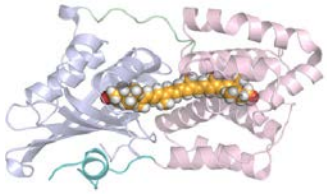
2017 Photosynthetic Systems Principal Investigators Meeting

Marriott Washingtonian Center, Gaithersburg, MD
November 13 to 15, 2017



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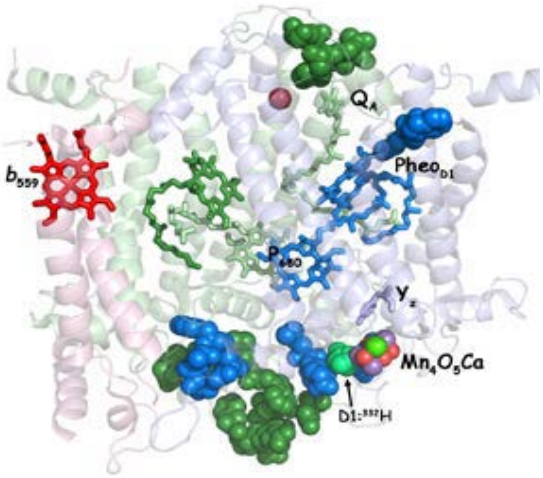

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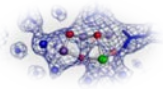

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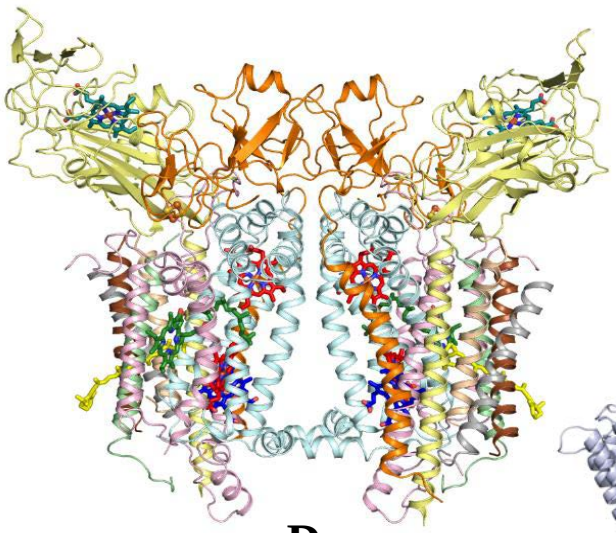

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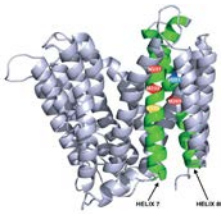

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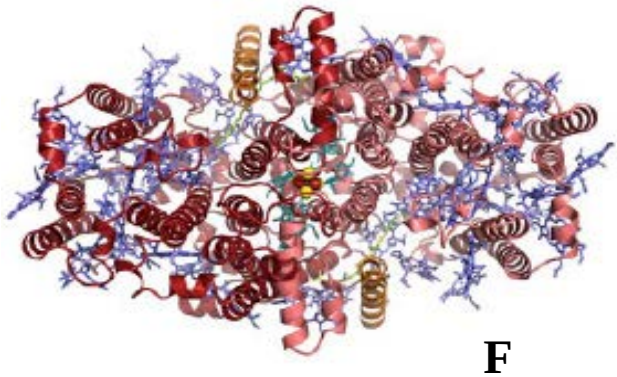

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Basic Energy Sciences Office,
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Cover Art
The cover art is taken from the abstracts of meeting participants: A – Warren Beck, B – Terry Bricker, C – Junko Yano, D – William Cramer, E – Fevzi Daldal, F – Kevin Redding

Acknowledgement and Disclaimer
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 is a record of the 5th biennial meeting of the principal investigators funded by Photosynthetic Systems, a program offered by the Chemical Sciences, Geosciences, and Biosciences (CSGB) Division in the Office of Basic Energy Sciences (BES), U.S. Department of Energy (DOE). CSGB supports basic biochemical research relevant to energy technology through Photosynthetic Systems and Physical Biosciences, two complementary programs established in 2007. These programs, along with the Solar Photochemistry program, comprise the CSGB Photochemistry and Biochemistry Team, a coordinated group of programs supporting areas of basic research that have been central to the science mission of the DOE since its earliest days.

The abstracts in this volume describe research at the leading edge of understanding natural photosynthesis, a process that exhibits great structural and chemical diversity across the biological world but that uniformly captures and stores solar energy with unmatched efficiency. The high caliber of the research in these abstracts reflects the talent, dedication, and industry of the principal investigators who make Photosynthetic Systems a vibrant, innovative funding program with growing relevance to many of the challenges facing our nation now and in the future.

The purpose of this meeting is to report research accomplishments made in recent years and to foster the exchange of scientific knowledge among all participants. Accordingly, the meeting is designed to promote sharing of new ideas and methodologies; facilitate cooperation and collaboration among research groups; challenge old paradigms with new; and provide opportunities to interact with program managers and staff of the DOE. In keeping with this purpose, questions and ideas from meeting participants are welcome at all times.

We thank Diane Marceau of DOE BES and Connie Lansdon and Tim Ledford of Oak Ridge Institute for Science and Education (ORISE) for their invaluable work planning and successfully executing the logistics of this meeting. Without their help, the meeting would not have occurred.

Stephen K. Herbert, Program Manager, Photosynthetic Systems, DOE BES
Robert J. Stack, Program Manager, Physical Biosciences, DOE BES
B. Gail Mclean, Team Lead, Photochemistry and Biochemistry Team, DOE BES

Links to Sections

(Click on the links below to go to sections of this document)

2017 Photosynthetic Systems Principal Investigators Meeting

Gaithersburg Marriott, Washingtonian Center, Gaithersburg, MD
November 13 to 15

Agenda

Sunday, November 12

3:00 – 6:00PM *Registration*

Monday, November 13

7:30 – 8:30AM *Continental Breakfast*

8:30 – 9:00AM Overview of the Chemical Sciences, Geosciences, and Biosciences Division
Bruce Garrett, Division Director

Session I: *Light Harvesting. Moderator: Warren Beck, Michigan State University*

09:00 –9:30AM Site-specific characterization of plastocyanin and the impact of binding with cytochrome f via infrared spectroscopy
Megan Thielges, Indiana University

09:30 – 10:00AM Characterization of picosecond structural dynamics of photosynthetic proteins: directionality and collectivity transitions in the FMO and orange carotenoid proteins.
Andrea Markelz, State University of New York at Buffalo

10:00 – 10:30AM *Coffee Break*

10:30 – 11:00AM Understanding bilin-based light perception in cyanobacterial photoreceptors.
J. Clark Lagarias, University of California, Davis

11:00 – 11:30AM Functional models of photosynthetic light harvesting systems templated by self-assembling proteins.
Matthew Francis, University of California, Berkeley

Monday, November 13

Session II: *Electron Transport 1. Moderator: Chris Kirmaier, Washington University*

11:30 – 12:00AM	Studies of photosynthetic reaction centers and biomimetic systems **Marilyn Gunner**, City College of New York
12:00 – 12:30PM	FTIR studies of photosynthetic oxygen production **Rick Debus**, University of California at Davis
12:30 – 03:30PM	*Lunch and Afternoon Break*

Session III: *Electron Transport 2: Bob Blankenship, Washington University*

03:30 – 4:00PM	Pulse EPR studies of substrate binding at the OEC of Photosystem II **David Britt**, University of California, Davis
04:00 – 04:30PM	Elucidating the principles that control proton-coupled electron transfer Reactions in the Photosynthetic Protein Photosystem II **K V Lakshmi**, Rennselaer Polytechnic Institute
04:30 –05:00PM	Structure-based studies on energy transduction in the cytochrome b6f complex. **William Cramer**, Purdue University
05:00 – 05:30PM	Fundamental research aimed at diverting excess reducing power in photosynthesis to orthogonal metabolic pathways **John Golbeck**, Pennsylvania State University
05:30 – 07:30PM	*Program Dinner*
07:30 – 10:00PM	*Poster Session 1: Odd-numbered posters* (Refreshments may be purchased at the hotel bar.)

Tuesday, November 14

07:30 – 08:30AM *Continental Breakfast*

08:30 – 09:00AM Program Update
Steve Herbert, Program Manager, Photosynthetic Systems program

09:00 – 10:00AM DOE's Nanoscience Research Centers
George Maracas, Scientific User Facilities Division, Office of Basic Energy Sciences

10:00 – 10:30AM *Coffee Break*

Session IV: *Electron Transport 3. Moderator: Bob Stack, Physical Biosciences program*

10:30 – 11:00AM Photosynthetic biohybrid systems for solar hydrogen production
Lisa Utschig, Argonne National Laboratory

11:00 – 11:30AM Photosynthetic energy transduction
Paul King, National Renewable Energy Laboratory

11:30 – 12:30PM Featured Crossover Talk: Light-driven nitrogenase
Lance Seefeldt, Utah State University

12:30 – 03:30PM *Lunch and Afternoon Break*

03:30 – 04:30PM Open Forum with DOE BES Program Managers
Steve Herbert, Gail McLean, Bob Stack

Session V: *CO_2 assimilation. Moderator: Asaph Cousins, Washington State University*

04:30 – 05:00PM Structure and function of the CO_2 uptake NDH-1 complexes in cyanobacteria
Rob Burnap, Oklahoma State University

05:00 – 05:30PM Structure and function of Rubisco activase from higher plants
Rebekka Wachter, Arizona State University

Tuesday, November 14

05:30 – 07:30PM *Dinner on your own*

07:30 – 10:00PM Poster Session 2: Even-numbered posters
(Refreshments may be purchased at the hotel bar.)

Wednesday, November 15

7:30 – 8:30AM *Continental Breakfast*

Session VI: *Self Assembly. Moderator: Kathy Osteryoung, Michigan State University*

08:30 –9:00AM Redox processes that regulate assembly and repair of the photosynthetic apparatus in cyanobacteria.
Himadri Pakrasi, Washington University

09:00 – 09:30AM Thylakoid assembly and folded protein transport by the chloroplast twin arginine translocation (cpTat) pathway.
Carole Dabney-Smith, Miami University

09:30 – 10:00AM TBA
Steven Theg, University of California at Davis

10:00 – 10:30AM *Coffee Break*

10:30 – 11:00AM Novel mechanism regulating H_2 photoproduction by *Chlamydomonas reinhardtii* upon transition from dark anaerobiosis to light aerobiosis
Maria Ghirardi, National Renewable Energy Laboratory

11:00 – 11:30AM TBA
Helmut Kirchhoff, Washington State University

11:30 – 12:00PM *Final Discussion and Meeting Ends*

Posters

Posters are listed alphabetically by presenter. Odd numbered posters will be presented Monday evening. Even-numbered posters will be presented Tuesday evening.

1. Tracking photochemical and photophysical processes of Photosystem I via multidimensional electronic and vibrational spectroscopic methods.
   Jessica Anna, University of Pennsylvania

2. Water oxidation in Photosystem II characterized by a combined computational and experimental study of $^{16}O/^{18}O$ and H/D kinetic isotope effects
   Victor Batista, Yale University

3. Energy transfer and radiationless decay in light-harvesting proteins
   Warren Beck, Michigan State University

4. Regulation of thylakoid lipid biosynthesis in plants
   Christoph Benning, Michigan State University

5. Molecular mechanisms of action of the cyanobacterial orange carotenoid protein
   Bob Blankenship, Washington University

6. Photodamage and repair in higher plant photosynthesis
   Terry Bricker, Louisiana State University

7. *Synechocystis* Flv1 and Flv2 flavodiiron proteins function in acclimation of photosynthetic electron transfer to changing electron flow
   Katherine Brown, National Renewable Energy Lab

8. Substitution of the D1-N87 site in Photosystem II of cyanobacteria mimics the chloride-binding characteristics of spinach Photosystem II
   Gary Brudvig, Yale University

9. Light energy transduction in green sulfur bacteria
   Daniel Canniffe, Pennsylvania State University

10. Photosynthetic reduction of carbon dioxide: biochemical analysis of Rubisco, phosphoenolpyruvate carboxylase, and carbonic anhydrase
    Asaph Cousins, Washington State University

11. Thylakoid assembly and folded protein transport by the chloroplast twin arginine translocation pathway
    Carole-Dabney-Smith, Miami University

12. Membrane-attached electron carriers in photosynthesis and respiration: biogenesis of a cytochrome complex from a facultative photosynthetic bacterium
    Fevzi Daldal, University of Pennsylvania

Posters, continued

13. Resolving protein-semiquinone interactions by advanced EPR spectroscopy: the Q_A and Q_B sites of the bacterial reaction center
Sergei Dikanov, University of Illinois, Urbana-Champaign

14. Regulation of photosynthetic light harvesting I
Graham Fleming, Lawrence Berkeley National Laboratory

15. Modular, controllable biomimetic systems to elucidate chromophore-protein interactions that facilitate photosynthetic light harvesting
Naomi Ginsberg, Lawrence Berkeley National Laboratory

16. Time-resolved step-scan FTIR differences spectroscopy for the study of Photosystem I
Gary Hastings, Georgia State University

17. Protein targeting to the chloroplast thylakoid membrane: structure and function of a targeting complex
Ralph Henry, University of Arkansas

18. Photosynthetic energy capture, conversion, and storage: from fundamental mechanisms to modular engineering. Project B: Construction and operation of the biological solar panel
Greg Howe, Michigan State University

19. Mutants of light harvesting antennas and reaction centers: disorder, excitonic structure, electron transfer, and excitation energy transfer dynamics
Ryszard Jankowiak, Kansas State University

20. Characterization and engineering subcellular and cellular modules for photosynthetic productivity
Cheryl Kerfeld, Michigan State University

21. Controlling electron transfer pathways in photosynthetic proteins
Christine Kirmaier, Washington University

22. The dynamic energy budget of photosynthesis
David Kramer, Michigan State University

23. Photosynthetic energy capture, conversion, and storage: From fundamental mechanisms to modular engineering. Project A: Robust photosynthesis in dynamic environments
David Kramer, Michigan State University

24. Non-canonical iron-sulfur clusters and electron transfer
Carolyn Lubner, National Renewable Energy Laboratory

Posters, continued

25. Molecular genetic analysis of Fe and Mn homeostasis in green algae
Sabeeha Merchant, University of California, Los Angeles

26. Mechanistic principles for hydrogen catalysis and proton-coupled electron transfer by [FeFe]-hydrogenase
David Mulder, National Renewable Energy Laboratory

27. Molecular chemistry for photosynthetic biohybrids
Karen Mulfort, Argonne National Lab

28. Regulation of photosynthetic light harvesting II
Kris Niyogi, Lawrence Berkeley National Lab

29. Multidimensional spectroscopies for probing coherence and charge separation in Photosystem II
Jennifer Ogilvie, University of Michigan

30. The chloroplast division machine: toward a mechanistic and structural model
Katherine Osteryoung, Michigan State University

31. Spin-quantum effects as a tool for resolving optimal pathways for proton-coupled ET in photosynthesis
Oleg Poluektov, Argonne National Laboratory

32. The homodimeric photosynthetic reaction center of *Heliobacterium modesticaldum*
Kevin Redding, Arizona State University

33. Accessing structure and dynamics of photosynthetic pigment-protein complexes by time-resolved circular dichroism spectroscopy
Sergei Savikhin, Purdue University

34. Interrogating protein-protein association through spectroscopic studies of model membranes
Gabriela Schlau-Cohen, Massachusetts Institute of Technology

35. Correlating structure and charge accumulating function in photosynthesis and photosynthetic hybrids
David Tiede, Argonne National Laboratory

36. Signal transduction pathways of chloroplast quality control
Jesse Woodson, The Salk Institute

Posters, continued

37. Electronic structure of the Mn cluster in Photosystem II: X-ray absorption and emission spectroscopy using an XFEL
Vittal Yachandra, Lawrence Berkeley National Laboratory

38. Taking snapshots of photosynthetic water oxidation: room temperature structure of Photosystem II using crystallography at an XFEL
Junko Yano, Lawrence Berkeley National Laboratory

Abstracts

Abstracts for all talks and posters are given in alphabetical order by the PIs last name beginning on the next page.

Tracking Photochemical and Photophysical Processes of Photosystem I Via Multidimensional Electronic and Vibrational Spectroscopic Methods

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Email: jmanna@sas.upenn.edu; Web: http://annagroup.chem.upenn.edu/

Overall research goals: The overarching goal of the project is to elucidate the mechanism of energy and electron transfer that governs the efficient charge separation in cyanobacterial photosystem I complexes through applying ultrafast multidimensional spectroscopies in the visible and mid-IR spectral regions. Progress towards this goal will be made through three objectives: (1) map pathways and determine timescales of energy transfer among light harvesting chlorophylls, red chlorophylls, and the reaction center; (2) determine the identity of the initial charge separated state; and (3) investigate protein-cofactor interactions. By applying multidimensional spectroscopies in the visible and mid-IR spectral regions to photosystem I, we will alleviate spectral congestion, allowing for a more direct determination of energy transfer pathways and further insight into protein-cofactor interactions that may be important for charge separation and electron transfer.

Significant achievements 2016-2017: *Instrument Construction:* We have completed the construction of our two-dimensional (2D) electronic spectrometer and have performed initial measurements on BODIPY laser dyes to confirm proper functioning of the instrument and develop data analysis codes. From these initial studies, we have demonstrated a technique for extracting the dynamic Stokes shift from 2D electronic spectra, establishing a new method for extracting system-bath interactions from congested spectra. *Energy Transfer in PSI:* 2D electronic spectra of photosystem I complexes isolated from two different cyanobacteria were obtained (Fig. 1). To interpret the 2D spectra we applied a global analysis to extract the 2D Decay Associated Spectra (2D-DAS). The information obtained from the 2D-DAS spectra is currently being used to inform 2D-Evolutionary Associated Spectra (2D-EAS) in order to extract rate constants associated with energy transfer among light harvesting chlorophylls, red chlorophylls, and the reaction center. *Protein-Phylloquinone Interactions:* As a first step to investigating

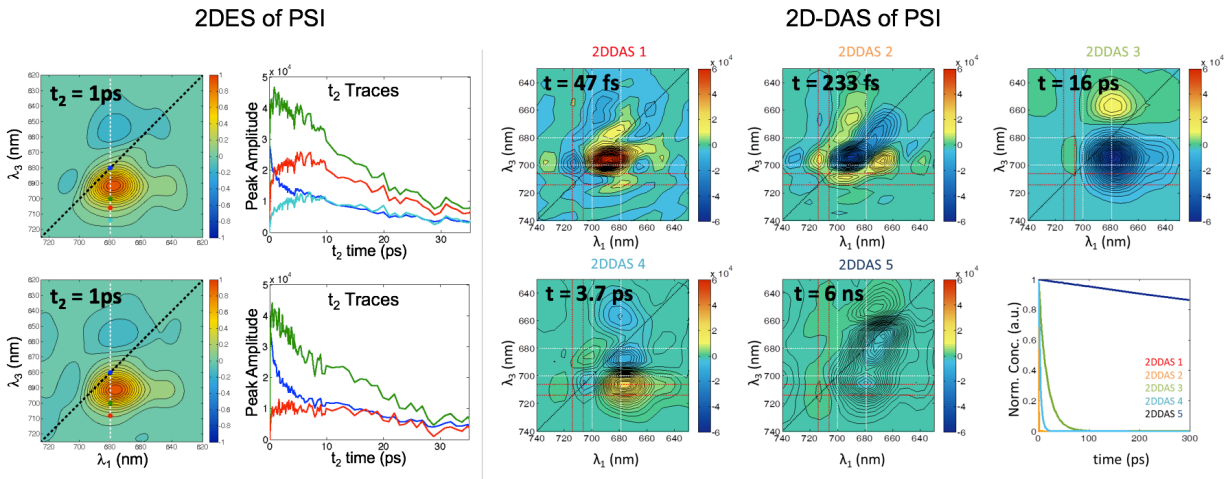

Figure 1. Left Panel: 2DES spectra of photosystem I isolated from PCC 6803 (top) and PCC 7002 (bottom) are shown in the first panel for t_2 = 1 ps. Middle Panel: Traces taken at different points in the 2D spectra. As the waiting time increases we see a growth and decay associated with excitation at 680 nm and detection in the red chlorophyll region. Right Panel: 2D-DAS spectra for photosystem I isolated from PC6803. 5 components are observed. Positive peaks correspond to a growth and negative peaks to a decay. The timescales associated with the DAS are indicated on the 2D-DAS spectra. 2D-DAS 1 and 2D-DAS 2 indicate energy equilibration in the bulk antenna. The position and sign of the peaks in 2D-DAS 4 indicate energy transfer to and from the red chlorophylls occurring on the ~4 ps timescale.

phylloquinone-protein interactions we have begun to characterize the vibrational modes of phylloquinone in various hydrogen bonding solvents. Using a combination of linear FTIR spectroscopy, molecular dynamics simulations, and quantum chemical calculations, we have assigned spectral features associated with hydrogen bonding to the carbonyl groups of the phylloquinone. We find that the relative intensities of carbonyl spectral features can be used as reporters for the asymmetry in hydrogen bonding to the phylloquinone carbonyl groups.

Science objectives for 2017-2018:

- We will apply a global kinetic analysis to the 2D electronic spectra and extract 2D-Evolutionary Associated Spectra in order to begin to determine the timescales of energy transfer and charge separation in photosystem I. Through comparison of the extracted rate constants we will gain further insight into whether or not cyanobacterial photosystem I is trap-limited or transfer-to-trap-limited. As a next step, 2D electronic spectroscopy will be performed on photosystem I complexes for which the reaction centers are chemically closed. These experiments could lead to further insight into the role that red chlorophylls play in light harvesting.
- We will expand on our phylloquinone work by applying 2D infrared spectroscopy to phylloquinones in various hydrogen bonding solvents. We will extract the dynamics associated with phylloquinone hydrogen bonding through analyzing the time-dependent changes in cross peaks and spectral line shapes in the 2D infrared spectra. From the molecular dynamics simulations performed to interpret the FTIR spectra, we will extract frequency-frequency correlation functions that will be used to model the 2D infrared spectra enabling a molecular level interpretation of our experimental results. These experiments will serve as a first step to applying 2D infrared spectroscopy to investigate phylloquinone hydrogen bonding dynamics in photosystem I complexes.
- Applying mixed spectral multidimensional spectroscopies, including 2D electronic vibrational (2DEV) spectroscopy and transient 2D infrared spectroscopy (T-2DIR) to photosystem I may help to (1) identify the initial charge separated state and (2) resolve how system-bath interactions (including hydrogen bonding) change upon the absorption of a photon. As a first step towards applying 2DEV and T-2DIR spectroscopy to photosystem I, we will construct these spectrometers by interfacing our 2D infrared spectrometer and 2D electronic spectrometer. We will perform initial experiments on metal-carbonyl systems to ensure that the experimental apparatus is functioning properly and develop data processing and analysis codes.

References to work supported by this project 2016-2017:

1. Y. Lee, S. Das, R.M. Malamakal, S. Meloni, D.M. Chenoweth, J. M. Anna, "Ultrafast Solvation Dynamics and Vibrational Coherences of Halogenated BODIPY Derivatives Revealed Through Two-Dimensional Electronic Spectroscopy" Journal of the American Chemical Society, Article ASAP (DOI**:** 10.1021/jacs.7b08558), (2017)

Water Oxidation in Photosystem II Characterized by a Combined Computational and Experimental Study of $^{16}O/^{18}O$ and H/D Kinetic Isotope Effects

Ke R. Yang, Sahr Khan, Gary W. Brudvig and Victor S. Batista

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Abstract. The mechanism of water oxidation in photosystem II has remained elusive to direct characterization by experimental and theoretical studies. Mechanisms based on water nucleophilic attack (WNA) onto a terminal oxyl radical and oxyl-oxo coupling (OOC) have been proposed. However, they have not been assessed as directly compared to experimental observables and calculations of kinetic isotope effects. Here, we report QM/MM calculations of O-O bond formation and direct comparisons to the experimentally determined $^{16}O/^{18}O$ and H/D kinetic isotope effects (KIE) and the catalytic cycle of water oxidation turnover frequency (one cycle per 1–2 ms). We find that the WNA mechanism involves a reaction time of 1 ms, as predicted by transition state theory ($\Delta G^{\ddagger}$=13.3 kcal mol^{-1}), and KIE values of 1.025 for $^{16}O/^{18}O$ and 2.025 H/D, consistent with experiments. In contrast, the OOC mechanism predicts observables that are inconsistent with experimental measurements, including a reaction time of 70 ns ($\Delta G^{\ddagger}$=7.7 kcal mol^{-1}), $^{16}O/^{18}O$ KIE of 1.036, and H/D KIE of 0.914. Therefore, the reported study represents the first consistent characterization of the O-O bond formation mechanism in PS II, shedding light on the machinery of water oxidation by natural photosynthesis which can provide valuable guidelines for the development of artificial photosynthetic systems.

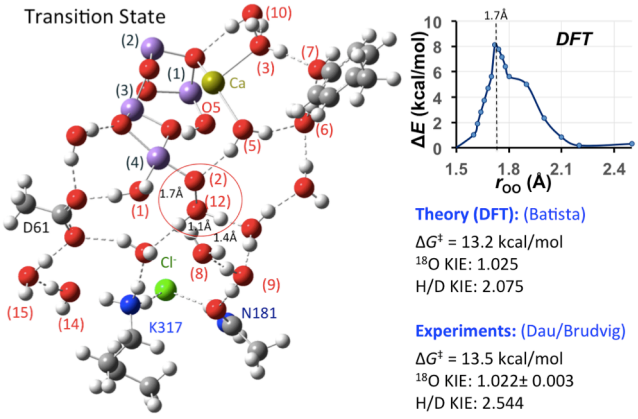

Figure 1: QM/MM model of the transition state for O-O bond formation at the OEC of PSII, based on the water nucleophilic attack (WNA) mechanism (left) and comparison to experimental kinetic data (right), including $^{16}O/^{18}O$ and H/D kinetic isotope effects (KIE) and activation free energy values based on transition state theory.

Publications supported by Grant # DESC-0001423 (VSB) during FY 2015-2017:

[1] M. Askerka, G. W. Brudvig, and V. S. Batista, "The O2-Evolving Complex of Photosystem II: Recent Insights from Quantum Mechanics/Molecular Mechanics (QM/MM), Extended X-ray Absorption Fine Structure (EXAFS), and

Femtosecond X-ray Crystallography Data.," *Acc. Chem. Res.* **50**: 41–48, (2017).
[2] M. Amin, M. Askerka, V. S. Batista, G. W. Brudvig, and M. R. Gunner, "X-ray Free Electron Laser Radiation Damage through the S-State Cycle of the Oxygen-Evolving Complex of Photosystem II.," *J. Phys. Chem. B*, p. acs.jpcb.7b08371, Sep. 2017.
[3] J. Wang, M. Askerka, G. W. Brudvig, and V. S. Batista, "Insights into Photosystem II from Isomorphous Difference Fourier Maps of Femtosecond X-ray Diffraction Data and Quantum Mechanics/Molecular Mechanics Structural Models", *ACS energy Lett.*, **2**: 397–407 (2017).
[4] M. Askerka, J. Wang, D. J. Vinyard, G. W. Brudvig, and V. S. Batista. "Energetics of the S_2 State Spin Isomers of the Oxygen-Evolving Complex of Photosystem II", *J. Phys. Chem. B* **121**: 1020-1025 (2017).
[5] J. Wang, M. Askerka, G. W. Brudvig, V. S. Batista, "Crystallographic Data Support the Carousel Mechanism of Water Supply to the Oxygen-Evolving Complex of Photosystem II *ACS Energy* **2**: 2299-2306 (2017).
[6] J. Wang, G. W. Brudvig, V. S. Batista and P. B. Moore, "On the Relationship Between Cumulative Correlation Coefficients and the Quality of Crystallographic Data Sets" *Protein Sci.* xxx: xxxx-xxxx (2017) [DOI: 10.1002/pro.3314].
[7] D. J. Vinyard, M. Askerka, R. J. Debus, V. S. Batista, and G. W. Brudvig, "Ammonia Binding in the Second Coordination Sphere of the Oxygen-Evolving Complex of Photosystem II.," *Biochemistry* **55**: 4432–4436 (2016).
[8] M. Askerka, J. Ho, E. R. Batista, J. A. Gascón, and V. S. Batista, "The MOD-QM/MM Method: Applications to Studies of Photosystem II and DNA G-Quadruplexes.," *Methods Enzymol.* **577**: 443–481 (2016).
[9] M. Askerka, J. Wang, D. J. Vinyard, G. W. Brudvig and V. S. Batista , "The S_3 State of the O_2-Evolving Complex of Photosystem II: Insights from QM/MM, EXAFS and Femtosecond X-ray Diffraction" *Biochemistry* **55**: 981-984 (2016).
[10] M. Askerka, D. J. Vinyard, G. W. Brudvig, and V. S. Batista, "NH_3 Binding to the S_2 State of the O_2-Evolving Complex of Photosystem II: Analogue to H_2O Binding during the $S_2 \rightarrow S_3$ Transition.," *Biochemistry* **54**: 5783–5786 (2015).
[11] L.Vogt, M. Zahid Ertem, R. Pal, G. W. Brudvig and V. S. Batista, "Computational Insights on Crystal Structures of the Oxygen-Evolving Complex of Photosystem II with either Ca^{2+} or Ca^{2+} Substituted by Sr^{2+}" *Biochemistry* **54**: 820-825 (2015).
[12] M. Askerka, J. Wang, G. W. Brudvig and V. S. Batista, "Analysis of the Radiation-Damage-Free X-ray Structure of Photosystem II in Light of EXAFS and QM/MM Data", *Biochemistry* **54**: 1713-1716 (2015).
[13] I. Rivalta, K. R. Yang, G. W. Brudvig and V. S. Batista, "Triplet Oxygen Evolution Catalyzed by a Biomimetic Oxomanganese Complex: Functional Role of the Carboxylate Buffer", *ACS Catalysis* **5**: 2384-2390 (2015).
[14] M. Askerka, D. Vinyard, G. W. Brudvig and V. S. Batista, "NH_3 Binding to the S_2 State of the Oxygen Evolving Complex of Photosystem II: Analogue to H_2O Binding during the S_2-S_3 Transition", *Biochemistry* **54**: 5783–5786 (2015).
[15] S. Khan, K. Yang, M. Zahid Ertem, V. S. Batista and G. W. Brudvig, "Mechanism of Manganese-Catalyzed Oxygen Evolution from Experimental and Theoretical Analyses of ^{13}O Kinetic Isotope Effects", *ACS Catalysis* **5**: 7104-7113 (2015).

Energy Transfer and Radiationless Decay in Light-Harvesting Proteins

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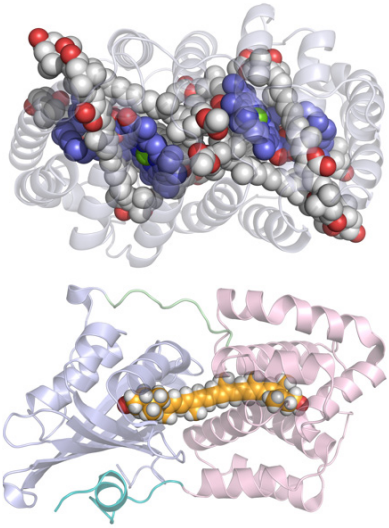

Overall research goals: This project will determine the photophysical and photochemical mechanisms that are involved in excitation energy transfer and photoprotection in two systems that bind carbonyl-substituted carotenoids:

1. the peridinin–chlorophyll *a* protein (PCP), a mid-visible light-harvesting complex in marine dinoflagellates in which peridinin serves as the principal light absorber and as an excitation energy donor to chlorophyll *a* acceptors.
2. the orange carotenoid protein (OCP), which mediates nonphotochemical quenching (NPQ) mechanisms in cyanobacteria using 3'-hydroxyechinenone (3'-hECN) or canthaxanthin (CAN) as a quencher of bilin excited states in the phycobilisome.

In both systems, the role of the electron withdrawing character of the carotenoid's carbonyl substituent is considered crucial in energy transfer and photoprotection mechanisms because it promotes the formation of intramolecular charge-transfer (ICT) character, which increases the efficiency of energy transfer and produces a low-lying electronic state that can serve as a quenching state. The planned experiments employ two-dimensional electronic spectroscopy (2DES) with broadband (<10 fs) pulses to characterize nonradiative decay and energy transfer mechanisms in PCP, OCP, and in solutions of their component carotenoids.

Significant achievements 2015–2017:

- Nonradiative decay mechanisms for β-carotene and peridinin in solution were characterized with heterodyne transient grating spectroscopy (3–5). A distorted conformation of the S_2 ($1^1B_u^+$) state, S_x, was identified as a key nonradiative decay intermediate. The solvent dependence of the lifetime of S_x for peridinin indicates that large-amplitude torsional and pyramidal motions of the conjugated polyene backbone promote the formation of ICT character and control the rate of nonradiative decay to the S_1 ($2^1A_g^-$) state (5).
- Heterodyne transient grating (6) and broadband 2DES spectra (7) were obtained for PCP from wild-type preparations containing Chl *a* and from reconstituted preparations containing Chl *b*. The results provide direct evidence for the involvement of quantum coherence in extraordinarily fast energy transfer pathways between peridinins and between peridinin and Chls. The quantum coherence is very short lived, however, due to the action of strongly coupled vibrational motions that promote formation of the S_x state and ICT character in <20 fs (6). The resulting enhanced system–bath coupling causes dynamic exciton localization and the onset of energy transfer from peridinin to Chl via the incoherent, Förster mechanism (6,7).

- Continuous-wave fluorescence spectra were observed for the first time in OCP preparations containining 3'-hydroxyechinenone (3hECN) and canthaxanthin (CAN) (8). The quantum yield of fluorescence is enhanced for 3hECN because it makes a hydrogen bond between its 3'-hydroxyl group and the peptide carbonyl of Leu37. This finding indicates that large-amplitude distortions of the β-cyclohexene end rings and of the conjugated polyene backbone of the bound carotenoid initiate photoactivation of OCP to the quenching OCP^R state.

Science objectives for 2017–2018:

- Determination of peridinin–peridinin energy transfer pathways from 2DES signals from PCP with Chl *a* or Chl *b*.
- Determination of nonradiative decay pathways for carotenoids in OCP^O using 2DES spectra.
- Determination of the role of ground-state conformers in the photoactivation mechanism and photoactivation quantum yield for OCP^R using fluorescence excitation and anisotropy spectra.

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Regulation of Thylakoid Lipid Biosynthesis in Plants

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Overall Research Goal: The conversion of sun light into chemical energy by plant photosynthesis requires a specialized photosynthetic membrane forming the thylakoids inside chloroplasts. The focus here is on the polar lipid components of the photosynthetic membrane, *i.e*. biosynthesis, turnover and regulation, thereof, and specific function. The long-term objective is to gain a mechanistic understanding of the apparent self-assembly and self-repair of the photosynthetic membrane and the essential role polar lipids play in this process.

Significant Achievements (2016 – 2017):

1. Membrane lipid biosynthesis and turnover have to be finely tuned to build and maintain the photosynthetic membrane. In recent years, we increasingly focused on the function of chloroplast lipases in addition to lipid-biosynthetic enzymes. We determined the biochemical and physiological function of an Arabidopsis thylakoid membrane-associated lipase, PLASTID LIPASE 1 (PLIP1), which is a phospholipase A_1. In vivo, PLIP1 hydrolyzes polyunsaturated acyl groups from a unique chloroplast-specific phosphatidylglycerol that contains $16{:}1^{\Delta 3trans}$ as its second acyl group (carbon number: double bond, here 3 carbons from the carboxyl end in *trans* configuration). The *PLIP1* gene is highly expressed in seeds, and *plip1* mutant seeds contain less oil and exhibit delayed germination. PLIP1 releases acyl groups that upon export from the chloroplast, incorporate into phosphatidylcholine and ultimately enter seed triacylglycerol. Thus, $16{:}1^{\Delta 3trans}$ uniquely labels a small but biochemically active plastid phosphatidylglycerol pool in Arabidopsis developing embryos, which is subject to PLIP1 activity, thereby contributing a fraction of the polyunsaturated fatty acids present in the seed oil. Based on these findings, a novel hypothesis was developed ascribing a role for acyl exchange on chloroplast lipids to serve acyl export and seed oil biosynthesis. The Arabidopsis genome also encodes two paralogs of PLIP1, PLIP2 and 3. Both are also located in the chloroplasts but have different suborganellar locations. PLIP3 primarily uses phosphatidylglycerol as substrates, while PLIP3 prefers monogalactosyldiacylglycerol. The expression of *PLIP2* and *3* is induced by abscisic acid. We are currently investigating the role of these two chloroplast lipases in stress adaptation.

2. We are investigating the biosynthesis and function of a specific chloroplast-localized lipid species of phosphatidylglycerol (PG) containing the unusual acyl group, $16{:}1^{\Delta 3t}$. We identified a thylakoid redox protein PEROXIREDOXIN Q (PRXQ) in Arabidopsis that is required for FATTY ACID DESATURASE4 (FAD4)-mediated synthesis of $16{:}1^{\Delta 3t}$. FAD4, a PG-specific desaturase that is also likely located in the thylakoid membranes, is evolutionary distinct from other membrane desaturases and its 16:1t product is found in almost all seed plants. PRXQ was previously shown to reduce harmful reactive oxygen species to water in Arabidopsis through the activity of two cysteines. Site-directed mutagenesis of these active-site cysteines could not fully restore $16{:}1^{\Delta 3t}$ levels in *prxq* transgenic lines, demonstrating its reducing activity is required for FAD4 to function. To test whether FAD4 required disulfide bonds that could be reduced by PRXQ, we mutated FAD4 cysteines and only found one cysteine required for $16{:}1^{\Delta 3t}$ synthesis. Therefore, assuming FAD4 is a dimer, it might require an inter-monomer disulfide bond to be fully active, and PRXQ might alter the redox status of FAD4 as a means to regulate its capacity to capture oxygen via desaturation. We are investigating this redox mechanism and whether this pathway is an outlet for plants to relieve potential oxygen-mediated metabolite damage as a byproduct of photosynthesis.

