluster.
- We have used Mössbauer spectroscopy to gain insight into the diamagnetic FeS cluster states associated with HydF; sample analysis has confirmed the results from EPR spectroscopy and shows the presence of $[2Fe\text{-}2S]^{2+/1+}$ cluster states. Moreover, Mössbauer spectral analysis has provided evidence for an all-ferrous $[2Fe\text{-}2S]^{0}$ cluster on $HydF^{G\Delta E}$ and $HydF^{\Delta EG}$. This result is incredibly exciting as it represents, to the best of our knowledge, the first existence of this cluster type that is achievable with physiological reducing agents.
- We have collaborated with Sandra and Gareth Eaton at U. Denver to apply advanced EPR techniques and relaxation enhancement calculations to probe the proximity of [2Fe2S] and [4Fe-4S] clusters on HydF, and we have coupled spectral analysis with gel filtration to probe the quaternary structural state of the EPR samples themselves. Results show strong support for these FeS cluster species being associated with the dimeric state of HydF.
- We have complemented our detailed FTIR study of $HydF^{EG}$ with a computational effort directed toward developing a model for the structure of the cofactor in "loaded" HydF. Our experimental and computational analysis has provided support for the Fe ions of the 2Fe subcluster precursor complex both being coordinatively saturated when bound to HydF.
- We have demonstrated that $HydF^{E\Delta G}$, but not $HydF^{G\Delta E}$, is capable of activating HydA to a small extent; activation ability can be increased via addition of free CO or free CN. This result provides support for the modification of a [2Fe-2S] cluster framework on HydF first by HydE, via addition of the bridging dithiomethylamine, and then HydG via addition of CO and CN.
- We have obtained FTIR spectra of $HydF^{G\Delta E}$ that demonstrate the presence of CO ligands on this protein. This result suggests that HydF captures HydG-derived products.
- We have successfully loaded the "dangler" iron onto the C-terminal [4Fe-4S] cluster on HydG, and have shown that occupancy of the dangler site correlates with the production of free CO in levels that exceed the amount of HydG present.

- We have transitioned all of our Hyd proteins to strep-tagged versions in order to eliminate potential artifacts from the metal-binding His_6 affinity tag.

Science objectives for 2016-2017:

- Complete a detailed characterization of the cluster states on HydF under different reducing conditions using Mössbauer spectroscopy.
- Use Mössbauer spectroscopy, FTIR, EPR, and mass spectrometry to define the cluster species present on $HydF^{E\Delta G}$ and $HydF^{G\Delta E}$.
- Use activity assays and EPR, FTIR, and Mössbauer spectroscopic studies to characterize HydF variants in which conserved amino acids have been changed, in order to probe their specific roles in 2Fe precursor assembly and/or delivery to HydA.
- Determine the oligomeric state of HydF that interacts with HydE and HydG.
- Determine whether the dangler site on HydG becomes part of a "synthon" for delivery to HydF, or alternatively whether it merely serves as a catalytic site for free CO formation. Replace the Fe ion at the dangler site with Ni(II) and Zn(II), respectively, and probe the ability of this substituted site to form free CO.
- Probe the cation-binding site of HydE and determine whether it could be a site for binding of a putative ammonium substrate, using EPR –monitored titration/competition experiments.
- Carry out rapid-freeze-quench EPR and ENDOR studies of HydG, in order to probe reaction intermediates that build up at the N- and C- terminal iron-sulfur clusters.

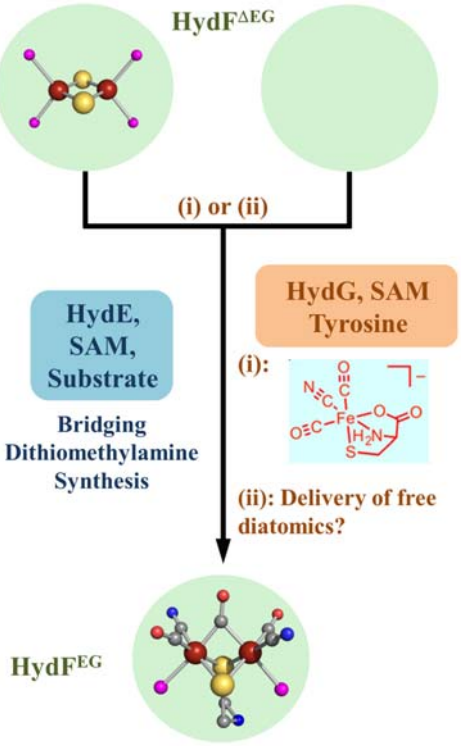

Fig. 1. Scheme detailing two possible pathways for 2Fe subcluster biosynthesis on HydF. (i) Cannibalization of a [2Fe-2S[cluster framework on HydF. (ii) Delivery of $Fe(CO)_2CN$ synthon units from HydG.

My scientific area(s) of expertise is/are: Bioinorganic chemistry, iron-sulfur clusters in biology, biological radical reactions, radical SAM enzymes.

To take my project to the next level, my ideal collaborator would have expertise in: Mössbauer spectroscopy, protein-protein interactions.

