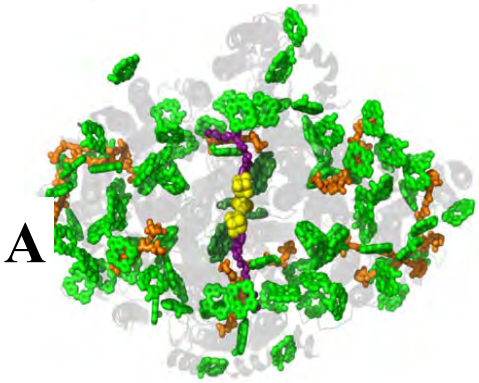
2019 Photosynthetic Systems Principal Investigators Meeting

Marriott Washingtonian Center, Gaithersburg, MD
October 21 to 23, 2019



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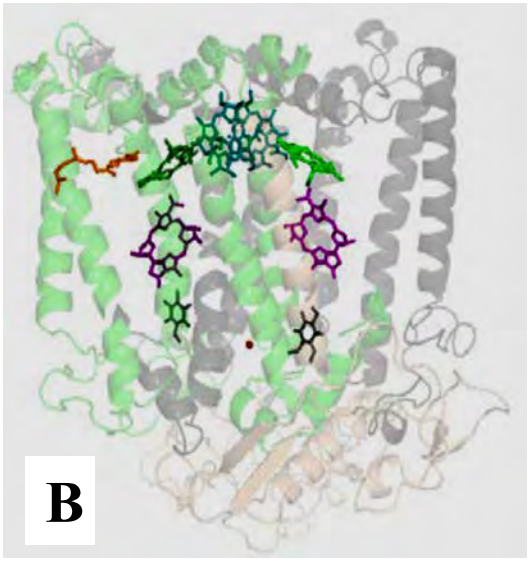

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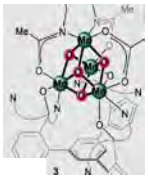

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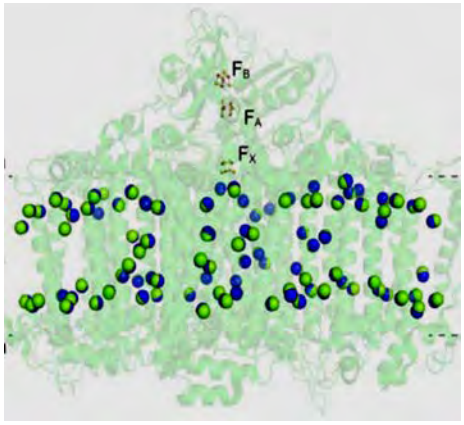

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Office of Basic Energy Sciences,
Division of Chemical Sciences,
Geosciences & Biosciences

Cover Art
Cover art is taken from the abstracts of meeting participants: A: Jessica Anna, B: Christine Kirmaier, C: David Britt, D: Megan Thielges, E: Jan Kern

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Foreword

This volume is a record of the 6th biennial meeting of the principal investigators funded by Photosynthetic Systems, a program of the Chemical Sciences, Geosciences, and Biosciences (CSGB) Division of the Office of Basic Energy Sciences (BES), U.S. Department of Energy (DOE). CSGB supports basic biochemistry 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 inception.

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 the vibrant and innovative science funding program that it is.

The purpose of this meeting is to report recent research accomplishments and foster exchange of scientific knowledge and insights among all participants. Accordingly, the meeting is designed to promote sharing of new results and methodologies, facilitate cooperation and collaboration, 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 Teresa Crockett of BES and Connie Lansdon of the Oak Ridge Institute for Science and Education (ORISE) for their competent planning and successful execution of meeting logistics. 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)