3. In plant lipid metabolism, the synthesis of many intermediates or end products appears often overdetermined with multiple synthesis pathways acting in parallel. Lipid metabolism is also dynamic with interorganelle transport, turnover, and remodeling of lipids. To explore this complexity in vivo, we probed lipid metabolism in Arabidopsis by heterologously expressing a coding sequence for a fatty acid desaturase from *Physcomittrella patens* (Δ6D), which places a double bond after the 6th carbon from the carboxyl end of an acyl group attached to phosphatidylcholine at its *sn*-2 glyceryl position. Phosphatidylcholine is a key intermediate in plant lipid metabolism as it is modified and converted to precursors for other lipids throughout the plant cell. Taking advantage of the exclusive location of Δ6D in the endoplasmic reticulum (ER) and its known substrate specificity for one of the two acyl groups on phosphatidylcholine, we were able to "tag and track" the distribution of lipids within multiple compartments and their remodeling in transgenic lines of different genetic backgrounds. Key findings were the presence of ER-derived precursors in plastid phosphatidylglycerol and prevalent acyl editing of thylakoid lipids derived from multiple pathways. A paper describing this work is currently under review.

4. We are studying the lipid regulatory role of a rhomboid protease presumed to be located in the inner chloroplast envelope membrane of Arabidopsis. A loss-of-function mutant has a lipid phenotype similar to the *ats1* mutant compromised in plastid lipid assembly with increased import of lipid precursors from the ER. Pulse chase label experiments with acetate show strong reduction in the labeling of the predominant thylakoid lipid monogalactosyldiacylglycerol in the chloroplast. We are characterizing the recombinant protein and are in the process of identifying the specific protein target(s) for this protease.

Science Objectives for (2017-2018):

1. We will complete the analysis of two paralogs of PLIP1, PLIP2 and 3, elucidating their biochemical reaction and their role in abiotic stress resistance.
2. We will determine the role of peroxiredoxin Q in the biosynthesis of 16:1$^{\Delta 3t}$ and its interaction with FAD4. In addition we plan to determine the reaction mechanism for FAD4 to pursue preliminary evidence that this mechanism may deviate from that of classic fatty acid desaturases.
3. Using *prxq* and *fad4* mutants, we will explore the role of plastid PG, which contains 16:1$^{\Delta 3t}$, in the mitigation of ROS damage in the photosynthetic membrane.
4. We will study the role of a chloroplast membrane-associated rhomboid protease in the regulation of thylakoid lipid metabolism.

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Molecular Mechanism of Action of the Cyanobacterial Orange Carotenoid Protein

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Overall research goals:

This project centers on the Orange Carotenoid Protein (OCP) that is found in cyanobacteria and quenches excited states in the phycobilisome (PBS) antenna complex. The OCP is a soluble photoactive protein and the first identified photoactive protein that binds a carotenoid molecule as the pigment. The OCP is found in an inactive orange form in the dark but is converted into a red form by excess light. The red form binds to the PBS and quenches excited PBS chromophores, providing photoprotection. A second protein called the Fluorescence Recovery Protein (FRP) serves to restore the active red form of OCP to the inactive orange form. The overall goal of this research project is to give us a much-improved understanding of the photoactivation and mechanism of action of this important photoprotection process at the molecular level and also improve our understanding of its physiological roles in cyanobacterial adaptation to changing environmental conditions.

Significant achievements:

Functional Domains of OCP. The Orange Carotenoid Protein (OCP) can be cleanly cleaved into N and C-terminal domains by trypsin treatment at low temperature (Fig. 1). The stability and unfolding of these domains was probed using native mass spectrometry with collisional activation as well as ion mobility analysis. The results show that the NTD releases its bound carotenoid without forming any intermediates and the CTD is resistant to unfolding upon collisional energy ramping.

Oligomerization State of OCP and FRP. Native mass spectrometry was used to investigate the oligomerization state of OCP and FRP as a function of concentration. OCP was found to be present as a monomer at low concentrations and to form dimers and higher oligomers at high concentrations (Fig. 2), whereas FRP was found to be dimeric even at low concentrations.

Science objectives for 2017-2018:

We are currently focusing on obtaining a better understanding of the interaction site of the OCP with the phycobilisome, using a variety of phycobilisome mutants, chemical crosslinking and mass spectrometry analysis.

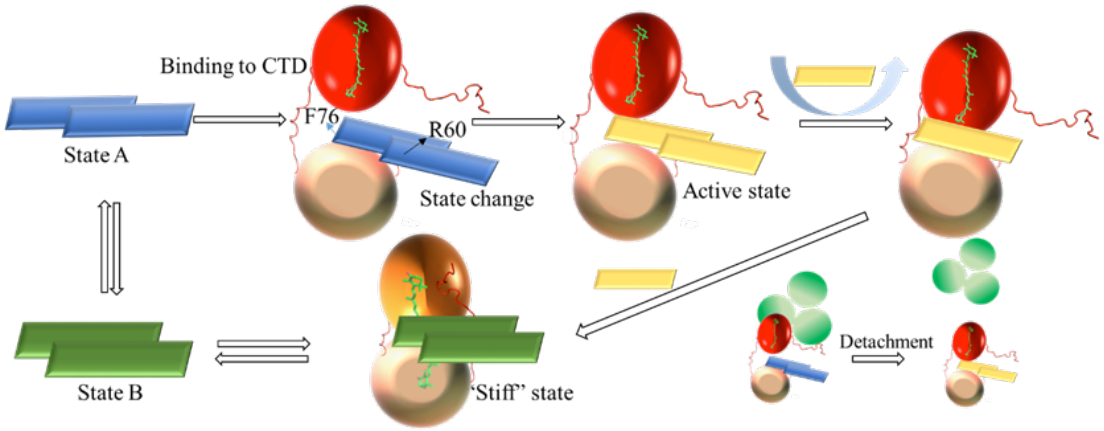

Figure 1. Proposed model of the interaction between FRP and OCP. FRP is shown as a rectangle with different colors corresponding to different states. The OCP NTD is shown as a red sphere in the red state and an orange sphere in the orange state. The OCP CTD is shown as a wheat-colored sphere. The APC core from the PBS is shown as three green circles. In the first step, dimeric FRP approaches and binds the CTD, inducing a conformational change in FRP (likely unfolding of the head region) and allowing its binding to the NTD. dFRP forms a stable complex with OCPr by bridging the two domains. At the stage, OCPr can be detached from PBS by FRP. The cooperative action of the linker region and the N-terminal arm facilitates the dissociation of the FRP dimer. Monomeric FRP is more flexible and effectively facilitates the closing up of the two OCP domains. Finally, the accelerated conversion process reaches completion, and dFRP weakly associates with OCPo around the linker region. The "capped" N-terminal arm on the CTD inhibits the dFRP from entering the cavity.

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Photodamage and Repair in Higher Plant Photosynthesis

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Overall research goals: Our proposal centers on two principal aims. First, we will identify the sites of reactive oxygen species (ROS) generation in the linear chain electron transport complexes. ROS are responsible for damage to Photosystem II (PS II) and Photosystem I (PS I) and are produced in significant quantities by the cytochrome b_6f complex (b_6f complex). With respect to PS II, we have hypothesized that amino acid residues in the vicinity of the sites of ROS production would be particularly susceptible to ROS modification, and this hypothesis has been strongly supported by recent experiments. We will extend our studies to the b_6f complex and PS I in higher plants, both identifying residues which exhibit native oxidative modifications and examining the formation of newly oxidized residues under stress and/or photoinhibitory conditions. Second, we will examine PS II repair complexes in higher plants, using non-detergent styrene-maleic acid copolymer (SMA) solubilization methods.

Significant achievements 2016-2017: 1. The timecourse for formation of oxidative modifications in PS II (Fig. 1, Left Panel) during photoinhibition was determined. Our findings indicate that during the photoinhibition, oxidative damage in the vicinity of the Mn_4O_5Ca cluster precedes damage occurring on the reducing-side of the photosystem (i.e. in the vicinity of $Pheo_{D1}$, Q_A and Q_B). In particular, D1:^{332}H, a ligand to Mn1 of the cluster, is oxidized quite early in the photoinhibition timecourse (Kale, et al., 2017). 2. Initial investigations examining the oxidative modifications natively present in the spinach b_6f complex were completed (Taylor et al., 2017). Our results indicated that numerous residues, principally localized near *p*-side cofactors and Chl *a*, were oxidatively modified (Fig. 1, Right Panel). We hypothesize that these sites are sources for ROS generation in the spinach cytochrome b_6f complex.

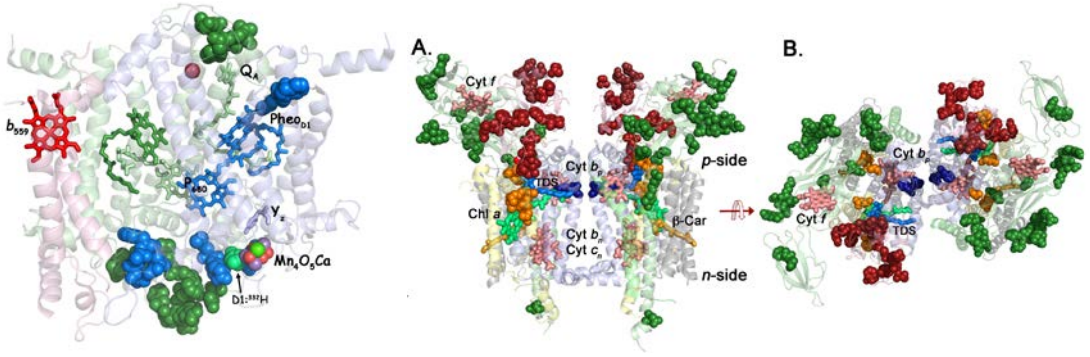

Figure 1. Left Panel: Oxidative modification of Amino Acid Residues in PS II. Subunits: D1, pale blue; D2, pale green; cytochrome b_{559}, pink. Modified residues are shown as darker spheres and mapped onto the spinach PS II structure. PS II cofactors are labeled and shown in stick representation. **Right Panel: Oxidized Residues in the Spinach b_6f Complex.** A. Side view of complex from within the plane of the membrane. B. Lumenal (*p*-side) view of the complex. Subunits: PetA (pale green), PetB (pale blue), PetC (pink), PetD (pale yellow) and the small subunits (grey). Modified residues are shown as darker spheres and were mapped onto their corresponding locations within the *Chlamydomonas reinhardtii* b_6f structure. Cofactors and TDS are shown in stick representation and are labeled.

Science objectives for 2016-2017:

- Continue examination of oxidative modifications in the spinach cytochrome b_6f complex. We will begin $^{18}O_2$ studies that will allow the elucidation of the timecourse of oxidative events occurring in the complex.
- Continue characterization of PS II assembly complexes obtained after SMA solubilization of photoinhibited spinach.
- Initiate studies identifying natively oxidative modifications in PS I. Perform preliminary studies examining PS I photoinhibition under chilling conditions in cucumber.
- Initiate experiments utilizing protein crosslinking to examine the molecular interaction between the cytochrome b_6f complex and Ferredoxin-$NADP^+$ oxidoreductase.

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Pulse EPR Studies of Substrate Binding at the OEC of Photosystem II

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We are interested in studying the mechanism of water oxidation by the Mn-Ca oxygen evolving complex of Photosystem II. Even with the current high-resolution X-ray structures, there is ambiguity in the substrate binding motif of the oxygen evolving complex. A core issue here is that the substrate, water, is also the solvent, and there are multiple potential water sites in close proximity to the OEC, as well as water-derived oxygen bridges within the cluster. High resolution pulse EPR methods such at electron nuclear double resonance (ENDOR), and 1 and 2 dimensional Fourier Transform methods (ESEEM and HYSCORE), can be used to probe the geometrical and electronic structure of ligands to the OEC, including water along with water analogs and inhibitors. In particular, we have been revisiting the mode of ammonia binding to the OEC, both monitoring the electronic structure of the two available ammonia nitrogen isotopes, ^{14}N and ^{15}N, as well as the proximity of both exchangeable and non-exchangeable hydrogen hydrogen sites at the cluster before and after ammonia binding. Implications as to the mechanism of water oxidation will be discussed. In addition, advantages of carrying out pulse EPR studies at multiple frequencies will be discussed, including new pulse EPR instrument developments at 130 and 263 GHz frequencies in the CalEPR laboratory at UC-Davis.

Synechocystis Flv1 and Flv3 Flavodiiron Proteins Function in Acclimation of Photosynthetic Electron Transfer to Changing Electron Flow

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Overall research goals: The objective of this area of research is to study how flavodiiron enzymes contribute to the peripheral redox reactions that support cellular adaption to changing photochemical flux in *Synechocystis* sp. PCC 6803. The physiological role of the soluble flavodiiron enzymes Flv1 and Flv3 has been previously characterized as a protective mechanism for the photosynthetic electron transfer (PET) chain in extreme conditions of high and fluctuating light, but the role of these enzymes under normal growth conditions, and their contributions to the general modulation of cellular redox state remain to be elucidated. We hypothesize that these enzymes may play a broader role in modulating the flux of photochemical energy to acclimate cells to changing conditions. We investigated the biochemical properties of Flv1 and Flv3 and the electron flow through the PET chain in wild-type (WT) and Δ*flv3*, Δ*flv1* and Δ*flv1*Δ*flv3* strains of *Synechocystis* sp. PCC 6803.

Significant achievements 2015-2017: Recombinantly produced Flv1 and Flv3 were purified and found to form homodimers. Both Flv1 and Flv3 were shown to catalyze the reduction of O_2 by direct oxidation of either NADH or NADPH. The K_M values for pyridine nucleotide oxidation for both enzymes were found to be adapted for catalyzing O_2 reduction at the physiological concentrations of reduced pyridine nucleotides. This result suggests that Flv1 and Flv3 have a broad role in managing the pyridine nucleotide pools during photosynthetic growth. The apparent tuning of the Flv K_M for optimal use of both NADH and NADPH suggests that these enzymes function beyond the previously described photosystem I (PSI) protection role in high and fluctuating light, since PSI protection is usually achieved through NADPH oxidation to relieve acceptor side limitations. *In vivo*, the three deletion strains exhibited reduced photosynthetic O_2 evolution capacity, suggesting a limitation on PSI acceptor availability, and a lack of function by either Flv homodimer *in vivo*. Long-term monitoring of PSI oxidation state showed distinct adaptations in Δ*flv3*, Δ*flv1* and Δ*flv1*Δ*flv3* strains compared to WT, and suggest that Flv catalyzed oxidation of reduced pyridine nucleotides and reduction of O_2 are essential in the adaptation of PET during the

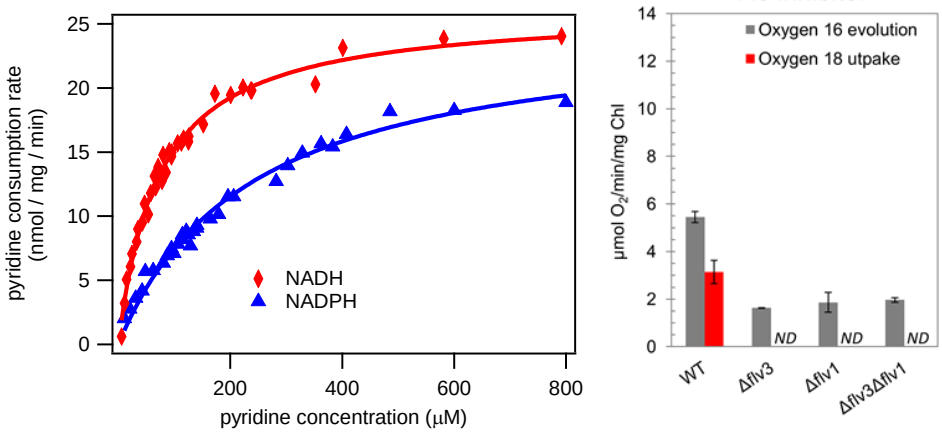

Figure 1. Left Panel: Pyridine nucleotide consumption rate by Flavodiiron 3 in air saturated buffer: Right Panel: O_2 evolution and consumption rates by wild-type (WT), Δ*flv3*, Δ*flv1* and Δ*flv1*Δ*flv3* strains of *Synechocystis* sp. PCC 6803

onset of light exposure. These results support our hypothesis that Flv1 and Flv3 have a broader function in maintaining electron flow as photochemical flux changes under dynamic growth conditions.

Science objectives for 2018-2019:

- The mechanism of O_2 reduction and the biophysical properties of cyanobacterial Flavodiiron proteins will be investigated by stopped-flow, UV-Vis spectroscopy, and H/D and $^{16}O/^{18}O$ isotope kinetics. We will use spectroelectrochemistry and redox titration EPR to measure the reduction potentials of the flavins (FMN) and diiron site to ascertain whether the different Flv proteins have differences in the thermodynamic properties.
- *Synechocystis* sp. PCC 6803 expresses 4 flavodiiron enzymes. Two, Flv2 and Flv4, have not yet been isolated and analyzed for the biochemical and kinetic properties. These enzymes will be recombinantly produced and purified and their activity and mechanism will be investigated for comparison to Flv1 and Flv3.
- The ability of flavodiiron enzymes to interact with redox partners other than pyridine nucleotides is now known. We are investigating the ability of reduced flavodoxin, ferredoxin and rubredoxin to function as electron donors to purified *Synechocystis* Flv's.

References to work supported by this project 2015-2017:

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Substitution of the D1-N87 Site in Photosystem II of Cyanobacteria Mimics the Chloride-Binding Characteristics of Spinach Photosystem II

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Abstract: Photosystem II (PSII) catalyzes sunlight-driven oxidation of water at the O_2-evolving complex (OEC) during photosynthesis, supplying nearly all the O_2 in our biosphere. An analysis of amino-acid residues in and around the OEC has identified residue 87 in the D1 subunit of PSII as the only significant difference between PSII in cyanobacteria and higher plants. We substituted the D1-N87 residue in the cyanobacterium *Synechocystis* sp. PCC 6803 (wild-type) with alanine, present in higher plants, or with aspartic acid. EPR spectra of the S_2 state and flash-induced FTIR spectra of both D1-N87A and D1-N87D PSII core complexes exhibited characteristics similar to those of wild-type *Synechocystis* PSII core complexes. However, flash-induced oxygen-evolution studies revealed decreased cycling efficiency of the D1-N87D variant, whereas the cycling efficiency of the D1-N87A PSII variant was similar to that of wild-type PSII. Steady-state O_2-evolution activity assays revealed that the alanine substitution of the D1-87 residue perturbs the chloride-binding site in the proton-exit channel. These findings provide new insight into the role of the D1-N87 residue in the water-oxidation mechanism and explain the difference in the chloride-binding properties of cyanobacterial and higher-plant PSII.

Overall research goals: The objective of this project is to characterize the water-oxidation chemistry catalyzed by the Mn_4CaO_5 cluster in the oxygen-evolving complex (OEC) of photosystem II (PSII) by using biophysical and spectroscopic methods to analyze the effects of point mutations, isotopic composition and inhibitors. Goals of the research are to determine how the local protein environment facilitates the water-oxidation activity. EPR spectroscopy and isotope effect measurements will be used to characterize the structure of the OEC and the mechanism of water oxidation. The effects of point mutations near the OEC that are hypothesized to perturb the mechanism of water oxidation and/or the hydrogen-bonding network surrounding the OEC will be determined, and studies of small molecule water analogues and anions will be carried out to gain insight into the substrate (water) and chloride binding sites.

Science objectives for 2017-2018:

- We aim to probe the mechanism of O-O bond formation by measuring $^{16}O/^{18}O$ kinetic isotope effects (KIE) of OEC turnover. These values will be measured when OEC turnover is limiting, in selected point mutants with slow O_2-release kinetics, and in the presence of D_2O.
- We will also determine and compare the effective pK_a's and H/D KIE's of point mutants in the hydrogen-bonding network surrounding the OEC to determine bottlenecks in proton release.
- Studies of small molecule water analogues and anions will be carried out to gain insight into the substrate (water) and chloride binding sites.
- Ammonia binds in two sites in the OEC: Site A directly on Mn and a second-shell site that is competitive with Cl^- and stabilizes the $g = 4.1$ S_2 state (Site B). We will investigate the "Site B" ammonia-binding site in point mutants that prevent Cl^- binding (D2-K317A and D1-N181A).

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Structure and Function of the CO_2 uptake NDH-1 Complexes in Cyanobacteria

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Overall research goals: The overall goal is to understand the CO_2 uptake and scavenging systems that are unique to cyanobacteria and enable them to concentrate CO_2 in the active site of Rubisco. Cyanobacteria are considered as ideal vehicles for numerous bioengineering applications ranging from CO_2 mitigation to the production of biofuels and chemicals. Importantly, the cyanobacteria have evolved a mechanism for scavenging CO_2 that could be exploited in engineering efforts aimed at the reduction of CO_2 emissions. This is because cyanobacteria have evolved enzymes that use redox energy to hydrate CO_2 to form bicarbonate. The enzymes could be used directly for bioengineered CO_2 absorption or used in the establishment of design principals for the fabrication of biomimetic CO_2 absorption devices. This project is basic research to understand cyanobacterial high affinity **CO_2-concentrating mechanism** (**CCM**), which efficiently supplies CO_2 to the photosynthetic mechanism of cyanobacteria. Essentially, it functions as a 'supercharger' for CO_2 by concentrating it within the cytoplasm of the cell, thereby saturating the active sites of the CO_2-fixation enzymes and thus increasing the efficiency of photosynthesis. After helping better define the suite of enzymes that constitute the CCM, the project is now focusing on the redox enzymes involved in CO_2-uptake. Specifically, we are using the lab expertise to understand the **specialized NDH-1 complexes that catalyze this powerful CO_2 uptake** mechanism.

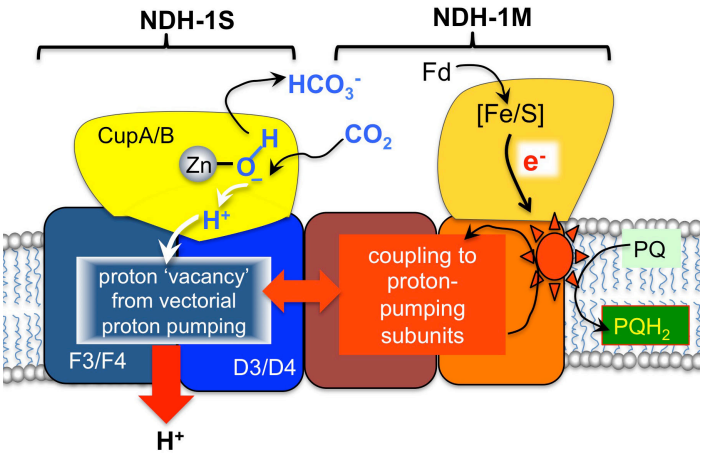

Figure 1. Hypothesis for electron transfer coupled to proton pumping coupled to CO_2-hydration. Hypothetical metal center, possibly Zn^{2+} ion. CupA/B may provide all or some of the ligands (likely His and/or Cys) to the metal center. The metal facilitates the de-protonation of substrate H_2O forming a hydroxide capable of nucleophilic attack upon incoming CO_2 as in the case of carbonic anhydrases. Proton pumping activity removes H^+ from the active site to trap the deprotonated metal hydroxide formed upon deprotonation of the metal-bound H_2O.

Virtually nothing is known about their mechanism apart from the fact that they are physically connected to NDH-1 complexes. A combined molecular genetic, biochemical, and biophysical approach is being used to address specific hypotheses on their mechanism. The **central hypothesis** is that a metal-containing carbonic anhydrase is situated adjacent to the proton pumping domain(s) of the NDH-1 complex, which drives the $H_2O + CO_2 \rightleftharpoons HCO_3^- + H^+$ reaction to the right by removal of the proton from the active site of CO_2-hydration.

Significant Achievements 2015-2017: **Construction and testing of a mutagenesis system.** The goal has been to develop the mutagenesis system to act as the genetic foundation to probe the structure-function relationships of the NDH-$1_{3/4}$ complexes regarding their CO_2-hydration and

putative proton pumping activities. After knocking out the full set of CUP genes (natively constitutive and inducible), an integration vector system was developed to re-introduce the CUP genes at a non-native (ectopic) location and under the constitutive expression of the rubisco promoter. The resultant ectopic construct strain was then tested for the ability to restore high affinity CO_2 uptake by measuring O_2 evolution while titrating in inorganic carbon.

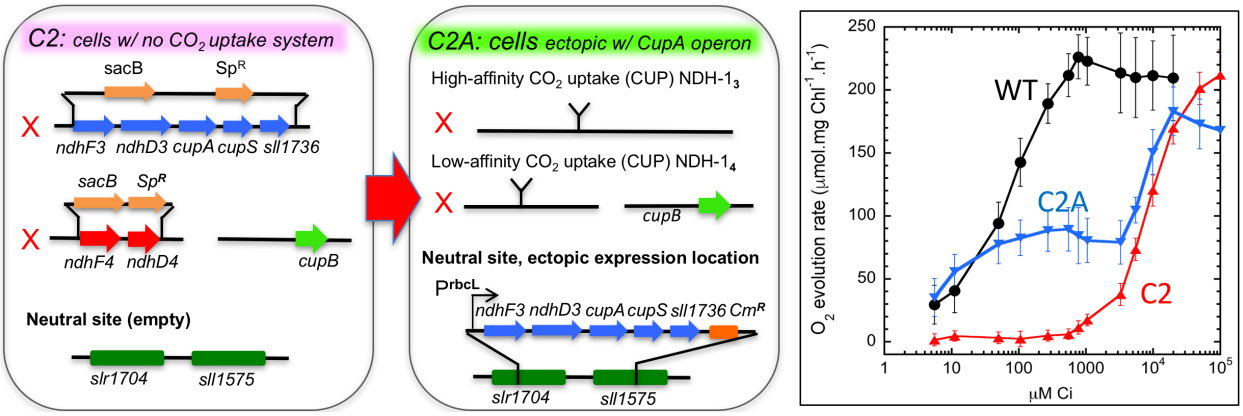

Figure 2. Cup-less strain, C2 and transformant C2A (Left Panels). The marker-less strain C2 lacks the gene regions for NDH-$1_{3,4}$ was the recipient of the ectopic construct where CUP operon (NDH-1_3) is under the control of a rubisco promoter integrated on a neutral site (C2 + CupA operon →C2A). **The C2A strain has a constitutive expression the high affinity, low flux NDH-1_3 system** (blue) compared to the full NDH-1_3 plus NDH-1_4 knockout (red), yet cells are incapable of both high affinity and high flux uptake observed in the wild-type (black) (**Right Panel**).

Other efforts include: 1) Heterologous expression, purification of the CupA protein for the production of antibodies, 2) development of spectroscopic assays to monitor proton pumping activity *in vivo*; 3) Continue analysis of regulation of CCM in biotechnological strains.

Science Objectives 2017-2018.

Site-directed mutagenesis of CupA protein and the NDH-1_3 proton pumping subunits. Use the above mutagenesis system to target amino acids to evaluate hypothesized involvement of His and/or Cys residues as ligands to coordinate the metal ion responsible for the proton-coupled carbonic anhydrase activity. In addition, the NdhD3 and NdhF3 subunits tinteract with CupA Because our hypothesis is that proton pumping evacuates the active site of protons to trap a deprotonated intermediate of CO_2 hydration (Fig. 1), we are also testing the role of the putative proton pumping subunits of the NDH-1_3 complex, which is feasible since these are also part of the ectopically engineered cupA operon in C2A (Fig. 2) and can be mutagenized.

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Light Energy Transduction in Green Sulfur Bacteria

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Overall research goals: The current research objectives are to identify genes encoding the few remaining unknown enzymes involved in the biosynthesis of (bacterio)chlorophyll ((B)Chl) and carotenoid pigments in phototrophic bacteria: (1) identify the enzyme responsible for the reduction of the common carotenoid precursors neurosporene and lycopene, producing unique dihydroneurosporene and dihydrolycopene in the BChl *b*-utilizing purple bacterium *Blastochloris* (*Blc*.) *viridis*, as well as assigning function to the gene products of additional paralogous '*crt*' genes in the recently sequenced genome; (2) identify the enzyme responsible for the production of dihydrochlorobactene in the green sulfur bacterium (GSB) *Chlorobaculum* (*Cba*.) *tepidum*, and analyze the distribution of this gene throughout the phylum *Chlorobi*; (3) identify the enzyme(s) responsible for the transformation of a methyl group into the formyl group at C7 on BChl *e*, a pigment that can assemble into chlorosomes, supramolecular, nanotubular structures acting as the major light-harvesting complexes in GSB. A similar reaction, a methyl to formyl group transformation at C7, allows plants to synthesize Chl *b*. However, no ortholog of the plant gene is found in the genomes of BChl *e*-producing GSB. Moreover, the plant enzyme uses molecular oxygen as a substrate, but GSB are obligate anaerobes and thus cannot use O_2 as a substrate.

Significant achievements 2016-2017: We have assigned functions to all of the predicted *crt* genes in the *Blc. viridis* genome, and ascertained that this organism contains a variant of the BChl biosynthesis gene *bchP*, encoding an enzyme that both reduces the alcohol moiety of BChl *b*, and reduces the 1,2 double bonds of neurosporene and lycopene, producing unique carotenoids in this organism. Many GSB contain a paralog of *bchP*, designated as *bchO*. We have determined that the enzyme encoded by *bchO* from both *Cba. tepidum* and *Cba. limnaeum* is a carotenoid 1,2 reductase, and that BchO is responsible for production of dihydrochlorobactene in *Cba. tepidum*.

Deletion of *bciD* in *Cba. limnaeum* prevents synthesis of BChl *e* while the strain accumulates BChl *c*, which has a methyl group at C-7. *In vitro* assays with recombinant BciD demonstrate that it is able to catalyze methyl to formyl transformations on both bacteriochlorophyllide *c* and bacteriochlorophyllide *d* in the absence of O_2, via sequential hydroxylations of the C7 methyl group, followed by spontaneous dehydration of the *geminal* diol to yield the formyl groups of BChl *e* and BChl *f*, respectively.

Scientific objectives for 2017-2018:

- The genome of *Blc. viridis* contains remnants of a more complex carotenoid biosynthesis pathway that is common to most purple bacteria. We aim to determine the selective advantage gained by the mutation of *bchP*, and thus the use of dihydrocarotenoids, in *Blc. viridis* by restoring the common pathway and comparing the carotenoid-to-BChl energy transfer efficiencies in modified strains to those of the type strain.
- The brown-colored GSB *Cba. limnaeum* contains paralogous genes encoding enzymes involved in modification of both the C8 and C17 positions. We aim to determine the substrate specificities of these enzymes and identify any potential redundancy via genetic manipulation of GSB strains and further *in vitro* analysis.
- The enzyme catalyzing the oxidation of the C3-vinyl group of Chl *a* to a formyl group, yielding Chl *d*, is the single remaining unassigned step involved in the biosynthesis of natural (B)Chls. Chl *d* has strong far-red absorbing properties, and thus identification of the 'Chl *d* synthase' will provide the opportunity for expansion of photosynthesis into the far-red region of the spectrum in engineered cyanobacteria and plants.

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Photosynthetic Reduction of Carbon Dioxide: Biochemical Analysis of Rubisco, Phospho*enol*pyruvate Carboxylase and Carbonic Anhydrase.

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Overall research goals

- The *overall objective* of our proposal is to determine the structure and functional control of Rubisco and PEPC kinetics, and the influence of CA activity on the availability of substrate (HCO_3^- or CO_2) for PEPC and Rubisco, respectively.
- The *central hypothesis* is that there is a change in the kinetic parameters that determine Rubisco specificity response to temperature, that specific PEPC amino acids control variation in HCO_3^- kinetics (K_P), and that CA activity limits the capacity of CO_2 reduction at high temperatures in both C_4 and C_3 plants.
- Our *specific aims* are 1) Determine the individual kinetic parameters driving the temperature response of Rubisco specificity; 2) Determine if specific amino acid differences between C_4 and C_3 isoforms of PEPC drive differences in K_P.; Determine how changes in leaf CA activity drive the temperature response of photosynthesis.

Significant achievements 2016-2017

T**he Temperature Response of Rubisco kinetics: breakpoints and implications for reaction mechanisms.** Membrane inlet mass spectrometry (MIMS) is a tool which may offer benefits over traditional methods for determining individual rate constants of the Rubisco reaction mechanism, as it can directly monitor concentration changes in CO_2, O_2, and their isotopologs. We measured the temperature responses of Rubisco kinetic parameters from *Arabidopsis thaliana* using Radiolabel and MIMS methods. The two methods provided highly comparable parameters above 25 °C, but temperature responses deviated at low temperature as MIMS derived catalytic rates of carboxylation, oxygenation, and specificity were found to have thermal breakpoints. We highlight the variability and uncertainty surrounding breakpoints in the Rubisco temperature response and relevance of individual reaction mechanisms to potential breakpoints.

Breakpoint in the Temperature Response of Rubisco Kinetic Parameters: Insights from Carbon Isotope Fractionation While Rubisco breakpoints have been declared as universal to all measurements of Rubisco temperature responses, they are often not observed or reported. We measured the temperature response of the maximum rate of carboxylation (V_{cmax}), maximum rate of oxygenation (V_{omax}), the Michaelis constant for CO_2 (K_C) and O_2 (K_O), and the specificity of the enzyme for CO_2 over O_2 ($S_{C/O}$) from 10 to 40 °C of Rubisco from *Oryza sativa*. Our measurements confirm the previously identified breakpoint in V_{cmax} and suggest a previously unreported breakpoint in *O. sativa* for V_{omax}, both occurring at 20 °C. Previous temperature models of the elementary reactions describing V_{cmax} and V_{omax} suggested that observed breakpoints are possibly due to changes in the energy barriers associated with CO_2 addition and cleavage of a carbon-carbon bond leading to product formation. These models suggest possible changes to the isotopic carbon discrimination factor for Rubisco with temperature. Therefore, we tested this hypothesis by measuring the temperature response of CO_2 discrimination by Rubisco, where changes in discrimination may occur due to changes in the elementary steps of the reaction mechanism. However, Rubisco discrimination in *O. sativa* was constant with temperature, maintaining a value of ~28 ‰, suggesting that breakpoints were not associated with changes to elementary rate constants, suggesting instead they are associated with continual deactivation of the enzyme with decreasing temperatures.

HCO_3^- kinetics of PEPC from a C_3 and C_4 Plant. We have characterized the HCO_3^- kinetic properties of PEPC from *Flavaria. trinervia (C4)* and *F. pringlei (C3)* (Ft966, Fp966, respectively) and chimeric PEPC exchange of a key serine for an alanine in the C_4 enzyme (Ft966S/A), and the alanine for a serine in

the C_3 enzyme (Fp966A/S). The C_4 enzyme has a lower K_p than the C_4 enzyme, which appears to be influenced by the serine to alanine amino acid substitution.

Temperature Response of Mesophyll Conductance in C_4 plants. Mesophyll conductance (g_m) is an important factor limiting rates of C_3 photosynthesis and may also restrict rates of C_4 photosynthesis. However, there is little information on C_4 g_m because it has been difficult to estimate. We use two methods to derive the temperature responses of g_m in C_4 species. The first method ($\Delta^{18}O$) combines measurements of gas exchange with models and measurements of ^{18}O discrimination. The second method (*in-vitro* V_{pmax}) derives g_m by retrofitting models of C_4 photosynthesis and ^{13}C discrimination with gas exchange, kinetic constants, and *in-vitro* V_{pmax} measurements. Our study demonstrates that: 1) the two described methods are suitable to calculate g_m in C_4 species; 2) g_m values in C_4 plants are similar to high-end values reported for C_3 species; and 3) g_m increases with temperature analogous to reports for C_3 species and the response is species specific. These results confirm that g_m should be included in descriptions of C_4 photosynthesis.

Science objectives for 2017-2018

1) Publish research on the genetic reduction of PEPC and Rubisco in *S. viridis*. We are currently preparing two manuscripts on the temperature response of C_4 photosynthesis in these plants.
2) Publish the temperature response of C_4 photosynthesis in *Zea mays* with reduced CA activity.
3) In collaboration with Tom Brutnell at the Danforth Center and JGI we will leverage the parallel evolution of PEPC in the grass family to identify novel allelic variants that define key amino acid changes from a wide range of C_3 and C_4 species. PEPC alleles synthesized by JGI will be expressed in *E. coli* and tested for enzymatic activity and kinetic properties.

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Structure-Based Studies on Energy Transduction in the Cytochrome b_6f Complex

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General Objective of Project: The general goal of the project is to use the details of the high resolution (2.5 Å) crystal structure (**PDB 4OGQ**) of the cytochrome b_6f complex to guide further understanding of its functions in electron transport and energy transduction.

Initially, the role of particular amino acids in the cytochrome complex, which are implicated from the trans-membrane structure in the determination of the rate-limiting step in electron transport, is being studied by site-directed mutagenesis and assay of resulting function. The rate-limiting step(s) in the overall electron transport network is (are) associated with the transfer of plastoquinol from the photosystem II reaction center to the b_6f complex and its subsequent oxidation and coupled proton transfer to the p-side (lumen) phase. Subsequently, it is planned to bbtain an atomic structure of a plant (e. g., pea) b_6f complex; (**iii**) Determine the function(s) of the b_6f complex associated with photosystem I – linked cyclic electron transport; (**iv**) test predictions from the crystal structure regarding amino acid residues that function in trans-membrane proton translocation.

Summary of Progress on Specific Project Objectives of the Initial Project (I. Initial Project: modification by site-directed mutagenesis of a rate-limitation in non-cyclic electron transport; started 09/15/17).

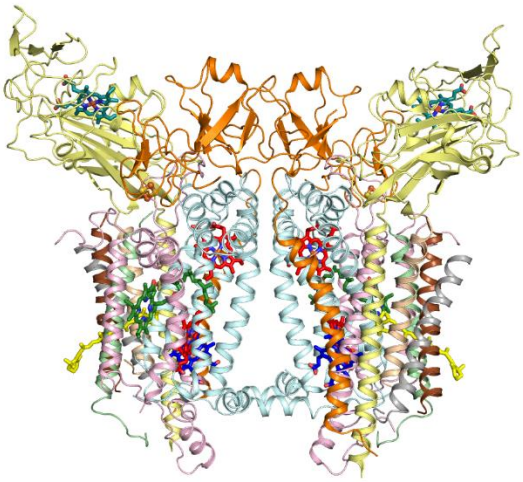
Quinol oxidation at the p-side of the b_6f complex (2.5 Å, **Fig. 1**) occurs at the end of an 11 Å portal that terminates near the 2Fe-2S cluster, as shown in the structure of the complex in the presence of the quinone analog inhibitor, tridecyl-sigmatellin (**PDB 2E76**). A structure-based rate limitation of plastoquinol oxidation is proposed to be a consequence of the required passage of the quinol/quinone passing through this narrow portal. The portal is bounded by the TM F α-helix of subunit IV (**Fig. 2**). Proline residues conserved at positions 105 and 112 in cyanobacteria, the green alga *C. reinhardtii*, and plants, define positions of kinks in this helix. These 2 Pro residues, 105 and 112 in the sequence, cause a bend in the F-helix of the SuIV subunit, away from the C-helix in the cyt *b* subunit, and contribute to the aperture of the portal that leads to the Qp site for electron-proton donation to the [2Fe-2S] center. Site-directed mutagenesis in the cyanobacterium *Synechococcu*s PCC7002 is being utilized to (**i**) enlarge this channel by changing the position of the two proline residues in the F-helix, or (**ii**) by replacing an upstream amino acid with a proline, Pro 105 and/or Pro 112, which are changed to a small residue Ala. The latter change is predicted to cause removal of the bend of the F-helix, and a decrease in size of the Q-portal and the over-all rate of noncyclic electron transport.