Publications supported by this project (2016-2019):

1. E. M. Shepard, A. S. Byer, J. N. Betz, J. W. Peters, J. B. Broderick, "A redox active [2Fe-2S] cluster on the hydrogenase maturase HydF," Biochemistry **55**, 3514-3527 (2016). DOI 10.1021/acs.biochem.6b00528

Hyperthermophilic Multiprotein Complexes and Pathways for Energy Conservation and Catalysis

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Overall research goals: The focus of this research is non-covalent multi-protein complexes that are involved in novel mechanisms of energy conservation and catalysis. The protein complexes under study have the remarkable property of being synthesized (self-assembling) at temperatures near 100°C in so-called hyperthermophilic microorganisms. Moreover, they are involved in the conversion of low potential reducing equivalents into gaseous end products with concomitant energy conservation in the form of ion gradients. Conversion of low potential reductant to a useable form of energy is a fundamental issue in all reaction systems that utilize light or produce biofuels.

Significant achievements [2015-2016]: A focus of this research is the 14-subunit respiratory H_2-evolving membrane-bound NiFe-hydrogenase (MBH) of the archaeon *Pyrococcus furiosus* (Pfu) that conserves energy by ion pumping. We genetically-engineered the production of a tagged catalytically-active sub-complex that was purified and characterized. Crystals of the complex have now been obtained (in collaboration with J. Peters, MSU) and structural characterization is in progress. In addition, the enzyme is currently being examined by cryoEM (with H. Li, Brookhaven). We also carried out a combined NRVS and DFT study of Pfu ferredoxin, the physiological electron donor for MBH, and a Mössbauer study of Ni-substituted Pfu rubredoxin (with S. Cramer, UC Davis). A second focus of this research is the cytoplasmic NiFe-hydrogenase of Pfu termed SHI. We believe this enzyme will be the pre-eminent model NiFe-hydrogenase for determining detailed mechanistic insights into H_2 production at the atomic level. We devised a high yield purification of the affinity-tagged version of SHI and also identified an immature form, which provided insights into how this complex metalloenzyme is synthesized. Purified SHI was used a) in a synthetic pathway for H_2-production from biomass (with P. Zhang, VPI), b) for the first electrochemical study of oxygen-inactivation of a group 3 NiFe-hydrogenase (with A. Jones, ASU), and c) for the first in-depth analysis of the proton-coupled electron transfer mechanism and catalytic bias of any NiFe-hydrogenase by photo-triggered nanosecond infrared spectroscopy (with B. Dyer, Emory). Perspectives on the properties and production of SHI, of the general properties of NiFe- and FeFe-hydrogenases, and on the role of geochemistry and energetics in the evolution of modern respiratory complexes from a proton-reducing ancestral hydrogenase of the MBH-type found in Pfu were also published. In non-published on-going work, our most significant achievements were a) the solubilization and purification of the intact 13-subunit membrane-bound respiratory oxidoreductase MBX (S-MBX), which is involved in the reduction of elemental sulfur (S^0), and b) the production and purification of an affinity-tagged catalytically active 5-subunit sub-complex of MBX (C-MBX). Characterization of S- and C-MBX is in progress with the specific goal of determining the physiological reaction of this respiratory complex. In addition, the membrane bound thiosulfate reductase from another hyperthermophile, *Pyrobaculum aerophilum*, was heterologously produced in Pfu. Interestingly, the activity of the enzyme was determined by the relative concentrations of Mo and W in the growth medium, where high Mo stimulated activity. The His-tagged enzymes have been purified and characterization is in progress.

Science objectives for 2016-2017:

- To determine the physiological reaction of MBX
- To characterize the 13-subunit S-MBX and the 5-subunit C-MBX forms of MBX by biochemical and spectroscopic approaches.
- To characterize the thiosulfate reductase and other membrane complexes that are heterologously produced in tagged forms in Pfu by biochemical and spectroscopic means.

My scientific area(s) of expertise is/are: anaerobic biochemistry and metalloenzymes.

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

Publications supported by this project 2015-2016:

1. McTernan, P. M., Chandrayan, S. K., Wu, C.-H., and Adams, M. W. W. (2015) "Engineering the respiratory membrane-bound hydrogenase of the hyperthermophilic archaeon *Pyrococcus furiosus* and characterization of the catalytically-active cytoplasmic subcomplex" *Prot. Eng. Design Select.* **28,** 1-8
2. Lauterbach, L., Gee. L. B., Pelmenschikov, V., Jenney, F. E., Kamali, S, Yoda, Y., Adams, M. W. W. and Cramer, S. P. (2016) "Characterization of the $[3Fe\text{-}4S]^{0/+1}$ cluster from the D14C variant of *Pyrococcus furiosus* ferredoxin *via* combined NRVS and DFT analyses" *Dalton Trans.* **45,** 7215-7219
3. Chandrayan, S. K., Wu, C.-H., McTernan, P. M. and Adams, M. W. W. (2015) "High yield purification of a tagged cytoplasmic [NiFe]-hydrogenase and a catalytically-active nickel-free intermediate form" *Prot. Express. Purif.* **107,** 90-94
4. Rollin, J. A., del Campo, J. M., Myung, S., Sun, F, Bakovic, A. E., Castro, R. L., Chandrayan, S., Adams, M. W. W., Senger, R. S., and Zhang, H-.Y. (2015) "High-yield hydrogen production from biomass by in vitro metabolic engineering: mixed sugars co-utilization and kinetic modeling" *Proc. Natl. Acad. Sci. USA* **112,** 4964-4969
5. Kwan, P., McIntosh, C. L., Hopkins, R. C., Chandrayan, S. K., Wu, C.-H., Adams, M. W. W. and Jones, A. K. (2015) "The [NiFe]-hydrogenase of *Pyrococcus furiosus* exhibits a new type of oxygen-tolerance" *J. Am. Chem. Soc.* **137,** 13556-13565
6. Wu, C.-H., Mcternan, P. M., Walter, M. and Adams, M. W. W. (2015) "Production and application of a soluble hydrogenase from *Pyrococcus furiosus*" *Archaea,* 912582
7. Peters, J. W., Schut, G. J., Boyd, E. S., Mulder, D. W., Shepard, E. M., Broderick, J. B., King, P. W. and Adams, M. W. W. (2015) "[FeFe]- and [NiFe]-hydrogenase diversity, mechanism, and maturation" *Biochim. Biophys. Acta* **1853,** 1350-1369
8. Greene, B. L., Wu, C.-H., Adams, M. W. W. and Dyer, R. B. (2015) "Mechanism and bias of a [NiFe] hydrogenase revealed by photo-triggered nanosecond infrared spectroscopy" *J. Am. Chem. Soc.* **137,** 4558-4566
9. Greene, B. L., Wu, C.-H., Vansuch, G. E., Adams, M. W. W. and Dyer, R. B. (2016) "Proton inventory and dynamics in the Ni_a-S to Ni_a-C transition of a [NiFe]-hydrogenase" *Biochemistry* **55,** 1813-1825
10. Schut, G. J., Zadvornyy, O., Wu, C.-H., Peters, J. W., Boyd, E. S. and Adams, M. W. W. (2016) "The role of geochemistry and energetics in the evolution of modern respiratory complexes from a proton-reducing ancestor" *Biochim. Biophys. Acta* **1857,** 958-970
11. Gee, L. B., Lin, C. Y., Jenney, F. E. Jr, Adams, M. W. W., Yoda, Y., Masuda, R., Saito, M., Kobayashi, Y., Tamasaku, K., Lerche, M., Seto, M., Riordan, C. G., Ploskonka, A., Cramer, S. P. and Lauterbach, L. (2016) "Synchrotron-based nickel Mössbauer spectroscopy" *Inorg Chem.* **55,** 6866-6872