2019 Photosynthetic Systems Principal Investigators Meeting

Gaithersburg Marriott, Washingtonian Center, Gaithersburg, MD. October 21 to 23

AGENDA

Monday, October 21

7:30 – 8:30AM	*Continental Breakfast*
8:30 – 9:00AM	Overview: Chemical Sciences, Geosciences, and Biosciences Division **Bruce Garrett,** Division Director, Office of Basic Energy Sciences
	Section I: *Light Harvesting* ***Moderator:*** *Warren Beck, Michigan State University*
09:00 –9:30AM	Tracking Photochemical and Photophysical Processes of Photosystem I via Multidimensional Electronic and Vibrational Spectroscopic Methods **Jessica Anna,** University of Pennsylvania
09:30 – 10:00AM	Identifying the Energy Transfer Dynamics of Light-Harvesting Complexes in Membrane Nanodiscs **Gabriela Schlau-Cohen**, Massachusetts Institute of Technology
10:00 – 10:30AM	Revealing Excitonic Structure and Charge Transfer in Photosynthetic Proteins by Time-Resolved Circular Dichroism Spectroscopy **Sergei Savikhin**, Purdue University
10:30 – 11:00AM	*Coffee Break*
11:00 – 11:30AM	Multidimensional Spectroscopies for Probing Coherence and Charge Separation in Photosystem II **Jennifer Ogilvie**, University of Michigan
11:30 – 12:10PM	Regulation of Photosynthetic Light Harvesting **Graham Fleming**, U. California, Berkeley, Lawrence Berkeley Nat'l Lab **Kris Niyogi**, U. California, Berkeley & Lawrence Berkeley Nat'l Lab
	Section II: *Electron and Proton Transport* ***Moderator:*** *Marilyn Gunner, City U. of New York*
12:10 – 12:40PM	Visualizing Structural Dynamics in Photosynthetic Reaction Centers on the Femtosecond to Seconds Time Scale **Jan Kern**, Lawrence Berkeley National Lab
12:40 – 03:00PM	*Lunch and Afternoon Break*

Monday, October 21, continued

03:00 – 3:30PM High Field EPR Studies of the Spin=3 S_3 State of the OEC and an Analogous $Mn(IV)_4$ Model Complex
David Britt, University of California, Davis

03:30 – 04:00PM FTIR Studies of Photosynthetic Oxygen Production
Rick Debus, University of California, Riverside

04:00 –04:30PM Insights into Proton-Transfer Pathways During Water Oxidation in Photosystem II
Gary Brudvig, Yale University

04:30 – 05:10PM Controlling Electron Transport Pathways in Photosynthetic Reaction Centers
Christine Kirmaier, Washington University
Philip Laible, Argonne National Lab

05:10 – 05:40PM Performance and Regulation of Photosystem II Energy Conversion in Oxygenic Phototrophs: From PSII Crystals to Living Cells
Chuck Dismukes, Rutgers University

05:40 – 07:30PM *Program Dinner*

07:30 – 10:00PM *Poster Session 1*
(**Odd numbered posters** will be presented. Presenters and titles are listed at end of the agenda. Refreshments may be purchased at the hotel bar.)

Tuesday, October 22

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

Section II: *Electron Transport, continued*
Moderator: *Himadri Pakrasi, Washington University*

08:30 – 09:00AM Photodamage and Repair in Higher Plant Photosynthesis
Terry Bricker, Louisiana State University

09:00 – 9:30AM Structure-Function of the Cytochrome b_6f Lipoprotein Complex
Bill Cramer, Purdue University

9:30 – 10:00AM Time-Resolved Step-Scan FTIR Difference Spectroscopy for the Study of PS I with Different Quinones Incorporated into the A1 Binding Site.
Gary Hastings, Georgia State University

Tuesday, October 22, cont'd

10:00 – 10:30AM The Type I Homodimeric Reaction Center in *Heliobacterium modesticaldum*
Kevin Redding, Arizona State University

10:30 – 11:00AM *Coffee Break*

11:00 – 11:30AM Structure of the NDH: The Complex I-like Molecule of Photosynthesis
Karen Davies, Lawrence Berkeley National Lab

11:30 – 12:00PM Cooperative Control of the O_2 Reduction Reaction by Photosynthetic Flavodiiron Proteins
Katherine Brown, National Renewable Energy Lab

12:00 – 12:30PM Tracking Sites for Multiscale Photosynthetic Function Using X-ray Scattering and Electron Microscopy Imaging Techniques
David Tiede, Argonne National Lab

12:30 – 03:00PM *Lunch and Afternoon Break*

Section II: *Electron Transport, continued*
Moderator: *Cara Lubner, National Renewable Energy Lab*

03:00 – 03:30PM Electron Valves and a Novel Molecular Switch Controls Electron Flow across Cytochrome b_6f to Protect PS I from Photoinhibition
Arthur Grossman, Carnegie Institution of Washington

03:30 – 04:00PM Regulation of Sustained Cyclic Electron Flow (CEF) in the Photopsychrophile *Chlamydomonas* sp. UWO241
Rachel Morgan-Kiss, Miami University

04:00 – 04:30PM MSU-DOE Plant Research Laboratory: Subproject A, Robust Photosynthesis in Dynamic Environments
David Kramer, Michigan State University

04:30 – 05:00PM Photosynthetic Reduction of Carbon Dioxide: Biochemical Analysis of Rubisco, Phospho*enol*pyruvate Carboxylase and Carbonic Anhydrase.
Asaph Cousins, Washington State University

05:00 – 05:30PM MSU-DOE Plant Research Laboratory: Subproject C, Characterizing and Engineering Subcellular and Cellular Modules for Photosynthetic Productivity
Cheryl Kerfeld, Michigan State University

05:30 – 07:30PM *Dinner on your own*

Tuesday, October 22, cont'd

07:30 – 10:00PM Poster Session 2

(**Even numbered posters** will be presented. Presenters and titles are listed at the end of the agenda. Refreshments may be purchased at the hotel bar.)