Specific progress thus far: (1) the mutation P105 –> A105 in the F-helix of subunit IV (petD gene) has been generated; it has been ligated into a pUC19 plasmid digested with NdeI and EcoRI; E. coli is being transformed; the plasmid will be extracted from an antibiotic-resistant colony and transformed into Synecococcus PCC 7002; electron transport activity will be assayed by the kinetics of reduction of flash-induced oxidation of cytochrome *f* (**Fig. 3**).

Planned Projects: (**ii**) Obtain an atomic structure, by crystallography or cryo-electron microscopy of a plant (e. g., pea) b_6f complex; (**iii**) Determine participation and functions of the b_6f complex associated with, or linked to, the pathway of photosystem I – linked cyclic electron transport/phosphorylation; (**iv**) Test predictions from the crystal structure regarding amino acid residues likely to participate in the pathway of trans-membrane H^+ transfer coupled to electron transfer.

Fig. 1 (top) Cytochrome b_6f Complex Symmetric 240 kDa Dimer; per monomer: 13 TMH, 7 prosthetic groups [(5 redox, 4 hemes + 1 [2Fe-2S] cluster); 1 β-Carotene, 1 Chl].

Fig. 2 (bottom) Anticipated effect on the rate of cytochrome f reduction of residue changes created by site-directed mutagenesis centered on the C- and F-helices of the b6f complex on the plastoquinone entrance portal. (top) Residue changes that would enlarge the quinone would result in an increase in the rate of cyt f reduction; (b) (bottom) residue changes that would constrict the portal would decrease the rate of cyt f reduction.



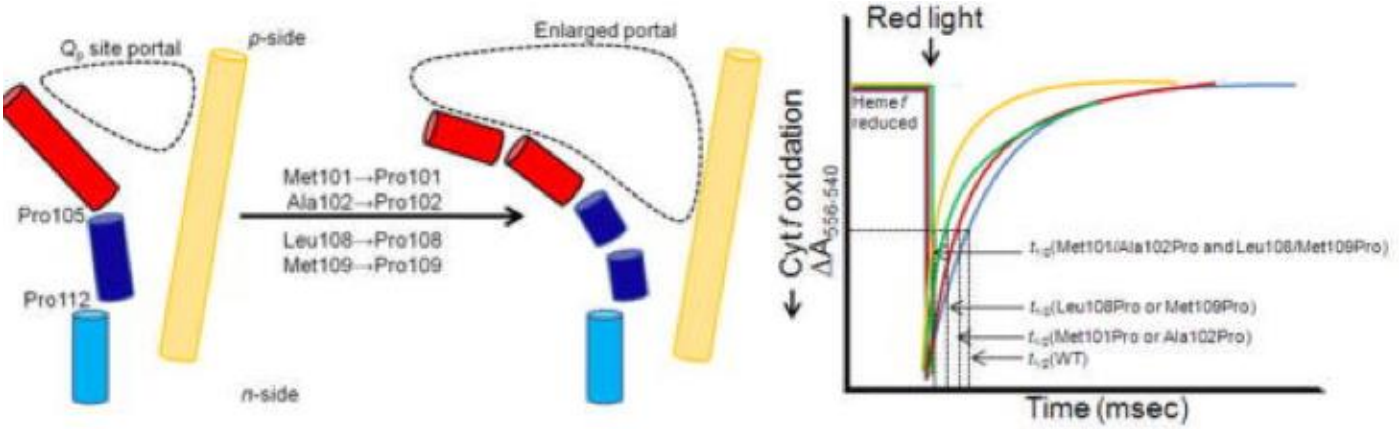
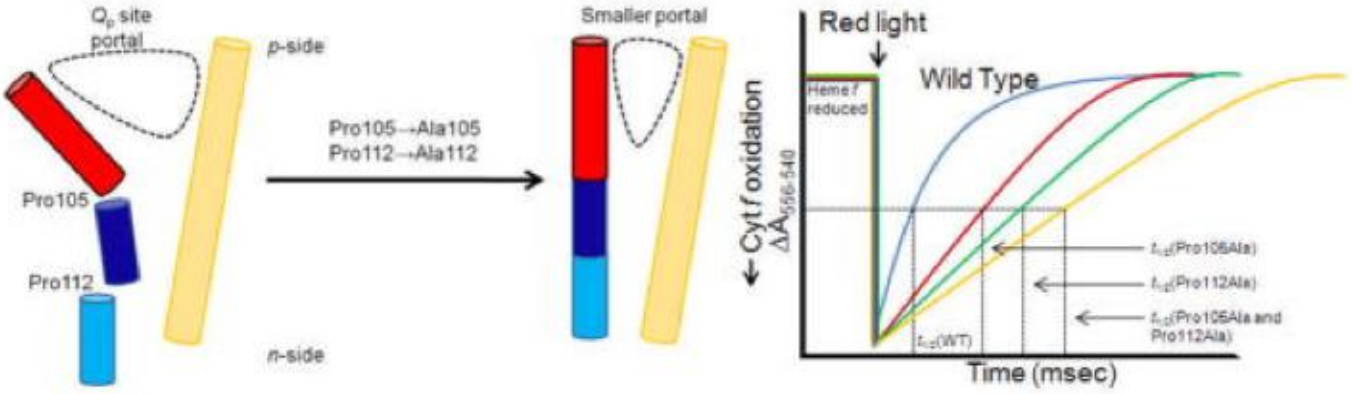


Fig 6B



Thylakoid Assembly and Folded Protein Transport by the Chloroplast Twin Arginine Translocation (cpTat) Pathway

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Overall research goals: The research objectives are to study the mechanisms of protein transport into the lumen of thylakoids by the chloroplast Twin Arginine Transport (cpTat) pathway by: (1) identifying the cpTat component(s) that interact with the mature domain of the precursor during transport; (2) determine the organization of the cpTat translocon; and (3) compare topology of cpTat component, Tha4 in thylakoids during active transport and at rest. This particular protein transport pathway is predicted to translocate ~50% of the lumen proteins. Understanding cpTat system mechanism in chloroplasts will lead to a better understanding of the biogenesis and assembly of photosynthetic membranes potentially providing a means to engineer photosynthetic complexes into synthetic membranes for energy production.

Significant achievements 2015-2017: Proteins destined for the thylakoid lumen of chloroplasts are nuclear-encoded, synthesized in the cytoplasm, and must cross three membranes *en route* to their final destination. The chloroplast Twin Arginine Translocation (cpTat) system facilitates transport of about half of all proteins that cross the thylakoid membrane in chloroplasts. Known mechanistic features of the cpTat system are drastically different from other known translocation systems, notably in its formation of a transient complex composed of thylakoid proteins cpTatC, Hcf106, and Tha4 to transport fully folded proteins utilizing only the proton motive force (pmf) for energy. cpTat and Hcf106 form a receptor complex, which has been thought to be a static interaction and binds the signal sequence on the precursor and Tha4 is thought to play a role in generating the point of passage for the precursor during the transport process. However, key details such as the structure and composition of the translocation pore are still unknown. First, we demonstrate that imported cpTatC and integrated Hcf106 are able to interact with each other through disulfide bonds (**Fig. 1**). In order to probe the interactions between cpTatC and Hcf106 using biochemical methods, we needed to establish that incorporated cpTat proteins will assemble into functional complexes. This is significant because it demonstrates that cpTatC complexes are dynamic and cpTatC and Hcf106 partners can move in and out of the complex. Little is known about cpTatC-Hcf106-Tha4 interaction during the transport cycle. We hypothesize that the receptor complex protein Hcf106, acts as a nucleator for Tha4 assembly in the presence of precursor and a pmf. Two cysteines placed in the transmembrane domain of Hcf106 interacts with both cpTatC and Tha4 (**Fig. 2**). We also hypothesize that the translocation point of passage includes all three cpTat proteins because we can show an interaction between the backend of the mature domain of the precursor with cpTatC. This is significant because current models question the participation of cpTatC in forming the point of passage. We have therefore established that we can use *in vitro* expressed exogenous forms of cpTat proteins in native thylakoid to query the organization of the cpTat receptor and translocase.

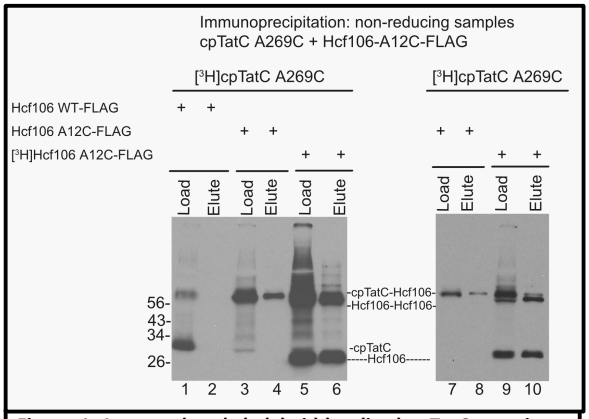

Figure 1. Imported and thylakoid-localized cpTatC associates with Hcf106 integrated into the same isolated thylakoids. The 57kDa band is a cpTatC-Hcf106 adduct. Radiolabeled cpTatCA269C was imported into isolated chloroplasts and thylakoid harvested by hypotonic lysis. Those thylakoids were used for integration of labeled or unlabeled Hcf106 as indicated in the left side of the panel. After cross-linking, non-reduced samples were subjected to immunoprecipitation with beads labeled with antibodies against the FLAG tag. The right panel is the same as lanes 3-6 of the left panel, except samples were diluted an additional 1:10 prior to SDS-PAGE.

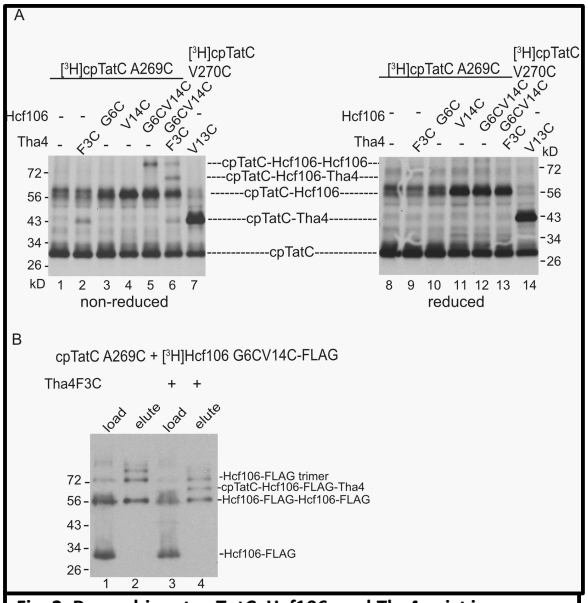

Fig. 2. Recombinant cpTatC, Hcf106, and Tha4 exist in one complex and can be cross-linked together through Hcf106. (A) Thylakoid membranes containing [^{3}H]cpTatC single Cys variants were incubated with unlabeled Hcf106 or Tha4 single or double Cys variants or with both Hcf106 and Tha4 and subjected to crosslinking by oxidation. Samples were analyzed by non-reducing and reducing SDS-PAGE and fluorography. Predicted dimers and trimers are indicated. (B) Immunoprecipitation of Hcf106-FLAG-cpTatC-Tha4 trimer. [^{3}H]cpTatC A269C was imported into thylakoids followed by the integration of unlabeled Hcf106-FLAG. Samples were then washed and integrated with unlabeled Tha4 or Mock and subjected to cross-linking. After cross-linking, non-reduced samples were subjected to immunoprecipitation with FLAG-beads.

Science objectives for 2017-2018:

- We do not know when the interaction occurs between the precursor mature domain and cpTatC. Is the interaction prior to transport, during transport, or post transport? Is the interaction timing dependent upon the position of the Cys residue on Hcf106 or Tha4? For example, does the timing of the precursor mature domain interaction change if the Cys is on the N terminus (e.g., the *trans* side of the membrane) or the C-proximal portion of the APH (*cis* side of the membrane)? Further investigations with modified assays will allow us to investigate the nature and timing of the interaction.
- We are continuing our investigation of interactions between all three cpTat components simultaneously using doubly Cys-substituted cpTat components to map contacts between Tha4, Hcf106 and cpTatC in the same complex, which has not been demonstrated to date.
- Biochemical analysis of Tha4 and Hcf106 topology suggests a N_{out}-C_{in} topology but we do consistently see topologies that suggest a mixed population of orientations. We are currently using EPR spectrometry to probe the environment and topology of the cpTat components, Tha4 and Hcf106, in model membranes.

References to work supported by this project 2015-2017:

1. Q. Ma, K. Fite, and C. Dabney-Smith. (2015) Cysteine scanning and cross-linking study reveals the oligomeric property of Hcf106 in the chloroplast Tat system. BBRC. In review.
2. Q. Ma and C. Dabney-Smith. Organization of the receptor complex of the Twin Arginine Transport pathway in thylakoids of chloroplasts. Plant Physiology. In review.

Membrane-attached Electron Carriers in Photosynthesis and Respiration: Biogenesis of a cytochrome complex from a facultative photosynthetic bacterium

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Overall research goals: Our long term aim is to contribute to the understanding of biological energy transduction during photosynthesis (Ps) and respiration (Res). We study cytochromes (cyts), which are heme containing electron transfer proteins, acting as key components during these pathways. The focus of our work is the biogenesis of cyt complexes from *Rhodobacter species*. The cbb_3-type cyt *c* oxidase (cbb_3-Cox), which is critical for both Ps and Res growth, is our model system. Its biogenesis requires two distinct maturation processes: **1-** Covalent ligation of heme to the apocyt subunits via the **Ccm** (cyt *c* maturation system I) complex. Ccm is widespread among bacteria, archaea, plant mitochondria and red algae. It is composed of nine membrane proteins (CcmABCDEFGHI) that carry out the covalent ligation of heme *b* to the apocyt subunits of cbb_3-Cox. **2-** Acquisition, trafficking and insertion of copper (Cu) into the active site of the catalytic subunit of cbb_3-Cox. In *Rhodobacter* species, Cu trafficking occurs via a high-affinity (*CcoA-dependent*) and a low-affinity (*CcoA-independent*) Cu acquisition pathways. Our overall goal is the characterization of the Ccm and Cu insertion processes that are essential for cbb_3-Cox biogenesis.

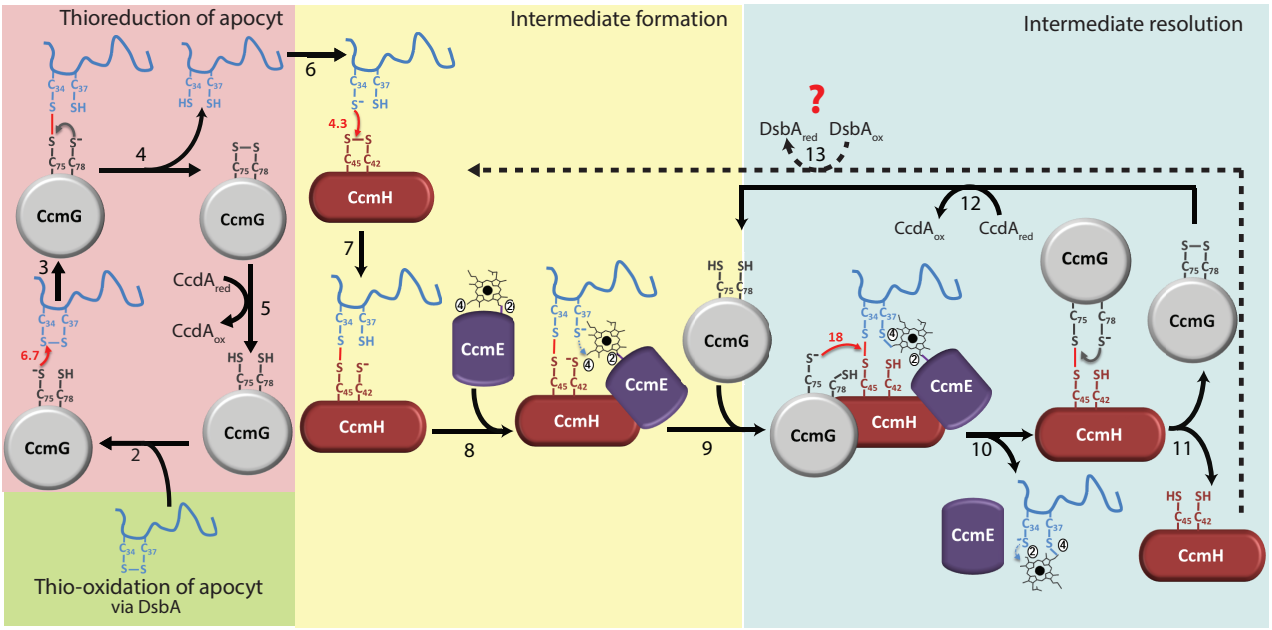

Fig. 1**. Thioreduction of apocyt *c* and stereo-specific heme ligation during Ccm** (apocyt c_1 used as an example). Upon translocation of the apocyt c_1 to the periplasm, DsbA oxidizes it, and then CcmG forms a mixed disulfide bond with it (**steps 2-3**). Resolution of this bond results in reduced apocyt c_1 and oxidized CcmG (**step 4**) (re-reduced by CcdA, **step 5**). Reduced apocyt c_1 and oxidized CcmH (**step 6**) forms an intermediate in which apocyt c_1 C34 and C45 of CcmH are engaged in a mixed disulfide (**step 7**). This step defines the ***stereo-specificity of heme ligation.*** Next, CcmE-heme interacts with CcmH and apocyt c_1 (**step 8**) and yields the first thioether bond between the available C-ter C37 of apocyt c_1 and vinyl-4 of heme. The CcmH-apocyt c_1-heme-CcmE intermediate thus formed is ***resolved efficiently*** by CcmG (**step 9**), which forms a mixed disulfide with CcmH and releases apocyt c_1 (**step 10**). The N-ter C34 of apocyt c_1 then forms the second thioether bond with vinyl-2 of heme, and is released from CcmE (possibly via CcmF, not shown). Resolution of the CcmG-CcmH mixed disulfide bond yields reduced CcmH and oxidized CcmG (**step 11**). As in (**step 5**), CcdA recycles oxidized CcmG (**step 12**), and CcmH is oxidized possibly via DsbA (**step 13**, dotted line) completing the process. See Verissimo *et al*, (2017) for a more complete description of this process.

Significant achievement 2016-2017:

A- Ccm process: Our major achievement was the molecular definition of the thioredox pathway during Ccm, including the sequence of the thioredox reactions between the Ccm components and apocyt *c*, and the identity of their reactive Cys residues. **1**- We showed that apocyt *c* together with CcmG, CcmF, CcmH and CcmI forms a CcmFGHI-apocyt *c* complex; **2**- We measured the rates of thiol-disulfide exchange reactions between selected Cys pairs of the Ccm components involved in these reactions, using purified Cys variants of these proteins. **3**- We established that CcmG reduces the disulfide bond of apocyt *c*, and efficiently resolves the mixed disulfide bond formed between the C45 of CcmH and C34 of apocyt *c*. Overall findings, depicted in **Fig. 1**, established for the first time that *CcmG* confers *efficiency* to the thioreduction pathway that occurs during the Ccm process, and that *CcmH* is responsible for the *stereo-specificity* of heme-apocyt *c* ligation. We believe that these are significant contributions to our understanding of the molecular mechanisms governing the Ccm process, in particular, of the universal stereo-specific heme ligation reaction.

B- Cu incorporation into *cbb*$_3$-Cox; Biogenesis of *cbb*$_3$-Cox also requires the insertion of a Cu atom into its active site to form the catalytic heme-Cu$_B$ binuclear center. Previously, we discovered that a novel Major Facilitator Superfamily (MFS)-type bacterial Cu importer (CcoA) is required for this process. CcoA is the prototype of bacterial Cu importers, and its discovery being recent, it is not yet well characterized. We now defined the universally conserved Met and His residues of CcoA, determined the topology of its transmembrane helices carrying these residues, and substituted them with amino acids that do not ligand metal atoms. Characterization of these mutants for Cu uptake and *cbb*$_3$-Cox activity demonstrated that Met233 and His261 of CcoA are essential, and Met237 and Met265 are important for Cu uptake and *cbb*$_3$-Cox activity. These findings now set the stage for further studies of CcoA-dependent and CcoA–independent Cu trafficking pathways to *cbb*$_3$-Cox.

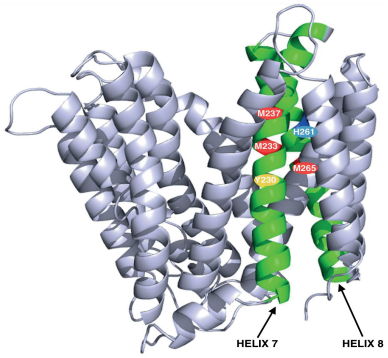

Fig. 2. CcoA and its conserved residues as putative Cu ligands.

Science objectives for 2017-2019: The following specific aims are being pursued:

- Probe the occurrence of a Ccm supercomplex in *R. capsulatus* membranes.
- Characterizations of the high affinity (*CcoA-dependent*) Cu delivery pathway to *cbb*$_3$-Cox.
- Characterizations of the low affinity (*CcoA-independent*) Cu delivery pathway to *cbb*$_3$-Cox.

References to the works related to Ccm and Cu studies supported by this project 2016-2017

- Khalfaoui-Hassani, B., Verissimo, A. F., Shroff, N. P., Ekici, S., Trasnea, P-I, Utz, M., Koch, H-G and **Daldal, F.** (2016). Biogenesis of cytochrome *c* complexes: From insertion of redox cofactors to assembly of different subunits. In: *Cytochrome Complexes: Evolution, Structures, Energy Transduction and Signaling*. Edited by Cramer, W. and Kallas, T. Springer.
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- Trasnea, P-I., Utz M., Khalfaoui-Hassani, B., Lagies, S., **Daldal, F**. and Koch, H-G. (2016). Cooperation between two periplasmic copper chaperones is required for full activity of the *cbb*$_3$-type cyt *c* oxidase and copper homeostasis in *R. capsulatus*. *Mol. Micro*. **100**: 345-61.
- Verissimo A. F, Khalfaoui-Hassani B., Hwang, J., Steimle, S., Selamoglu, N., Sanders, C., Khatchikian, C. E. and **Daldal, F**. (2017). The thioreduction component CcmG confers efficiency and the heme ligation component CcmH ensures stereo-specificity during cytochome *c* maturation. *J. Biol. Chem*. **292**: 13154-13167.

FTIR Studies of Photosynthetic Oxygen Production

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Overall research goals: The project's primary aims are to (i) further delineate the dominant water access and proton egress pathways that link the Mn_4CaO_5 cluster in Photosystem II with the thylakoid lumen and (ii) characterize the influence of specific residues on the water molecules that serve as substrate or as participants in the networks of hydrogen bonds that make up these pathways. Infrared spectroscopy is being employed to analyze mutant Photosystem II core complexes having substitutions at residues identified crystallographically or computationally as interacting with the Mn_4CaO_5 cluster and the water molecules in its vicinity, as well as altered wild-type Photosystem II core complexes.

Significant achievements 2016-2017: Considerable evidence supports identifying one of the two substrate waters for O_2 formation as the Mn_4CaO_5 cluster's O5 oxo bridge. The identity of the second substrate water is less clear. In one set of models, the second substrate is the Mn-bound water, W2. In another set of models, the second substrate is the Ca^{2+}-bound water, W3. In both sets of models, a deprotonated form of the second substrate moves to a position next to O5 during the S_2 to S_3 transition. To differentiate between these models, we have employed FTIR difference spectroscopy to identify the vibrational modes of hydrogen-bonded water molecules that are altered by substituting Sr^{2+} for Ca^{2+}. This substitution substantially altered the δ(DOD) mode of only a single water: the water molecule whose δ(DOD) mode is eliminated during the S_2 to S_3 transition (the 1239(-) cm^{-1} feature in the S_3-S_2 spectrum of Ca^{2+}-PSII - Figure 1). The only waters whose positions are altered substantially in the crystallographic and computational studies of Sr^{2+}-substituted PSII are W3 and W4 (the two water ligands of the Ca^{2+} ion), and W5, the water that forms hydrogen bonds with W3 and the Mn4-bound W2. Consequently, our data are most consistent with identifying the Ca^{2+}-bound W3 as the substrate that moves in deprotonated form to a position next to O5 during the S_2 to S_3 transition (*2*). We suggest that W5 moves to the coordination position on Ca^{2+} vacated by W3. If so, the 1239(-) cm^{-1} feature in the S_3-S_2 spectrum of Ca^{2+}-PSII should correspond to the loss of the δ(DOD) mode of W5.

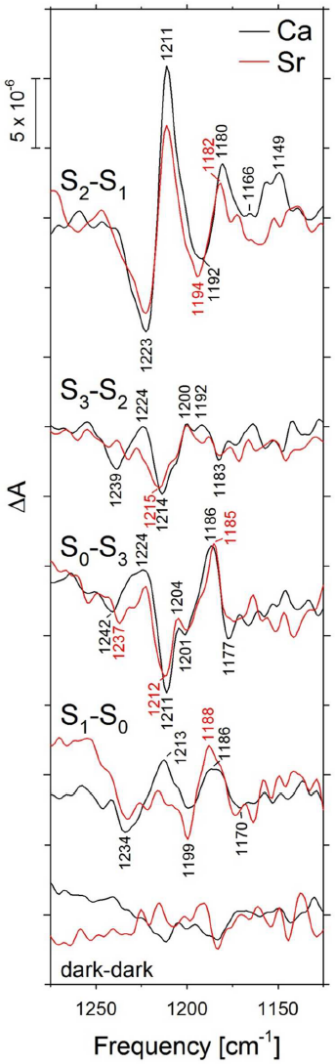

Figure 1. $D_2{}^{16}O$–$D_2{}^{18}O$ double-difference spectra of PSII core complexes containing Ca^{2+} (black) or Sr^{2+} (red) in the D-O-D bending region. From (*2*).

D1-V185 contacts numerous water molecules located between tyrosine Y_Z and D1-D61, including W5. The mutation D1-V185N decreases the efficiency of O_2 formation and dramatically decreases the rate of O_2 release, features in common with mutations of D1-D61. In collaboration with R. L. Burnap and coworkers at Oklahoma State University, we have examined the FTIR properties of the D1-V185N mutant. This mutation alters the same δ(DOD) mode that is altered by substituting Sr^{2+} for Ca^{2+} (Figure 2). This observation provides further evidence that W3 is the substrate water that moves next to O5 during the S_2 to S_3 transition and that W3 is replaced by W5.

Ammonia is a substrate water analogue and binds to two sites near

the Mn_4CaO_5 cluster. The primary site is on Mn4 in place of W1. Ammonia bound to this site generates an altered multiline EPR signal in the S_2 state. The secondary site is competitive with Cl^-. Studies of the D2-K317A mutant performed in collaboration with G. W. Brudvig and coworkers at Yale University provided evidence that this site is remote from the Cl^- site: at this site, ammonia competes with D1-D61 as a hydrogen bond acceptor to W1 (*1*).

D1-87 is Asn in cyanobacteria and Ala in higher plants. Studies of the D1-N87A and D1-N87D mutants in collaboration with G. W. Brudvig and coworkers provided evidence that this residue participates in a network of hydrogen bonds that forms a water entry channel leading to the Mn_4CaO_5 cluster.

D1-S169 forms a hydrogen bond with a water (Wx) that forms a hydrogen bond with the Mn_4CaO5 cluster's O4 oxo bridge. In models identifying W2 as the second substrate, Wx replaces W2 after W2 moves next to O5. Studies of the D1-S169A mutant in collaboration with G. W. Brudvig and coworkers provided evidence that this residue participates in a network of hydrogen bonds that participates in substrate water entry and/or proton egress.

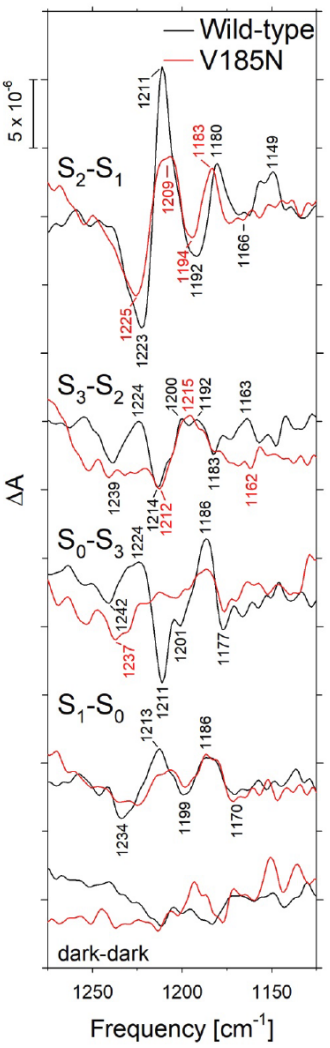

Figure 2. $D_2{}^{16}O–D_2{}^{18}O$ double-difference spectra of wild-type (black) and D1-V185N (red) PSII core complexes in the D-O-D bending region.

<u>Science objectives for 2017-2018:</u>

- Record D-O-D spectra of the D1-S169A mutant to determine if the δ(DOD) mode that is altered by Sr^{2+}/Ca^{2+} exchange and by the D1-V185N mutation is also altered by the D1-S169A mutation. If yes, then identifying W2 as the second substrate remains viable.
- A network of hydrogen bonds extending from Y_Z past D1-N298 has been proposed to serve as a proton egress pathway that dominates during the S_2 to S_3 transition. To test this proposal, we have initiated a study of the D1-N298A mutant. These studies will be completed and extended to other residues in the same network.
- Conduct time-resolved IR measurements to monitor the rearrangement of amino acid residues, water molecules, and hydrogen bond networks during the individual S state transitions, starting initially with the D1-D61A, D1-S169A, and D1-N298A mutations, in part to determine if different proton egress pathways dominate during different S state transitions. This work is being performed in collaboration with H. Dau at the Freie Universität of Berlin in cooperation with R. L. Burnap and coworkers at Oklahoma State University.
- Collaborative studies of the binding of water substrate analogs at or near the Mn_4CaO_5 cluster will continue to be pursued with R. D. Britt and coworkers at UC Davis.

<u>References to work supported by this project 2016-2017:</u>

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2. Kim, C. J. and Debus, R. J. (2017) "Evidence from FTIR Difference Spectroscopy That a Substrate H_2O Molecule for O_2 Formation in Photosystem II Is Provided by the Ca Ion of the Catalytic Mn_4CaO_5 Cluster," *Biochemistry 56*, 2558-2570.

Resolving protein-semiquinone interactions by advanced EPR spectroscopy: The Q_A and Q_B sites of the bacterial reaction center.

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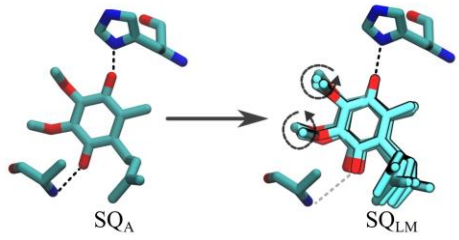
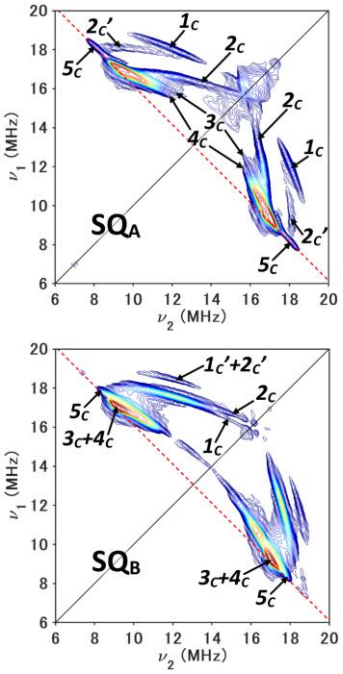
Overall research goals: Our focus is on use of modern, high-resolution EPR spectroscopy to explore the catalytic domains trapped in states with semiquinone (SQ) as an intermediate. The catalytic sites we propose to study, - the Q_A and Q_B sites of the bacterial reaction center (RC), the Q_H site of the bo_3 quinol oxidase, and the Q_i site of the bc_1 complex, - all operate using ubiquinone, 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 2015-2017:

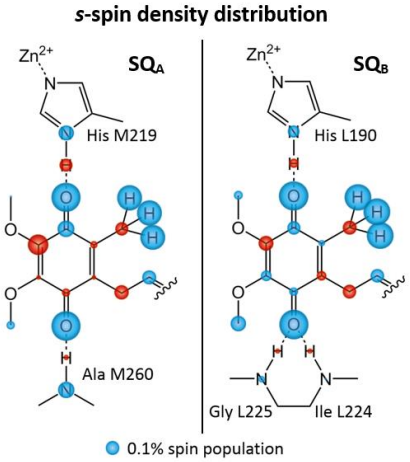
Regulation of the primary quinone binding conformation by the H Subunit in RC from Rb. sphaeroides. Unlike photosystem II (PSII) in higher plants, RCs from *Proteobacteria* have an additional peripheral membrane subunit "H". The H subunit is necessary for photosynthetic growth, but can be removed chemically *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 interquinone 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. (Ref. 1)

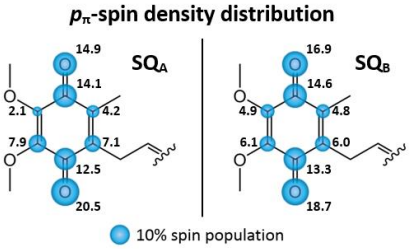




The complete spin density distribution in SQ_A and SQ_B by ^{13}C labeling. The spin density distribution of an organic or biological radical provides a unique signature of its electronic structure, in particular the electron density of the singly occupied molecular orbital (SOMO). Determining the complete electron spin density distribution for protein-bound radicals, even with advanced pulsed EPR methods, is a formidable task. Here we report a strategy to overcome this problem combining multifrequency HYSCORE and ENDOR measurements on site-specifically ^{13}C-labeled samples with DFT calculations. As a demonstration of this approach, pulsed EPR experiments are performed on the primary Q_A and secondary Q_B ubisemiquinones of the RC from *Rhodobacter sphaeroides* ^{13}C-labeled at the ring and tail positions. Despite the large number of nuclei interacting with the unpaired electron in these samples, two-dimensional X- and Q-band HYSCORE (see figure) and orientation selective Q-band ENDOR resolve and

allow for a characterization of the eight expected ^{13}C resonances from significantly different hyperfine tensors for both SQs. Six tensors with notable hyperfine anisotropy are assigned to ring carbons C_1 through C_6. The remaining ^{13}C couplings lack a strong anisotropic component and are associated with the two carbons of the isoprenoid tail. The results of this study are then combined with previously reported ^{1}H (5'-methyl, hydrogen bonds), ^{13}C (methoxy, 5'-methyl), ^{17}O (carbonyl), and ^{14}N (H-bond donors) couplings to construct an extensive map of the *s*- and p_π-orbital spin density distribution across the quinone ring carbons, their substituents, and the hydrogen bonds for SQ_A and SQ_B (figure). This work lays a foundation for understanding the relationship between the electronic structure of SQs and their functional properties, and introduces new techniques for mapping out the spin density distribution that are readily applicable to other systems. (Ref. 7)





Science objectives for 2017-2018:

- ^{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 investigate the influence of mutations on SQ_A in RC and SQ_H in cyt bo_3. We will focus on the M265I(T,S,N) mutants in RC and on two nonpolar residues Ile102 and Met78 that are part of the Q_H binding pocket in cyt bo_3.

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Regulation of Photosynthetic Light Harvesting

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Overall research goals: Photosynthetic light-harvesting systems are regulated, protected against photo-oxidative damage, self-assembled into supercomplexes, and rely on a subtle balance of electronic interactions to produce a highly robust system. In this project, we apply a multidisciplinary approach that combines genetic and biochemical techniques with ultrafast spectroscopy and modeling to understand the mechanisms by which oxygenic photosynthetic organisms regulate the efficiency of light harvesting in Photosystem II. In response to fluctuations in light intensity, non-photochemical quenching (NPQ) mechanisms that regulate photosynthetic light harvesting are induced. In algae and plants, the qE type of NPQ is turned on and off rapidly by changes in thylakoid lumen pH, whereas the slower qE type downregulates PSII during long-term light stress. Our specific aims are (1) to identify new components involved in different types of NPQ through genetic analysis, (2) to elucidate the mechanisms, locations, and timescales of NPQ using fluorescence lifetime and transient absorption snapshot spectroscopies, advanced imaging, and biochemical approaches, and (3) to develop mechanistic models of NPQ that can predict how to optimize NPQ and thereby improve photosynthesis.

Significant achievements 2015-2017:
The engineered Arabidopsis lines that have a lutein-lutein epoxide cycle, or a standard VAZ (Violaxanthin-Antheraxanthin-Zeaxanthin) cycle enable us to isolate the relative contributions of lutein and Zeaxanthin to qE in wild type plants. This work involved fluorescence lifetime snapshot studies of five mutants and the wild type along with modeling work based on our earlier "engineering" model of qE. On a per molecule basis Zeaxanthin is about 10 times more effective a quencher than lutein, and when both are present there is a synergistic effect suggesting some type of cooperatively. To explore the role of Zeaxanthin in more detail we developed snapshot transient absorption spectroscopy to complement snapshot fluorescence lifetime measurements. We detected the appearance of the Zea radical cation in spinach thylakoids under high light conditions and showed that the signal is not present when DTSSP is added. DTSSP is a cross linking agent that is thought to inhibit monomerization of PsbS. We developed a coarse grained model of the grana membrane on the 200 nm x 200 nm length scale (~10,000 chlorophyll (Chl) molecules) and reproduced the experimental fluorescence decay with no adjustable parameters. In an unquenched membrane the diffusion length of an excitation is 50 nm, as reaction centers close through excitation and qE is turned on, a competition begins between quenching at open RCs and at qE sites. The experimental fluorescence snapshot data can all be understood via the idea that the diffusion length is the control parameter for qE quenching. This controls the number of Chl that can reach a given reaction center. This is the

link between the microscopic dynamics within pigment-protein complexes and the mesoscopic dynamics that control photosynthetic yields.