Utilization of protein film electrochemistry to characterize the mechanisms imparting aerotolerance and bidirectionality in soluble, multimeric [NiFe]-hydrogenases

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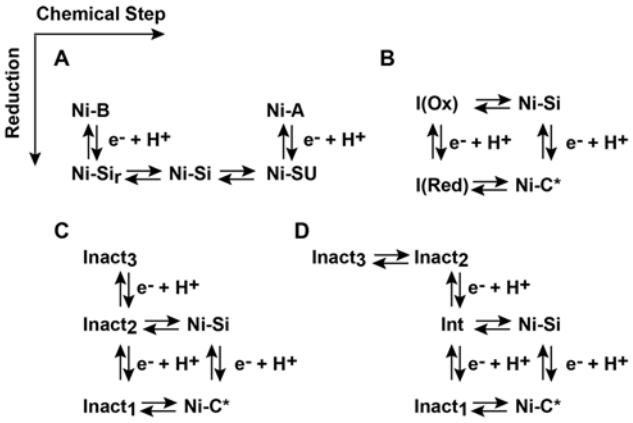
Overall research goals:

The goal of this project is to define structure/function relationships controlling the reactivity of soluble [NiFe]-hydrogenases (SH) as a model for energetically relevant multielectron redox catalysis. Specific aims are to understand the factors that determine (1) catalytic bias, i.e. ratio of oxidative to reductive catalytic activity and (2) reactions with oxygen and oxygen-tolerance. This fundamental knowledge should prove beneficial to predicting and controlling reactivity in other redox enzymes.



Significant achievements (2012-2016):

In collaboration with Prof. Michael Adams (UGA), the hydrogenase electrocatalytic activity of SHI from *Pyrococcus furiosus* (PfSHI), an oxygen-tolerant [NiFe]-hydrogenase, has been determined under a broad range of conditions. Redox catalytic bias, the preference of a redox catalysts for reactivity in the forward or reverse direction, is an important and largely unexplored property. We have shown that the catalytic bias of PfSHI depends strongly both on enzyme quaternary structure and solution temperature. These results suggest that proton reduction includes a temperature-dependent, rate-limiting step. Furthermore, coupling of the active site to other protein domains may impact catalytic rate. This is surprised since most models of [NiFe]-hydrogenases assume that intramolecular electron transfer is fast enough not to impact catalysis.

Although previous work demonstrated that [NiFe]-hydrogenases form two inactive states upon oxidation (Figure panels A and B), we have shown that PfSHI forms three inactive states. Figure panels C and D show schemes consistent with the PfSHI data. This is a remarkable result since oxygen-tolerant hydrogenases were previously thought to form only one inactive state. It also raises new questions regarding the chemical identity of these states and the mechanism of oxygen-tolerance in this enzyme.

Science objectives for 2016-2017:

- Characterize the electrocatalytic properties of the hydrogenase subdimer of *Pf*SHI. In particular, catalytic bias as a function of temperature and reactivity in the presence of oxygen will be investigated and compared to the holo-enzyme to determine whether the diaphorase plays a role in determining bias.
- Preliminary evidence suggests that catalytic properties are being controlled at sites remote from the catalytic active site. This may be accessory [FeS] clusters. This hypothesis will be evaluated by electrocatalytic characterization of protein variants with substitutions in the accessory cluster domains.

- The soluble [NiFe]-hydrogenases are bifunctional with a diaphorase domain as well as the hydrogenase. Interactions between the two reactions have not been probed. Electrocatalytic properties of the diaphorase portion of PfSHI as well as the closely related *Synechocystis* [NiFe]-hydrogenase will be determined, and the ability of pyridine nucleotides to modulate hydrogenase activity articulated.