Wednesday, October 23

7:30 – 8:30AM *Continental Breakfast*

Section III: *Self Assembly*

Moderator: *Christoph Benning, Plant Research Laboratory, Michigan State University*

08:30 –9:00AM Membrane-attached Electron Carriers in Photosynthetic and Respiration: Biogenesis of a Cytochrome Complex from a Facultative Photosynthetic Bacterium
Fevzi Daldal, University of Pennsylvania

09:00 – 09:30AM Photophosphorylation Requires Thiol-Disulfide Transactions at the Thylakoid Membrane
Patrice Hamel, The Ohio State University

09:30 – 10:00AM MSU-DOE Plant Research Laboratory: Subproject B, Integrating Energy Supply and Demand in the Biological Solar Panel
Gregg Howe, Michigan State University

10:00 – 10:30AM Signal Transduction Pathways of Chloroplast Quality Control
Jesse Woodson, Arizona State University

10:30 – 11:00AM Photoreceptors and Photosynthesis, Past and Future: It's All About the Bilin
Clark Lagarias, University of California, Davis

11:00 – 11:30AM *Coffee Break*

11:30 – 12:00PM Photosynthetic Systems Program Update
Steve Herbert, Gail Mclean, Bob Stack, DOE Program Mangers

12:00PM *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. Energy Transfer and Radiationless Decay in Light Harvesting Proteins
   Warren Beck, Michigan State University

2. Structure and Function of the CO_2 Uptake NDH-1 Complexes in Cyanobacteria
   Rob L. Burnap, Oklahoma State University

3. Resolving Protein-Semiquinone Interactions by Advanced EPR Spectroscopy
   Sergei Dikanov, University of Illinois, Urbana-Champaign

4. Using Singlet-Singlet Annihilation to Track Energy Transfer Pathways within Chlorophyll Pools of Cyanobacterial Photosystem I in its Native Environment
   Greg Engel, University of Chicago

5. Functional Models of Photosynthetic Light Harvesting Systems Templated by Self-Assembling Proteins
   Matt Francis, Lawrence Berkeley National Lab

6. Towards Structure Determination of the Photosynthetic Supercomplex to Understand the Regulation of Sustained Cyclic Electron Flow (CEF) in the Photopsychrophile *Chlamydomonas* sp UWO241
   Petra Fromme, Arizona State University

7. Fundamental Research Aimed at Diverting Excess Reducing Power in Photosynthesis to Orthogonal Metabolic Pathways
   Michael Gorka, Pennsylvania State University

8. Role of Protons and Substrate Waters near the OEC of Photosystem II
   Marilyn Gunner, City College of New York

9. Protein Targeting to the Chloroplast Thylakoid Membrane: Structure and Function of a Targeting Complex
   Colin Heyes, University of Arkansas

10. Modulation of State Transitions by Flavodiiron Activity in *Synechocystis* PCC 6803
    Paul King, National Renewable Energy Laboratory

11. Mechanistic Understanding of Ion Fluxes in Photosynthetic Thylakoid Membranes
    Helmut Kirchhoff, Washington State University

Posters, cont'd

12. Elucidating the Principles that Control Proton-Coupled Electron Transfer Reactions in the Photosynthetic Protein, Photosystem II
    K.**V. Lakshmi**, Rensselaer Polytechnic Institute

13. Characterizing Rubisco by Phylogeny-Informed Mutagenesis
    Myat Lin, Cornell University

14. Molecular Mechanisms of Action of the Cyanobacterial Orange Carotenoid Protein
    Haijun Liu, Washington University

15. Analysis of the Effect of Flavodiiron 1 and 3 on the Function of Photosystem I and Photo-Driven Electron Flux in *Synechocystis sp.* PCC 6803
    Cara Lubner, National Renewable Energy Lab

16. Evidence of Intramolecular Structural Stabilization in Light Activated State of Orange Carotenoid Protein
    Andrea Markelz, University of Buffalo

17. Defining the Structure and Reactivity of the HoxEFU Enzyme Complex from *Synechocystis sp.*PCC 6803
    David Mulder, National Renewable Energy Lab

18. A Novel Chlorophyll Protein Complex in the Repair Cycle of Photosystem II
    Himadri Pakrasi, Washington University

19. Electron Flow, O_2 reduction, and Cellular Redox Balancing: Crosstalk between Chloroplasts and Mitochondria
    Matt Posewitz, Colorado School of Mines