Science objectives for 2017-2018:

- Extend the snapshot transient absorption method to *Arabidopsis* to give access to the NPQ mutants available.
- Use the "no minors" mutant to explore locations of specific qE mechanisms.
- Elucidate the roles of PsbS and LHCSR in mosses where both are present but can be relatively knocked out
- Continue our atomistic molecular dynamics simulation of PsbS in a lipid bilayer.
- Incorporate additional components into the membrane model, including PsbS and PSI
- Extend fluorescence snapshot method by using periodic modulation schemes to reveal timescale of various NPQ processes.

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1. Multiscale Model of Photosystem II Light Harvesting in the Thylakoid Membrane of Plants, K. Amarnath, D.I.G. Bennett, A.R. Schneider, G. R. Fleming, PNAS, 113, 5, pp 1156-1161 (2015).
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3. Identification of pH-sensing sites in the light harvesting complex stress-related 3 protein essential for triggering non-photochemical quenching in Chamydonas reinhardtii, M. Ballotari, T.B. Truong, E. De Re, E. Erickson, G.R. Stella, R. Bassi, G.R. Fleming, K.K. Niyogi, J. Bio. Chem. 291, 14, pp 7334 (2016).
4. Two mechanisms for dissipation of excess light in monomeric and trimeric light-harvesting complexes. L. Dall'Ostoa, S. Cazzanigaa, M. Bressana, D. Zigmantas, D. Palečekb, K. Židekb, K.K. Niyogi, G.R. Fleming, R. Bassi. Nature Plants 3:17033 (2017)
5. The Contributions of 49ers to the Measurements and Models of Ultrafast Photosynthetic Energy Transfer. G.R. Fleming. Photosynthesis Research DOI 10.1007/s11120-017-0360-4 (2017).
6. Dissecting and modeling zeaxanthin- and lutein-dependent non-photochemical quenching in Arabidopsis thaliana. M. Leuenberger, J.M. Morris, A. Chan, L. Leonelli, K.K. Niyogi, and G.R. Fleming. PNAS 114, E7009-E7014 (2017).

Functional Models of Photosynthetic Light Harvesting Systems Templated By Self-Assembling Proteins

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Overall research goals: Photosynthetic organisms rely on a series of complex light harvesting systems to absorb broad spectrum light and transfer the resulting excitations to the reaction centers. Much progress has been made toward understanding how these systems function, but a complete and detailed understanding of photosynthetic light harvesting has remained elusive due to our inability to determine the individual contributions of the many functional components that must work in concert to yield higher-level function. Additionally, the effects of protein dynamics and conformational disorder on the performance of these systems are poorly understood and often overlooked. This program is developing a highly adaptable set of protein-based scaffolds that can position multiple chromophores into artificial light harvesting arrays. By tuning the structures of the proteins and the chromophores, a large number of experimental variables, such as pigment structures, orientations, distances to other chromophores, and interactions with the protein environment, can be explored systematically. By comparing sparsely labeled systems to fully-labeled analogs, the site energies arising from the interactions of the chromophores with the protein environments can readily be distinguished from the electronic interactions that govern energy transfer. The rich spectroscopic data are interpreted using extended electronic structure calculations and molecular dynamics simulations that span multiple time and length scales. The models and approaches developed through this program are informing the design of new artificial light harvesting systems and also suggesting the features responsible for the high quantum efficiencies exhibited in natural photosynthesis.

Significant achievements 2014-2017: Two self-assembling protein scaffolds have been developed for the construction of pigment arrays with different geometries and multiplicities. These are a new C_2-symmetric double disk structure based on the tobacco mosaic virus coat protein and a trimeric assembly with deep surface grooves based on the thermostable MTH1491 protein. In both cases, chemistry has been developed to attach a variety of chromophores to different positions on the protein surfaces. Using these constructs:

- Ginsberg, Francis, and Geissler showed that hydrated nanoscale protein cavities are a promising new way to mimic the tight protein pockets for chromophores in natural light harvesting systems. Time-resolved spectroscopic data showed that interior pigments experienced hindered chromophore motion to allow for more prolonged and efficient energy transfer (publication 1).
- Using computer simulations, Geissler helped to rationalize the complex dynamics observed (by Ginsberg and Francis) in a protein-chromophore complex by ultrafast spectroscopy. Specifically, they found that solvent dynamics inside a protein complex vary strongly with location in ways that mirror experimental measurements (publication 1).
- Ginsberg has developed an ultrafast STED imaging technique that could allow the diffusion of excitons to be mapped with unprecedented spatial and temporal resolution (publication 2).
- Francis has developed new method for the purification of protein assemblies labelled with different numbers of chromophores (publication 3).
- Francis and Ginsberg have completed a systematic study of the ability of conformationally constrained linkers to direct the orientations and restrict the motions of protein-bound chromophores.

- The electronic structures of unique chromophore monomers and dimers have been studied using GW/BSE and TDDFT. Additionally, Neaton has completed the first study benchmarking the GW/BSE approach against higher-level quantum chemistry methods (publications 4-8).
- Using theory and simulation, Geissler has explored several fundamental issues of driven nonequilibrium dynamics in mesoscale systems, with general implications for energy transfer kinetics in protein-chromophore systems driven by irradiation. This work has shown that the thermodynamic efficiency in such systems can be a strongly fluctuating quantity with statistics that have interesting and general features. Dynamical phase transitions have also been found to occur in such systems even with very simple underlying kinetic rules (publications 9-11).

Science objectives for 2017-2019:

- Confined protein-embedded-chromophore assemblies will be generated to lock chromophore orientation and control competing kinetic pathways.
- Synthetically tailored linking groups will be used to explore the effects of chromophore-protein interactions and fluctuating environments on energy transfer efficiency.
- Functional mesoscale architectures will be generated to study and optimize inter-assembly energy transfer and morphological bottlenecks.
- A time-resolved ultrafast stimulated emission depletion (TRUSTED) approach will be used to map energy migration through large chromophore assemblies with high spatial and temporal resolution.
- Computational efforts will continue to simulate the bath fluctuations that occur in both natural and synthetic light harvesting systems.

References to work supported by this project 2014-2017:

1. Noriega, R.; Finley, D. T.; Haberstroh, J.; **Geissler, P. L.**; **Francis, M. B.**; **Ginsberg, N. S.** "Manipulating Excited State Dynamics Of Light Harvesting Chromophores Through Restricted Motions In A Hydrated Nanoscale Protein Cavity" *Journal of Physical Chemistry B* **2015**, *119*, 6963.
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11. Vaikuntanathan, S.; Gingrich, T. R.; **Geissler, P. L.** "Dynamic phase transitions in simple driven kinetic networks" *Physical Review E* **2014**, *89*, 062108.

Novel Mechanism Regulating H_2 photoproduction by *Chlamydomonas reinhardtii* upon transition from dark anaerobiosis to light aerobiosis

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Overall research goal: The overall research goal of this project is to understand how photosynthetic microbes balance photosynthate generation with utilization in order to adapt to fluctuating growth conditions, nutrient stress, and changes in carbon metabolism. The specific objectives of the presented work are to develop an understanding of the electron flow pathways and biochemical networks that *Chlamydomonas* uses to adapt to the dark-to-light transition that precedes CO_2 fixation, particularly the hydrogenase-mediated reaction.

Significant achievements 2016-2017:
Adaptation of the green alga *Chlamydomonas reinhardtii* to light/aerobiosis following prolonged dark/anaerobiosis is a dynamic process that involves the temporal expression of various Alternative Electron Flow pathways responsible for maintaining a desirable ATP/NADPH ratio. This research focuses on the hydrogenase pathway, which is active only under anaerobic conditions and is responsible for acting as a temporary valve for excess reductant when the culture is transferred from dark/anaerobiosis to light/aerobiosis, before CO_2 fixation is activated. Our latest research revealed a *novel regulatory mechanism, based on the activity of prolyl-4-hydroxylases that affects the maturation of the hydrogenase enzyme*; as a result, the non-mature form of the enzyme accumulates, H_2 photoproduction levels following anaerobic induction are reduced, and in vitro hydrogenase activity using methyl viologen as the electron donor is significantly decreased. Additionally, transcripts encoding the maturation protein HYDEF are not up-regulated during anaerobiosis as in the wild-type strain. These findings support the hypothesis that the disruption of P4H gene impacts the maturation of hydrogenase by the HYDEF protein. Since hydrogenase activity is not completely absent in the mutant, one or more other P4Hs may be able to perform its function as well, but with lower efficiency. This work raises questions related to the temporal process of apo-protein translocation into the chloroplast, its N-terminal processing, and insertion of the catalytic cluster that may be relevant to other metal-containing proteins as well.

Science objectives for 2017-2018:
- Investigate the mechanism by which proyl-4-hydroxylases regulate the maturation of algal hydrogenase, by purifying the enzyme and identifying its substrate and interacting partners in vitro.
- Deconvolute hydrogenase maturation steps from gene translation to enzyme maturation and activation.

References to work supported by this project 2016-2017:
- Seth Noone, Kathleen Ratcliff, ReAnna Davis, Venkatamaran Subramanian, Jonathan Meuser, Mathew C. Posewitz, Paul W. King, and Maria L. Ghirardi (2017). Expression

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- Melissa Cano, Steven Holland, Juliana Artier, Rob Burnap, Maria Ghirardi, John A. Morgan and Jianping Yu. Glycogen synthesis and metabolite overflow contribute to energy balancing in cyanobacteria. *In review at Joule*.
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Modular, Controllable Biomimetic Systems to Elucidate Chromophore-Protein Interactions that Facilitate Photosynthetic Light Harvesting

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Overall research goals: In natural photosynthesis, light harvesting complexes have evolved to shuttle excitons among their multiple protein-bound chlorophyll and carotenoid pigments with astonishingly high efficiency. The complex environment of each pigment is unique, on account of specific steric and electrostatic interactions with protein residues and with any exposed aqueous regions at a given binding site. Elucidating nature's design principles for crafting these pigments' surroundings in order to enable the high efficiency of photosynthetic energy transfer is extremely challenging with naturally occurring pigment-protein complexes because the pigments electronically couple to one another. Spectroscopic observables convolve the effects of pigment-pigment interactions with pigment-environment interactions, precluding an understanding of how the pigment environment protects excitons from non-radiative decay and enhances the efficiency of energy transport compared to pigment aggregates in the absence of a protein 'scaffold'. Furthermore, perturbing the intricate configurations of photosynthetic organisms by removing pigments or mutating protein residues often destabilizes the overall molecular architecture or alters multiple variables at a time. To address this fundamental challenge, this program employs modular, synthetically controllable biomimetic light harvesting complexes to test hypotheses regarding the evolutionary strategies in natural photosynthesis. By controlling the pigment labeling density of a set of self-assembling protein monomers derived from viral capsids, pigment environments approximating those in natural photosynthesis may be examined both in the absence and presence of interchromophore coupling. Ultrafast spectroscopic measurements are combined with molecular dynamics simulations to obtain a detailed molecular picture of chromophores' dynamic interactions with their surroundings as a function of pigment placement and of configurational constraints achieved through engineered linkages to the protein. The energetics of the pigments are also computed with excited state density functional theory.

Significant achievements 2014-2017: In the past funding cycle we were able to construct biomimics of natural light harvesting complexes with sophisticated chromophore attachments that begin to more closely approximate the conditions of those found in natural systems. For example, instead of tethering dyes to the exterior of a globular protein, we embedded them in a hydrated protein cavity and developed a library of short, constraining linkers to keep pigments closer to protein residues. Many different permutations of these configurations were studied with transient absorption and fluorescence measurements and molecular dynamics simulations. Some configurations slowed the excited state dynamics by as much as 25x, which could approximate nature's design principle for preserving excitation energy to enable transport over longer distances with lower losses. The comparative nature of our studies allows us to use and correlate retardation in excited state dynamics time scales and in simulated protein-chromophore interactions to determine the extent to which our systems are able to inform us on features adopted by natural light harvesting.

In a series of collaborative experiments exploring *confinement* of sparsely labeling chromophores at different location within a hydrated nanoscale protein cavity we found that cavity confinement effects substantially slow the excited state solvation and nuclear relaxation, intramolecular rearrangements, and lifetime of the chromophore.[1] Because the water molecules in the cavity are necessarily proximal to the cavity protein surfaces, we hypothesize that these water molecules act as an extension of the protein side chains themselves to mimic the more conformal protein pockets often found in natural light harvesting

complexes. This geometry that we have discovered could therefore serve as a proxy for conformal pockets in light harvesting proteins that is far more modular and synthetically controllable in order to perform comparative studies.

In another series of four-way collaborative experiments we combined and compared the effects of confinement and dye linker *constraints* in sparsely labeled self-assembled protein complexes on chromophore photophysics and molecular dynamics (see Fig. 1; *in preparation*). We developed the ability to control chromophore orientation, conformational constriction, and distance to the protein surface. We found that we are able to finely tune the degree of retardation in the chromophore's excited state evolution by using linker engineering to restrict chromophore motion within different biomolecular hydration shells on the exterior surface of the protein, where protein motion and hindered water reorientation dictate the dye's environmental dynamics. The study confirmed that the above-mentioned confinement effect is very strong, dominating the effect on photophysical time scales compared to linker engineering strategies. The linker engineering employed nevertheless serves as a means to carefully control the relative position and orientation of chromophores with respect to one another, which is another essential element to mimicking natural light harvesting complexes close enough that experiments on the mimics can inform on natural light harvesting strategies.

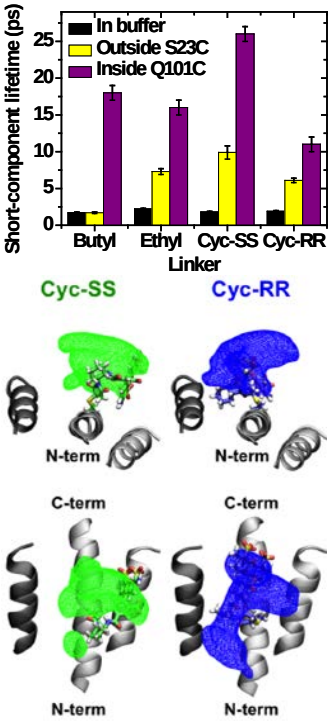

Figure 1. Comparison of ps transient absorption lifetimes of rhodamine dye with 3 different environments and 4 different linkers. Molecular simulations show close proximity of dyes to the protein surface for 2 configurations.

Last, we developed an experimental formalism to measure long range excitonic energy transport that should be suitable for natural and artificial biomimetic light harvesting protein arrays. The approach converts super-resolution fluorescence microscopy into an ultrafast measurement to track exciton population redistribution on its native nanometer and picosecond scales and was benchmarked on a conducting polymer solid model system.[2]

Science objectives for 2017-2018:

- The project will transition from a focus on studies of sparsely labeled protein complexes to fully labeled ones in order to use the developed confinement and constraining strategies to configure chromophores with respect to one another in geometries and environments that well approximate natural photosynthesis.
- Additional strategies to 'sandwich' pigments closely related to the pyrrole structures of chlorophylls within proteins will be pursued and compared to already developed configurations to assess which will be best to mimic and inform on natural photosynthesis while preserving the modularity that enables comparative studies.
- Ultrafast spectroscopy and computation of fully labeled protein assemblies will be compared to the previously obtained sparsely labeled results in order to reveal how fluctuating chromophore environments may enhance or interfere with excitation energy transfer.

References to work supported by this project: 2015-2017:

1. Noriega, R.; Finley, D. T.; Haberstroh, J.; **Geissler, P. L.**; **Francis, M. B.**; **Ginsberg, N. S.** "Manipulating Excited State Dynamics Of Light Harvesting Chromophores Through Restricted Motions In A Hydrated Nanoscale Protein Cavity" *Journal of Physical Chemistry B* **2015**, *119*, 6963.
2. Penwell, S. B.; Ginsberg, L. D. S.; Noriega, R.; **Ginsberg, N. S.** "Resolving Ultrafast Exciton Migration in Organic Solids at the Nanoscale" *Nature Materials* **2017** Advanced Online Publication, DOI: 10.1038/NMAT4975.

Fundamental Research Aimed at Diverting Excess Reducing Power in Photosynthesis to Orthogonal Metabolic Pathways

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Overall research goal: The objective of this project is to engineer novel biochemical pathways *in vivo* in the cyanobacterium *Synechococcus* sp. PCC 7002 such that excess reducing power generated in photosynthesis will be diverted into orthogonal metabolic pathways. The problem is that photosynthesis saturates several hours before and after the solar meridian due to downstream rate limitations, and this excess solar energy must be dissipated safely to heat through non-photochemical quenching (NPQ) mechanisms. This adaptation allows survival under light stress conditions, but it also represents a significant loss in photosynthetic yield. We propose that this excess energy can productively utilized by diverting the electrons at the reducing side of Photosystem I (PS I) to alternative enzymes when normal metabolic pathways are saturated. Our goal is not to build a device, but to carry out fundamental biochemical, biophysical, and physiological studies so as to provide proof of concept for a new way of extracting electrons *in vivo* on the reducing site of PS I.

Significant Achievements 2014-2017: The method to accomplish this relies on new coupling (bio)chemistries that we have developed with previous DOE funding. In the last funding cycle, we have 1) substituted a molecular wire with a dicluster ferredoxin (Fd_m) that was modified to contain two externally facing cysteine residues in our previous PS I-wire-H_2ase nanoconstruct, and we have demonstrated that the PS I-Fd_m-Pt nanoparticle and PS I-Fd_m-H_2ase nanoconstructs generate hydrogen in the light, and 2) placed a naphthoquinone-containing molecular wire construct in the A_{1B} and A_{1B} binding sites of PS I so that when the end of the wire is connected to a Pt nanoparticle or a modified H_2ase enzyme, hydrogen is generated at high rates in the light.

Science objectives for 2017-2018: Our first research objective is to express a PS I-Fd_m-H_2ase nanoconstruct *in vivo* in a cyanobacterium such as *Synechococcus* sp. PCC 7002. With funding from the DOE, we have developed a method to tether PS I to a [FeFe]-hydrogenase enzyme through their redox-active [4Fe-4S] clusters using a 1,8-octanethiol molecular wire. The wire tethers the F_B cluster of PS I and the distal FeS cluster of the hydrogenase in tunneling distance so that the electron can to pass from one enzyme to another. We have recently generated an *in vivo* variant of PS I that substitutes Gly for Cys_{13}, making it possible to attach a sulfhydryl-containing molecular wire to the F_B cluster without the need to remove PsaC with urea and rebind a Gly_{13} variant of PsaC to the resulting P_{700}-F_X cores. By substituting a modified dicluster ferredoxin that contains external-facing Cys residues near the iron-sulfur clusters, all three components - a modified PS I, a modified dicluster Fd_m, and a modified [FeFe]-H_2ase - can be expressed in the interior of the cell. This module should assemble *in vivo*; the result would provide a new mechanism to produce a reduced product at the highest possible quantum efficiencies by tapping the electron directly on the reducing side of PS I.

Our second research objective is to build a passive switch into PS I that will automatically divert excess electrons to an orthogonal metabolic pathway when the normal metabolic pathway becomes saturated. The core idea for the passive switch is depicted in **Figure 1**. The electron on the A_1 quinone in PS I would have two options. Under sub-saturating light conditions, the electron would

proceed forward to metabolic pathways by proceeding through the F_X, F_B and F_A clusters and on to ferredoxin and $NADP^+$. The alternative route of proceeding through the wire to the orthogonal metabolic pathway (e.g., hydrogenase) would not be taken simply because of the distance to the attached enzyme is longer than the distance to the F_X cluster. However, when sunlight is in excess, the F_X, F_B and F_A iron-sulfur clusters and the A_1 quinones would backfill with electrons, and the electron would now proceed through the (otherwise unfavorable) molecular wire to the orthogonal fuel-producing pathway. This would constitute a true passive switch: based solely on kinetics and on whether the acceptor side of PS I is filled would the electrons proceed either through the FeS clusters to normal metabolism or through the molecular wire to the orthogonal metabolic or fuel-producing pathway. The energy otherwise lost through NPQ would thereby be tapped for useful work. Our goal is to demonstrate proof-of-concept for a new way of utilizing excess reducing power by diverting electrons into an alternative pathway in lieu of being dissipated to heat.

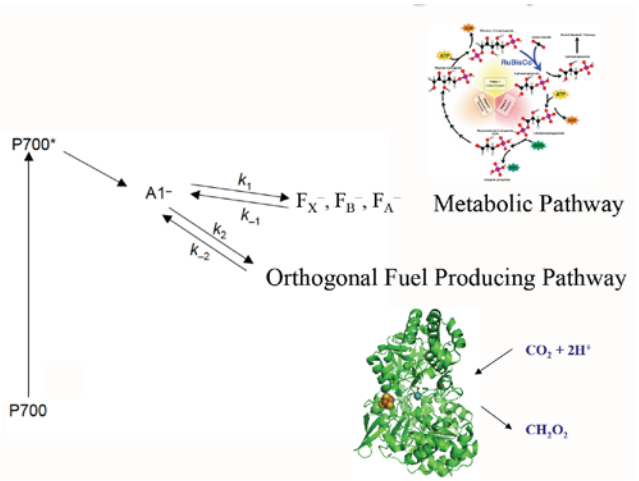

Figure 1. The idea of the passive switch to divert excess electrons to an orthogonal fuel-producing pathway. Under normal physiological conditions, the electron would proceed through k_1 to the FeS clusters to fuel normal metabolic pathways. Under excess light, electrons would back up in the FeS clusters and A_1 quinone, and any additional electrons would proceed through through k_2 to the orthogonal fuel-producing pathway. The switch would be entirely passive, depending solely on the presence of excess light.

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Studies of Photosynthetic Reaction Centers and Biomimetic Systems

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Overall research goals:

The OEC is a Mn_4CaO_5 complex that catalyzes the oxidation of two waters to O_2. This is accomplished without generating dangerous oxygen redox intermediates by accumulating 4 holes in the OEC so that water can be oxidized in one step. The OEC intermediates are described as S-states, from S_0 (most reduced) to S_4 (most oxidized). The stable state is S_1. The catalytic S_4 state is unstable and cannot be trapped experimentally for study. Thus, computational approaches provide important tools for understanding the chemical reaction. Our efforts focus on computational analysis of the structure and reactions in Photosystem II (PSII), in particular the Oxygen Evolving Complex (OEC) and quinone reactions. We have established a collaborative approach to carry out fundamental studies that can be extended to other natural photosynthetic proteins and biomemetic systems. The methods incorporate Classical Electrostatics with Monte Carlo (MCCE) simulations for calculations of pK_as, electrochemical midpoints and ligand binding (Gunner) and quantum mechanics and quantum mechanics/molecular mechanics (QM/MM) analysis (Batista). We have used experiments monitoring the OEC reactions (Brudvig) and novel analysis of the structure (Wang, Amin) to determine how the OEC functions and how its function is modulated by the PSII protein.

Significant achievements 2015-2017:

The key questions we have addressed are: 1) What are the changes in structure of the complex as it moves through the S states. QM/MM has been used to generate optimized structures in each S state from S_{-2} to S_3. Work is ongoing on the structural evolution in S_4. 2) How small ligands such as the required chloride or inhibitory ammonia act. Their binding sites in different S-states and how they modify the equilibrium between the redox states or as function of pH has been calculated; 3) How proton loss aids in redox leveling so that the OEC electron affinity does not increase as the complex becomes more oxidized. MCCE calculations suggest a role for W2, E65 and E329; 4) The role of the Ca is accessed by studies of substitution of this ion by Sr. Structures were optimized with QM/MM with each cation in the resting state (S_1) and in more reduced states (S_0, S_{-1}, and S_{-2}). Comparison with experimental data, shows that the X-ray crystal structures with either Ca^{2+} or Sr^{2+} are most consistent with the S_{-2} state (i.e., Mn_4[III,III,III,II] with O4 and O5 protonated). As expected, the QM/MM models show that Ca^{2+}/Sr^{2+} substitution results in the elongation of the heterocation bonds and the displacement of terminal waters W3 and W4. The hydrogen-bonded W5 is displaced in all S states with Sr^{2+}, suggesting W5 may play a critical role in water oxidation. 5) How much remains to be learned from the current experimental structures is addressed by in-depth analysis of the published electron density maps for the different s-ray structures (Wang) and by ab initio QM analysis of the behavior of the OEC core bombarded by the radiation from a free electron laser used in obtaining crystal structures (Amin). In each case, experimental measurements are used as a test of computational results.

Science objectives for 2018-2021:

The goals can be divided into 3 interrelated sets of questions.

1) At the center is the chemistry of oxygen evolution itself in the $S_3 \rightarrow S_4 \rightarrow S_0$ transition. Analysis by QM/MM will investigate the rate-determining step of water oxidation in photosystem

II as characterized by a combined computational and experimental analysis of the $^{16}O/^{18}O$ Kinetic isotope effect. Other efforts will be focus on the nature of intermediates generated upon deprotonation of the S_2 state and the effect chloride on PCET mechanisms along the catalytic cycle.

2) The questions of how OEC utilizes the protein to achieve rapid proton loss and redox leveling through the S-state cycle will be evaluated with combined classical MC and MD analysis given OEC structures optimized with QM/MM. Improved analysis of the relative redox potentials for the Ems for the state transitions will be used to evaluate the role of proton loss from specific sites (such as W1, W2, E65, E329) on redox leveling in the S-state cycle (from S_0 to S_3) prior to reaction. The proton transfer pathways will be determined in each S-state using a combination of MD and MCCE in wild-type PSII and in a selected series of mutants that have been shown to modify the oxygen evolving reactions. The effects of these reactions on the proton loss and the electrochemical midpoints for the S-state transitions will also be determined.

3) The improving structures of the OEC has provided data that is amenable to additional analysis providing new suggestions, which can be evaluated with simulation and experiment. Combined analysis of the high-resolution electron microscopy with XFELS X-ray crystal structures has suggested the presence of additional bound ions in the structure or multiple conformations in critical locations. The relative stability of local site ionization or ion binding will be evaluated with classical and QM/MM simulations.

Reference to the work supported by this project 2016-2017:

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11 papers published 2015;
2 papers currently submitted.

Time resolved step-scan FTIR difference spectroscopy for the study of photosystem I with different quinones incorporated into the A_1 binding site

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Overall research goals: The goal of this project is to gain a molecular-level understanding of the factors that modulate the functional properties of quinones that occupy the A_1 binding site in photosystem I (PSI).

To address this goal we are using microsecond time-resolved step-scan Fourier transform infrared (FTIR) difference spectroscopy to study photosystem I particles in which many foreign quinones have been incorporated into the A_1 binding site.

Bands in the time-resolved infrared difference spectra report on single molecular bonds of the quinones in the A_1 protein binding site. Assigning bands in experimental spectra to molecular bonds can be difficult, however. To aid in spectral band interpretation and assignment a second goal is to also develop computational methods in order to simulate the experimental spectra. In particular, ONIOM-type (QM:MM) quantum chemical methods are being developed and employed.

Significant achievements 2017- -: Recently we have used time-resolved optical spectroscopy to probe the dynamics of electron transfer in PSI with several different quinones incorporated. Through modeling the bioenergetics, using the experimental data as input, we were able to show that $P700^+A_1^-$ radical pair recombination occurs in the inverted region, in native PSI at room temperature. Furthermore, we were able to show that this inverted region mechanism was a factor contributing to the very high efficiency of solar energy conversion in photosystem I [Makita and Hastings (2017). *Proc. Nat. Acad. Sci.* 114, 9267-9272]. The bioenergetics in PSI outlined in this paper will be correlated to structural details we will uncover in the current project.

Time-resolved step-scan Fourier transform infrared (FTIR) difference spectra have been obtained using PSI particles with phylloquinone (PhQ) and 2-methyl naphthoquinone (2MNQ) incorporated. An initial molecular model was constructed and ONIOM-type, QM:MM calculations were undertaken in order to simulate the experimental spectra (Makita, Rohani, Zhao and Hastings (2017). *Biochim. Biophys. Acta* 1858, 804-813). In particular, this published work introduced a new type of computational method for calculating vibrational frequencies of quinones in protein binding sites, a so-called "three-layer ONIOM method". The advantages of this particular methodology were outlined and will form the basis of future studies (see below).

Science objectives for 2017-2018:

- We have produced time-resolved FTIR difference spectra using intact PSI particles with PhQ and 2MNQ incorporated into the A_1 binding site. The next steps are to obtain similar spectra for PSI samples using two isotope labeling strategies that will allow us to better distinguish FTIR bands associated with the pigment (the quinone) and the protein. First we will incorporate specifically ^{18}O labeled PhQ and 2MNQ into unlabeled PSI particles. Secondly we will incorporate unlabeled PhQ and 2MNQ into fully ^{13}C labeled PSI particles. Spectra obtained from this work, when compared to that already undertaken, should allow a clearer delineation on the nature of the bands in the spectra. This experimental work will also help drive the computational components of the project forward.
- All work so far has been undertaken using intact PSI particles. For a variety of reasons, it will be worthwhile undertaking similar work using PSI particles in which the terminal electron acceptors have been removed, and use what are commonly referred to as P700-F_X particles.

- We have previously published a basic outline for QM:MM type calculation of the vibrational properties of pigments in protein binding sites, and on relating calculation to experimental spectra. More inclusive molecular models will be constructed and considered, to help establish which molecular parameters (and computational parameters) are most important for spectral simulation.
- Electron paramagnetic resonance (EPR) spectroscopy has also been used to study PSI particles with a range of different quinones incorporated. In the coming year we aim to use the same molecular models (as used in vibrational frequency calculations) to also calculate EPR spectroscopic parameters. Using the same molecular models to simulate both EPR and FTIR spectroscopic data is new, and will very stringently test the applicability of the computational approach.
- To further extend our understanding of the structural and functional properties of quinones in the A_1 binding site in PSI we will produce time-resolved FTIR difference spectra using PSI particles with dimethyl naphthoquinone (DMNQ) and plastoquinone (PQ) incorporated into the A_1 binding site. Associated calculations to simulate the FTIR and EPR spectroscopic data will also be undertaken. PQ is a benzoquinone (BQ) analogue. To better understand PSI with PQ incorporated a range of BQ analogues will be incorporated into PSI.

References to work supported by this project:

Project began Sept. 2017.

Protein Targeting To The Chloroplast Thylakoid Membrane:
Structure and Function of a Targeting Complex

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Overall research goals: Chloroplast signal recognition particle (cpSRP) is a heterodimer composed of an evolutionarily conserved 54-kDa GTPase (cpSRP54) and a unique 43-kDa subunit (cpSRP43) responsible for delivering light-harvesting chlorophyll binding proteins (LHC) to the thylakoid membrane. Our central hypothesis is that vectorial targeting of LHC proteins to the thylakoid membrane is dictated by changes in the structural dynamics of cpSRP43 upon sequential interaction with each of its binding partners to ensure that cpSRP43 efficiently associates with the Alb3 insertase only when at the membrane carrying LHC targeting cargo. In this context, we are examining the following specific aims:

Aim 1: Determine structural changes in cpSRP43 at each step of the targeting pathway.
Aim 2: Determine kinetics and thermodynamics of cpSRP43 and cpSRP43-containing complexes with its various binding partners (cpSRP54, LHC, cpFtsY, Alb3-Cterm).
Aim 3: Determine the Alb3-Cterm-cpSRP43 binding interface and the Alb3 Cterm-bound structure of cpSRP43.

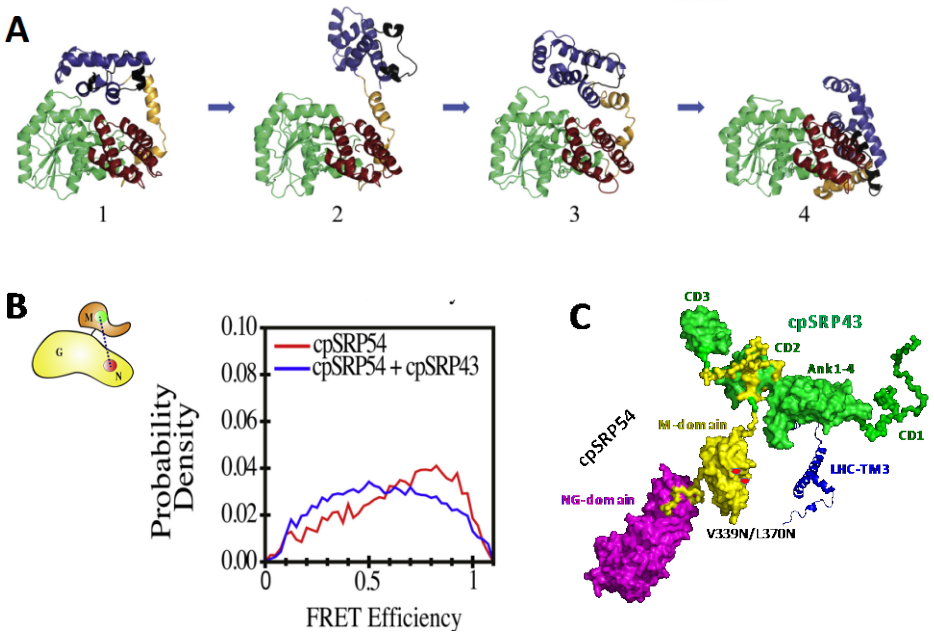

Figure 1. (**A**) Results of an all atom molecular dynamic simulation shows the position shift of the M-domain (blue) in cpSRP54 from a coupled state (1) to an uncoupled state (2) and subsequent interactions that bring it back to a coupled state. (**B**) Dye molecules (red and green donor and acceptor) placed at specific sites in the cpSRP54 N and M domains were used to conduct smFRET with cpSRP54 alone and bound to cpSRP43, which caused a change in cpSRP54 domain organization to a low FRET state. (**C**) The structure of cpSRP from existing domain structures and SAXS data is shown in a low FRET state observed in B. The hypothesized position of LHC-TM3 near the cpSRP54 M-domain is indicated based on the know interaction site of LHCs on cpSRP43. A double point mutation in cpSRP54 M-domain is indicated, which causes a loss of cpSRP-LHC targeting complex formation except in LHCs lacking TM3.

Significant achievements during funding year 1 (2016-2017):
We developed and examined an in silico three-dimensional model of the structure of cpSRP54 by homology modeling using cytosolic homologs and SAXS. Model selection was guided by results from single-molecule Förster resonance energy transfer experiments (smFRET). Small angle x-ray scattering showed that the linking region between the GTPase (NG-domain) and methionine-rich (M-domain) domains, an M-domain loop, and the cpSRP43 binding C-terminal extension of cpSRP54 are predominantly disordered.

In addition to revealing the presence of multiple cpSRP54 conformations, our approach identified a new low FRET conformation induced by interaction with cpSRP43. It was hypothesized that a functional consequence of cpSRP43-induced domain reorganization might be to promote binding of cpSRP54 M-domain to transmembrane domain 3 (TM3) of LHC, already positioned by an LHC-cpSRP43 interaction that initiates cpSRP-LHC targeting complex formation. Functional assays demonstrated that a functional cpSRP54 M-domain was only required for targeting complex formation when LHC targeting substrates contained TM3. Removal of TM3 from the LHC targeting substrate eliminated the need for a functional cpSRP54 M-domain to form a cpSRP-LHC targeting complex.

Science objectives for 2017-2018:

- From our cpSRP54 structural models, we are currently exploring hypothesized interactions between charged residues in cpSRP54 N-domain and residues in cpSRP43 CD1, which would be stabilized by binding of LHC targeting substrate and favor a cpSRP54 structure that exposes its binding interface for a membrane receptor, cpFtsY. This hypothesis is consistent with our previous observation showing that CD1 mutants of cpSRP43 form cpSRP-LHC targeting complex, but fail to support LHC insertion into thylakoids. We are using FRET dyes placed near the cpSRP43 (CD1)-cpSRP54 (N-domain) interaction sites to examine the influence of LHC binding on FRET efficiency. Stabilization of this interaction by LHC binding would increase FRET efficiency and support a mechanistic role of LHC binding in stabilizing a cpSRP54 structure necessary for the step wise progression of LHC targeting substrates to the thylakoid.
- The C-terminal domain of Albino-3 (Alb3-Cterm) is intrinsically disordered. Biophysical studies reveal that portions of Alb3-Cterm are structured and we hypothesize that the ordered structural elements in Alb3-Cterm serve as recognition sites to promote specific interaction with cpSRP43. In this context, we are using a wide-array of experimental techniques including, site-directed mutagenesis, multidimensional NMR spectroscopy, isothermal titration calorimetry, FRET analysis and *in silico* molecular modeling to characterize the structured elements in Alb3-Cterm and examine how the structural order facilitates specific recognition and interaction between Alb3-Cterm and cpSRP. We believe that understanding the interplay of the structural forces that govern the specificity of interaction between Alb3-Cterm and cpSRP is critical to decipher the molecular mechanism underlying the integration of LCHs into the thylakoid membrane.

References to work supported by this project during year 1 funding (2016-2017):

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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

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Overall research goals: Biological processes involved in the conversion of sunlight into energy-dense organic compounds are highly integrated across multiple temporal and spatial scales, ranging from photon capture by membrane-embedded photosynthetic complexes to biochemical pathways that partition reduced carbon to various metabolic sinks. The overarching objective of Project B is to work collaboratively between multiple labs and across disciplines to better understand photosynthesis as an assembly of functional modules that comprise the biological solar panel. One focus of this collaborative endeavor involves the use of engineered plant and cyanobacterial systems to study how shifts in carbon partitioning are sensed, and to understand how alterations in sink demand influence the activity and composition of the photosynthetic apparatus. A key aspect of this subproject employs the "growth *versus* defense" paradigm to understand processes governing carbon partitioning in Arabidopsis leaves.