My scientific area(s) of expertise is/are: redox chemistry, electrochemistry, bioinorganic chemistry

To take my project to the next level, my ideal collaborator would have expertise in: Synthetic biology, expression of multicofactor-containing redox enzymes, culture of unusual microorganisms, surface spectroscopy

Publications supported by this project 2012-2016:

1. P. Kwan, C. L. McIntosh, D. P. Jennings, R. C. Hopkins, S. K. Chandrayan, C.-H. Wu, M. W. W. Adams, A. K. Jones, "The [NiFe]-Hydrogenase of *Pyrococcus furiosus* Exhibits a New Type of Oxygen Tolerance." J. Am. Chem. Soc. 137, 13556 (2015). DOI: 10.1021/jacs.5b07680
2. R. Pal, J. A. Laureanti, T. L. Groy, A. K. Jones, R. J. Trovitch, "Hydrogen production from water using a bis(imino)pyridine molybdenum electrocatalyst". Chem. Commun., 52, 11555 (2016). DOI: 10.1039/c6cc04946j

Mechanistic Studies on the Activation of Hydrogen by Algal [FeFe]-Hydrogenase

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Paul W. King, Principal Investigator, NREL
Michael W. Ratzloff, Staff Scientist, NREL
Katherine A. Brown, Staff Scientist, NREL
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Overall research goals:

The overarching goal of this project is to establish a deep understanding of the mechanisms of redox enzymes that function in photosynthetic energy transduction networks. The photosynthetic production of H_2 in green algae is catalyzed by [FeFe]-hydrogenases that couple to low potential reductant pools to help maintain electron flow under anaerobic-aerobic transitions. The active site, complex bridged metallocofactor H-cluster of these enzymes is finely tuned to operate in narrow thermodynamic window by the means of unique proton-coupled electron-transfer (PCET) chemistry. By defining spectroscopic signatures of catalytic intermediates, reduced hydride states, and developing the mechanism of catalysis through integrated spectroscopy, we aim to reveal fundamental properties of PCET mechanisms. The information gained through these studies will shed light on how photosynthetic organisms control the generation and use of photosynthate as well as inform on how to refine and tune properties of synthetic catalysts for efficient H_2 production.

Significant achievements (2014-2016):

We determined the primary electronic and biophysical properties of the Fe-hydride intermediate of the [FeFe]-hydrogenase from *Chlamydomonas reinhardtii* (CrHydA1). Up until this point, fast PCET has challenged direct characterization of the critical H-cluster intermediate. We took advantage of altered proton-transfer kinetics in a variant of CrHydA1 (C169S) where the primary proton donor (Cys residue 169) to the H-cluster is changed to a Ser residue to enrich for the otherwise fast-lived hydride intermediate. Initial hydrogen-deuterium isotope FTIR and DFT analysis suggest the presence of a terminal hydride bound to the distal Fe site of the 2Fe subcluster ($[2Fe]_H$) of the H-cluster. Using a combination of Mössbauer, EPR, FTIR and DFT, we demonstrated that H_2 activation proceeds on an H-cluster intermediate consisting of a diferrous $[2Fe]_H$ coupled to a paramagnetic $[4Fe–4S]_H^+$ subcluster. In addition, the reduction potential of the hydride intermediate was determined to lie at the formal potential of the H^+/H_2 couple, confirming its role as the key H_2 activation step in the catalytic cycle. Overall, the findings show how the H-cluster works in concert with the protein environment to enable facile PCET through positioning of the electron rich hydride proximal to a proton-exchangeable group and a electron relay [4Fe-4S] cluster.

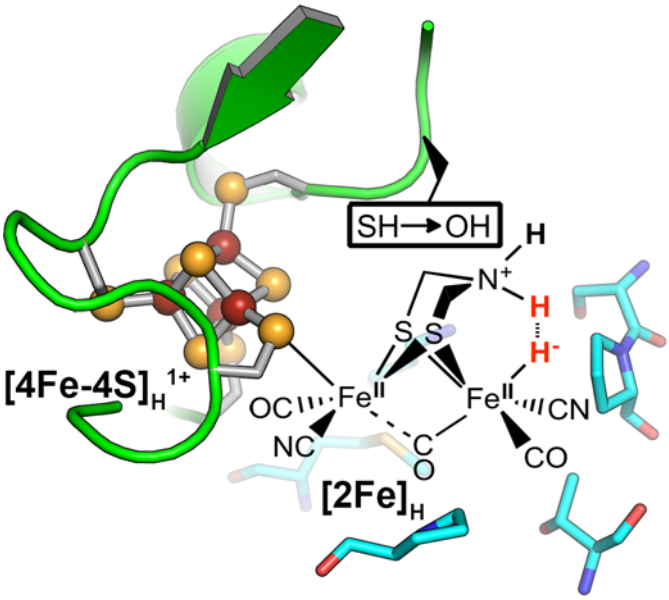

Figure 1. Model of the hydride intermediate of [FeFe]-hyrdrogenase enchriched for in the C169S variant.