20. Spin-Correlated Radical Pairs as Quantum Sensors for Resolving Structure Function Relationship in Photosynthetic Proteins
    Oleg Poluektov, Argonne National Laboratory

21. Lipid Chaperoning of a Thylakoid Membrane Signal Peptidase whose Stability is Modified by the Protonmotive Force
    Steve Theg, University of California, Davis

22. Mechanisms for Tuning Protein Electron Transfer Investigated via Site-Specific Linear and Two-Dimensional Infrared Spectroscopy
    Megan Thielges, Indiana University

23. Photosynthetic Biohybrid Systems for Studying Mechanisms of Solar Energy Conversion
    Lisa Utschig, Argonne National Lab

Posters, cont'd

24. Assembly and Repair of the Photosystem II Reaction Center
    David Vinyard, Louisiana State University

25. Single Photon Studies of Quantum Efficiency of Photosynthetic Light Harvesting
    Birgitta Whaley, University of California, Berkeley

26. Electronic Structure of the Mn Cluster in Photosystem II Using and XFEL
    Vittal Yachandra, Lawrence Berkeley National Lab

27. Structures of the Intermediates of Kok's Photosynthetic Water Oxidation Clock
    Junko Yano, Lawrence Berkeley National Lab

28. Regulation of Sustained Cyclic Electron Flow (CEF) in the Photopsychrophile *Chlamydomonas* sp. UWO241
    Ru Zhang, Donald Danforth Center, St. Louis

Abstracts (alphabetically by presenter)

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. This will allow for a more direct determination of energy transfer pathways and provide further insight into protein-cofactor interactions that may be important for charge separation and electron transfer.

Significant achievements 2017-2019: *Energy Transfer in PSI:* We have applied two-dimensional electronic spectroscopy (2DES) to PSI complexes isolated from two different cyanobacteria differing in red chlorophyll content. From a global analysis of the 2D spectra, we observe two pathways of energy equilibration involving the red chlorophylls occurring on different timescales. We recently published these findings. To expand on these studies, we have obtained 2DES of PSI complexes with chemically oxidized and reduced reaction centers in order to compare the kinetics and pathways of energy transfer as a function of reaction center oxidation state. *Protein-Cofactor Interactions:* We have characterized the vibrational modes of phylloquinone in hydrogen bonding and non-hydrogen bonding solvents using a combination of FTIR spectroscopy, molecular dynamics simulations,

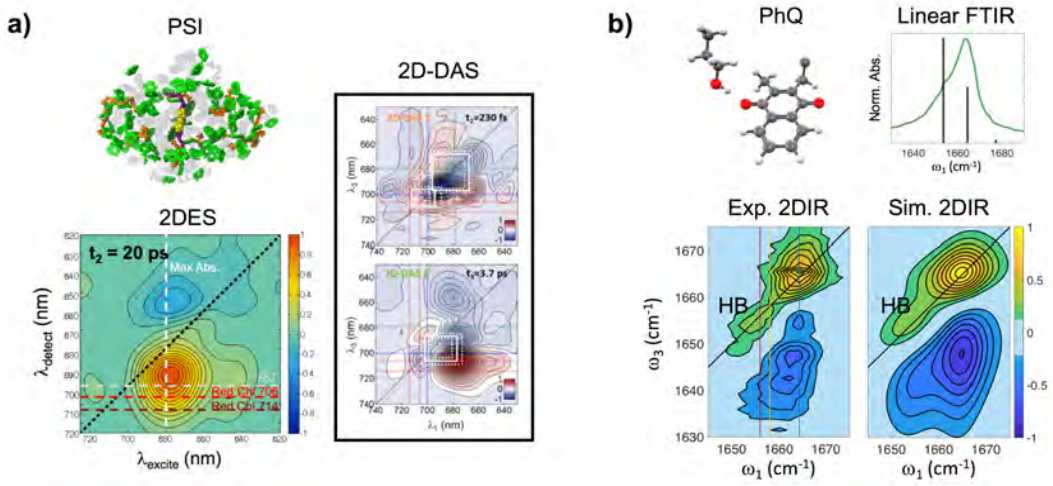

Figure 1. (a) A representative 2DES spectrum of photosystem I isolated from PCC 6803 is shown at t_2 = 20 ps. Performing a global analysis on the 2DES spectra we extract 2D-DAS. The 2D-DAS associated with equilibration pathways involving the red chlorophylls of PSI from PCC 6803 are shown. (b) We performed DFT calculations and linear FTIR spectra on phylloquinone (PhQ) in hydrogen bonding solvents. The optimized geometry of PhQ with a hydrogen bonded solvent molecule is shown along with the calculated FTIR spectrum (black sticks) and experimental FTIR spectrum of PhQ in hexanol (green). The experimental 2DIR spectra of PhQ in hexane is shown for a waiting time of t_2=500 fs (left) along with the simulated 2DIR spectrum (right). Through comparison of the simulated and experimental 2DIR spectra we determine the anharmonicities along with the bath correlation times.