Significant achievements 2016-2017

1. We constructed a series of constitutive jasmonate response mutants that exhibit increased levels of leaf defense concomitant with restricted growth. The level of growth-defense antagonism in these lines was genetically "tuned" through mutation of an increasing number of JAZ repressor proteins. These *jaz* mutants are being used to study complex relationships between photosynthesis, growth, and changes in carbon partitioning to major metabolic sinks.

2. A *jaz* quintuple mutant (*jazQ*) was used for a genetic suppressor screen to identify mutants in which leaf growth inhibition was eliminated. This screen identified two distinct groups of mutants. One group exhibited robust growth and complete loss of defense; these phenotypes were attributed to mutations in genes required for jasmonate biosynthesis and signaling. More interestingly, the second group of mutants exhibited robust leaf growth but maintained increased flux of metabolism into leaf defense compounds. Identification of the causal mutation in one such suppressor line revealed a null mutation in the gene encoding the red light receptor phytochrome B (phyB). The discovery that *jazQ phyB* plants grow and defend well at the time same demonstrates that growth-defense tradeoffs observed in *jazQ* are not caused by simple diversion of limited photosynthetic products from growth to defense. This work also highlights a key role for photoreceptors and light quality in the control of carbon allocation.

3. The *jazQ* mutation partially rescued the low photosynthetic capacity of *phyB* leaves, which results in part from limitation in Rubisco activity. *phyB* leaves were also thinner than those of wild type, and this architectural feature was retained in *jazQ phyB*. Because of the greater projected leaf area available to intercept light (due to longer petioles and flatter, thinner leaves), the whole-plant photosynthetic rate in *jazQ phyB* plants was greater than that of either the *phyB* or *jazQ* parents. The costs associated with construction of *jazQ phyB* leaves may therefore be lowered through increased partitioning of carbon to leaf area at the expense of leaf thickness.

4. We demonstrated that the reduction of leaf growth by *jazQ* results from constitutive activity of the three related transcription factors, MYC2/3/4. These regulators strongly repress the

expression of genes required for various photosynthetic processes. Consistent with this, gas exchange experiments showed that *myc2/3/4* triple mutants had higher CO_2 assimilation rate per unit leaf area than wild-type. These findings implicate MYC2/3/4 as negative regulators of photosynthesis, and highlight a new mechanism to modulate photosynthesis during plant acclimation to changing environmental conditions.

5. We used *jazQ* as a chassis to deplete five additional JAZ repressors, thus increasing the strength of growth-defense tradeoffs. Remarkably, strong partitioning of photoassimilates to defense compounds in this *jaz* decuple mutant (*jazD*) resulted in depletion of transient carbon reserves (starch and sucrose), increased rates of respiration, and hallmark signs of carbon starvation. Despite the fact that *jazD* leaves had normal photosynthetic efficiency (on a leaf area basis) as determined by leaf ^{13}C discrimination analysis and gas exchange, the accumulated levels of thylakoid-bound photosynthetic proteins were significantly reduced in *jazD* leaves. These findings suggest the existence of a mechanism to reduce resource investment in photosynthesis without compromising photosynthetic efficiency.

Science objectives for 2017-2018:

1. We will investigate the mechanisms by which increased jasmonate signaling, as a proxy for increased metabolic sink demand, modulates photosynthetic efficiency. This will be achieved by studying the effects of *jazD* on protein, pigment and lipid composition of thylakoid membranes, and also by determining effects on chloroplast size, number, and positioning.

2. We will investigate the mechanism of carbon starvation in *jazD*, including quantitative analysis of the effects of *jaz* and *phyB* mutation on various metabolic sinks.

3. We will characterize *jazD* suppressor mutants and identify the molecular basis of at least one genetic lesion capable of uncoupling growth-defense tradeoffs.

References to work supported by this project 2016-2017:

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Abramson, B. W., Kachel, B., Kramer, D. M., & Ducat, D. C. (2016) Increased photochemical efficiency in cyanobacteria via an engineered sucrose sink. *Plant and Cell Physiology* 57, 2451-2460. Selected as Editor's Choice for this issue of *PCP*.

Major IT, Yoshida Y, Campos ML, Kapali G, Xin XF, Sugimoto K, Ferreira DO, He SY, Howe GA (2017) Regulation of growth-defense balance by the JAZ-MYC transcriptional module. *New Phytologist* 215: 1533-1547. Subject of a Commentary in this issue of *New Phytol*.

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Mutants of light harvesting antennas and reaction centers: disorder, excitonic structure, electron transfer, and excitation energy transfer dynamics.

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Overall research goals: Unravel mutation induced effects and their impact on excitonic structure and relevant photochemical/nonphotochemical processes in various photosynthetic complexes. Use high-resolution spectroscopic techniques and excitonic calculations to provide more insight into the relationship between structure-function relationship and dynamics in intact and mutated light harvesting antennas and reaction centers. The following issues are being explored: i) mutation induced shifts of pigment site energies; ii) altered inhomogeneity; iii) spectral density and electron-phonon couplings; iv) protein energy landscape; and v) electron transfer (ET) and excitation energy transfer (EET) dynamics. We also anticipate to provide a more comprehensive relationship between hole-burning (HB) and 2DES methodologies. Better understanding of mutation induced effect (at molecular level) on the excitonic structure and ET/EET processes may help to design better artificial photosynthetic systems.

Significant achievements (2016 - 2017):

- ***Towards an understanding of the excitonic structure of the CP47 antenna protein complex of PSII revealed via circularly polarized luminescence*** **(Publ. 1).** There was no consensus concerning the nature of the low-energy state(s) nor chlorophyll (Chl) site energies in this important photosynthetic antenna. Although, we raised concerns regarding the estimations of Chl site energies obtained from modeling studies of various types of CP47 optical spectra **[Publ. 2]** new assignments reported in the literature based on the shape of the circularly polarized luminescence (CPL) spectrum necessitated our comments. We exposed the heterogeneous nature of the recently studied complexes and argued that the published composite nature of the CPL does not represent an intact CP47 protein. A new information on the excitonic structure of intact and destabilized CP47 complexes and their lowest energy state(s) was provided.
- ***Mutation-induced changes in the protein environment and site energies in the (M)L214G mutant of the Rhodobacter sphaeroides bacterial reaction center*** **(Publ. 3).** This work focused on the low-T photochemical (transient) HB spectra within the P870 absorption band, and their theoretical analysis, for the (M)L214G mutant of the *Rb. sphaeroides* bacterial (bRC). To provide insight into system-bath interactions of the BChl *a* special pair in the mutated bRC, the optical lineshape function for the P870 band was calculated numerically. Based on the modeling studies, we demonstrated that (M)L214G mutation led to a heterogeneous population of bRCs with modified (increased) total electron-phonon coupling strength of the special pair BChl *a* and larger inhomogeneous broadening. Specifically, we showed that after mutation in the (M)L214G bRC a large fraction (~50%) of the bacteriopheophytin (H_A) chromophores shifted red and the 800 nm absorption band broadened, while the remaining fraction of H_A cofactors retained nearly the same site energy as H_A in the wild-type bRC. Modeling using these two subpopulations allowed for simultaneous fits of the absorption and nonresonant (transient) HB spectra of the mutant bRC in the charge neutral, oxidized, and charge-separated states using the Frenkel exciton Hamiltonian providing new insight into the mutant's complex electronic structure and electron transfer dynamics.
- ***Conformational complexity and light-induced photoconversion in the LH2 antenna of the purple sulfur bacterium Alc. vinosum revealed by HB spectroscopy*** **(Publs 4 and 5).** The protein conformational complexity of the B800-850 LH2 complexes from the purple sulfur bacterium *Alc. vinosum* has been revealed. We demonstrated the presence of two conformations of B850 bacteriochlorophylls (BChls), referred to as conformations 1 and 2, and two conformations of B800 BChls, denoted as $B800_R$ and $B800_B$. The energy differences between average site energies of conformations 1 and 2, and $B800_R$ and $B800_B$ are ~200 cm^{-1}. Although conformations 1 and 2 of the B850 chromophores, and $B800_R$ and $B800_B$, exist in the ground state, selective excitation led to $1 \rightarrow 2$ and $B800_R \rightarrow B800_B$ phototransformations. An exciton model with dichotomous protein conformation disorder has been developed. We showed that both experimental data and the modeling study support a two-site model with strongly and weakly hydrogen-bonded B850 and B800 BChls, which under illumination undergo conformational changes, most likely caused by proton dynamics (rearrangement of H-bonds).

- ***The Fenna-Matthews-Olson (FMO) trimer and its mutant analogs*** **(Publs 6-8).** Using experimentally determined shapes for the spectral densities we simulated optical spectra of for both the intact and destabilized FMO trimers, as well as several FMO mutants. Simultaneous fits of multiple low-temperature spectra provided new Hamiltonians. For example, we showed that Y16F mutant is a mixture of FMO complexes with three independent low-energy traps (located near 817, 821, and 826 nm), in agreement with a measured composite emission and HB spectra. These traps belong to three mutated FMO subpopulations characterized by significantly modified low-energy excitonic states. Hamiltonians for the two major subpopulations (Sub_{821} and Sub_{817}) provided new insight into extensive changes induced by the single point mutation in the vicinity of BChl 3 (*where* tyrosine 16 (Y16) was replaced with phenylalanine F16). We showed that the average decay from the higher exciton state(s) in Y16F mutant depends on frequency and occurs on a picosecond time scale.

Science objectives for 2017-2018:

- Explore the role of bath fluctuations in the non-linear spectra in *Rb. sphaeroides*. Show that vertical cross sections of the simulated 2D spectra (with all populations in the lowest excited state) will reveal transient HB spectra excited resonantly within the B-band in agreement with experiment, providing new insight into environmental fluctuation parameters on charge separated configurations of molecular excited states.
- Explain the nature of persistent holes burned into the P-band of WT *Rb. sphaeroides* and its triple L(L131)H+L(M160)H+F(M197)H mutant with a very low quantum yield of charge separation.
- Complete experimental/modeling studies of reconstituted CP29 complexes and its A2, B3, and YF(135) mutants. Describe their underlying excitonic structure and dynamics.
- Using our new FMO Hamiltonian calculate the 2D electronic spectroscopy frequency map and transient HB spectra (using the vertical cross sections) to provide insight into coherent and dissipation effects in this important protein complex.
- Complete spectroscopic and modeling studies of C49A and C353A FMO mutants.
- Elucidate the nature of low-energy Chls in WT and mutants of PSI trimers of *Synechocystis.*

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Characterizing and Engineering Subcellular and Cellular Modules for Photosynthetic Productivity

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Overall research goals: Project C of the MSU-DOE Plant Research Laboratory was initiated in 2014 with the overarching goal of gaining a fundamental mechanistic understanding of cyanobacterial photosynthesis--the single cell natural solar panel--that can be applied to guide strategies for engineering improvements in primary productivity.

Significant achievements 2015-2017: We have focused on the structural, functional and interconnectivity of two cyanobacterial modules, light harvesting and the carboxysome, and also on a modular photoactive protein, the Orange Carotenoid Protein. These modules are prominent components of cyanobacterial photosynthesis and possess structural features broadly useful for bioengineering applications.

Science objectives for 2015-2017:

- Understand the regulation, constitution, organization of the carboxysome in the context of cell size and morphology in response to the environment.
- Using our knowledge of the three dimensional structures and progress in developing new architectures from these building blocks, realize the potential of BMC domains to construct designer metabolic scaffolding *in vivo* and *ex vivo*.
- Devise new ways of controlling communication between modules by repurposing a photoactive protein that responds to light intensity.

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Photosynthetic Energy Transduction

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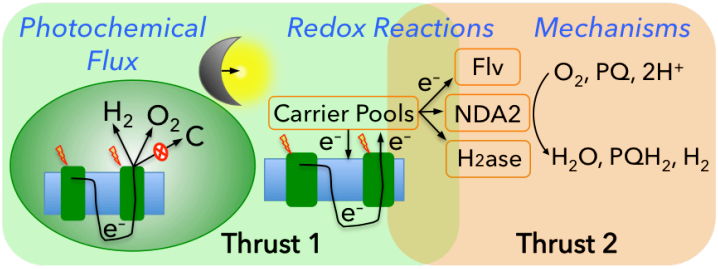

Overall research goals:

The overall goal of this program is to develop an understanding of how peripheral redox reactions integrate with core photosynthetic electron transfer (PET) to enable acclimation to changes in photochemical flux. Acclimation is complex, involving numerous electron transfer pathways that are regulated, and have unique reaction kinetics, redox partners and substrate reactivities. The collective contribution of peripheral redox reactions to photochemical electron flow affords enormous flexibility to cells to maintain redox homeostasis. Remodeling of photosynthetic complexes, and the differential regulation of biochemical pathways, redox enzymes and electron carriers results from coordinated responses, and the mechanistic basis of control is complex. The aim of our work is to identify the biosynthetic processes that establish electron flow pathways, define the redox enzyme networks and catalytic activities, and establish models for the integration of peripheral electron flow with photosynthetic electron transport during acclimation.

Significant achievements (2016-2017):

Photosynthetic energy utilization in mutants with altered carbon allocation pathways.

- A putative prolyl-4-hydroxylase mutant (AHP5) of *Chlamydomonas reinhardtii* was studied as a biochemical regulatory model of H_2 photo-production. We have determined that P4H is required for *hydEF* expression, and that in the AHP5 strain hydrogenase accumulates in the unprocessed form of Hyd structural proteins. The results implicate that Hyd translocation and processing are coupled to maturation during biosynthesis.

Biochemical studies of Synechocystis flavodiiron (Flv) enzymes.

- We have investigated photochemical properties of Δ*flv1*, Δ*flv3* and Δ*flv1*Δ*flv3* strains of *Synechocystis*. All deletion strains exhibited reduced photosynthetic O_2 evolution capacity that was restored by addition of methyl viologen, indicating a limitation on photosystem I (PSI) acceptor availability. Long-term monitoring of PSI oxidation state showed that Flv1 and Flv3 function not only in acclimation to stress, but more generally in establishing electron transport upon illumination.
- Purified recombinantly expressed *Synechocystis* Flv1 and Flv3 to homogeneity for biochemical and kinetic analysis. Flv1 and Flv3 purified as homodimers and catalyzed the reduction of O_2 in the presence of either NADH or NADPH, with K_m values in the range of 70-90 μM or 175-220 μM, respectively, which match the cellular steady-state levels of reduced pyridine nucleotides.

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

- The NDH complexes, HOX [NiFe]-hydrogenase, and some [FeFe]-hydrogenases have a unique site-differentiated His1Cys3 [4Fe-4S] cluster that functions in electron transfer at interfaces or with soluble donors. A domain fragment of [FeFe]-hydrogenase that coordinates this cluster was

prepared, and EPR and square-wave voltammetry measurements showed the reduced cluster has S = 1/2 and S = 3/2 spin states and an unusually low reduction potential of $E_m^{8.8}$ = -570 mV. The biophysical and thermodynamic properties frame how cluster properties are tuned to modulate intermolecular electron transfer, relevant under changing photochemical flux conditions.

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.

Science objectives for 2017-2018:

- Complete a NRVS analysis of the ^{57}Fe enriched proton-transfer mutant of [FeFe]-hydrogenase poised in the Fe-hydride state, determine the Fe-H vibrational modes and computational models of extended H-bonding with the pendant amine. Complete Mössbauer analysis of the electronic structure of diamagnetic H-cluster states observed at more positive reduction potentials.
- Complete biochemical, thermodynamic and kinetic studies of cyanobacterial Flv proteins, and test redox coupling reactions of Flv's with non-pyridine nucleotide electron donors including flavodoxin and rubredoxin, and conduct mapping of the dimer interaction surface by mass-spectrometry. Further define the function of Flv's in photosynthetic electron transport in knock-out strains using photochemical and P700 kinetic measurements.
- Define whether the HOX hydrogenase diaphorase domain functions in electron transfer reactions with soluble electron donor-acceptors in modulating electron flow around photosystem I, as well as with other photosynthetic electron transport components. Isolate and analyze NADH dehydrogenase I subunits (NdhS) to test for interactions with HOX.

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Controlling Electron Transfer Pathways in Photosynthetic Proteins

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Overall research goals: Photosynthetic reaction centers (RCs) are protein-cofactor complexes that convert light energy into chemical energy in a series of extremely efficient electron transfer (ET) reactions that accomplish transmembrane charge separation. RCs reveal two symmetry-related branches of cofactors called A and B (Fig. 1) that are functionally asymmetric. In RCs from photosynthetic bacteria, only the A cofactors are used for light-induced electron transfer. The goal is to produce such an RC that instead uses the B side preferentially and efficiently. RCs from the purple photosynthetic bacteria *Rhodobacter (R.) capsulatus* and *R. sphaeroides* with targeted sites of saturation mutagenesis are rapidly screened for high-yield transmembrane, B-side charge separation with limited competing charge recombination (Fig. 2). We have developed semi-directed molecular evolution approaches that streamline mutagenesis and RC isolation as well as high-throughput time-resolved spectroscopic screening assays. By design, a large number of RC variants can be examined for the yield of $P^+Q_B^-$ arising from exclusive use of the B-side cofactors. The principles realized will be broadly applicable, such as aiding design of biomimetic systems that are competent in light-induced ET.

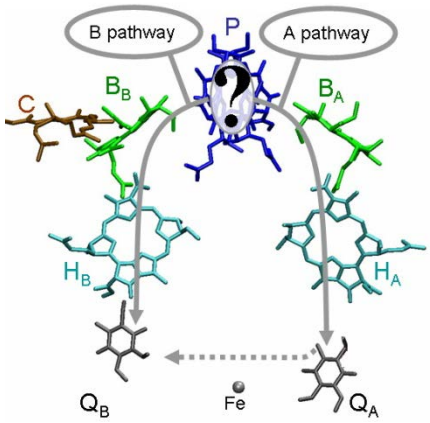

Fig. 1. *Arrangement of cofactors in the bacterial RC.*

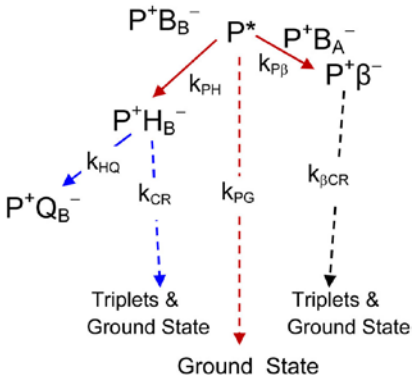

Fig. 2. *States, target processes, and key $P^+H_B^-$ branching point.*

Significant achievements 9/2015 – 9/2017: Improvements to our mutagenesis and screening methods have boosted our inventory to >800 mutants. They are comprised of 29 mutant sets involving saturation mutagenesis at target sites plus a number of individual substitutions. Of these, ~600 expressed RCs at sufficiently high levels to be purified and screened. Fig. 3 shows the yields of B-side formation of $P^+Q_B^-$ in a subset of nearly 400 mutants relative to wild type (~100%, via Q_A), as determined by a millisecond screening assay. The colors of the bars reflect different RC templates used and different residues targeted. Many mutants utilize a scaffold (YFHV; Fig. 3, inset) that displays substantial B-pathway activity in the assay (Fig. 3; main, yellow bar). Specific key accomplishments during the past two years are as follows:

- Generated and screened six *R. capsulatus* sets with mutations near H_B, B_B and/or B_A. The rates and yields of B-side ET steps $P^* \rightarrow P^+H_B^-$ and $P^+H_B^- \rightarrow P^+Q_B^-$ and competing deactivation processes give increased insight into engineering a high overall yield of transmembrane charge separation.

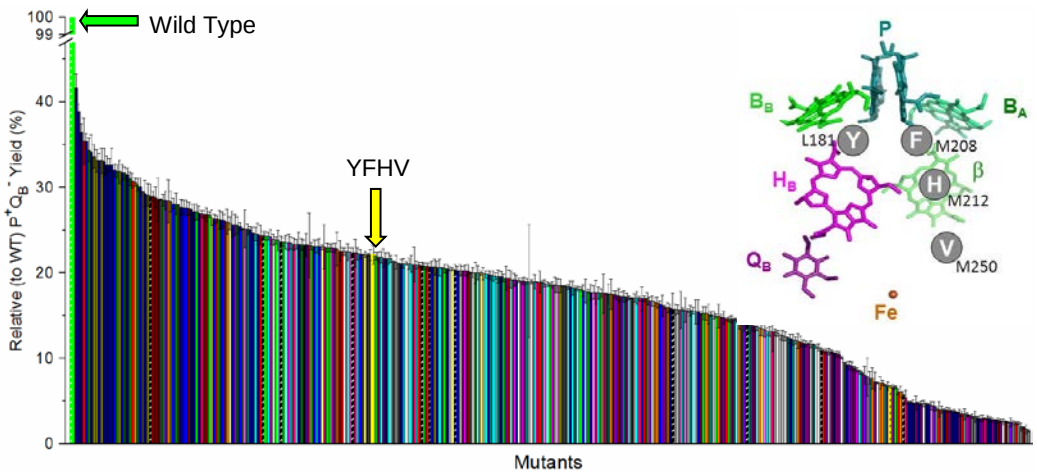

Fig. 3. *Yield of $P^+Q_B^-$ relative to wild type in ~400 mutants of two species as assayed by a millisecond screening assay (main panel); the YFHV template RC (top right).*

- Developed a cassette-based mutagenesis system for the *R. sphaeroides* RC. Comparison of the first two sets of *R. sphaeroides* mutants revealed differences from the analogous *R. capsulatus* sets. A *R. sphaeroides* mutant showed one of the highest yields of $P^+H_B^-$ formation (~60%) reported to date.
- Found excellent agreement in the *relative ordering* of $P^+Q_B^-$ yield from B-side ET for ~150 *R. capsulatus* mutant RCs screened using 15-ps excitation flashes with the plate-based ms assay (to minimize potential photochemical recycling) compared to prior results using 7-ns flashes.
- Generated two sets of *R. capsulatus* mutants with saturation mutagenesis at M180His (the ligand to B_B) affording RCs that retain B_B or replace it with a bacteriopheophytin (Φ_B).
- Employed 'tryptophan scanning' on a host of sites near and/or between H_B and Q_B to examine the potential for an electron conduit and/or electronic tuning to facilitate secondary ET. Probed a Phe-rich region in the vicinity of H_A for comparison.
- Obtained and genotypically characterized photocompetent revertants. Analyzed the unique photochemistry of RCs purified from revertant strains.

Science objectives for 2018:

- Iterate combinations of mutations, pairing favorable substitutions with those in new regions.
- Mine datasets to correlate properties of substituted residues with observed photochemistry.
- Select photocompetent, phenotypic revertants that grow via B-side cofactors exclusively.

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2. *Species Differences in Unlocking B-side Electron Transfer in Bacterial Reaction Centers*, N. P. Dylla, K. M. Faries, R. M. Wyllie, A. M. Swenson, D. K. Hanson, D. Holten, C. Kirmaier and P. D. Laible, *FEBS Lett.*, **2016**, *590*, 2515–2526.
3. *Manipulating the Energetics and Rates of Electron Transfer in* Rhodobacter capsulatus *Reaction Centers with Asymmetric Pigment Content.* K. M. Faries, N. P. Dylla, D. K. Hanson, D. Holten, P. D. Laible and C. Kirmaier, *J. Phys. Chem. B*, **2017**, *121*, 6989–7004.

The Dynamic Energy Budget of Photosynthesis

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Overall research goals: Photosynthesis must balance how much energy is stored in ATP and NADPH to *precisely* meet the ratio required for biochemical demands. If this balancing does not occur, the system will fail, leading to photodamage. It is also critical for efforts to improve the efficiency of photosynthesis by introducing CO_2 concentrating mechanisms, altering metabolism or biosynthetic pathways to shunt energy to alternative products. These balancing processes must also be extremely robust to contend with the rapid and unpredictable fluctuations in environmental conditions and metabolic demands that occur in nature. This proposal takes advantage of recent developments from our current work to address several key questions about the mechanisms of these balancing acts, how they are regulated and why they are critical for maintaining photosynthesis. Specifically, we will address 1) the basic mechanisms of Cyclic Electron Flow (CEF) that increase the production of ATP relative to NADPH; 2) how CEF is regulated at the level of the key enzyme, the NDH complex; and 3) the mechanisms by which photodamage occurs when ATP/NADPH output is perturbed.

Significant achievements 2016-2017: We have made progress on all proposed aims, resulting in six publications during the 2016-2017 period [1-7]. In addition, we are currently preparing a new paper on the role of chloroplast NTT transporters in maintaining the photosynthetic energy budget and on the regulation of the NDH complex by ATP (see below). The most important published outcomes include 1) the demonstration that [1, 5] the chloroplast NDH complex is a thermodynamically reversible proton pump enables highly efficient ATP production by cyclic electron flow (CEF); 2) The NDH complex may run "in reverse" under some physiological conditions [1, 2], allowing for buffering of the ATP/NADPH budget; 3) Strand et al. [5] presents our recent results demonstrating that the ferredoxin-quinone reductase (FQR) pathway for CEF is regulated by a redox (thiol) switch; 4) Fisher et al. [6] describes a model in which proton extraction allows the cytochrome b_6f and bc1 complexes to catalyze energy storage through bifurcated electron transfer reactions; 5) Carrillo et al. [7] describes the unexpected finding that the chloroplast proton circuit is controlled at low light is controlled by the NTRC (rather than the thioredoxin) system.

Important recent results include: 1) We have demonstrsted that the NDH complex is directly regulated by ATP, which competes for the ferredoxin binding site. This discovery provides a direct functional link between activation of CEF and metabolic processes, to restore metabolic imbalances in ATP/NADPH production and consumption; 2) In collaboration with Dr. Tom Sharkey we have identified mutants and metabolic conditions in which the ATP/NADPH balance is perturbed. The direct involvement of ATP in regulating NDH can readily explain these phenomena. 3) Our previous work showed that H_2O_2 activates CEF through the NDH complex, but it was unclear whether this occurred through direct modification of CEF enzymes or indirectly through effects on metabolic processes. The most recent result from the Sharkey collaboration points to H_2O_2 activating a new bypass reaction in the Calvin-Benson cycle that consumes ATP, our hypothesis is that the decreased stromal ATP will lead to activation of NDH; 4) To test these models in vivo, under natural conditions, we have developed a new method for probing LEF and CEF that can be readily deployed using the PhotosynQ platform. We are currently accumulating CEF data from many field and lab experiments around the world; 5) We

have preliminary data indicating successful transformation of *Amaranthus hybridis*, our proposed model system for CEF; 6) We have demonstrated that in isolated thylakoids, under conditions when CEF through FQR is active, the reduction of cytochrome b hemes in the b_6f complex is extremely slow. These results appear to eliminate the major model for CEF proposed by Minagawa and coworkers. 7) Development of a purification protocol for *A. hybridus* NDH which demonstrates NADPH/Fd:plastoquinone oxidoreductase activity (preparations published elsewhere are quinone-reductase inactive); 8) *in situ* 77K chlorophyll fluorescence spectroscopic (and proteomic) analysis of *A. hybridus* and *Arabidopsis thaliana* NDH/Photosystem I-supercomplexes in a novel large-scale clear native PAGE setup.

Science objectives for 2017-2018:

- Determine the structural bases for regulation of NDH complex by ATP;
- Develop a computational model that integrates ATP regulation of NDH to test its impact on balancing the chloroplast energy budget;
- Successfully transform Amaranthus with constructs that inactivate NDH to test for its involvement in C4 photosynthesis;
- Map the occurrence of CEF under field conditions and assess its importance for photosynthetic productivity and resilience.

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

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Overall research goals: Project A focuses on one of the grand challenges in basic energy science, understanding how the components of natural photosynthesis are integrated into living organisms. In other words, we want to know how photosynthetic organisms operate as self-organizing molecular assemblies that respond to diverse, highly dynamic and unpredictable challenges of natural environments. Project A has three highly interactive components: 1) Development of enabling technologies to enhance our ability to probe and understanding energy capture and storage in vivo under environmental conditions relevant to the field; 2) Understanding processes that support efficient photosynthesis in dynamic environments; and 3) Disseminating knowledge and technology to enable the broader community of energy researchers.

Significant achievements 2016-2017: In 2016-2017 the project made substantial progress on all components, resulting in 16 published papers [1-16]. Because of lack of space, we present selected highlights of these achievements below.

Enabling technologies. Part of our work aims to develop enabling technologies that allow researchers to overcome current technological limitations and gain unique insights into biological energy storage. In this regard, we increased the capabilities of our Dynamic Environmental Phenotype Imager, which is being used by many of the PRL research projects to assess the impact of fluctuating environmental conditions on photosynthesis [1, 3, 6-8, 10-13, 17]. We developed a new method for probing photoprotective mechanisms that is much faster, field deployable and less prone to certain artifacts than previous methods [2]. We released 500 new field-deployable photosynthesis instruments (MultispeQ 1.0, photosynq.org /technology) which have been used to accumulated over 670,000 photosynthesis datasets around the world. Finally, we developed an approach called Spectroanalysis in native gels (SING) that measures multiple spectroscopic properties of thylakoid preparations directly in native polyacrylamide gel electrophoresis gels, enabling unprecedented resolution of photosynthetic complexes, both in terms of the spectroscopic and functional details, as well as the ability to distinguish separate complexes and thus test their functional connections. As a demonstration, we used the method to reveal a previously unseen Chlamydomonas photosystem I-containing megacomplex that is specifically produced at low light.

A new "Achilles Heel" of photosynthesis. Using both phenotyping platforms, we showed that, under fluctuating light conditions plants experience in the field, generates large spikes in thylakoid electric field that accelerates photosystem II recombination, inducing $^1O_2$ production and photosystem II photodamage [7, 10], as illustrated in the figure below.

The importance of photosynthetic energy balancing mechanisms to accommodate altered metabolic demands [1]. Using DEPI, we conducted a detailed analysis of photorespiratory and metabolic mutants, including a large library of peroxisomal mutants. One major outcomes of these studies suggest strong links between imbalances in metabolic demands for ATP and NADPH, the regulation of the ATP synthase, the production of H_2O_2 and the activation of cyclic electron flow (CEF) to rebalance the ATP/NADPH budget.

NTRC regulates ATP synthase at low light [12]. Results from DEPI led us to identify a second (unexpected) redox regulator the chloroplast NADPH thioredoxin reductase C (NTRC), which operates specifically at low light.

The ATP synthase as the Safety Brake of Photosynthesis. Photosynthesis must be regulated to balance the need for efficient harvesting of energy with the avoidance of photodamage. Our work [5] shows that the ATP synthase plays a central role in this balancing,

by restricting the efflux of protons from the thylakoid lumen, increasing lumen acidity and activating photoprotective mechanisms, but at the cost of lower rates of electron flow. We also found that disabling this regulation can increase efficiency under mild conditions, but results in severe damage to both photosystems I and II under production-like conditions.

Science objectives for 2017-2018: We will focus on understanding the factors that limit the storage of photosynthetic energy. In particular, we have developed field-deployable methods to assess what processes limit the rate at which nonphotochemical quenching (NPQ) and other regulatory systems respond to fluctuating in environmental conditions, taking advantage of natural and induced genetic variations.

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Understanding bilin-based light perception in cyanobacterial photoreceptors

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Overall research goals: We seek to understand the structural basis of light utilization by phytochromes and cyanobacteriochromes (CBCRs), bilin-based photosensors of the phytochrome superfamily. Our ongoing studies examine how light absorption by representative members of this family is tuned by the protein while also providing fundamental insight into the role of the protein scaffold for regulating bilin photophysics and photochemistry. This project combines approaches including ultrafast characterization of photochemical processes, biochemical analysis of photoconversion, and NMR spectroscopy to understand these processes at the molecular level in solution. In the long term, the lessons learned will inform approaches for re-engineering natural bilin-based systems to maximize light capture and/or to minimize overexcitation. We envisage that tools developed under this project will find applications in a wide variety of oxygenic photosynthetic species to tailor more efficient, sustainable, and carbon-neutral biological capture of sunlight and its conversion and storage as chemical energy in a changing light environment.

Significant achievements, 2015-2017: The Lagarias lab has continued to explore the astounding natural diversity of CBCRs (publications #1, #3, #7, #10, #11). Highlights include discovery of the first CBCRs detecting far-red to near-infrared light (#7), successful re-engineering of the model red/green CBCR NpR6012g4 to exhibit a green/blue photocycle with only three amino acid substitutions (#11), and first publication of a multi-domain CBCR system (#12) that provides a model for function of frequently observed tandem CBCR arrays. The Larsen lab has published (#2) the first in a series of four papers characterizing primary and secondary dynamics during forward and reverse photoconversion of nine red/green CBCRs including NpR6102g4. This work established a conserved reaction pathway in primary forward photoconversion for CBCRs in this subfamily, but with varying quantum yields and timescales. The Ames lab has published solution NMR studies of chromophore configuration, protein-chromophore interactions, and secondary structure assignments for NpR6012g4 in both photostates (#4, #5, #8, #9). This work has now been leveraged to determine solution NMR structures for NpR6012g4 in both photostates (now in preparation). Remarkably, we observe structural heterogeneity at key, conserved residues that can be directly linked to spectroscopic and photochemical heterogeneity in this protein.

Science objectives, 2017-2018:

1. We seek to use NpR6012g4 and the closely related NpF2164g5 to identify structural factors that determine the fate of excited populations. NpF2164g5 fails to photoconvert, but pilot experiments have identified a substitution that restores minimal activity. The converse substitution in NpR6012g4 reduces the efficiency of photoconversion.
2. We seek to use red/green CBCRs as a model for understanding how heterogeneity can be controlled in photoproteins. We have identified two additional NpR6012g4 relatives that

have adopted different strategies in response to loss of one of the structurally heterogeneous residues identified in the structures.

3. We seek to combine data on dynamics with solution structures to identify residues that raise or lower photochemical quantum yield in red/green CBCRs.

References to work supported by this project, 2015-2017:

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Elucidating the Principles that Control Proton-Coupled Electron Transfer Reactions in the Photosynthetic Protein, Photosystem II.

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Overall Research Goals. The solar water-splitting protein complex, photosystem II (PSII), catalyzes one of the most energetically demanding reactions in Nature by using light energy to drive a catalyst capable of oxidizing water. Proton-coupled electron transfer (PCET) reactions, which are exquisitely tuned by smart protein matrix effects, are central to the water-splitting chemistry of PSII. Elucidating the water-splitting chemistry of PSII is of major importance in designing bio-inspired catalytic systems for solar fuels production. However, the details of PCET processes are not yet understood because of the inability of conventional methods to directly probe the reactions. A major challenge is to develop methods to directly probe PCET reactions to understand the structural requirements for minimizing the energetic penalty for multiple charge transfers. The objective of our research program is to understand the tuning and regulation of PCET reactions of PSII and to elucidate their role in the early charge-transfer steps of photosynthesis. We are determining the factors that control the coupling of proton (PT) and electron transfer (ET) pathways in PSII by the application of state-of-the-art multi-dimensional and multi-frequency electron paramagnetic resonance (EPR) spectroscopy methods.

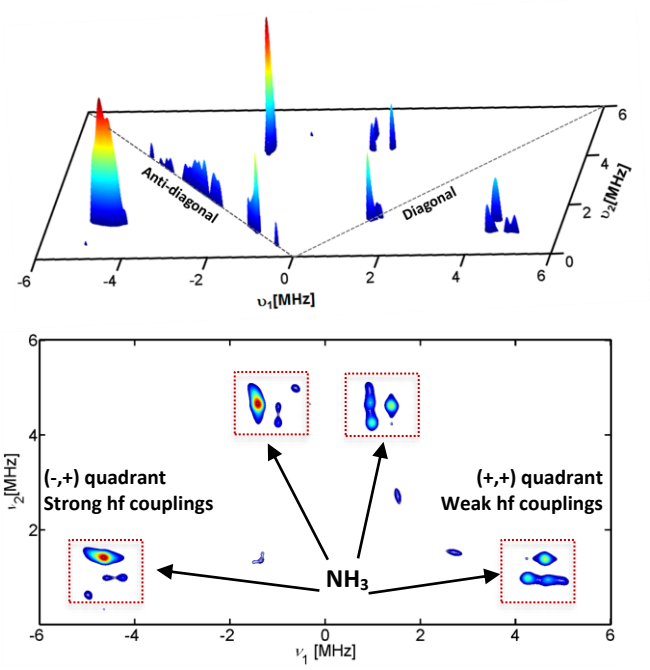

Figure 1. (A) Three- and (B) two-dimensional projections of the 2D ^{14}N HYSCORE spectrum of the annealed S_2 state of ammonia-treated PSII. The spectra display distinct cross-peaks that arise from the electron-nuclear hyperfine (hf) interactions of ammonia that is directly coordinated to the Mn_4Ca-oxo cluster in the annealed S_2 state of ammonia-bound PSII.

Significant Achievements 2016-17. (A) The mechanism of substrate binding in the oxygen-evolving complex of photosystem II. The light-driven four-electron water oxidation reaction occurs at the tetranuclear manganese-calcium-oxo (Mn_4Ca-oxo) cluster that is present in the oxygen-evolving complex (OEC) of PSII. The binding of substrate water molecules at the Mn_4Ca-oxo cluster is central to the water-oxidation chemistry of PSII. There have been previous efforts to interrogate the substrate binding sites in the OEC of PSII. However, the assignment and mechanism of substrate binding is unclear due to the inability of conventional experimental methods to directly probe these sites. Studies of ammonia binding provide valuable insights into

the active site for water coordination in the OEC as water and ammonia are both electronic and structural analogues. Based on computational models of ammonia binding in the S_2 state, a recent study by Batista, Brudvig and coworkers has proposed a carrousel mechanism for the binding of substrate water molecules in the OEC of PSII.[1] We have developed high-resolution two-dimensional (2D) hyperfine sublevel correlation spectroscopy methods[2-6] that provide direct 'snapshots' of the binding of NH_3 (Figure 1) and/or water molecules in the S_2 state of the Mn_4Ca-oxo cluster in NH_3-treated and wild-type PSII from *Synechocystis* PCC 6803. These results, for the first time, unambiguously assign the binding sites of ammonia and substrate water molecules in the OEC of PSII.