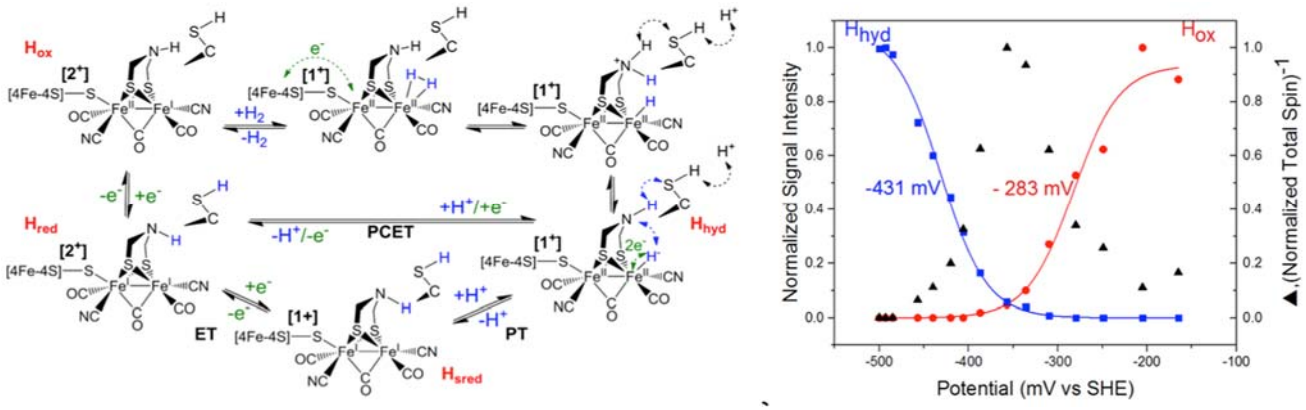

Figure 2. Left: Proposed model for the catalytic cycle for H_2 activation by [FeFe]-hydrogenase. **Right:** Nernst curves for H_{hyd} and H_{ox} catalytic intermediates based on EPR potentiometric titrations of C169S CrHydA1.

Science objectives for 2016-2017:

- Further characterize the electronic, vibrational, and structural properties of the proposed hydride intermediate enriched in C169S CrHydA1 by application of pulse EPR along with nuclear resonance vibrational spectroscopy and hydrogen-deuterium exchange.
- Describe PCET transitions of the H-cluster between reduced and oxidized states with aim to fill in gaps of electronic states of catalytic intermediates and also higher oxidation states by preparation of samples at poised redox potentials for analysis by Mössbauer spectroscopy.
- Explore structure-function relationships of catalytic intermediates by utilizing [FeFe]-hydrogenase:nanoparticle photobiohybrids for controlled optical triggered spectroscopy.
- Extend underlying principles and themes of PCET chemistry taken from hydrogenase studies to other enzymes functioning in redox biochemistry and energy transduction networks.

My scientific area(s) of expertise is/are: Anaerobic biochemistry, metalloenzymes and hydrogenases, biophysical and biochemical characterization, EPR and FTIR spectroscopy, structural biology and protein chemistry, and enzyme mechanisms.

To take my project to the next level, my ideal collaborator would have expertise in: Mössbauer spectroscopy, DFT calculations (eg. Dr. Yisong Guo, Carnegie Mellon University), advanced spectroscopic techniques for characterization of metal-hydrides (eg. Dr. Stephen Cramer, UC Davis), biological electron-transfer and hydride-transfer mechanisms (eg. Dr. Russ Hille, UC Riverside).

Publications supported by this project 2014-2016:

1. D.W. Mulder, M.W. Ratzloff, M. Bruschi, C. Greco, E. Koonce, J.W. Peters and P.W. King. "Investigations on the role of proton-coupled electron transfer in hydrogen activation by [FeFe]-hydrogenase." *J. Am. Chem. Soc.* 136:15394. (2014).
2. K.D. Swanson, M.W. Ratzloff, D.W. Mulder, J. Artz, S. Ghose, A. Hoffman, S. White, O.A. Zadvornyy, J.B. Broderick, B. Bothner, P.W. King, J.W. Peters. "[FeFe]-hydrogenase oxygen inactivation is initiated by the modification and degradation of the H cluster 2Fe subcluster." *J. Am. Chem. Soc.* 137:1809. (2014).
3. J.W. Peters, G.J. Schut, E.S. Boyd, D.W. Mulder, E.M. Shepard, J.B. Broderick, P.W. King, M.W. Adams. "[FeFe]-and [NiFe]-hydrogenase diversity, mechanism, and maturation." *BBA-Mol. Cell. Res.* 21853: 1350-1369. (2015).
4. S., Morra, S., Maurelli, M., M. Chiesa, D.W. Mulder, M.W. Ratzloff, E. Giamello, P.W. King, G. Gilardi, F. Valetti. "The effect of a C298D mutation in CaHydA [FeFe]-hydrogenase: Insights into the protein-metal cluster interaction by EPR and FTIR spectroscopic investigation." *BBA-Bioenergetics*, 1857:98-106. (2016).
5. D.W. Mulder, Y. Guo, M.W. Ratzloff, P.W. King. "Catalytic proton reduction at the H-cluster of [FeFe]-hydrogenase." *Nat. Chem.* submitted. (2016).

Session VIII

Elucidating Biological Energy Transduction from Ammonia

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Overall research goals:

While most organisms metabolize carbon-based chemical fuel, a select few organisms evolved to derive sufficient biological energy from the six-electron aerobic oxidation of NH_3 to NO_2^-. This process, referred to as nitrification, involves two remarkable steps. The first is the hydroxylation of NH_3 by the enzyme NH_3 monooxygenase (AMO) to form NH_2OH. AMO uses a Cu cofactor to activate the strong, 107 kcal/mol N–H bond of NH_3 using O_2 as the oxidant. The second step involves the oxidation of NH_2OH to NO_2^- by NH_2OH oxidoreductase (HAO). The oxidation of NH_2OH to NO_2^- by HAO is a four-electron process requiring proton management. HAO uses a heme cofactor, heme P460, which is unique in its ability to directly remove electrons from substrate bound to its Fe center.