quantum chemical calculations, and two-dimensional infrared spectroscopy (2DIR). We find that hydrogen bonding acts to decouple the carbonyl stretching modes and leads to changes in their anharmonicities. *Instrument Construction:* We have completed the construction of our two-dimensional electronic vibrational (2DEV) spectrometer, obtaining 2DEV spectra of the N719 laser dye to test and compare different referencing schemes for noise reduction.

Science objectives for 2019-2020:

- We will continue to expand our global analysis toolbox to analyze 2DES spectra of oxidized and reduced PSI complexes, with the goal of extracting rate constants associated with energy transfer and charge separation in the two different complexes. In addition, we will interface a cryostat with our 2DES spectrometer to obtain low-temperature spectra. The low-temperature measurements should aid in resolving energy transfer pathways and oscillatory components.
- Applying mixed spectral multidimensional spectroscopies, including 2D electronic vibrational spectroscopy (2DEV) 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. Though we have completed the construction of our 2DEV spectrometer, before applying 2DEV to PSI we will perform experiments on model metal-carbonyl systems. These systems have a large absorption in the visible region coupled with large oscillator strength and narrow peaks in the mid-IR carbonyl stretching region. Given these spectral features, the metal carbonyl systems will serve as models to ensure that our newly constructed experimental apparatus is functioning properly and to establish data analysis procedures. We will then move on to obtain 2DEV spectra of chlorophyll a molecules in different solvent environments and aggregated states. These studies will eventually aid in the interpretation of 2DEV spectral profiles of PSI.
- A thorough characterization of the vibrational potential energy surface of PhQ in hydrogen bonding solvents will aid in the interpretation of the visible pump mid-IR probe based experiments (2DEV and T-2DIR) to be performed on the PSI complexes. As such, we will further our analysis of the 2DIR spectra of PhQ in different hydrogen bonding solvents to include a larger spectral region, focusing on how hydrogen bonding disrupts coupling between the carbonyl stretching modes and ring bending modes.

References to work supported by this project 2016-2019:

1. Y. Lee, S. Das, R.M. Malamakal, S. Meloni, D.M. Chenoweth, J.M. Anna*, Ultrafast Solvation Dynamics and Vibrational Coherences of Halogenated Boron-Dipyrromethene Derivatives Revealed through Two-Dimensional Electronic Spectroscopy" *J. Am. Chem. Soc.*, (2017), 139 (41), 14733-14742. https://doi.org/10.1021/jacs.7b08558
2. Y. Lee, M. Gorka, J. H. Golbeck, J.M. Anna*, Ultrafast Energy Transfer Involving the Red Chlorophylls of Cyanobacterial Photosystem I Probed Through Two-Dimensional Electronic Spectroscopy, *J. Am. Chem. Soc.*, (2018) 140 (37) 11631-11638. https://doi.org/10.1021/jacs.8b04593
3. R. Gera, S.L. Meloni, J.M. Anna*, Unraveling Confined Dynamics of Guests Trapped in Self-Assembled Pd_6L_4 Nanocages by Ultrafast Mid-IR Polarization-Dependent Spectroscopy, *J. Phys. Chem. Lett.*, 2019, *10* (3), 413–418. https://doi.org/10.1021/acs.jpclett.8b03485 (Live Slides invitation)
4. A. Jain, A. Petit, J.M. Anna, J.E. Subotnik*, Simple and Efficient Theoretical Approach to Compute 2D Optical Spectra, *J. Phys. Chem. B*, 2019, *123* (7), 1602–1617. https://doi.org/10.1021/acs.jpcb.8b08674

Elucidating mechanisms of Photosystem I assembly and repair

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Overall research goals: The broad goal of this project is to advance understanding of mechanisms underlying PSI assembly and homeostasis. PSI is among the most complex macromolecular structures in nature, consisting of ~19 different proteins that scaffold approximately 200 prosthetic groups. Given its complex structure, hydrophobic nature, and the oxidation sensitivity of its prosthetic groups, it is anticipated that accessory proteins orchestrate PSI assembly to promote productive interactions, prevent or resolve non-productive interactions, and prevent deleterious reactions involving prosthetic groups. This project builds on the prior discovery of seven proteins that promote PSI assembly. Several of these have been shown to reside in multimeric complexes, and a handful of interactions among PSI assembly factors and PSI subunits have been reported. However, in no case has the biochemical role of a PSI assembly factor been established.