(B) The functional specificity of the tyrosine residues of PSII: Mechanism of proton-coupled electron transfer at Y_Z and Y_D. The D1 and D2 polypeptides that form the core of PSII each contain a redox-active tyrosine residue, Y_Z and Y_D, respectively. Recent models invoke Y_Z in the O_2 evolution reaction as an abstractor of protons and/or hydrogen atoms from the substrate water molecules in the OEC. In contrast, Y_D does not participate in rapid electron transfer in the O_2 evolution reaction. We have developed pulsed 2D HYSCORE and high-frequency electron nuclear double resonance (HF ENDOR) spectroscopy methods to structurally characterize the $Y_Z^\bullet$ and $Y_D^\bullet$ PCET intermediates of PSII. These studies provide direct 'snapshots' of functional PCET intermediates and, for the first time, makes it possible to detail the mechanism of PCET in biological solar energy transduction.[7] We have also used quantum mechanical calculations to elucidate the smart matrix effects from the surrounding protein environment that determine the distinct functional tuning of the Y_Z and Y_D residues of PSII.[8]

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Science Objectives for 2017-18. (i) We have unambiguously determined the mechanism of substrate binding in the S_2 state of the OEC in ammonia-bound PSII. We plan to extend these studies to investigate genetic variants of PSII that are proposed to influence substrate binding in the OEC. (ii) We are investigating the other S-states of the OEC of PSII to better understand the structural and electronic requirements for the water oxidation reaction. (iii) We plan to develop experimental models to further elucidate the mechanism of light-induced PCET at the redox-active tyrosine residues of PSII.

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3. C. S. Coates, R. Chatterjee, S. Milikisiyants and K. V. Lakshmi "The Tuning of PCET Reactions at Redox-active Tyrosine Residues: A Comparison of the Tyrosine-Z and Tyrosine-D Residues of Photosystem II" (2017) Manuscript Submitted.
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Non-Canonical Iron-Sulfur Clusters and Electron Transfer

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Overall research goals: The photosynthetic energy transduction core program is developing an understanding of how peripheral redox reactions integrate with photosynthetic electron transfer (PET) by establishing a mechanistic foundation for the chemical reactions that control the flux of photochemical energy and its conversion into reduced compounds. This area of effort includes clearly defining the dynamic pathways that interface with PET, as well as interrogating the enzymes and mechanisms that comprise these pathways. Many redox enzymes responsible for transferring reducing equivalents within PET employ iron-sulfur clusters and flavin cofactors. Elucidating the fundamental properties of how redox enzymes tune these sites for the efficient and directed transfer of electrons should provide insight into why and how certain pathways are favored under changing conditions experienced during photosynthesis. Additionally, it may help to explain the observations of supramolecular complexes involving several photosynthetic chain components and their corresponding mechanisms of action.

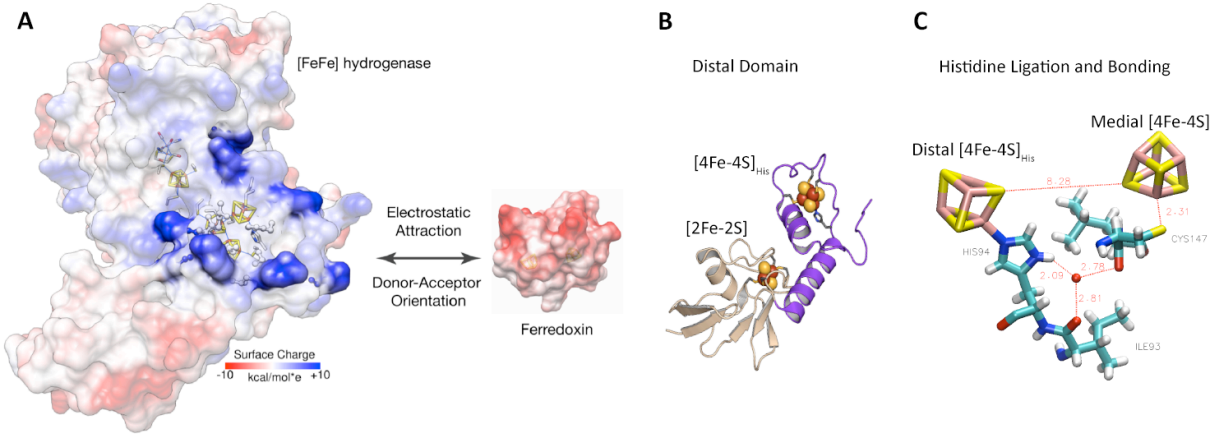

Figure 1. A) [FeFe]-hydrogenase with iron-sulfur clusters exposed. Surface charge shows binding interface of distal iron-sulfur clusters with ferredoxin. B) [FeFe]-hydrogenase distal iron-sulfur cluster domain containing [2Fe-2S] and site-differentiated [4Fe-4S] clusters. C) Histidine ligation of the distal [4Fe-4S] cluster and resulting hydrogen bonding interactions with the medial [4Fe-4S] cluster.

Significant achievements 2015-2017: It is appearing that one level of control in the distribution of reducing equivalents lies in the specific environment surrounding an iron-sulfur cluster cofactor. Several redox enzymes contain site-differentiated iron-sulfur clusters in which one of the canonical cysteine ligands is exchanged to a histidine ligand. While the most commonly accepted result of this variation is a modulation of reduction potential, our studies indicate that there are more profound effects on the cofactor's electronic and magnetic properties, likely important for controlling the rate and fidelity of intermolecular electron transfer. These features were elucidated from EPR, Raman, and electrochemical studies of a distal domain construct of *Clostridium acetobutylicum* [FeFe]-hydrogenase containing only the two most surface located clusters: a [2Fe-2S] and the site-differentiated [4Fe-4S] (Fig. 1 A, B). Additionally, mutation of the complete enzyme to form a canonical [4Fe-4S] cluster results in dramatic effects on the active site electronic structure

implicating a role for the site-differentiated cluster, and F cluster chain, in mediating long-range interactions between soluble redox partners and the active site (Fig.1 A, C) during electron transfer.

Science objectives for 2017-2018:

- Our current work provides insights into how the unique electronic and magnetic properties of site-differentiated iron-sulfur clusters contributed to electron transfer mechanisms through effects on coupling and reduction potentials. Biochemical and photobiohybrid experiments will be conducted to measure the effects of the unique ligation on interfacial electron transfer rates between the distal [4Fe-4S] cluster and either the physiological redox partners, ferredoxin and flavodoxin, or a photoactive nanoparticle, respectively.
- Using the techniques we have developed to examine site-differentiated and extremely low potential iron-sulfur clusters, we will apply this knowledge to the diaphorase domain (HoxEFU) of the cyanobacterial Hox [NiFe]-hydrogenase. HoxEFU has been shown to form a functional and stable complex, however its mechanism of action as well as its redox partners have remained uncharacterized. We will investigate whether HoxEFU employs similar electronic and magnetic features, due to the strong resemblance of HoxF to the distal domain, to direct electron flow towards specific PET pathways. How these features contribute to the formation and function of supramolecular interactions of HoxEFU with various components of the PET chain (ferredoxin, flavodoxin, flavodiiron, NDH-1 and PSI) and their corresponding electron transfer mechanisms will also be explored.

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Characterization of picosecond structural dynamics of Photosynthesis Proteins: Directionality and Collectivity transitions in FMO and Orange Carotenoid Protein

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Overall research goals: The research objectives are to study the role of intramolecular protein dynamics on the picosecond time scale in electron energy transfer dynamics in FMO and in photoswitching to the photoprotective state for orange carotenoid protein (OCP) by using unique spectroscopic techniques and simulation approaches namely: (1) anisotropic terahertz microscopy (ATM) which directly characterizes the intramolecular vibrations as a function of photoexcitation and temperature; (2) solution phase temperature dependent terahertz spectroscopy to characterize the effective resilience and motional collectivity; (3) MD simulations to calculate the changes in the vibrational collectivity and the anisotropic spectra for the identification of motions that impact EET or dominate energy dissipation.(1-4)

Significant achievements 2016-2017: FMO. The picosecond time scale corresponds to long range vibrational motions of the protein scaffold that directly impact conformational change and energy dissipation, and could provide a means of coherence observed for FMO. The phonon bath of the protein scaffold is particularly important to characterize for the proper modeling of EET, however previous techniques relied on cryogenic measurements. Our recently developed technique, Polarization Varying ATM (PV-ATM) was used to measure the intramolecular vibrations of FMO as a function of temperature over the 150-295K range. PV-ATM measurements show considerable structure at low temperature that rapidly dissipates as the temperature goes above 200K in the 5-60 cm^{-1} (.15 – 2.00 THz) range. The temperature of the rapid decline coincides with the dynamical transition of the biological water. We interpret the rapid decline as the lifting of steric constraints on the protein motions imposed by the immobile water. This result has significance for the B-factor measurements using X-ray crystallography. Previous interpretation focused on the net dynamics of proteins, but our results suggest that it is only those motions that are constrained by the frozen water channels of the crystal that will undergo the transition. On the other hand, of particular relevance to FMO is a higher frequency resonant anisotropic absorption band that does not disappear with increasing temperature, but in fact blue shifts! The resonance goes from 85 cm^{-1} at 150 K to 110 cm^{-1} at 300 K nearly linearly up to 250 K, and then approaches an asymptotic value at higher temperatures. The result immediately confirms that the spectral density and overlap of scaffold vibrations with the electronic energy level differences is temperature dependent. The temperature dependence is not affected by the solvent dynamical transition, indicating that the vibrations do not involve large displacements into the water channels of the crystal.

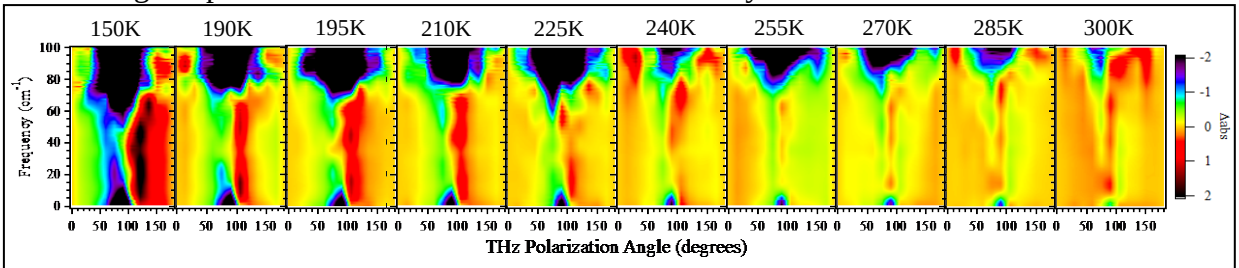

Figure 1. **Te**mperature Dependent PV-ATM of FMO showing substantial shifts in intramolecular vibrations

OCP: Photo-protection is crucial for photosynthesis efficiency. Cyanobacteria have evolved a unique photo-protection mechanism mediated by Orange Carotenoid Protein (OCP). OCP binds a

single ketocarotenoid as the chromophore, essential to its photo-protective function. Under strong green-blue (or white) illumination or high chaotrope concentration, OCP converts from the orange state OCP^O to the activated or photo-protective red state OCP^R. The OCP^R facilitates dissipation of excess energy via direct interaction with allophycocyanin (APC) cores of the light-harvesting antenna Phycobilisome (PB). Picosecond intramolecular dynamics are critical to the photo-protective conformational switching, energy transfer between the APC and OCP, and energy dissipation. In particular intramolecular vibrations at THz frequencies can both provide efficient access to intermediate state conformations and couple to embedded chromophore vibrations for energy dissipation. Here we characterize global picosecond flexibility using temperature dependent terahertz spectroscopy on OCP solutions. The THz absorbance decreases and structural resilience increases in the photoactive state. The dynamical turn on temperature for picosecond dynamics shifts from 200K in OCP^O to 250K in OCP^R, signifying a substantial increase in vibrational collectivity and structural stability. To characterize the nature of the intramolecular vibrations in more detail, we employ PV-ATM. **For the first time we demonstrate intramolecular vibrational changes with photoexcitation.** In particular we find an increase in vibrational directionality in the photo-activated OCP in the 60-72 cm^{-1} and 85-100 cm^{-1} bands. In addition, the orientation of the vibrational motions switches for the 38-48 cm^{-1} band. We suggest that the increased dynamical collectivity and directionality changes with photo-state contribute to OCP efficiently binding and interacting with the APC complex to optimize photo-protective function.

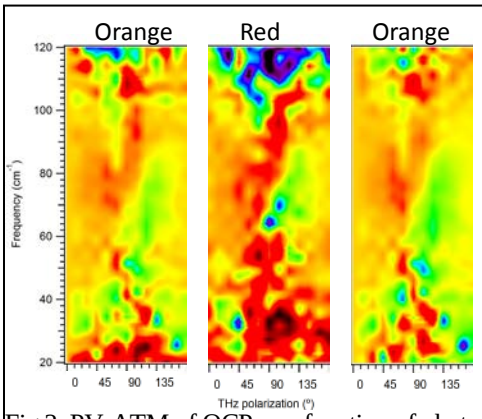

Fig.2. PV-ATM of OCP as a function of photo state. These are the first measurements of a photo induced switching of intramolecular protein vibrations.

Science objectives for 2017-2018:

- Additional FMO PV-ATM measurements for different species/mutants and with H/D exchange to enable identification of the spectral features. It is also essential to perform solution phase measurements to characterize net collectivity changes with temperature.
- Extensive calculations of the intramolecular vibrations and the anisotropic spectra as a function of photoexcitation and deuteration are planned.
- Based on the success with OCP we will attempt FMO photoswitching with smaller crystals. We will also collaborate with NeaSpec in Munich and the Huber lab at the University of Regensburg to use ultra high resolution near field THz to measure micro crystals.
- How OCP internal dynamics impact interaction with APC needs to be established. We will work with the Yang group to explore mutations remote from the binding site to examine how they affect the fluorescent quenching versus intramolecular dynamics.

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Molecular genetic analysis of Fe and Mn homeostasis in green algae

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Overall research goals: Iron is an essential nutrient for nearly all forms of life. The photosynthetic apparatus is rich in iron containing proteins, and iron deficiency therefore impacts primary productivity. In previous work, we showed that in iron-poor medium, Chlamydomonas operates in iron economy mode, involving increased assimilation, reduced utilization, and recycling, while in iron-rich medium, Chlamydomonas operates in iron luxury mode, involving decreased assimilation, a full complement of iron proteins, and increased storage. Carbon metabolism interacts with iron homeostasis pathways so that the photosynthetic apparatus is sacrificed when a source of reduced carbon (acetate) is available, while it is maintained when cells are grown solely with CO_2. In prior work, transcriptomic analyses had identified several candidate genes whose expression is increased in Chlamydomonas under iron-poor conditions (e.g. 1). Last year, we a) undertook functional analyses of some of these genes. In the present project period, we b) used a reverse genetic approach to distinguish the functions of candidate metal transporters and related proteins, and c) undertook a comparative survey to assess conservation of these iron-deficiency targets in algae and plants. We also d) developed a system to monitor Mn accumulation in Chlamydomonas.

Significant achievements 2015-2017
a) We had identified three heme oxygenases of Chlamydomonas: two conventional enzymes related to ER-localized animal enzymes and plastid localized plant enzymes, whose functions we analyzed in collaboration with the Lagarias group (5), and a new one (related to bacterial IsdG) that is present in a subset of algae. In collaboration with Eric Skaar at Vanderbilt University, the new protein was shown to catalyze heme degradation in vitro (3). The expression of this presumed bacterially-derived protein is increased in iron-deficient Chlamydomonas cells, but its function in iron homeostasis remains obscure.
b) Candidate loss-of-function mutants were identified from the Jonikas collection in a number of genes. Each of the genotypes was verified by molecular analysis, following which the strains were tested for growth or chlorosis in response to poor iron nutrition. No phenotypes were evident for most genes, possibly reflecting some redundancy in function. Mutants with phenotypes include *fre1*, *ctp1*, *fox1* and *nramp1*. FRE1 is a putative ferric reductase. Two allelic strains carrying independent mutations lack ferric and cupric reductase activity and show a conditional growth phenotype on iron-poor medium. One *ctp1* allele displays poor growth in a situation of Fe and Cu deficiency, making *CTP1* a prime candidate for the gene affected in the *crd2* mutant. The *nramp1* mutant accumulates 25% less Mn than does the corresponding wild-type, which is consistent with NRAMP1 serving as at least one route for Mn assimilation. In previous work, we had noted that *NRAMP1* showed increased expression in Mn deficiency.
c) *Dunaliella* spp. are halotolerant volvocine algae related to Chlamydomonas, with commercial application as sources of carotenoids. Laboratory species studied to date, *D. bardawil* and *D. salina* are strict photoautotrophs. We discovered that this lineage expresses a flavodoxin (as do many cyanobacteria), which allows the organism to reduce its iron quota and hence grow on very low iron in the environment (4, and unpublished).
Phylogenomics identified other green algae, including *Chromochloris zofingiensis*, that encode a flavodoxin. We have also built gene co-expression networks for Chlamydomonas and Arabidopsis using all available RNAseq and microarray data, respectively, to help identify missing components of metal homeostasis. Additional networks are being generated for evolutionary intermediates Physcomitrella and ferns.

d) As cells enter stationary phase, they hyper-accumulate Mn. We collaborated with the Pett-Ridge laboratory at LLNL and Si Chen at ANL to localize the Mn to the acidocalcisome (an acidic vacuole containing Ca and polyphosphate). Mutants that are blocked in polyphosphate synthesis (*vtc* strains) have low Ca and do not hyper-accumulate Mn. Nevertheless, spectroscopic analyses with Tim Stemmler at Wayne State U. indicate that the Mn is not associated with polyphosphate.
e) Our expertise with iron quantitation (post-doctoral researcher Stefan Schmollinger) was useful in a collaborative study with a colleague at UCLA, Siavash Kurdistani, in which the importance of ER-mitochondria communications in Fe homeostasis in *Saccharomyces cerevisiae* was established (2).

Science objectives for 2017-2018

- More detailed phenotyping at the molecular level, especially of the *nramp1* mutant (e.g. to compare photosynthetic parameters) is in progress. We are also seeking additional alleles of select loci to strengthen the conclusions. We have discovered that besides carbon source, Fe accumulation is influenced by multiple variables in the growth parameters (such as cell density, pH). A systematic analysis of Fe content and Fe protein profile (e.g. ferredoxin, ferritin) as a function of light, cell density, pH, O_2, carbon source will be undertaken for Chlamydomonas to enable more reproducible and reliable outcomes.

- Comparative transcriptomics and proteomics for two *Dunaliella* species is underway. This will allow us to understand acclimation / adaptation to Fe-deficiency beyond a single laboratory reference organism, especially in the context of the flavodoxin replacement of ferredoxin.

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Mechanistic Principles for Hydrogen Catalysis and Proton-Coupled Electron-Transfer by [FeFe]-hydrogenase

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Overall research goals: This projects seeks to establish a mechanistic basis for the hydrogen activation reaction catalyzed by [FeFe]-hydrogenase by investigating the composition of the active-site H-cluster intermediates, mechanisms of proton-transfer and electron-transfer, thermodynamics of individual reaction steps, and electron-injection from external redox partners. The goal is to reveal overarching principles for biological transformation of photochemical potential into chemical bonds and how this is accomplished through the tuning of protein cofactors by surrounding environment and external redox partners. In doing this, we aim to establish a fundamental understanding of the biochemical mechanisms of redox enzymes that function in photosynthetic energy transduction networks. As such, the $2e^- + 2H^+ \rightleftarrows H_2$ reaction is critical to microbial energy conservation, and H_2 as a whole plays an underlying role in the regulation of cellular energetics. The outcome of this work will help reveal how H_2 metabolism is controlled during adaptive transitions of photosynthetic energy transduction and inspire the design of more efficient organometallic complexes for artificial photosynthetic systems and fuel cells.

Significant achievements 2015-2017: We have been investigating the mechanism of H_2 catalysis by the photosynthetic [FeFe]-hydrogenase from *Chlamydomonas reinhardtii* (CrHydA1). Fast PCET and oxygen sensitivity has challenged research efforts to define properties of reduced, hydride bound forms of the catalytic H-cluster. By means of a proton-transfer variant (C169S CrHydA), an otherwise short-lived, key hydride intermediate (H_{hyd}) was trapped for spectroscopic characterization. Analysis by EPR, FT-IR, Mossbauer (collaboration with Prof. Alex Guo at Carnegie Mellon University) and DFT, we were able to reveal the electronic and geometric configuration of the Fe–hydride bond formed during heterolytic H_2 bond activation. Spectroelectrochemical studies showed significant shifts in the oxidation-reduction midpoint potentials of certain H-cluster intermediates compared to the wild-type enzyme, demonstrating tuning of the catalytic cofactor by the secondary coordination sphere. NRVS studies by collaboration with Prof. Stephen Cramer at UC-Davis also revealed a shift in the energy of the Fe-hydride peak position relative to the native enzyme, suggesting an alteration of the electronic properties of the H-cluster in the variant. Overall, the results reveal the inner workings of PCET by [FeFe]-hydrogenase through extended interactions of an electron rich hydride with the H-cluster and protein environment.

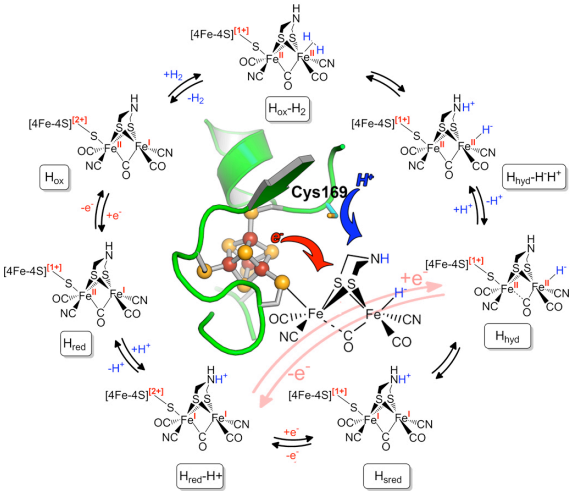

Figure 1. H-cluster active site of [FeFe]-hydrogenase with terminal hydride formation (center) and mechanism of catalysis featuring the H_{hyd} intermediate in reversible H_2 formation.

We have been investigating the mechanism of electron-injection and electron-transfer to the H-cluster using photocatalytic complexes consisting of [FeFe]-hydrogenase from *Clostridium acetobutylicum* (CaI) and CdSe nanocrystals. Compared to the algal CrHydA1, Ca1 contains additional FeS clusters (F-clusters) that function in electron-transfer to the H-cluster. With collaboration of Prof. Gordana Dukovic's group at CU-Boulder, we applied a series of light-drive experiments on the photocatlaytic complexes to probe the thermodynamics of H-cluster redox transitions and entry point of electrons into the enzyme. Rapid scan IR made it possible to detect formation of H-cluster redox intermediates during catalytic proton reduction while the EPR followed the reduction to the F-cluster conduit. Together, the results show how the F-clusters functions both as a conduit and a reservoir to drive catalysis. They also demonstrate that the initial $H_{ox} \rightarrow H_{red}$ transition step of catalytic proton reduction proceeds by concerted PCET.

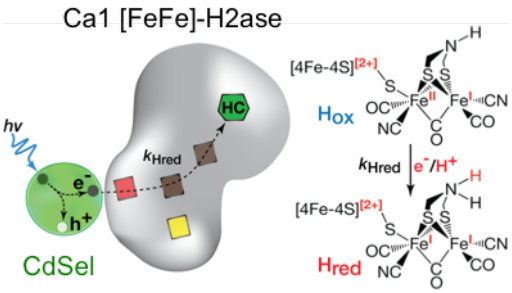

Figure 2. Photocatalytic CdSel:Ca1 complex and H-cluster reduction scheme for the initial proton-reduction step of H_2 catalysis.

Science objectives for 2017-2018:

- Develop mechanistic models that describe the individual PCET transitions of the H-cluster and protonation states of different intermediates. Utilize cryogenic IR spectroscopy, along with hydrogen-deuterium exchange and alteration of pH, to decipher the order of protonation and electron-steps for H_2 activation. Apply light-triggered FT-IR experiments to probe kinetics of H-cluster intermediates redox transitions.
- Resolve the electronic states of catalytic intermediates that are EPR silent and also higher oxidation states of the H-cluster by the preparation of samples at poised redox potentials with analysis by Mössbauer studies in conjunction with EPR, FT-IR and DFT modeling with Prof. Guo at Carnegie Mellon University.

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Molecular Chemistry for Photosynthetic Biohybrids

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Overall research goals

The objective of this research program is to develop a portfolio of synthetic light-harvesting and catalytic modules that are designed for integration within photosynthetic proteins and enable the creation of hybrid architectures that combine synthetic chemical and photosynthetic redox protein functionalities. By manipulating the physical and electronic properties of the individual molecular modules as well as the molecule-protein binding chemistry, we aim to understand how the photoinduced one-electron/hole transfer steps from a molecular photosensitizer leads to the charge accumulation at a molecular catalyst that is required to drive multi-electron redox chemistry. A long-term goal is to understand the physical-chemical design principles underlying the use of photosynthetic proteins as hosts to facilitate non-native, complex chemical transformations using mild conditions (i.e. temperature, pressure, pH) and abundant and sustainable reagents (i.e. light, water, CO_2).

Significant achievements 2015-2017

Several molecular photosensitizers and catalysts were synthesized for use as modules in the development of biohybrid photocatalysts and photosynthetic biomimetics (Figure 1). Bidirectional electron transfer kinetics to and from a specifically-functionalized $Ru(bpy)_3^{2+}$ analog and the heme cofactors of PpcA, a multi-heme cytochrome protein, was shown to occur over four orders of magnitude depending on labeling location. The $Ru(bpy)_3^{2+}$ photosensitizer module was also used in

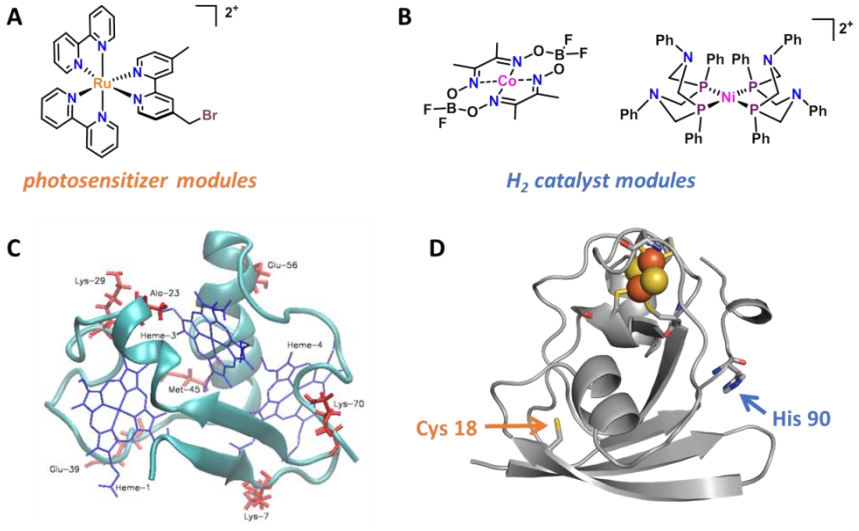

Figure 1. A) Chemical structure of $Ru(bpy)_3^{2+}$-based photosensitizer module used in biomimetics and biohybrids. B) Chemical structure of Co(II) and Ni(II) proton reduction catalyst modules used in biohybrid photocatalysts. C) Crystal structure of tri-heme PpcA cytochrome from *G. sulfurreducens* showing the heme I,III, IV nomenclature and locations selected for site-directed cysteine mutagenesis and subsequent $Ru(bpy)_3^{2+}$ labeling. D) Crystal structure of ferredoxin highlighting location of Cys 18 used for $Ru(bpy)_3^{2+}$ binding, Fe_2S_2 cofactor, and His 90 which binds Co(II) catalyst module.

tandem with well-known molecular proton reduction catalysts based on cobalt and nickel in biohybrid constructs using ferredoxin or flavodoxin. Remarkably, the cobalt and nickel catalysts were highly active for photocatalytic proton reduction in near-neutral water when integrated into the biohybrid architectures, conditions that are completely inactive for the molecular system, demonstrating that the protein imparts unique stability and activity to the molecular modules.

Science objectives for 2018-2020

- Toward the goal of developing an all earth-abundant biohybrid photocatalyst architecture, we plan to use Cu(I)diimine complexes to replace the benchmark $Ru(bpy)_3^{2+}$ photosensitizer modules. Our group has recently been exploring the photoinduced kinetics of heteroleptic Cu(I)diimine complexes (*Dalton Trans.* **2016**, *45*, 9871-9883; *Dalton Trans.*, **2017**, *46*, 13088-13100). This work has shown that the heteroleptic ligand coordination around the Cu(I) center provides an enormous degree of synthetic versatility with which to introduce protein-labeling functional groups as well as tunability in the ground state optical and redox properties and excited state kinetics. Preliminary results show that epoxide-decorated Cu(I) photosensitizers bind quantitatively to Cys 18 of ferredoxin and suggest that they could also initiate photo-induced electron transfer to proton reduction catalyst modules.
- A significant drawback of previously used molecular catalyst modules is their relative instability, particularly in aqueous environments. Our group has recently developed a new molecular Co(II)polypyridyl catalyst for proton reduction which is highly active at moderate pH levels for *days* when photo-driven by $Ru(bpy)_3^{2+}$ using ascorbate as a sacrificial electron donor. Preliminary synthetic work has demonstrated that we can asymmetrically functionalize the catalyst module with protein-labeling functional groups and we intend to test its behaviour as a module for biohybrid H_2 photocatalysis.

References to work supported by this project 2015-2017:

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Regulation of Photosynthetic Light Harvesting

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Overall research goals: Photosynthetic light-harvesting systems are regulated, protected against photo-oxidative damage, self-assembled into supercomplexes, and rely on a subtle balance of electronic interactions to produce a highly robust system. In this project, we apply a multidisciplinary approach that combines genetic and biochemical techniques with ultrafast spectroscopy and modeling to understand the mechanisms by which oxygenic photosynthetic organisms regulate the efficiency of light harvesting in Photosystem II. In response to fluctuations in light intensity, non-photochemical quenching (NPQ) mechanisms that regulate photosynthetic light harvesting are induced. In algae and plants, the qE type of NPQ is turned on and off rapidly by changes in thylakoid lumen pH, whereas the slower qI type downregulates PSII during long-term light stress. Our specific aims are (1) to identify new components involved in different types of NPQ through genetic analysis, (2) to elucidate the mechanisms, locations, and timescales of NPQ using fluorescence lifetime and transient absorption snapshot spectroscopies, advanced imaging, and biochemical approaches, and (3) to develop mechanistic models of NPQ that can predict how to optimize NPQ and thereby improve photosynthesis.

Significant achievements 2015-2017: Through analysis of the *npq1* mutant of *Chlamydomonas*, we discovered a new type of violaxanthin de-epoxidase (VDE) that converts violaxanthin into zeaxanthin in excess light. This enzyme, which we named CVDE for Chlorophycean VDE, evolved early in the green lineage and is located on the stromal side of the thylakoid membrane, rather than in the thylakoid lumen like the plant-type VDE. We confirmed our earlier hypothesis that LHCSR functions as a sensor of thylakoid lumen pH and identified three critical lumen-facing acidic amino acid residues that are necessary for this function *in vivo*. We also demonstrated that PsbS has a role in NPQ in *Chlamydomonas*, possibly by promoting conformational changes required for activation of LHCSR-dependent quenching. We isolated *npq* mutants of the green alga *Chromochloris zofingiensis* (formerly known as *Chlorella zofingiensis*) and the photosynthetic stramenopile *Nannochloropsis oceanica* CCMP1779, both of which have a substantially higher NPQ capacity compared to *Chlamydomonas* and *Arabidopsis*. We generated a high-quality genome assembly and high-light transcriptome for *Chromochloris zofingiensis* to facilitate analysis of the *npq* mutants. In *Nannochloropsis*, we isolated knockouts of genes encoding LHCSR-like (LHCX) proteins, VDE, ZEP, and a VDE-like enzyme called VDL. Using the *Nannochloropsis* ZEP, we engineered *Arabidopsis* lines that have a lutein epoxide cycle instead of a violaxanthin cycle and used them to show that the lutein epoxide cycle functions in qE and photoprotection. Analysis of the *NoM* mutant (in collaboration with Roberto Bassi) revealed that there are at least two mechanisms of qE in *Arabidopsis*, one occurring in the monomeric LHCs and the other occurring in the trimeric LHCII. Through a genetic screen in a *soq1 npq4* parental strain of *Arabidopsis*, we identified two other components involved in a slowly reversible (qI) type of photoprotective NPQ that occurs in LHCII.

Science objectives for 2017-2018:

- Sequence *LHCSR* and *PSBS* genes from *npq* mutants of *Chromochloris*.
- Perform structure-function analysis of LHCX1 in *Nannochloropsis* using knock-in mutations.

- Investigate the role of vaucheriaxanthin in photoprotection of *Nannochloropsis*.
- Determine the location of PsbS in photosystem II using single-particle cryo-EM.
- Reconstitute qE in proteoliposomes containing oriented LHCII, zeaxanthin, and PsbS.
- Elucidate the biophysical mechanism of the slowly reversible NPQ using transient absorption spectroscopy.

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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 (2016-2017)[1-5]:

In the past year we have worked on several fronts towards addressing the key open questions about the PSII RC. Our work has included new methods development, and a combination of multidimensional spectroscopy measurements and modelling as detailed below.

To uncover the charge-transfer states involved in the charge-separation in the PSII RC we have demonstrated 2D electronic Stark spectroscopy (2DESS) and transient-grating Stark spectroscopy (TGSS) on a model system[2]. We expect these methods to be widely applicable to studies of charge separation in other systems. To understand the spectral signatures of these new methods we have modelled an excitonic dimer with a single charge separated state, using parameters relevant to the PSII RC (see Figure 1). We have also improved upon our previous excitonic model of the PSII RC, using a complex time-dependent Redfield (ctR) theory for optical lineshapes[3]. We have collected 2DESS and TGSS data on the PSII RC and will use this data, as well as 2DES data to further refine our new PSII RC model and test charge separation mechanisms.

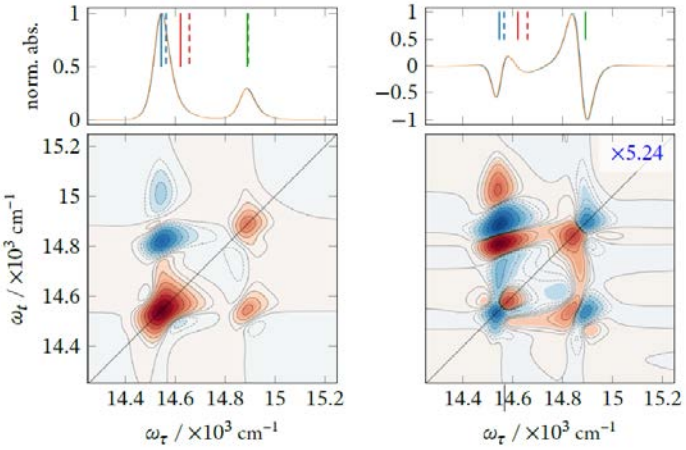

Figure 1. Simulated 2DES (lower left) and linear absorption (upper left) spectra of the $P_{D1}P_{D2}$ dimer with a single CT state. Corresponding simulated 2DESS (lower right) and 1D Stark spectrum (upper right). The lower exciton (blue solid line) consists of primarily P_{D2} and P_{D1} in the proportion 62:37:0:5% of P_{D2}:P_{D1}:CT. The upper exciton (green solid line) consists of 36:56:8% P_{D2}:P_{D1}:CT. The CT state is primarily localized on the second eigenstate (red solid line). The CT state is obscured in the 1D Stark spectrum but shows distinctive cross-peak features in the 2DESS spectrum.

Our previous 2DES experiments on the PSII RC primarily studied 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 complex (PSII CC) preparation[1]. Despite the predicted small electronic coupling in this system, we see extremely rapid energy transfer within the first 100 fs. 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[1].

We are continuing our studies of vibronic coherence in the PSII RC, aiming to probe the functional relevance of the coherence. We previously developed a two-color coherence spectroscopy method to provide high sensitivity measurements of coherent dynamics[4]. We have applied this approach to study the ~820 nm anion band of the PSII RC, and have observed coherent dynamics. As a control experiment, we have also studied chlorophyll a (Chl a) in this spectral range and have observed similar coherence signatures. We plan further studies probing anion signatures near 455 nm and in the mid-IR to determine whether we are seeing coherent formation of the charge separated state. We have made a careful comparison between the frequencies and 2D spectral signatures of coherence in Chl a and the PSII RC to assign the physical origin of the observed coherences. To better understand the coherence signatures of Chl a, we have also performed polarized two-dimensional electronic spectroscopy (P-2DES) on penta-coordinated Chl a. We resolve three electronic transitions with different polarizations in the Q-band, and measure the angles between these transitions, finding results that are qualitatively consistent with TD-DFT calculations. Our observations are consistent with strong mixing of the Q_x and Q_y transitions due to vibronic coupling.

Science objectives for 2017-2018:

- Make additional Stark 2DES measurements of the PSII RC and PSII cores and use our data to further 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 anion band experiments and studies of site-directed mutants.
- Perform our first 2D electronic-vibrational spectroscopy to help elucidate the charge separation mechanism of the PSII RC.

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The Chloroplast Division Machine: Towards a Mechanistic and Structural Model

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Overall research goals:

The division of chloroplasts during leaf growth and development increases chloroplast numbers and is essential for achieving maximum photosynthetic productivity. This process occurs by binary fission and is driven by a dynamic macromolecular complex with components located both inside and outside the organelle and in the intermembrane space. Our overarching goal is a deep mechanistic understanding of how the chloroplast division machinery operates. We have made significant progress towards elucidating the mechanisms governing the spatial regulation of division-site placement and dissecting the functions of several membrane proteins that coordinate the assembly of stromal and cytosolic contractile complexes across the two envelope membranes. Current work is aimed at delving more deeply into the biochemical underpinnings of these aspects of division to understand more fully how a key regulator of accurate division-site placement functions and gain new insight into the molecular architecture and functional interactions within the division complex.