Mastering the fundamental chemical principles underlying these reactions will fuel the development of novel catalysts for small molecule activation and selective, proton-coupled redox transformations. Moreover, mechanistic knowledge of AMO and HAO will spur the development of improved nitrification inhibitors that would alleviate economic and ecological burdens resulting from the nitrification of nitrogenous fertilizer. However, molecular level understanding of the mechanisms involved in nitrification has remained elusive. This is partly due to the difficulty of isolating sufficient quantities of AMO and HAO from nitrifying bacteria and archaea, which are slow-growing microbes that achieve low cell densities.

The goals of this project are to establish at a molecular level of detail the reaction mechanisms underlying the complete conversion of NH_3 to NO_2^- by AMO and HAO. This will be achieved using stopped-flow kinetics to follow the course of reactions and rapid freeze-quench methods to trap intermediates observed during via these experiments. Resting and intermediate species will be characterized using convention spectroscopies including UV/visible absorption and electron paramagnetic resonance spectroscopy as well as using cutting-edge, synchrotron-based high-resolution X-ray spectroscopies. Studies of AMO employ a recombinant *Mycobacterium smegmatis* expression system based on the pMycoFos shuttle fosmid. Studies of NH_2OH oxidation employ both *Nitrosomonas europaea* HAO as well as recombinantly expressed *N. europaea* cytochrome (cyt) P460.

Significant achievements (2015-2016):

- We have established that our *M. smegmatis* expression system produces active *Nitrosopumilus maritimus* AMO. This AMO can be modified using site directed mutagenesis, which we have used to pinpoint residues putatively involved in metal binding and/or O_2 activation (**Figure 1**). We have shown that O_2 activation is inhibited by AMO-specific inhibitors allylthiourea and acetylene.
- We have established a minimal mechanism for oxidation of NH_2OH by *N. europaea* cyt P460 (**Figure 2a**). We discovered that, contrary to early literature reports, the chemistry of cyt P460 differs from HAO in that no NO_2^- appears to be enzymatically produced.

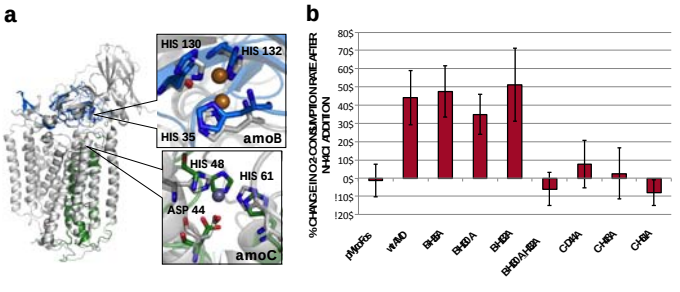

Figure 1. (a) Putative active site residues of *N. maritimus* AMO located by structural homology modeling template on *M. capsulatus* (Bath) particulate methane monooxygenase. (b) Results of O_2 respirometry induced by NH_4Cl addition to cultures of *M. smegmatis* expressing the *N. maritimus* AMO gene cluster.

We have characterized by UV/vis absorption and EPR the resting Fe^{III}–OH_2 form of cyt P460 as well as intermediates in the anaerobic oxidation of NH_2OH: Fe^{III}–NH_2OH, and $\{Fe–NO\}^6$. We showed that $\{Fe–NO\}^6$ itself reacts with NH_2OH in the rate-determining step to form N_2O (**Figure 2b**). These results establish the first direct link between NH_3 oxidation and environmental N_2O release.

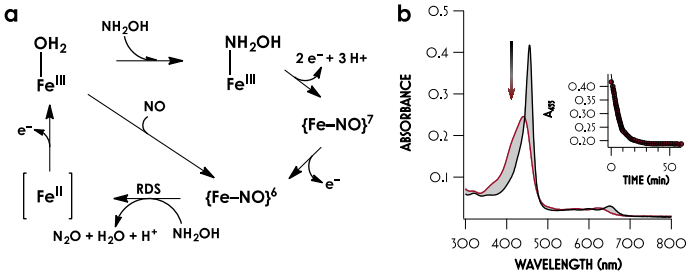

Figure 2. (a) Working experimental mechanism for N_2O formation from NH_2OH by *N. europaea* cyt P460. (b) A heme $\{Fe–NO\}^6$ species (black) reacts with 1 equiv. NH_2OH to form N_2O.

- We have obtained a 1.5 Å resolution X-ray crystal structure of the apo form of a putative non-heme Fe HAO from *Paracoccus denitrificans* (**Figure 3**). Preliminary data verified the competence of this enzyme for anaerobic Fe-dependent NH_2OH oxidation using chemical oxidants.

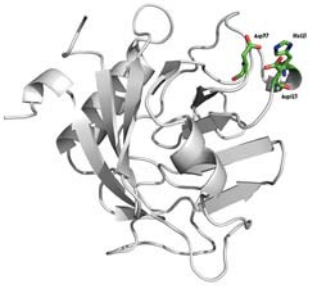

Figure 3. Undeposited 1.5 Å X-ray crystal structure of a putative non-heme HAO from *P. denitrificans*. The protein was crystallized in the apo form; putative metal binding residues are highlighted in green.