Our experiments derive from prior data suggesting that two assembly factors, PYG7 and PSA3, cooperate to facilitate the incorporation of PsaC early in PSI assembly. PsaC binds the two [4Fe-4S] clusters that serve as the terminal electron acceptors of PSI. PsaC is the core of PSI's "stromal ridge", and forms an intimate connection with stroma-exposed loops of the membrane-embedded PsaA/B reaction center. It is known that PsaC undergoes a large conformational change to establish these contacts and that PsaC's Fe-S clusters are particularly prone to oxidative damage. We hypothesized that PSA3 and PYG7 cooperate to mediate the incorporation of PsaC into PSI, that they do so by facilitating the conformational change in PsaC and/or by shielding its Fe-S clusters from oxidation, that PSA3's activity involves the redox-dependent binding of a peptide to an acidic groove on its surface, and that the PSA3/PYG7 cooperation extends to a role in post-assembly PSI protection or repair. We anticipate that these experiments will elucidate how PsaC, an oxygen-sensitive Fe-S protein near the heart of PSI, is chaperoned during its addition to the PSI reaction center, and how PsaC integrity is maintained in the face of challenging environmental conditions.

The Specific Aims are to:

1. Clarify the interplay between PSA3, PYG7, and PsaC during PSI biogenesis and homeostasis.
2. Seek proteins that collaborate with PSA3 and PYG7.
3. Elucidate the physical and functional network among PSI assembly factors.

Significant achievements 2018-2019:

• To test the roles of PSA3's predicted acidic groove and flanking cysteine pairs, we generated site-directed PSA3 mutants affecting each of these features: in one mutant, one cysteine in each pair was changed to serine, and in the other, twelve Glu or Asp residues lining the acidic groove were changed to Gln or Asn, respectively. To assess the effects of these changes on PSA3 function, we are determining whether they can substitute for the wild-type in an *in vivo* complementation assay. We introduced transgenes encoding each variant into the genome of Arabidopsis *psa3/+* plants. We identified eight transgenic lines for each variant, and allowed them to self- pollinate. Encouragingly, "complemented" homozygous *psa3* mutants grow slowly and exhibit elevated chlorophyll fluorescence, indicating that the mutations do disrupt PSA3 function. We are now bulking up these lines to allow comprehensive analysis of the phenotypes.

• To identify proteins that interact with PSA3 and PYG7, we took two approaches:

i) We used PSA3 and PYG7 as the bait in genome-wide yeast-two hybrid screens. The PSA3 screen came up empty, but the PYG7 screen detected a protein called HEMC, involved in chlorophyll synthesis. This seems like a plausible interaction partner, so we will determine whether we can validate this interaction using *in vivo* assays (e.g. BiFC).

ii) We immunoprecipitated PSA3 from gently solubilized thylakoid membranes and identified coimmunoprecipitating proteins by mass spectrometry. The results were highly concordant in two replicate experiments: the two most highly enriched proteins in both experiments are called THF1 and PTAC5, both of which have been studied previously but neither of which has a well-defined function. (Although these are abundant proteins, they have not been top hits in other coimmunoprecipitations we have performed with thylakoid membranes solubilized in the same manner.) PTAC5 harbors a type of zinc-finger domain that has been shown to have protein-disulfide isomerase activity in other contexts. This is intriguing in light of the cysteine pairs flanking PSA3's acidic groove. In addition, PsaC was highly enriched in the coIP dataset, and was more highly enriched than any other PSI subunit, consistent with the view that this is the focus of PSA3's activities. Tic21 involved in iron transport and homeostasis, and a protein-of-unknown-function that is coexpressed with Fe-S cluster assembly factors were also highly enriched, in accord with the hypothesis that PSA3's function pertains to PsaC's Fe-S clusters.

We had hypothesized that PYG7 and PSA3 cooperate. However, PYG7 was not found in either of the two PSA3 coIP datasets. Furthermore, our PYG7 Y2H screen did not recover PSA3, and vice versa. Finally, we learned about unpublished work on Chlamydomonas PYG7 that suggests PYG7 is not a PSA3 partner. Thus, we are steering away from our initial hypothesis that PYG7 and PSA3 interact directly.