Significant achievements 2016-2017:

1. Chloroplast division is initiated in the stroma by self-assembly of the cytoskeletal FtsZ proteins into a contractile "Z ring" at the midplastid division site (Fig. 1). Previously, we established that the chloroplast division protein Accumulation and Replication of Chloroplasts3 (ARC3) is part of a negative regulatory system that restricts Z-ring formation to the middle of the organelle, and that it does so by directly inhibiting FtsZ assembly. Using a fluorescent ARC3 fusion protein that complements an *arc3* mutant, we have found there are two pools of ARC3 in Arabidopsis. One is distributed evenly across the stroma; this pool likely prevents Z-ring formation away from the midplastid. A second pool is concentrated at the division site, where its assembly-inhibitory activity may facilitate Z-ring remodeling during chloroplast constriction (Fig. 1). We also found that ARC3 recruitment to the division site requires the inner envelope membrane protein PARALOG OF ARC6 (PARC6), whose stromal region interacts with both ARC3 and FtsZ. ARC3 bears a C-terminal domain called the MORN (membrane occupation and recognition nexus) domain that is required for ARC3-PARC6 interaction but prevents ARC3-

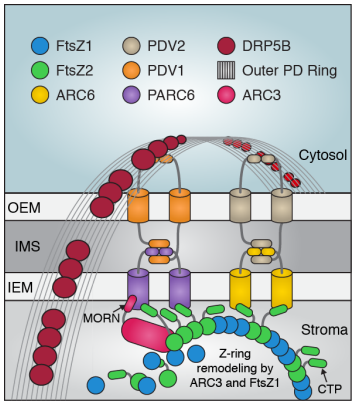

Figure 1. Working model of the chloroplast division complex and ARC3-PARC6-mediated Z-ring remodeling at the division site. PARC6 recruits ARC3 to the division site via interaction with the ARC3 MORN domain, enabling ARC3 to interact with FtsZ subunits in the Z ring. The latter interaction may be facilitated by interaction of PARC6 with the FtsZ2 C-terminal peptide (CTP) (Zhang et al., 2016). As an FtsZ assembly inhibitor, ARC3 activation at the division site promotes Z-ring remodeling. Dynamic remodeling of the Z ring probably also depends on FtsZ1 (Chen et al., 2017). PD ring, plastid-dividing ring; OEM, outer envelope membrane; IMS, intermembrane space; IEM, inner envelope membrane; MORN, ARC3 membrane occupation and recognition nexus domain of ARC3; CTP, FtsZ2 C-terminal peptide. Adapted from Chen et al., 2017.

FtsZ interaction, suggesting the former interaction sequesters the MORN domain, thereby enabling ARC3 to bind to FtsZ. Towards testing this hypothesis, we used a yeast three-hybrid system and found that full-length ARC3 bearing the MORN domain can interact with FtsZ in the presence of the stromal region of PARC6. Further, full-length ARC3 inhibits assembly of FtsZ filaments in a heterologous fission yeast system only in the presence of PARC6, consistent with our hypothesis. However, genetic data in Arabidopsis indicate that PARC6 also contributes to the regulation of Z-ring assembly independently of ARC3. Consistent with this finding, fluorescence recovery after photobleaching indicates that PARC6 alone enhances turnover of FtsZ filaments expressed in fission yeast. Our results suggest that the stromal region of PARC6 acts as a scaffold to bring ARC3 and FtsZ into close proximity, activates the inhibitory activity of ARC3 on Z-ring assembly by sequestering the MORN domain, and may directly promote Z-ring remodeling through an ARC3-independent activity.

2. The intermembrane space (IMS) region of PARC6 ($PARC6_{IMS}$) interacts with the IMS region of the outer envelope division protein PDV1 ($PDV1_{IMS}$) (Fig. 1). We purified, crystallized and determined the X-ray structure of $PARC6_{IMS}$ and found that it is very similar to that of $ARC6_{IMS}$. We also determined the structure of $PARC6_{IMS}$ in complex with $PDV1_{IMS}$. The data indicate how these two regions interact in the IMS, including revealing interactions involving conserved residues we've shown or hypothesized to be critical for PARC6 and PDV1 function.

Science objectives for 2017-2018:

- Complete analysis of the determinants of ARC3-PARC6-FtsZ interaction and their effects on FtsZ assembly in fission yeast and prepare a manuscript on this work.
- Study the mechanism of ARC3-mediated inhibition of FtsZ assembly using *in vitro* FtsZ assembly assays with purified ARC3.
- Purify the stromal regions of PARC6 and ARC6, determine their crystal structures, and compare their relative binding affinities for FtsZ.

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Redox processes that regulate assembly and repair of the photosynthetic apparatus in cyanobacteria

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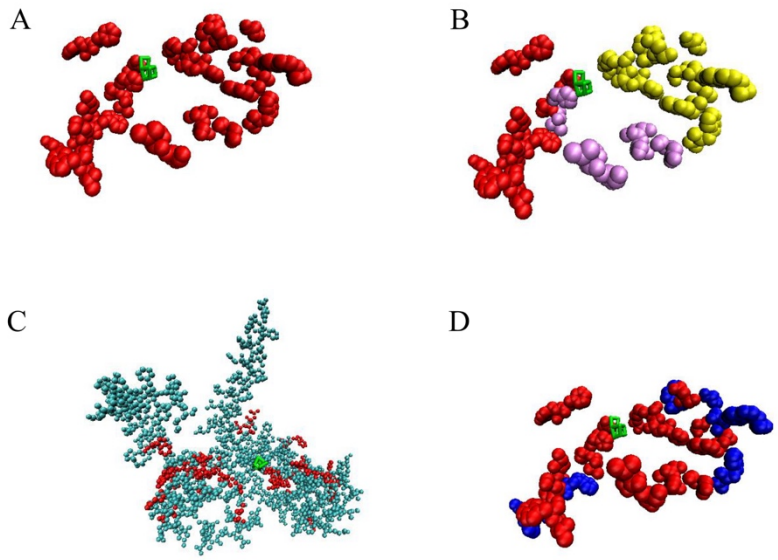
Overall research goal: The research objective of this project is to understand the mechanisms that regulate the biogenesis and repair of the photosynthetic apparatus, in particular Photosystem II (PSII), in cyanobacteria.

Significant achievements 2016-2017: During this period, we have focused on the assembly and repair of PSII in the cyanobacterium *Synechocystis* sp. PCC 6803. PSII, a unique membrane bound oxidoreductase, catalyzes light-driven oxidation of water to molecular oxygen. PSII produces reactive oxygen species (ROS), responsible for the frequent damage and turnover of this megacomplex that occur under physiological conditions. Such ROS are known to specifically modify PSII proteins. Using high-resolution tandem mass spectrometry, we identified oxidative modifications of 36 amino acid residues on the lumen side of PSII, in the core PSII proteins, D1, D2 and CP43 of the cyanobacterium *Synechocystis* sp. PCC 6803. Remarkably, these oxidized residues clustered into three nearly continuous formations, tracking the pathways of ROS diffusion from the manganese center all the way out to the surface of PSII. We suggest that these profiles of oxidized residues reveal the locations of water channels within PSII. Our results provide the most comprehensive experimental evidence to date of physiologically relevant oxidized residues in PSII, and illuminate three possible channels for water between the catalytic Mn cluster in the PSII complex and the bulk medium around it.



Lumen-side oxidized residues detected in this study. A. Depiction of the 42 lumen-side oxidized residues detected in this study (red). **B.** Same depiction as **A** but Arm 1 is shown in red, Arm 2 in purple, and Arm 3 in yellow. **C.** All of the 255 lumen-side residues that were covered by MS in this study. Some residues are obscured by others and are not visible in this view. Red: oxidized residues; cyan: non-oxidized residues. **D.** Same depiction as **A**, except that in this view, the surface-exposed residues are colored in blue and buried residues are colored in red. Throughout the figure, the Mn cluster is shown in green.

Science objectives for 2017-2018:

- Our findings have provided a comprehensive picture of all oxidized residues in the D1, D2, CP43 and CP47 proteins in PSII. These data also suggest the presence of three water channels in PSII.
- During the next nine months, we plan to focus on processes that directly influence the assembly of the manganese cluster in PSII.

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Spin-Quantum Effects as a Tool for Resolving Optimal Pathways for Proton-Coupled ET in Photosynthesis

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Overall research goals: Our research objectives focus on resolving fundamental mechanisms of light-induced proton-coupled electron transfer, charge-accumulation, and chemical energy conversion in photosynthesis and developing photosynthetic-hybrid systems for enhancing photosynthesis-driven solar energy conversion efficiency by: (1) developing new experimental methods to study light-induced electron and energy transfer in natural and hybrid photosynthetic systems using spin-quantum effects; (2) resolving mechanisms that allow reaction center proteins (RCs) to operate as media optimized for efficient proton-coupled electron transfer (PCET) reactions; (3) investigate structural dynamics associated with both activationless and conformationally gated ET processes; (4) investigate the response of the protein environment to light-induced charge separation. Understanding structure-function relationships in biological photosynthetic systems provides the basis to design advanced biohybrids in which the best features from nature are selectively used while the shortcomings of biology are bypassed.

Significant achievements 2015-2017: *Regulation of Electron Transfer in Type I Reaction Center Proteins.* Observation of the spin-correlation between donor and acceptor unpaired electrons upon light-induced charge separation opens a new avenue for exploiting PCET processes in natural photosynthesis. The significance of this spin-quantum phenomenon is in the sensitivity of the EPR spectra of spin-correlated radical pairs (SCRP) to the geometry and electronic structure of charge separated states as well as ET dynamics. This potential was completely revealed after developing a high-frequency time-resolved EPR technique which allows for complete resolution of the anisotropic spectra of SCRP. Using this approach we were able to definitively prove that under strongly reducing conditions ET in PSI proceeds down both nearly symmetrical cofactor branches (Figure 1). To prove that the reduction condition of the PSI preparation does not influence our conclusion on directionality of ET in PSI and to clarify the degree of the ET asymmetry, we have spectroscopically characterized biochemically modified PSI RCs wherein the terminal acceptor iron-sulfur centers, F_A/F_B, and F_X, have been sequentially removed to prevent secondary ET from phylloquinones (A_1) to F_x. For these modified RCs, we find that ET occurs along both A- and B- branches and the ratio of ET through the A- and B- branches is close to 1.

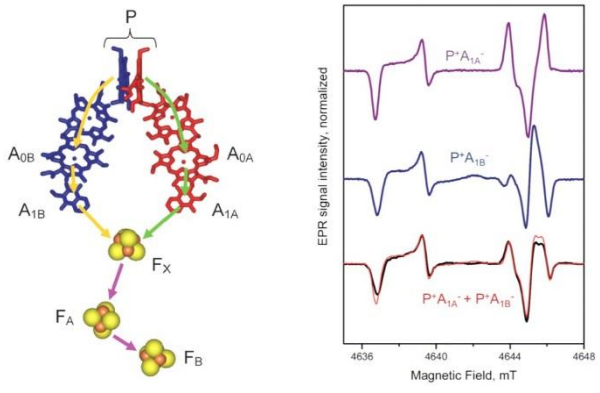

Figure 1. Schematic presentation of ET pathways in PSI and corresponding line-shapes of the spin-correlated high-frequency EPR

Imaging Proton-Coupled Electron Transfer Pathways. As we demonstrated in the previous funding period, spin-correlation upon light-

induced charge separation occurs not only between two electron spins but also between electron and nuclear spins. As a consequence, the time-resolved electron nuclear double resonance (ENDOR) spectra contain information on the distribution of the separated electrons spin densities in the body of proteins. Moreover, owing to transient features of the ENDOR technique, the spin density distribution can be followed in time and provides important information about the protein environment's response to photoinduced ET. Using this technique, we revealed that ET induces a small-scale reorganization at the level of the global H-bonding network. Orientation dependence of the matrix proton time-resolved ENDOR spectra of deuterated purple bacteria RCs in deuterated buffer allows us to identify a number of exchangeable protons involved in the ET process. These protons are located close to the primary acceptor pheophytin and belong to Trp100L and Glu104L amino acid residues. This finding supports our previous hypothesis that matrix relaxation responsible for the ET regulation in bacterial RCs occurs around the pheophytin cofactor.

Science objectives for 2017-2018:

- We will to continue to explore spin-quantum effects to clarify the mechanism of ET in photosynthetic systems. We demonstrated that ET process in PSI is bidirectional. In order to clarify what determines ET along one or another branch we will obtain and analyze the temperature dependence of spin-dynamics of entangled states in SCRP as a function of sample preparation.
- We are going to apply a suite of advanced EPR techniques in combination with isotopically labeled cyanobacterial membranes and isolated protein to characterize the coupling of RC primary photochemistry to secondary reaction sequences in thylakoid membranes.

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3. S. Soltau, J. Niklas, P. Dahlberg, K. Mulfort, O. Poluektov, L. Utschig, "Charge Separation Related to Photocatalytic H2 Production from a Ru-apoflavodoxin-Ni Biohybrid" *ACS Energy Letters*, **2017**, 2, 230-237.
4. M. Ortiz, S. Cho, J. Niklas, S. Kim, O. G. Poluektov, W. Zhang, G. Rumbles, J. Park "Through-Space Ultrafast Photoinduced Electron Transfer Dynamics of a C_{70}-Encapsulated Bisporphyrin Covalent Organic Polyhedron in a Low-Dielectric Medium" *JACS*, **2017**, 139, 4286−4289.
5. M. Bacchi, E. Veinberg, M. J. Field, J. Niklas, T. Matsui, D. M. Tiede, O. G. Poluektov, M. Ikeda-Saito, M. Fontecave, and V. Artero, "Artificial Hydrogenases Based on Cobaloximes and Heme Oxygenase", *ChemPlusChem*, **2016**, 81(10), 1083-10898.
6. S. R. Soltau, P. D. Dahlberg, J. Niklas, O. G. Poluektov, K. L. Mulfort, and L. M. Utschig "Ru-protein-Co biohybrids designed for solar hydrogen production: understanding electron transfer pathways related to photocatalytic function", *Chemical Science*, **2016**, 7, 7068-7078.
7. S. R. Soltau, J. Niklas, P. D. Dahlberg, D. M. Tiede, O. G. Poluektov, K. L. Mulfort, and L. M. Utschig, "Aqueous light-driven hydrogen production by a Ru-Ferredoxin-Co biohybrid", *Chem. Comm.*, **2015**, *51*, 10628-10631.
8. O. G. Poluektov, L. M. Utschig, "Low Temperature Bidirectional Electron Transfer in F_X removed photosystem I", *J. Phys. Chem. B*, **2015**, 119 (43), 13771-13776.

The Homodimeric Photosynthetic Reaction Center of *Heliobacterium modesticaldum*

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Overall research goals: The subject of this project is the heliobacterial reaction center (HbRC), which is a homodimeric Type I RC. The specific objectives are to (1) develop a genetic system for *H. modesticaldum*, (2) study modification of HbRC when the associated pigment is converted from bacteriochlorophyll (BChl) *g* to Chl *a*, (3) study the ferredoxins serving as electron acceptors, (4) test the ability of the HbRC to use quinones as alternate terminal electron acceptors, and (5) determine the structure of the HbRC.

Significant achievements 2015-2017:

1. Introduction of replicating plasmids into *H. modesticaldum* via conjugation of pre-methylated plasmids has now become routine in the lab. We have developed several modular shuttle vectors to allow cloning in *Escherichia coli* and subsequent transfer into *H. modesticaldum.*
2. BChl *g* is converted to 8^1-OH-Chl a_F on exposure to light and oxygen with an activation energy of 66 ± 2 kJ mol^{-1}. We have shown that the nonlinear loss of P_{800} photooxidiation and flavodoxin reduction, the biphasic backreaction kinetics, and the increased EPR line width of P_{800}^+ are consistent with a model in which the BChl *g*'/BChl *g*' and BChl *g*'/Chl a_F' special pairs are functional but the Chl a_F'/Chl a_F' special pair is not (Ferlez et al., 2015).
3. The reduction potentials of the major ferredoxins serving as electron acceptors in this species have been measured to be –480 ± 11 mV/–524 ± 13 mV for PshBI, –453 ± 6 mV/–527 ± 6 mV for PshBII, and –452 ± 5 mV/–533 ± 8 mV for HM1_2505 (Ferlez et al., 2016). In addition, the reduction potential of the F_X cluster has been measured (–504 ± 10 mV), allowing us to estimate the reduction potential of the A_0 cofactor (–850 mV).
4. We have demonstrated that the absence of a recombination triplet is a result of the fact that the zero-field splitting (ZFS) tensor of $^3P_{800}$ is maximally rhombic, which results in total cancelation of the absorptive and emissive polarization in randomly oriented samples (Ferlez et al., 2017).
5. We have created a system to incorporate the purified HbRC into liposomes containing the major phospholipids of heliobacterial membranes and menaquinone-9, as well as a lipid attached a Ni(II) complex. This allows us to decorate the vesicles with a hexahistidine-tagged cytochrome c_{553}, the native electron donor to the HbRC. We have characterized the interaction of the attached cytochrome with the HbRC after photo-oxidation. We have also found that illumination of such proteoliposomes results in reduction of menaquinone to menaquinol, which is dependent upon the attached cytochrome.
6. We have determined the structure of the HbRC to 2.2 Å resolution (Gisriel et al., 2017). In the process, we have discovered a new subunit consisting of a single transmembrane α-helix, which we have named PshX. The HbRC has the stoichiometry $(PshAPshX)_2$ and exhibits perfect C_2 symmetry (Figure 1). The structure lacks a tightly bound quinone, making it unique among all photosynthetic RCs structures. Not only is this the first homodimeric RC structure reported, it also contains 4 new molecules never before in the PDB: BChl *g*, BChl *g'*, 8^1-OH-Chl a_F, and 4,4'-diaponeurosporene.

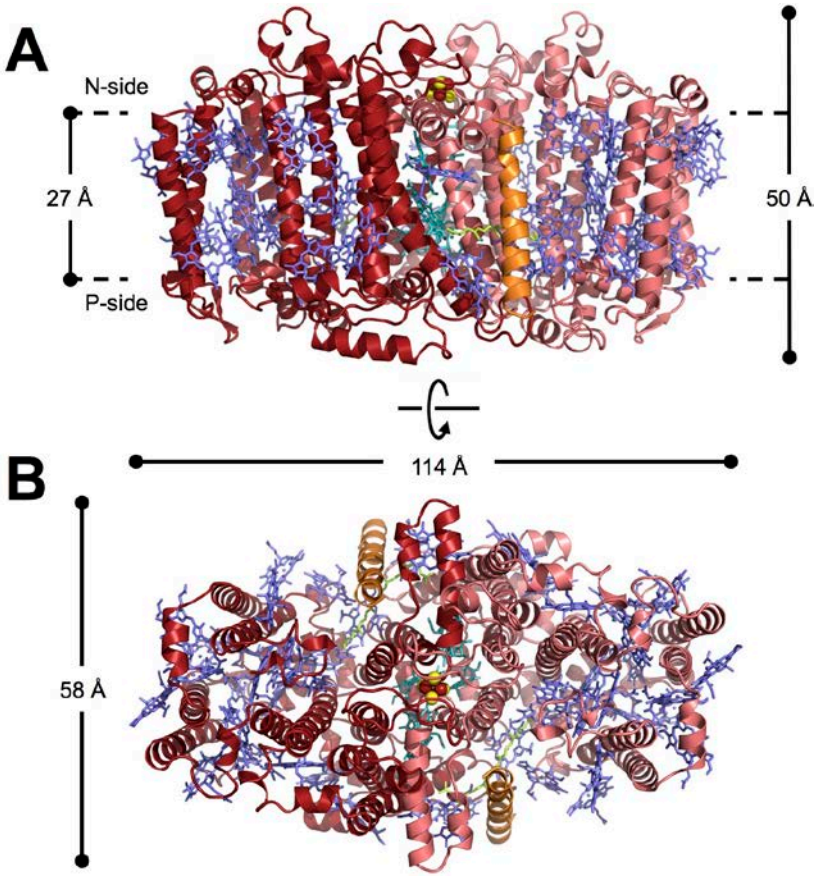

Figure 1. Overall structure of the HbRC viewed from the N-side (**A**) or within the membrane (**B**). The two PshA polypeptides are in red and pink. PshX subunits are in orange. Cofactor molecules are shown as stick models and teal (ET), blue (antenna), and lime (carotenoids). The [4Fe-4S] cluster is shown as yellow (S) and red (Fe) spheres. (B)Chl tails have been truncated for clarity.

Science objectives for 2017-2019:

1. Develop a system to modify the chromosome in a controlled manner (e.g. gene deletions).
2. Identify the genes and enzymes in the biochemical pathway for the biosynthesis of BChl *g* and 8^1-OH-Chl a_F, where F is the farnesyl tail.
3. Determine the structures and roles of the PshB1 and PshB2 ferredoxins – are they redundant or do they have specialized roles in the cell?
4. Finish characterization of the in proteoliposome-based quinone reduction system. Identify inhibitors that block the quinone reduction site.
5. Solve a new HbRC structure based on phasing from anomalous scattering from native S atoms.

References to work supported by this project 2015-2017:

1. Ferlez B, Dong W, Siavashi R, Redding K, Hou HJ, Golbeck JH, van der Est A (2015) The Effect of Bacteriochlorophyll *g* Oxidation on Energy and Electron Transfer in Reaction Centers from *Heliobacterium modesticaldum*. *J Phys Chem B*, **119**:13714-25.
2. Ferlez, B., Cowgill, J., Dong, W., Gisriel, C., Lin, S., Flores, M., Walters, K., Cetnar, D., Redding, K. E., and Golbeck, J. H. (2016) Thermodynamics of the Electron Acceptors in *Heliobacterium modesticaldum*: An Exemplar of an Early Homodimeric Type I Photosynthetic Reaction Center, *Biochem.* **55**:2358-2370.
3. Gisriel, C., I. Sarrou, B. Ferlez, J.H. Golbeck, K.E. Redding, R. Fromme. (2017). Structure of a Symmetric Photosynthetic Reaction Center-Photosystem. *Science* **357**:1021-1025.
4. Ferlez, B., Agostini, A., Carbonera, D., Golbeck, J. H., and van der Est, A. (2017) Triplet Charge Recombination in Heliobacterial Reaction Centers Does Not Produce a Spin-Polarized EPR Spectrum, *Z Phys Chem* **231**: 593-607.

Accessing Structure and Dynamics of Photosynthetic Pigment-Protein Complexes by Time-Resolved Circular Dichroism Spectroscopy

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Overall research goals: To introduce time-resolved circular dichroism (TRCD) spectroscopy to photosynthesis studies: (1) develop spectrometers capable of measuring weak transient circular dichroism (CD) signals associated with energy transduction in nanosecond to femtosecond time scales; (2) demonstrate the power of these spectrometers by studying excitonic states and their dynamics in a test system - the Fenna Matthews Olson (FMO) complex; (3) reveal the intrinsic charge separation dynamics in the photosystem I reaction center (PS I RC); (4) apply the method to other system where transient CD can provide unique information inaccessible by other techniques.

The conventional transient pump-probe spectroscopy distinguishes kinetic signals originating from different pigments based on their absorptive and emissive spectral properties. In the case of complexes that contain numerous molecules with overlapping energy levels (spectral congestion) distinguishing signals originating from different molecules is ambiguous and model dependent. The strongly coupled pigments in systems like the PS I RC and FMO complex, however, can be distinguished by their characteristic CD spectral signature, which is much stronger than that for weakly interacting pigments. The spectral shape and the sign of CD are very sensitive to mutual orientation of the interacting pigments. Thus, TRCD technique will be a powerful tool to study pigment-protein complexes that contain strongly coupled pigments.

While number of TRCD spectrometers have been proposed in past, their application has been limited to a few test samples with strong CD signatures. The goal of the proposed research was to advance TRCD technique by increasing its sensitivity by 1-2 orders of magnitude to measure weak CD signals from wide range of typical molecular systems and apply this technique to solve long standing scientific problems in photosynthesis.

Significant achievements 2016-2017: (1) The nanosecond TRCD spectrometer was built [3] that provides unprecedented sensitivity and enabled us to measure nanosecond triplet state dynamics of FMO for the first time (Fig. 1). As predicted, TRCD DADS (Fig. 1C) spectra are more structured than ordinary DADS (Fig. 1D) and more sensitive to the pigment arrangement within the protein. The data has been analyzed using existing excitonic models of FMO, the paper is in preparation.

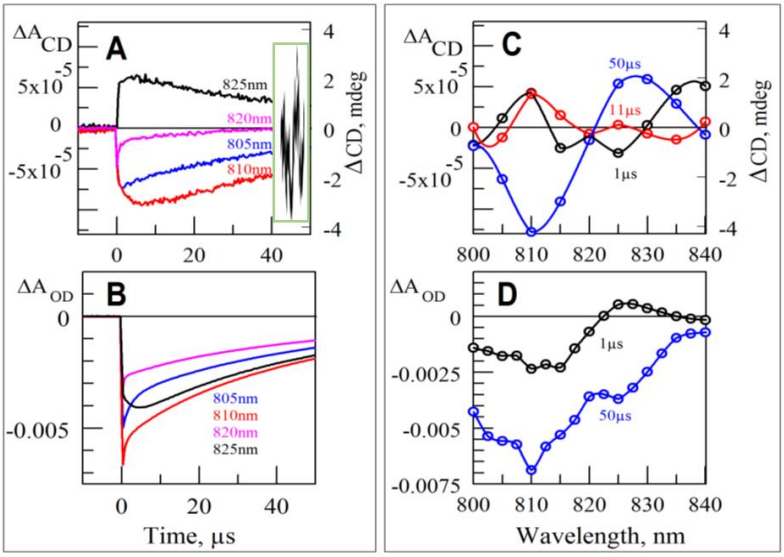

Figure 1. (A) TRCD kinetics measured for FMO at room temperature at several wavelengths after excitation into Q_x at 600 nm; (B) the respective ordinary absorption difference kinetics measured simultaneously with TRCD (notice noise level being below line thickness, an additional advantage of the new setup). Inset in green frame of (A) shows a typical noise of published TRCD spectrometer (from Lewis et al, J. Phys. Chem. **89**-289). (C) Decay associated difference spectra (DADS) obtained from ΔA_{CD} profiles and (D) ΔA_{OD} profiles.

(2) Similar experiments were performed with FMO at low temperatures (77 K). Low temperature measurements present an additional challenge since number of optical windows in cryostat add to CD signal of a sample. The spectra are obtained and data is processed.

(3) The TRCD spectrometer was updated with an option to measure steady state CD spectra in NIR region of spectrum; due to the use of dedicated NIR laser source its performance is dramatically better than that of a commercial CD spectrometer. The spectrometer was used to fully and precisely characterize new set of site-directed FMO mutants (from prof. Blankenship's group). The changes in ordinary absorption and CD associated with each mutation were analyzed in terms of existing exciton models of FMO. While absorption spectra changes for several mutants could be predicted by the existing exciton models of FMO reasonably well, in some cases (Fig. 2) changes in CD could not be reproduced indicating that current models (based predominantly on ordinary absorption data) assign diagonal energies to at least some pigments in FMO incorrectly [2].

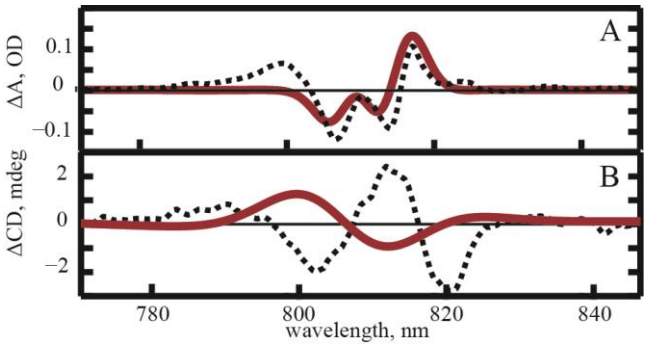

Fig. 2. Spectral changes in ordinary absorption (A) and CD (B) for mutation Q198V that affects predominantly BChl a #7 of FMO. Dashed lines – measurement, solid red line is signal expected using current FMO model. Notice that CD signal cannot be reproduced with a current set of diagonal energies proposed for FMO, while changes in ordinary absorption can be accounted for. Clearly the model needs a significant reassignment of diagonal energies to describe both ordinary absorption and CD.

(4) With slight modifications, the nanosecond TRCD design was adapted for femtosecond pump-probe setup and TRCD spectra of a test sample were measured demonstrating that this design used with femtosecond pump and probe pulses gives similarly high sensitivity. However, this work was put on hold - due to significant cuts in DOE funding it was decided to concentrate an on alternative TRCD design, which is riskier, but promises much greater potential (see the next paragraph).

(5) A construction of an alternative femtosecond TRCD spectrometer with dual-beam modulation and rf side-band detection is near completion. Unlike the design above, it will offer unmatched versality – in particular, a CD-modulated pump beam could be used to excite pigments with strong CD even in the presence of much stronger ordinary absorption from other pigments in the system, enabling, for example, direct excitation of the special pair in photosystem I (PS I) and remove spectral congestion, that prevents such experiments in ordinary ΔA spectrometers.

Science objectives for 2017-2018:

- Complete work on femtosecond TRCD and apply it to FMO studies. Develop proper excitonic modeling methods for fs TRCD signals. Investigate its feasibility for PS I charge transfer studies.
- Complete analysis of low-temperature FMO data and publish the results

References to work supported by this project 2016-2017:

1. S Bhaduri, V Stadnytskyi, SD Zakharov, S Saif Hasan, Łukasz Bujnowicz, Marcin Sarewicz, S Savikhin, Artur Osyczka, WA Cramer. "Pathways of Transmembrane Electron Transfer in Cytochrome bc Complexes: Dielectric Heterogeneity and Interheme Coulombic Interactions. The Journal of Physical Chemistry B 121, p. 975 (2017)
2. Rafael G Saer, Valentyn Stadnytskyi, Nikki C Magdaong, Carrie Goodson, Sergei Savikhin, Robert E Blankenship. "Probing the excitonic landscape of the Chlorobaculum tepidum Fenna-Matthews-Olson (FMO) complex: a mutagenesis approach". BBA 1858, p. 288 (2017)
3. V Stadnytskyi, GS Orf, RE Blankenship, S Savikhin. "Near shot-noise limited time-resolved circular dichroism pump-probe spectrometer". arXiv preprint arXiv:1706.07754 (2017)

Interrogating protein-protein association through spectroscopic studies of model membranes

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Overall research goals: The research goal of this project is to apply optical spectroscopy to model membrane-protein systems to measure energy and charge transfer dynamics within a controllable, well-defined near-native environment. We use ultrafast transient absorption spectroscopy to explore the dynamics in the following light-harvesting systems from purple bacteria: (1) LH2; (2) low-light LH2 with LH2; and (3) LH1 with the RC. Controlling membrane size, composition, and protein crowding within these membrane-protein systems achieves an experimental paradigm in which we can construct, piecewise, the photosynthetic light-harvesting network. These experiments should reveal how the local membrane environment impacts dynamics within a protein and between proteins as well as the role of the organization of the embedded proteins. Thus, these systems will enable exploration of how the photosynthetic membrane is constructed to give rise to long-distance energy transduction.

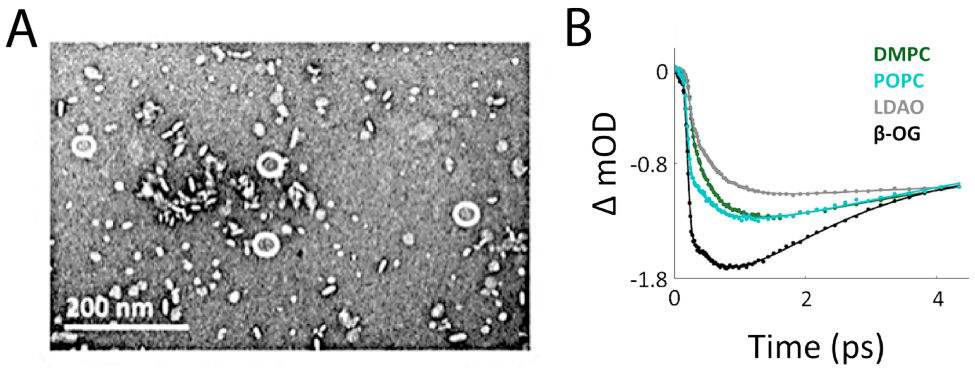

Figure 1. A: Successful incorporation of LH2 into membrane discs shown by TEM image of LH2 discs. Circles highlight individual discs: B: 800 nm pump - 850 nm probe transient absorption data of LH2 in DMPC membrane discs (green), POPC membrane discs (teal), LDAO detergent (grey), and β-OG detergent (black).

Science objectives for 2017-2020:

- Our experiments show that one energy transfer step in LH2, energy transfer between the two rings of pigments (B800 to B850), speeds up by 30% in the membrane environment versus the typical detergent environment. The faster rate is robust to lipid composition of the surrounding membrane. Based on experimental and theoretical results, this effect is assigned to tilting of the pigments in the B800 ring, which protrude from the protein structure. The molecular or mechanical interaction responsible for the perturbation may be investigated by changing membrane composition and pressure.
- The project will be expanded from incorporating single light-harvesting complexes into model membranes to multiple light-harvesting complexes, including both LH2 and low-light LH2. Energy transfer between these complexes will be explored with ultrafast spectroscopy.
- While previous work measured average protein-to-protein energy transfer rates within the native membrane, AFM studies revealed that the network is highly heterogeneous, which means that measured rates average over a distribution consisting of dramatically different values. Furthermore, a remarkable reorganization of the membrane with light conditions was observed,

including a change in the crowding of the light-harvesting complexes. Studying the effects of crowding will explore how light harvesting is robust to membrane heterogeneity.

- LH1 surrounds the RC in the membrane. Incorporating LH1-RC within a membrane shows how the energy transfer dynamics of the larger ring structure are affected by the membrane, as well as how the charge transfer dynamics of the embedded RC are protect by the surrounding LH1.

Light-Driven Nitrogenase

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Overall research goals: The goals of this project are to gain insights into the mechanism of N_2 reduction to NH_3 catalyzed by the enzyme nitrogenase using photoactivated nano-materials as the source of electrons. Nitrogenase reduction of N_2 requires energy input as ATP hydrolysis and low potential electrons. We have discovered that CdS nanorods can be used to inject electrons into nitrogenase to drive N_2 reduction without ATP and thus providing all of the energy for the reaction from light. These studies seek to gain molecular level insights into how light-driven N_2 reduction is achieved by these biohybrid systems of nanoparticles-nitrogenase. The findings are expected to provide foundational information about how to couple light to difficult reduction reactions.

Significant achievements 2016-2017: We discovered that it is possible to make a biohybrid between a CdS nanorod and the nitrogenase MoFe protein. This complex was shown to achieve light-driven N_2 reduction to NH_3 with high turnover frequency and turnover number. These findings now set up a suite of studies to understand how driving force, rates of electron delivery, and pathways work together to allow light-driven N_2 reduction.

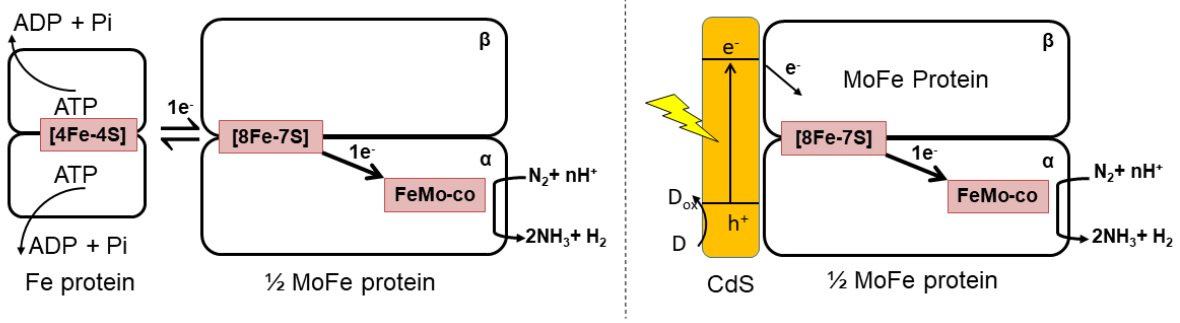

Figure 1. Left Panel: Schematic of nitrogenase proteins showing the delivery of electrons from the Fe protein to the MoFe protein driven by ATP hydrolysis. Right Panel: Schematic of the CdS nano-rod-MoFe protein biohybrid showing the light driven transfer of electrons into the MoFe protein protein to support N_2 reduction.

Science objectives for 2016-2019:

- Establish what effects the linker chemistry and nanorod diameter have on the rates and driving force of electron transfer into nitrogenase and how these parameters control the stoichiometries and rates of N_2 reduction. These studies provide an unprecedented opportunity to map the dependence of electron transfer rate and driving force on achieving N_2 reduction.
- Establish the roles of the P cluster in the nanorod-MoFe protein light-driven reaction. For the normally functioning enzyme, the P cluster operates as a mediator of electron flow to the active site FeMo-cofactor. It is unknown if the CdS nano-rods donate electrons through the P clusters. Unraveling the pathway for electron transfer will provide key insights into how the P cluster contributes to N_2 reduction.
- We will use light pulses to inject electrons at defined stoichiometry into nitrogenase to capture partially reduced states. These states can be freeze-trapped and investigated by a range of spectroscopic methods including advanced EPR techniques. We anticipate that such studies will provide insights into how nitrogenase accumulates electrons to achieve N_2 reduction. This will, in turn, provide key insights into the catalytic mechanism.

References to work supported by this project 2016-2017:

1. Brown, K. A., Harris, D. F., Wilker, M. B., Rasmussen, A., Khadka, N., Hamby, H., Keable, S., Dukovic, G., Peters, J. W., Seefeldt, L. C., and King, P. W. (2016) Light-driven dinitrogen reduction catalyzed by a CdS:nitrogenase MoFe protein biohybrid. *Science 352*, 448–450.

Site-specific Characterization of Plastocyanin and the Impact of Binding with Cytochrome *f* via Infrared Spectroscopy

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Overall research goals: Electron transfer reactions are central to life as they underlie the harvesting, storage, and retrieval of the energy required to sustain it. Unlike electron transfer reactions involving small molecules, biological electron transfer occurs between redox centers imbedded in proteins that contain electrostatically heterogeneous environments, adopt many possible conformational states, and undergo fluctuations on a wide range of timescales. It is well accepted that the reactivity of metal sites in proteins is tuned by protein-based ligands, and that formation of protein-protein complexes gates inter-protein electron transfer. However, the complexity of biological electron transfer has challenged rigorous characterization, and thus our understanding of remains incomplete. Our research program aims to overcome these challenges to delineate the mechanisms by which proteins mediate electron transfer, specifically focusing on electron transfer involving plastocyanin (Pc) and cytochrome (cyt) *f*, two key proteins of the photosynthetic apparatus. By taking advantage of the very fast inherent timescale of infrared (IR) spectroscopy and the high spatial resolution provided by the small size of IR chromophores, we are developing approaches for the rigorous characterization of localized environments anywhere in a protein and the measurement of the dynamics on even the fastest timescales. We combine linear and two-dimensional (2D) IR techniques with methods of biochemistry and chemical biology for placing frequency-resolved vibrational probe groups at specific sites to map out the varying environments and dynamics throughout proteins.