Science objectives for 2016-2017:

- We will adapt our AMO expression system to scale-up for protein purification. We hypothesize that accumulation of NH_2OH compromises cell growth. Our preliminary identification of a non-heme HAO gives us optimism that we can install this protein as a defense mechanism against cytotoxic NH_2OH.
- We will probe the early steps following NH_2OH binding to Fe in the cyt P460 mechanism. We will establish whether early oxidative steps are single- or concerted multi-electron in nature using stopped flow kinetics as well as laser-induced flash quench oxidation using phototrigger-labeled protein. We will use pH-dependence and isotope exchange to explore the proton-coupled nature of these reactions.
- We will ascertain how the cyt P460 protein scaffold gates oxidation of NH_2OH. We have identified two forms of $\{Fe–NO\}^7$ produced following NH_2OH oxidation that are differentiated by heme coordination number. We will determine how the enzyme controls Fe coordination to permit turnover of cyt P460.
- We will determine how O_2 leads to the production of NO_2^- by cyt P460. We hypothesize that NO is released during the oxidation, and that non-enzymatic aerobic oxidation of NO produces NO_2^-.
- We will purify HAO from *N. europaea* and carry out experiments paralleling our work to date with cyt P460. We have acquired three 15 L fermenters that will allow us to produce *N. europaea* at a sufficient scale to isolate useful quantities of HAO.
- We will further characterize the HAO reactivity of the putative non-heme HAO from *P. denitrificans.* Identification of a non-heme bacterial HAO will provide valuable information toward the discovery of the presently elusive archaeal HAO.

My scientific area(s) of expertise is/are: Bioinorganic chemistry, inorganic chemistry, inorganic spectroscopy, electronic structure calculations.

To take my project to the next level, my ideal collaborator would have expertise in: Microbial ecology, particularly the use of chemostats and large-scale (> 15 L fermenters) in cell culture.

Publications supported by this project 2015–2016:

1. J. D. Caranto, A. C. Vilbert, K. M. Lancaster, "*Nitrosomonas europaea* Cytochrome P460 Is a Direct Link between Nitrification and Nitrous Oxide Emission." *Proc. Natl. Acad. Sci. U.S.A.* (Under revision).

Energy Conservation During Methanogenesis in *Methanosarcina* Species

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Overall research goals:

Our efforts in this project are centered on two major goals: (1) Identification of the cellular localization and protein-protein interactions for components of the electron transport chains of *Methanosarcina barkeri* and *Methanosarcina acetivorans,* and (2) Characterization of the molecular mechanisms that govern flux between divergent branches of the electron transport chain in *M. barkeri*. Two additional goals will be pursued as time permits, including testing whether the evolutionarily divergent hydrogen dependent and independent electron transport chains are mutually exclusive, and examination of the cofactors required for specific methanogenic pathways.

Significant achievements (2016-present):

Most methanogenic archaea can produce methane by reducing CO_2 to CH_4 with electrons derived from H_2 via hydrogenase enzymes. However, some species, such as *Methanosarcina acetivorans,* are not able to use H_2 as a substrate for methanogenesis, nor do they exhibit hydrogenase activity under any condition examined. Intriguingly, the *M. acetivorans* genome encodes for the full set of hydrogenase genes found in the close relative *M. barkeri*, which grows well on H_2. Thus, it is widely believed that the M. acetivorans hydrogenases have been inactivated by mutation. To examine this further, we constructed expression plasmids for the methanophenazine-dependent (Vht) hydrogenases from *M. acetivorans* and *M. barkeri*. Contrary to our expectations, both hydrogenases are active in *M. barkeri,* whereas neither is active in *M. acetivorans*. Further, quantitative RT-PCR experiments show that both genes are transcribed in both organisms. We suggest two possible explanations for this surprising result: either *M. acetivorans* has an active mechanism to suppress hydrogenase activity, or it lacks a gene required for hydrogenase activity. Experiments are in progress to distinguish between these possibilities.

In separate experiments, we have begun to examine the interactions between redox partners, both direct and indirect, in the *M. barkeri* electron transport chain via two different approaches. In the first approach, we have constructed strains carrying fluorescence-labeled F_{420}-dependent (Frh) hydrogenase. Preliminary data show that the enzyme is localized to the cell membrane, despite previous evidence showing Frh to be a soluble cytoplasmic protein. We are currently constructing strains with similar tags on other electron transport chain components. In the second approach, we will establish the protein interaction networks for electron transport chain components using mass-spectrometry to identify proteins that co-purify with affinity tag-labeled proteins expressed in the native host. Towards this end we have designed a tandem affinity tag (TAP tag) to be appended to proteins of interest. We are currently constructing strains to test this approach in vivo.

Both approaches require the ability to genetically modify protein-encoding genes in the native host. While our current techniques are sufficient to this task, the strain constructions are complicated by the fact that the target genes are often essential and in multi-gene transcriptional units. As a result the process can be exceedingly slow. However, in the past six months we have adapted the recently developed CRISPR-Cas gene editing approach for use in *Methanosarcina*. This tool reduces the time needed to construct mutants by more than half (three weeks versus eight weeks), a feature that greatly enables genetic manipulation of these slow-growing organisms. Further, the method allows simultaneous construction of double mutants at high efficiency, exponentially amplifying time saved during strain constructions. Introduction of both insertions and deletions by homology directed repair (HDR) is remarkably efficient and precise, occurring at frequency of *ca.* 20% relative to the transformation efficiency, with the desired mutation being found in essentially all transformants

examined. This approach will be employed for all genetic tagging experiments going forward. It should be noted that development of the Cas9-editing tools was only partially supported by this award. Accordingly, the PI Metcalf (supported by this award) was involved in the design and development, while Dipti Nayak, a post-doc funded by a Life Sciences Research Foundation fellowship, was involved in the design and execution of the work.