<u>Science objectives for 2019-2020:</u>

- We will analyze phenotypes of *psa3* mutants expressing the acidic groove and cysteine PSA3 variants to determine how those features contribute to PSA3's functions. We will monitor phenotypes under standard laboratory conditions and under conditions that challenge PSI integrity: fluctuating light, low temperature, and high light intensity. These stress conditions will be imposed either from the time of germination (to assess impact on initial PSI biogenesis) or in mature plants that had developed under non-stress conditions. Phenotypes to be examined include: growth rate, PSI abundance, and fluorescence readouts of photosynthetic parameters.
- We will study the putative interacting partners we identified for PYG7 (HemC) and PSA3 (THF1, PTAC5, TIC21, and several others) by:

- using BiFC and coimmunoprecipitation to validate the interactions.
- exploring validated interactions with *in vitro* pulldown assays involving recombinant proteins.
- studying phenotypes of maize and Arabidopsis mutants lacking each of these proteins to gain insight into their functional interactions with PSA3.
- exploring whether disulfide bonds involving PSA3's cysteine pairs change in the *ptac5* mutant

We have decided not to continue study of YCF3, YCF4 or PYG7, beyond following up the intriguing PYG7 Y2H positive. I learned that the Takahashi group in Japan is intensively studying PYG7 in Chlamydomonas. Furthermore, they published a paper on Ycf3 and Ycf4 after our grant was approved for funding but before funding began, so they have a significant head start. As such, it seems prudent to focus our attention on PSA3.

Energy Transfer and Radiationless Decay in Light-Harvesting Proteins

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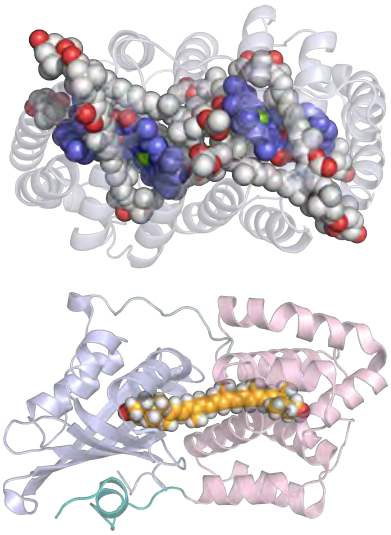

Overall research goals: This project has focused so far on the photophysical and photochemical mechanisms that are involved in excitation energy transfer and photoprotection in two systems that bind ketocarotenoids:

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

In both systems, the role of the electron withdrawing character of the carotenoid's carbonyl substituent is crucial in energy transfer and photoprotection mechanisms because it promotes the formation of intramolecular charge-transfer (ICT) character in the optically prepared S_2 state via activated torsional motions of the conjugated polyene. Two-dimensional electronic spectroscopy (2DES) with broadband (<10 fs) excitation pulses is used to characterize nonradiative decay and excitation energy transfer mechanisms in PCP, OCP, and in solutions of carotenoids. Additionally, fluorescence lineshape and anisotropy measurements as a function of temperature are employed to characterize activated torsional motions in carotenoids in solution and in OCP.

Significant achievements 2017–2019:

- First measurements of the spontaneous fluorescence line shape and quantum yield of ketocarotenoids in two OCP^O homologs. This work leads to the conclusion that the binding site in OCP^O lengthens the emissive lifetime and alters the photophysics of the ketocarotenoid via specific hydrogen-bonding interactions between the β-cyclohexene rings and groups in the C-terminal and N-terminal domains. These interactions are apparently crucial to the photophysical trigger that leads to the photoactivation event.
- First broadband two-dimensional electronic spectroscopy (2DES) experiments in PCP and in reconstituted PCP complexes containing chlorophyll *b*. This work detects peridinin-to-peridinin and peridinin-to-chlorophyll excitation energy transfer processes and the associated electronic coherences via off-diagonal cross peaks in the 2DES spectra. The results establish directly that a nonadiabatic energy transfer mechanism accounts for the majority of the excitation transfer yield in PCP.

Science objectives for 2019–2020:

- Determine the photophysical mechanism that initiates photoactivation from the resting OCP^O state to the active OCP^R state upon absorption of light by the intrinsic ketocarotenoid in OCP and how this mechanism enables the organism to sense the intensity and color of ambient illumination.
- Determine the photophysical mechanisms and pathways for excitation energy transfer, photoadaptation, and photoprotection in the intact phycobilisome, the latter including intrinsic light-induced trap formation and OCP^R-dependent quenching mechanisms.

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Photodamage and Repair in Higher Plant Photosynthesis

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Overall research goals: Our research 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 produced by all of the photosynthetic membrane protein complexes: Photosystem II (PS II), the cytochrome b_6f complex (b_6f complex), and Photosystem I (PS I). 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. Second, we will examine how the generation of ROS is modulated under stress conditions.