Significant achievements 2015-2017: We have applied this approach toward understanding the role of the usually long methionine axial ligation of blue copper proteins like Pc and its perturbation by binding to cyt *f*. Toward investigating the dynamics of plastocyanin, we have incorporated cyanophenylalanine at three distinct positions and used 2D IR spectroscopy to measure the site-specific variation in heterogeneity and dynamics. We have begun to characterize the dynamics when bound to cyt *f* to explicate the nature of the complex. In addition, we have worked toward improving the 2D IR approach and extend the experimental timescale by development of new probes with longer vibrational lifetimes. To date, we have examined the use of two novel IR probes, *p*-$^{13}C^{15}N$-cyanophenylalanine and *p*-cyano-selenophenylalanine.

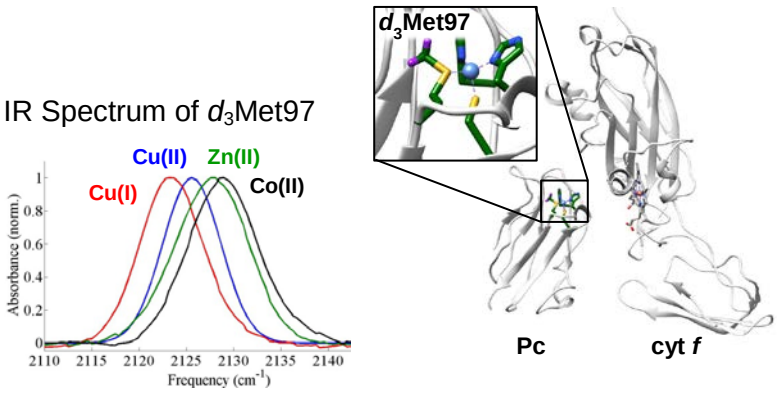

Figure 1. FT IR characterization of d_3Met97 of reduced, oxidized, and metal-subsititued Pc.

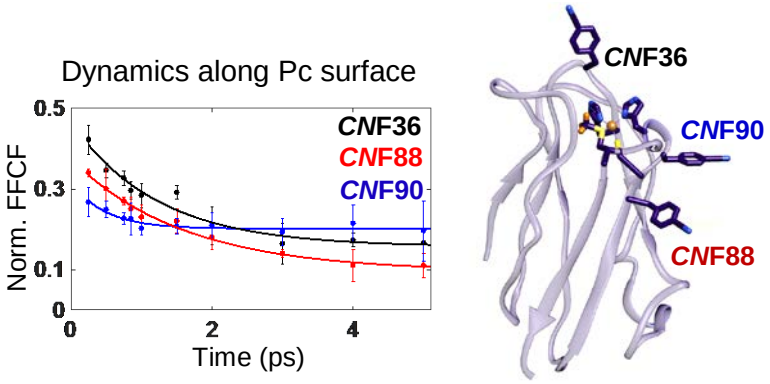

Figure 2. Site-specific dynamics of Pc measured via 2D IR spectroscopy.

Science objectives for 2017-2018:

- The site-specific characterization of the dynamics of Pc when bound to cyt *f* using cyanophenylalanine probes and 2D IR spectroscopy will be completed to generate a model of the Pc-cyt *f* complex.
- The investigation of localized changes in the environments and heterogeneity will be extended using Pc labeled site-specifically with d_2Tyr, which is in hand.
- The non-equilibrium 2D IR spectroscopy will be used to investigate the dynamics that occur upon photo-initiated reduction of Pc as a route toward investigation of the role of the protein scaffold in controlling the reorganization energy of electron transfer. The process will also be characterized on the ns and longer timescales using a recently built a quantum cascade laser-based spectrometer.
- The dynamics of the Pc-cyt *f* complex will be compared for mutants with altered surface charge to explore how the surface might be tailored to facilitate efficient electron transfer.

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Correlating Structure with Charge-Accumulating Function in Photosynthesis and Photosynthetic Hybrids

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Overall research goals: The objective of this research program is to investigate electron transfer mechanisms and structures that enable electron transfer chains to function as charge-accumulating linkages between primary light-driven charge-separation and multi-electron, proton-coupled solar energy conversion in photosynthesis and photosynthetic hybrids. Key questions that are investigated include the identification of mechanisms for electron transfer in multi-cofactor photosynthetic and biohybrid redox chains and visualization of sites on photosynthetic and biohybrid protein surfaces where accumulated reducing equivalents are accessible for subsequent energy conversion function. The program highlights the development of advanced synchrotron-based X-ray scattering and spectroscopy, correlated with multi-scale imaging approaches.

Significant achievements 2015-2017: *X-ray imaging of sites for electron transfer using metal redox chemistry.* In 2016 we initiated a new project to exploit reductive precipitation of platinum and other metal salts as a way to visualize locations on the surfaces photosynthetic and biohybrid proteins where accumulated reducing equivalents are accessible for subsequent energy conversion function. The approach is significant by offering a means to visualize redox active sites with respect to docking domains for partner redox proteins in photosynthetic electron transfer chains, and for visualizing activity topologies in intact membranes. X-ray scattering techniques were developed for structure characterization. X-ray scattering profiles measured for isolated PSI from *Synechocystis* and self-assembled photo-hydrogen evolving complexes formed with 3 nm mercaptosuccinic acid capped Pt nanoparticles, PSI-Pt. Small angle X-ray scattering profiles showed characteristic differences between PSI and PSI-Pt complexes which are qualitatively simulated in atomic models. The results demonstrate opportunities for identification of site for Pt nanoparticle binding by model structure refinement

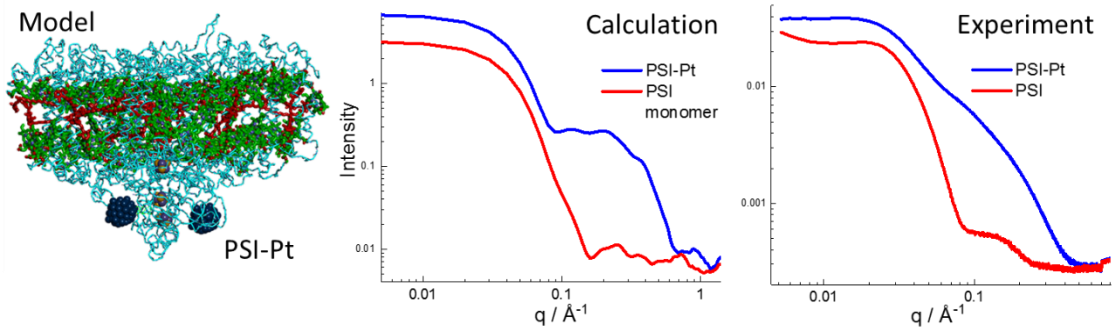

Figure 1. X-ray scattering characterization of PSI-Pt biohybrids. The left panel shows a model PSI-Pt structure composed a PSI monomer and two Pt_{55} nanoparticles positioned at candidate docking sites. The middle panel shows calculated X-ray scattering for the PSI monomer and PSI-Pt model. Calculations using trimer assemblies are on-going. The right panel shows experimental scattering for PSI and the PSI-Pt biohybrid.

through iterative model fitting to experimental X-ray scattering data.

Mechanisms mimicking primary reaction center photochemistry. In the completion phase of a project on characterization of light-induced electron transfer mechanisms in multi-heme c-cytochrome photo-hybrids, we demonstrated that light-induced electron transfer could be designed by site-directed mutagenesis to proceed along single or bifurcated, symmetry-related, electron transfer pathways, and function without loss of activity down to 2.8 K. Inter-heme electron tunnelling was measured to occur on the picosecond timescale and demonstrate the concept of a "protein nano-wire". These photosynthetic mimics duplicate the core features of primary photosynthesis and suggest the possible requirement for an electronic "gap" between primary and secondary electron carriers in design of the charge-accumulating electron transfer chains that link primary charge-separation with multi-electron, proton-coupled solar energy conversion.

Science objectives for 2017-2018:

- Develop computational methods for PSI-Pt nanoparticle structure refinement and quantitative fitting to experimental X-ray scattering data. We have previously developed algorithms for the simulation of X-ray scattering data and atomic pair distribution function analysis (PDF) using atomic models. These methods will be extended to allow model refinement of PSI trimer "super complexes" with Pt nanoparticles.
- X-ray scattering measurements and model structure analyses will be carried out on isolated PSI complexes following photo-reductive precipitation of platinum. These measurements will be designed to visualize locations on the PSI surface where accumulated reducing equivalents are accessible for electron transfer to secondary electron acceptors.
- A new research effort area will be initiated on the use of X-ray fluorescence microscopy, XFM, at the APS and cryo-electron microscopy at the Center for Nanoscale Science to monitor PSI activity across the topology of the thylakoid membrane.

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Photosynthetic Biohybrid Systems for Solar Hydrogen Production

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Overall research goals: Natural photosynthetic energy research is aimed at resolving fundamental mechanisms of photochemical energy conversion in photosynthetic proteins. These basic studies provide us with insight into how to use Nature's optimized photochemistry to drive non-native chemical reactions. Currently, we are designing new bio-inspired systems that capture and convert the sun's energy and store it in the energy-rich bond of hydrogen, a clean, carbon-neutral and renewable energy source. Specifically, we are creating a new class of small protein-based photocatalytic complexes that replicate essential design features of photosynthetic reaction centers (RCs) and enable the spectroscopic discernment of the structure and processes crucial to solar-driven proton reduction. Our combined effort at the forefront of biochemical and spectroscopic experimental approaches provides an opportunity for breakthroughs in the resolution of fundamental mechanisms for coupling photons to fuels in photosynthetic biohybrid systems, a necessary step forward in the development of optimized systems for solar fuels.

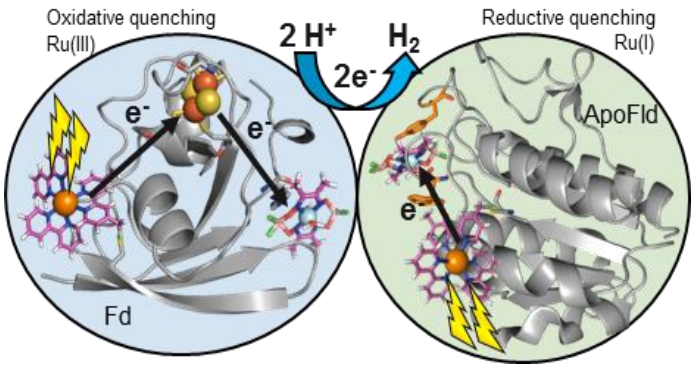

Figure 1 Schematic of different photosensitizer-protein-catalyst complexes that have distinctive pathways for capturing visible light and converting it to a fuel.

Significant achievements 2015-2017:
Biohybrids shed light on pathways to solar fuels. We have developed two systems for photocatalytic hydrogen production that both directly link a proton reduction catalyst $[Co(dmgBF_2)_2]$ and a photosensitizer molecule $[Ru(bpy)_3]^{2+}$ with the small electron transfer proteins ferredoxin and flavodoxin.(Figure 1) Placement of redox active electron transfer moieties at designed locations in the protein scaffolds enables rapid forward electron transfer and prohibits charge recombination. Through detailed transient optical spectroscopy and EPR studies two distinct mechanisms for electron transfer efficiency related to photocatalytic function were observed. Continued studies of biohybrid designs will provide a knowledge base about system catalysis function related to underlying electron transfer kinetic mechanisms and pathways.

Stabilization of molecular catalysts in water for solar fuels production. We have constructed a biohybrid that embeds a nickel diphosphine hydrogen evolution catalyst into the cofactor binding pocket of the electron shuttle protein, flavodoxin. The system is made photocatalytic by linking a protein cysteine residue to a ruthenium photosensitizer. Importantly, the protein environment enables the otherwise insoluble Ni(II) catalyst to perform photocatalysis in aqueous solution over a pH range of 3.5- 12.0.(Figure 2) For the first time, a reversible light-induced charge-separated

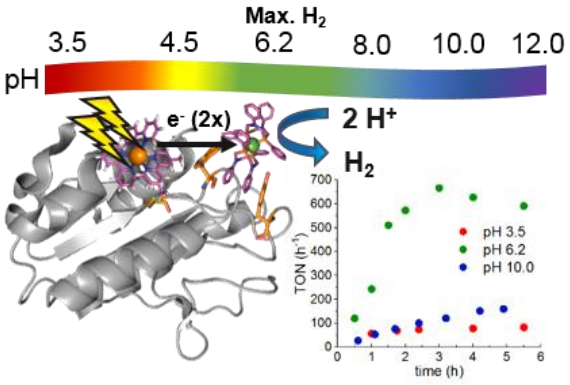

Figure 2 Schematic of a photosensitizer-protein-catalyst complex that functions across a wide pH range to produce hydrogen directly from water using light**.**

state involving a Ni(I) intermediate was directly monitored by EPR spectroscopy. Transient optical measurements reflect two conformation states, with a Ni(I) state formed in ~1.6 or ~185 µs that persists for several milliseconds as a long-lived charge-separated state facilitated by the protein matrix. The pH stability of the Ni catalyst in the protein environment is unprecedented and gives suggestions for further roles for proteins to support and protect important catalytic systems in aqueous environments through protein engineering and design.

Science objectives for 2018-2020:

- New biohybrids comprised of earth abundant Cu(I)diimine photosensitizer molecules and robust Co(II) polypyridyl catalysts will be developed with our small protein systems. PSI-Co(II) polypyridyl complexes will be explored for longevity of H_2 photocatalysis.
- A new direction involves examining the self-assembly of abiotic proton reduction catalysts to PSI within thylakoid membranes. A membrane environment provides opportunities for coupling reductive chemistry to intact electron transfer pathways, thereby removing the necessity of sacrificial donors. The feasibility of linking water oxidation chemistry at PSII to the reduction chemistry at PSI via soluble mediator molecules will be investigated.
- Using a unique combination of isotopically labeled cyanobacterial membranes and isolated proteins, we will develop EPR methods to characterize the coupling of RC primary photochemistry to secondary reaction sequences in thylakoid membranes.

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Structure and Function of Rubisco Activase from Higher Plants

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Overall research goals:

This project addresses the regulation of higher plant carbon assimilation by Rubisco activase (Rca), a chemo-mechanical motor protein essential in maintaining ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity. Rca is a ring-forming ATPase that plays a critical role in regulating the conversion of energy derived from photosynthetic electron transport into chemical storage forms. The goal of this project is to elucidate the relationship between Rca tertiary and quaternary structure and its ATPase and Rubisco reactivation activities. (1) A major research thrust is to unravel how Rca oligomerization is regulated by ligand binding, and how shifts in assembly equilibria modulate ATPase and Rubisco reactivation activities. (2) A second major objective is to characterize the physical interaction of Rca assemblies with the Rubisco client protein.

Significant achievements in 2015-2017:

1. Rca subunit exchange as a function of nucleotide

In vitro, Rca is known to undergo dynamic assembly-disassembly processes, with several oligomer stoichiometries coexisting over a broad concentration range. Therefore, changes in quaternary structure could play a role in Rubisco regulation. To examine the rate of subunit reorganization, we attached fluorescent labels to the C-termini of spinach β-Rca, and monitored subunit mixing by measuring energy transfer (FRET) as a function of nucleotide and divalent cation. Only dimeric units appeared to exchange. Poorly hydrolyzable substrate analogs provided locked complexes with high thermal stabilities (apparent T_m = 60°C) and estimated $t_{1/2}$ of at least 7 hours, whereas ATP-Mg provided tight assemblies with $t_{1/2}$ of 30-40 min, and ADP-Mg loose assemblies with $t_{1/2}$ of less than 15 min. Accumulation of ADP to 20% of total adenine nucleotide accelerated exchange substantially. An initial lag period was observed with ATP·Mg, indicating inhibition of subunit mixing at low ADP. The ADP K_i value was estimated to be larger than the K_m for ATP (0.772 $\pm$ 96 mM), suggesting that the equilibration rate is a function of the relative contributions of high- and low-affinity states. C-terminal crosslinking required the N-terminal extension to the AAA+ domain to generate covalent dimers, and provided additional evidence for different classes of sites. ***Based on these data, we propose that oligomer reorganization may be stalled during periods of high Rubisco reactivation activity, whereas changes in quaternary structure are stimulated by the accumulation of ADP at low light.***

2. Assembly pathway and oligomer activity of tobacco Rca

(a) ABEL trap. In collaboration with WE Moerner (Stanford), we have utilized single-molecule diffusometry to measure the size distribution of tobacco Rca oligomers under equilibrium conditions. Monomers, dimers and a tetramer/hexamer mixture were directly observed. Their fractional abundance was extracted as a function of protein concentration, and a reliable value for the monomer-dimer equilibrium constant (K_d^{m-d}) was determined. In the 1-10 μM range, ATPγS strongly promotes tetramer/hexamer formation, whereas trimers are not observed under any condition tested. In addition, dynamic assembly and disassembly processes of single complexes were directly observed in real time. The rate of subunit exchange was estimated to be ~0.1s^{-1} in the presence of ATP-γS, whereas with ADP, subunit exchange was enhanced more than 2-fold, indicating destabilization of Rca complexes.

(b) Fluorescence correlation spectroscopy (FCS). In parallel work, we have determined the full assembly pathway of tobacco Rca by use of FCS methods, and measured its ATPase activity as a function of subunit concentration. By incorporating information from single-molecule diffusometry (see

above), we were able to estimate K_d values for the dimer-tetramer, tetramer-hexamer and hexamer-24mer equilibria for both wild-type Rca and the R294V assembly mutant. We find that with ATP-γS and ADP·AlF_4, the hexamer dominates at 10 μM (70-90%), while a small amount of tetramer (25% or less) may accumulate. With ADP and the apo-protein, the tetramer does not accumulate at all, while hexamers and larger aggregates are favored. Surprisingly, active turnover of ATP stimulates tetramer accumulation to 25 - 40% at 2 μM Rca, while the hexamer accumulates to 50 - 60% at 5 μM Rca. Turnover measurements indicate that the ATPase activity decreases at a rate equal to the formation of large aggregates such as 24mers, which may serve as a storage mechanism of inactive Rca. Although maximum ATP hydrolysis coincides with the tetramer peak, the concentration-dependent activity curve follows neither the tetramer nor the hexamer. Rather, it appears that activity is highest when dimers, tetramers and hexamers coexist. Apparently, not one particular oligomeric species, but the equilibrium between three species is coupled to rapid ATP hydrolysis. ***Therefore, the data suggest a model in which ATP hydrolysis breaks the hexameric rings apart via dimer dissociation.*** According to this idea, a high level of activity would lead to the accumulation of tetrameric and dimeric species. These may diffuse through the stroma, then re-associate to hexamers for continued Rubisco reactivation.

3. Structure

(a) Electron microscopy (EM). Using the AAA^+ domain of spinach β-Rca, we have identified conditions that allow us to observe hexameric rings by negative-stain EM. In collaboration with Po-Lin Chiu (ASU), we have calculated preliminary class averages that can be fit to circular or ellipsoid particles. We will continue our screening efforts until class averages are obtained with sufficient homogeneity for cryo-EM studies. *(b) Attachment of Rca to DNA Origami.* To facilitate structure-function studies, we are collaborating with Hao Yan (ASU) to attach Rca and Rubisco to a flat sheet of DNA designed to carry spatially patterned attachment sites. Recently, we have succeeded in labeling tobacco Rca with single-stranded DNA and have demonstrated that full Rubisco reactivation activity is retained. Atomic force microscopic (AFM) images have demonstrated that these constructs can be attached to DNA origami by means of DNA hybridization.

Science objectives for 2017-2018:

- Monitor the magnesium-dependence of the Rca assembly process by FCS.
- Pursue electron microscopy studies for high resolution single-particle reconstructions.
- Attach both Rca and Rubisco to DNA origami to promote the formation of Rca-Rubisco complexes for EM studies.
- Test the function of the Rubisco C-tail in Rca-mediated reactivation.

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Signal Transduction Pathways of Chloroplast Quality Control

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Background: Chloroplasts (photosynthetic plastids) of higher plants contain about 3000 proteins of which more than 95% are encoded by nuclear genes. To avoid accumulation of reactive oxygen species (ROS) that are the inherent by-product of photosynthesis, gene expression of these spatially separated genomes is regulated by two-way signaling. Thus, while plastid differentiation and development are largely under nuclear control, developmentally arrested or damaged plastids can regulate expression of nuclear genes via retrograde signaling pathways. In previous years of DOE-funded study, we performed a number of genetic screens that implicated the chloroplast-localized tetrapyrrole biosynthesis pathway as the source of both positive and stress-related retrograde signals[3,4]. Using that work as a foundation, we have uncovered a new type of retrograde signal that leads to the selective removal of damaged chloroplasts from the cell (Figure 1[1,2]).

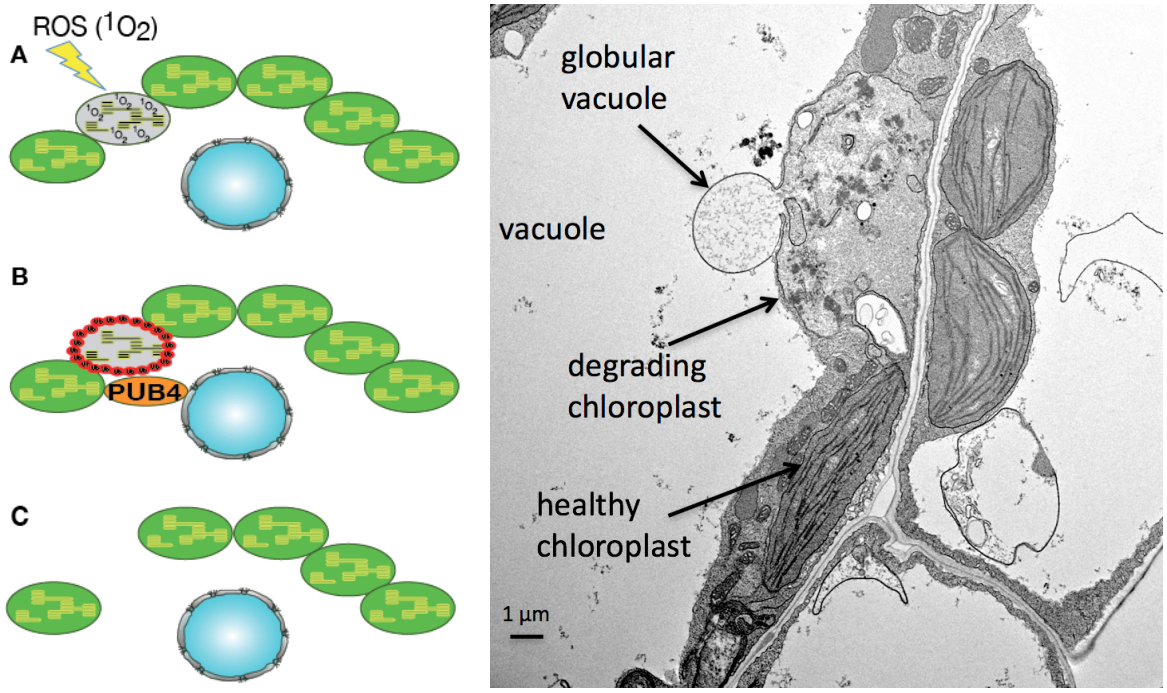

Figure 1: Left panel: Model for selective chloroplast degradation. A) During photosynthesis stress, singlet oxygen $^1O_2$ accumulates in individual chloroplasts. B) This leads to the ubiquitination of unidentified chloroplast membrane proteins. Although the mechanism is unknown, it involves the cytoplasmic E3 ubiquitin ligase PUB4. C) After ubiquitination, the chloroplast is subsequently degraded by an unidentified mechanism. Right panel: Transmission electron microscopy (TEM) image of an *Arabidopsis fc2* mutant cotyledon mesophyll cell. A degrading chloroplast can be seen blebbing into the central vacuole in an otherwise healthy cell.

Overall research goals: The major goal of this new proposal is to define the signal(s) and signaling pathways from chloroplasts that regulate selective chloroplast degradation, a process of which little is known. The proposed studies will integrate genetic, molecular, and biochemical approaches in *Arabidopsis* in an effort to understand these signaling events. The

successful completion of the proposed projects should allow us to begin to engineer these crucial pathways. Furthermore, the generation of new genotypes will ultimately influence our abilities to manipulate plant growth and development. Together this will aid in the understanding of the developmental control of photosynthesis, the chloroplast assembly/disassembly pathways, and how plants adapt to stressful conditions.

Significant achievements: We have recently reported that a chloroplast quality control pathway exists in plants[1,2]. By studying *plastid ferrochelatase 2* (*fc2*) mutants of *Arabidopsis* we have shown that a ROS burst can initiate a signal to selectively degrade damaged chloroplasts (Fig. 1). *fc2* mutants conditionally accumulate the photosensitizing heme/chlorophyll precursor Protoporphyrin-IX leading to the formation of singlet oxygen ($^1O_2$). Subsequently, chloroplast envelope proteins become ubiquitinated, which may be "marking" these chloroplasts for degradation in the central vacuole. The mechanism by which this occurs is not known, but by using a genetic screen, we identified the plant U-box 4 (PUB4) E3 ubiquitin ligase as playing a pivotal role in this process. The role of PUB4 may be to seek out stressed chloroplasts for ubiquitination and then rapid turn-over. As ubiquitination is known to regulate protein function and degradation, this offered a possible mechanism for how individual $^1O_2$-stressed chloroplasts may be "marked" for degradation. Such an organelle-autonomous signal allows for rapid on-site regulation of photosynthesis during stressful conditions.

Science objectives for 2017-2020:

- The mechanisms that trigger chloroplast ubiquitination and degradation are not understood, but accumulation of $^1O_2$ is associated with initiating the response. Chloroplast quality control is probably not regulated directly by $^1O_2$ as the short half-life of $^1O_2$ makes it unlikely to exit the chloroplast. Instead, a local change in chloroplast metabolism due to $^1O_2$-damage to chloroplast-localized metabolites may be the signal. As such, we have performed metabolomics profiling of stressed *fc2* mutants and identified potential signaling metabolites. We also propose to complete our genetic screen of *fts* mutants where 13 loci await characterization. We are currently analyzing the roles of these genes, proteins, and metabolites in chloroplast signaling.
- Our studies show that chloroplast proteins in *fc2* mutants become ubiquitinated prior to chloroplast degradation. This was in line with the fact that PUB4, an E3 ubiquitin ligase that may be facilitating the ubiquitination, was necessary for chloroplast-induced degradation. To understand this role of ubiquitination, we are in the process of identifying which proteins on the chloroplast are ubiquitinated, how that ubiquitination leads to chloroplast degradation, and what role PUB4 plays in the process. This will provide insight into the mechanisms of how chloroplasts direct their own degradation in response to damage.
- Genetic analysis of the *fc2* mutant has been extremely valuable in uncovering an important pathway that involves PUB4 and ubiquitination. Ultimately, however, we need to understand how chloroplast degradation and chloroplast quality control are used during natural conditions in the wild or in the field. For this reason, we are testing our model (Fig. 1) under excess light stress that naturally produces $^1O_2$ in the chloroplast.

References to work supported by previous DOE support:

1. J. D. Woodson 2016. Chloroplast quality control – Balancing energy production and stress. *New Phytologist*. 212:36-41.
2. J. D. Woodson, M. S. Joens, A. B. Sinson, J. Gilkerson, P. A. Salome, D. Weigel, J. A. Fitzpatrick, and J. Chory. 2015. Ubiquitin facilitates a quality control pathway that removes damaged chloroplasts. *Science*. 350:450-4.
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Electronic Structure of the Mn Cluster in Photosystem II: X-ray Absorption and Emission Spectroscopy using an XFEL

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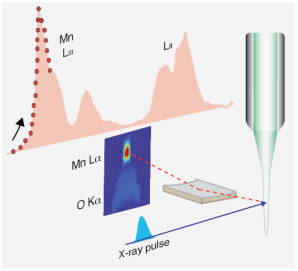
Research goals: The objective of this proposal is to understand the mechanism by which water is oxidized to dioxygen at the Mn_4CaO_5 site in photosystem II in plants, algae and cyanobacteria. We propose to develop and utilize new methodologies of X-ray spectroscopy and crystallography, using synchrotron and X-ray free electron sources, in our investigations of photosynthetic water oxidation. The specific subjects that are our focus are as follows: 1) Determine the molecular and electronic structural changes of the Mn_4CaO_5 cluster of the photosynthetic oxygen-evolving complex of Photosystem II as it advances through the intermediate states in the catalytic cycle. 2) Understand the interactions between the protein environment and the catalytic center that allow PS II to carry out the sequential events during the four-electron water oxidation reaction, under ambient conditions. 3) Application of X-ay spectroscopic techniques to study light absorption and catalysis in artificial photosynthetic systems.

Significant achievements 2016-2017:

The knowledge about the geometric structure of the Mn_4CaO_5 cluster has to be complemented by a detailed understanding of the electronic structure of the cluster to address key questions of the mechanism of photosynthetic water oxidation.



The reflection zone-plate emission spectrometer discriminates the weak Mn signal of PS II from the background of light atoms. PS II Sample is introduced with a liquid jet, and fs X-ray pulses from the XFEL enables the room temperature data collection possible.

1) We have combined the fs soft X-ray pulses from a XFEL with a novel 100 zone plate spectrometer and have obtained L-edge spectra from PS II and also Mn complexes. The zone plate spectrometer allows for discriminating between the Mn L-fluorescence signal from the predominant O K-emission background. During the course of these studies, we also demonstrated how L-edge XAS can be performed using the XFEL self-seeding scheme without the need of an additional beamline monochromator that is required when we use the XFEL standard self-amplified spontaneous emission (SASE) mode where the bandwidth can be as high as 5 eV in the soft X-ray regime.

2) Controlling the electron/hole flow between multiple sites, as in PS II, in inorganic systems may be a key issue for developing efficient catalysts and assembled systems. To simultaneously follow the chemistry at multiple sites by probing the element/orbital/spin-specific signals, we developed an energy dispersive XES detection scheme, enabling an XES spectrum of multiple elements to be measured in a single-shot mode. This approach provides data, free from temporal and normalization errors, and therefore is ideal to follow sequential chemistry occurs through multiple sites. We have applied this method to investigate a MnO_x-NiO_x based bifunctional electrocatalyst, that catalyzes both the oxygen reduction reaction and the oxygen evolution reaction. We simultaneously collected Mn and Ni XES as a function of the applied electrochemical potential, and showed the evolution of the electronic structure of two sites. We also collected time-resolved data from a $Ti^{IV}OMn^{II}$ charge

transfer chromophore unit, in which excited state ultra-fast fs dynamics, structural rearrangements, and, possibly, spin crossover processes are expected following metal-to-metal charge transfer.

Science objectives for 2017-2018:

We plan to use the Mn L-edge spectra at RT for capturing the electronic structural changes during the $S_3 \rightarrow [S_4] \rightarrow S_0$ transition of the Mn_4CaO_5. We also plan to collect data from a series of Mn inorganic complexes at SR/XFEL facilities.

We will develop room temperature EXAFS and XANES methods for PS II at XFELs, in addition to the Mn $K\beta_{1,3}$ and the ligand to valence $K\beta_{2,5}$ emission studies that are ongoing.

We are also continuing our efforts with developing stimulated emission methods for dilute transition metal solution studies.

References to work supported by this project 2016-2017:

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1. X-ray Free Electron Lasers, Applications in Materials, Chemistry and Biology, (Bergmann, U., Yachandra, V. K., Yano, J. eds.), The Royal Society of Chemistry, Cambridge, U.K. 2017.
2. Yano, J., *et al*. (2017) Enzymes and Models, in *XAFS Techniques for Catalysts, Nanomaterials, and Surfaces* (Iwasawa, Y., Asakura, K., Tada, M. eds.), Springer, Switzerland. pp 451-465.

Reviews

1. Lassalle-Kaiser, B., *et al*. (2017) In situ/Operando Studies of Electrocatalysts using Hard X-ray Spectroscopy, *J. Electron Spect. Rel. Phenom*. http://dx.doi.org/10.1016/j.elspec.2017.05.001

Publications

1. Zhang *et al*. (2017) Structural Insights into the Light-driven Auto-assembly Process of the Water-Oxidizing Mn_4CaO_5-Cluster in Photosystem II. *eLife*, DOI: 10.7554/eLife.26933.
2. Kubin, M., *et al*. (2017) Soft X-Ray Absorption Spectroscopy of Metalloproteins and High-Valent Metal-Complexes at Room Temperature Using Free-Electron Lasers. *Struct. Dyn*. **4**, 054307.
3. Fuller, F. D., *et al*. (2017) Drop-on-Demand Sample Delivery for Studying Biocatalysts in Action at X-ray Free-Electron Lasers. *Nat. Methods*, 14, 443-449.
4. Young, I. D., *et al*. (2016) Structure of Photosystem II and Substrate Binding at Room Temperature. *Nature*, **540**, 453-457.
5. Alonso-Mori, R., *et al*. (2016) Towards Characterization of Photo-Excited Electron Transfer and Catalysis in Natural and Artificial Systems Using XFELS. *Faraday Discuss*. **194**, 621-638.
6. Kroll, T., *et al*. (2016) X-ray Absorption Spectroscopy Using a Self-Seeded Soft X-ray Free-Electron Laser. *Opt. Exp*. **24**, 22469-22480.
7. 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 OEC of Photosystem II. *Chem. Sci*. **7**, 5236-5248.
8. Sauter, N. K., *et al*. (2016) No Observable Conformational Changes in PSII, *Nature* **533**, E1-E2.
9. Hatakeyama, M., *et al*. (2016) Structural Changes in the S_3 State of the Oxygen Evolving Complex in Photosystem II. *Chem. Phys. Lett*. **651**, 243-250.
10. Roessler, *et al*. (2016) Acoustic Injectors for Drop-on-Demand Serial Femtosecond Crystallography. *Structure*, **24**, 631-640.
11. Baxter, E. L., *et al*. (2016) High-Density Grids for Efficient Data Collection from Multiple Crystals. *Acta Cryst. D*, **72**, 2-11.
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Taking Snapshots of Photosynthetic Water Oxidation: Room Temperature Structure of Photosystem II Using Crystallography at an XFEL

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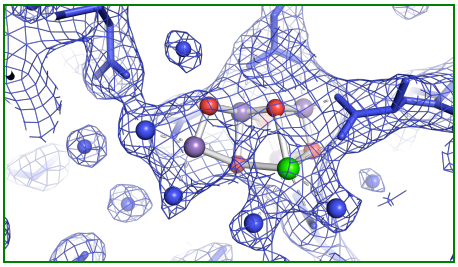
Research goals: The objective of this proposal is to understand the mechanism by which water is oxidized to dioxygen at the Mn_4CaO_5 site in photosystem II in plants, algae and cyanobacteria. We propose to develop and utilize new methodologies of X-ray spectroscopy and crystallography, using synchrotron and X-ray free electron sources, in our investigations of photosynthetic water oxidation. The specific subjects that are our focus are as follows: 1) Determine the molecular and electronic structural changes of the Mn_4CaO_5 cluster of the photosynthetic oxygen-evolving complex of Photosystem II as it advances through the intermediate states in the catalytic cycle. 2) Understand the interactions between the protein environment and the catalytic center that allow PS II to carry out the sequential events during the four-electron water oxidation reaction, under ambient conditions. 3) Application of X-ay spectroscopic techniques to study light absorption and catalysis in artificial photosynthetic systems.

Significant achievements 2016-2017:

We have developed the method for collecting X-ray Emission Spectroscopy and X-ray diffraction data simultaneously in a snap-shot mode at an XFEL using microcrystals and solution samples. The method has been successfully applied for studying the catalytic cycle of PS II with *in situ* multiple photo-excitations. In our recent work (Young et al, *Nature* 2016), we significantly improved the data collection efficiency and sample handling for the simultaneous XRD/XES study by introducing the *Drop on Tape* method (Fuller, et al., *Nat. Methods*, 2017), and increased the high-resolution fraction of the diffraction images. Together with the new crystallization protocol that gives rise to high quality diffraction images from 20-50 μm size crystals at room temperature (up to 2.0 Å or better), we have collected complete datasets for several different illumination conditions at 2.15-2.3 Å resolution cutoff. Accompanying XES data from crystals showed energy shifts comparable to the low-dose cryogenic synchrotron data, confirming the S-state advancement after each flash under the conditions of our XFEL experiment. The XES also provided an *in situ* way of characterizing the S-state population at each flash state.



Structure of the oxygen evolving Mn_4Ca complex in photosystem II at room temperature in a light-activated state, at ~2.15 Å resolution.

We determined the structure of the PS II complex at 2.25-3.0 Å resolution in three different states, the dark (S_1), the doubly illuminated (2F, S_3) and an ammonia bound 2F-NH_3 state. The room temperature data clearly showed an expansion of the dimeric complex along the membrane plane, leading to slightly larger distances between the Chl cofactors compared to cryogenic structures. The overall geometry of the OEC at RT resembled the cryogenic structures, but the 2.25 Å 2F structure showed some structural changes, namely a tilt of the Mn4-Mn3 axis with respect to the S_1 structure and some changes (0.1-0.3 Å) in positions of ligating waters and amino acids. We also showed that the W2 water binding site is preferably perturbed on NH_3 binding.

In our current XRD data, the metal positions (Mn and Ca) and terminal water ligands can be located. The isomorphous differences between the states show a clear indication of the atomic shifts in the Mn_4CaO_5 cluster and surrounding ligands, as well as water positions and acceptor quinone during the catalytic cycle. However, determining the precise location of the metal-bridged oxygens, which is a key for determining the reaction pathway, remains a challenge at the current data quality due to the presence of the neighboring heavy atoms.

Science objectives for 2017-2018:

We plan to collect simultaneous XRD at a higher resolution of ~2 Å that will enable identifying the oxygen atoms, and XES of all the S-states at room temperature, and follow the steps between the S-state transition in a time-resolved manner. The S_1 to S_2, S_2 to S_3 and S_3 to S_0 transition, and the time-points between these transitions will be the focus of these studies.

References to work supported by this project 2016-2017:

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1. X-ray Free Electron Lasers, Applications in Materials, Chemistry and Biology, (Bergmann, U., Yachandra, V. K., Yano, J. eds.), The Royal Society of Chemistry, Cambridge, U.K. 2017.
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