Science objectives for 2014-2015:

- Characterization of strains that express heterologous electron transport components
- Determine the protein interaction networks of selected electron transport components using TAP-tagged proteins
- Determine the cellular localization of SNAP-tagged proteins by super-resolution light microscopy.

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

To take my project to the next level, my ideal collaborator would have expertise in: Super-resolution microscopy.

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

1. J.R. Peterson; S. Thor; L. Kohler; P.R.A. Kohler; W.W. Metcalf; Z. Luthey-Schulten. Genome-Wide Gene Expression and RNA Half-Life Measurements allow Predictions of Regulation and Metabolic Behavior in *Methanosarcina acetivorans*. In revision.
2. Nayak, D.D. and W.W. Metcalf. Cas9-mediated genome editing in the methanogenic archaeon *Methanosarcina acetivorans*. Submitted.

Mechanism of Electron Bifurcation-BETCy EFRC

Cara Lubner, Staff Scientist, NREL
John W. Peters (MSU), Director
Paul W. King (NREL), Mike W.W. Adams (UGA), Anne K. Jones (ASU), Brian Bothner (MSU), Anne-Frances Miller (UK), Co-PI(s)
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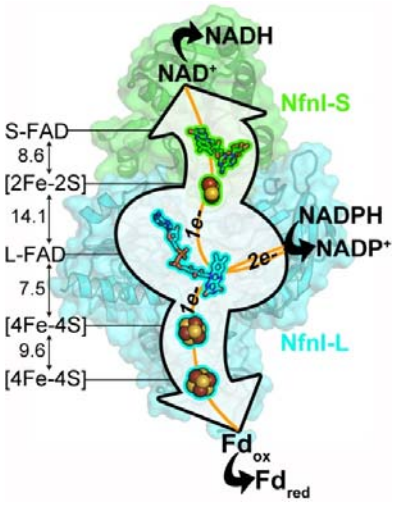
Overall research goals:

The focus of the Biological Electron Transfer and Catalysis (*BETCy*) EFRC research is elucidating the mechanisms of conversion of electrochemical potential into chemical bond energy.

Significant achievements (2014-2016):

First complete mechanistic description of a model bifurcating enzyme – Flavin-based electron bifurcation has recently gained acceptance as a fundamental mechanism of biological energy conservation, in addition to substrate-level phosphorylation and electron transport-linked phosphorylation. Bifurcation is fundamental to the biochemistry that drives microbial life at the thermodynamic limits observed for global anaerobic processes, including methanogenesis, acetogenesis and hydrogen metabolism. A key feature common to all bifurcating enzymes is the ability to use the free energy generated by an exergonic oxidation-reduction reaction to drive a coupled endergonic reaction, although the mechanism of how this is achieved remains unknown. This study demonstrates how the flavin-based bifurcating enzyme, NADH-dependent ferredoxin-$NADP^+$ oxidoreductase I (Nfn), catalyzes the formation of an energy-rich product, reduced ferredoxin (Fd), from the less energetic donor, NADPH, by coupling this reaction to the thermodynamically favorable reduction of NAD^+ by NADPH.



The data together indicate that the high barrier reaction is driven by the formation of an unstable flavin anionic semiquinone (ASQ) intermediate that promotes one of the electrons from the two-electron donor, NADPH, to a highly reduced state sufficient to reduce ferredoxin. This flavin intermediate has not been previously observed due to its high reactivity, however by utilizing ultrafast transient absorption spectroscopy we have measured this species to have a lifetime of 10 ps. Its characterization, along with the energetic landscape of the additional redox cofactors in Nfn, through application of electrochemical and electron paramagnetic studies allow us to construct a mechanistic understanding of how electrons are bifurcated over more than one volt of electrochemical potential, a range typically associated with photo-based biology.

Science objectives for 2016-2017:

- Our current work provides a template for evaluating how the broader structural context of bifurcating enzymes accomplishes catalytically demanding reactions. We will be applying this knowledge to the bifurcating Fix enzyme to elucidate the unique mechanistic features in a flavin rich enzyme.
- Identify how the protein environment tunes the properties of unique flavin sites to affect efficient two-electron or one-electron chemistries.

- Determine the role and mechanisms of proton transfer events and proton coupled electron transfer in the gating of electrons in the bifurcating enzyme, Nfn.

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

To take my project to the next level, my ideal collaborator would have expertise in: Our expertise spans from structural biology to biophysical spectroscopies such as transient absorption and electron paramagnetic spectroscopies and electrochemistry, which allows us to access the unique properties and features of bifurcating enzymes.

Publications supported by this project 2014-2016:

1. Cohen, A. E. *et al*. Goniometer-based femtosecond crystallography with X-ray free electron lasers. *Proceedings of the National Academy of Sciences of the United States of America* **111**, 17122-17127, doi:10.1073/pnas.1418733111 (2015).
2. Peters, J. W. *et al*. [FeFe]- and [NiFe]-hydrogenase diversity, mechanism, and maturation. *Biochimica et Biophysica Acta-Molecular Cell Research* **1853**, 1350-1369, doi:10.1016/j.bbamcr.2014.11.021 (2015).
3. Brown, K. A. *et al*. Light-driven dinitrogen reduction catalyzed by a CdS:nitrogenase MoFe protein biohybrid. *Science* **352,** 448-450, doi:10.1126/science.aaf2091 (2016).
4. Peters, J. W. *et al*. Electron bifurcation. *Current Opinion In Chemical Biology* **31**, 146-152, doi:10.1016/j.cbpa.2016.03.007 (2016).
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