Significant achievements 2017-2019: 1. 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. We hypothesize that these sites are sources for ROS generation in the spinach cytochrome b_6f complex. 2. We have completed studies examining natively oxidized residues in the spinach PS I-LHC I supercomplex. Interestingly, a well-defined group of oxidatively modified residues lead from the chl *a'* of P_{700} to the surface of the supercomplex (Fig. 1A). This result is consistent with the hypothesis that $^1O_2$ may be formed by the interaction of dioxygen with $^3P_{700}$. The observed modified residues may define a preferred ROS exit pathway vectoring $^1O_2$ from the vicinity of P_{700}. No similar group of oxidized residues is associated with the chl *a* of P_{700}. Additionally, Lhca1-Lhca4, which form the distal light-harvesting antennae for PS I, exhibit high degrees of oxidative modification (Fig. 1B). This observation suggests that a significant amount of $^1O_2$ is produced in the distal antennae. No similar groups of modified residues were observed associated with any of the proximal antennae chls associated with PsaA and PsaB.

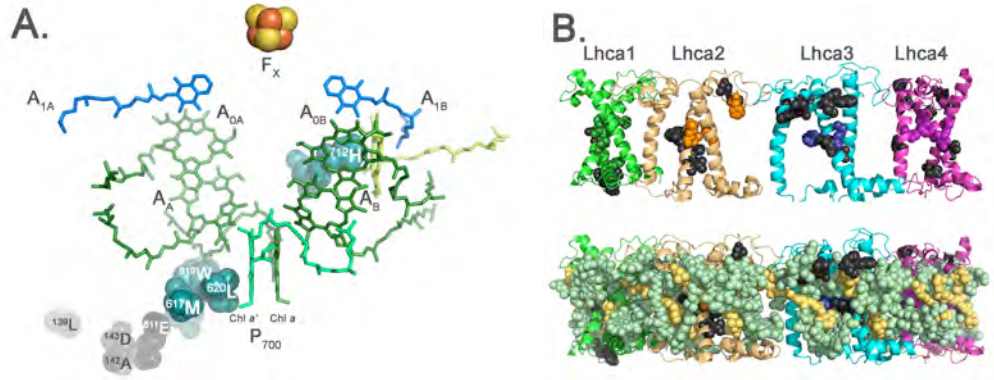

Figure 1. Oxidative modification of Amino Acid Residues in PS I. A. Modified residues in the vicinity of the redox active cofactors of the PsaA and PsaB subunits. Cofactors are shown as sticks, except for F_X, and are labeled. B. Modified residues in the Lhca1-Lhca4 distal antenna viewed within the plane of the membrane. Above, without cofactors. Below, with cofactors. Cofactors: chlorophyll, pale green; carotenoids, pale yellow. Modified residues are shown as darker spheres (black spheres are surface-exposed residues) and were mapped onto their corresponding locations within the *Pisum satvium* PS I-LHC I supercomplex (PDB: 5L8R).

Science objectives for 2019-2020:

- Continue characterization of PS II assembly complexes obtained after SMA solubilization of photoinhibited spinach.
- Continue characterization of PS I – LHC I – LHC II membranes.
- Perform studies examining PS I photoinhibition under both chilling and fluctuating light conditions in cucumber.
- Analyze Lhcb proteins for oxidative modifications.

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High Field EPR Studies of the spin=3 S_3 state of the OEC and an analogous $Mn(IV)_4$ model complex

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Overall research goals: Understanding the geometrical and electronic structure of the integer spin S_3 state (spin S=3) using high field (HF) EPR spectroscopy along with parallel development of high field and frequency spectrometers and techniques to underpin this research.

Significant achievements 2017-2019: Recent studies have focused on the S_3-state, one photooxidation event removed from O-O bond formation, but many questions remain regarding the electronic structure of the S_3-state. Here, we employ high-field electron paramagnetic resonance (EPR) to investigate the OEC poised in the S_3-state in *Synechocystis*. Pulse EPR reveals that two forms of the $S = 3$ S_3-state are present in *Synechocystis*. Based on the breadth of the S_3 EPR spectrum, two different zero-field splitting (ZFS) parameters, D, of ~ 0.2 and 0.3 cm^{-1} are determined. ELDOR-detected NMR (EDNMR) reveals that in both forms of S_3 all four Mn ions are in the Mn(IV) oxidation state and coordinatively saturated. The measured Mn(IV) hyperfine values (Mn1, Mn2 ~ 100 MHz and Mn3, Mn4 ~ 5 MHz) mirror those in *T. Elongatus* poised in the S_3-state. We also examine an $S = 3$ all Mn(IV) Mn_4O_4 cubane (collaboration with the T. Agapie group) with pulse EPR to contextualize our S_3 results in *Synechocystis*.

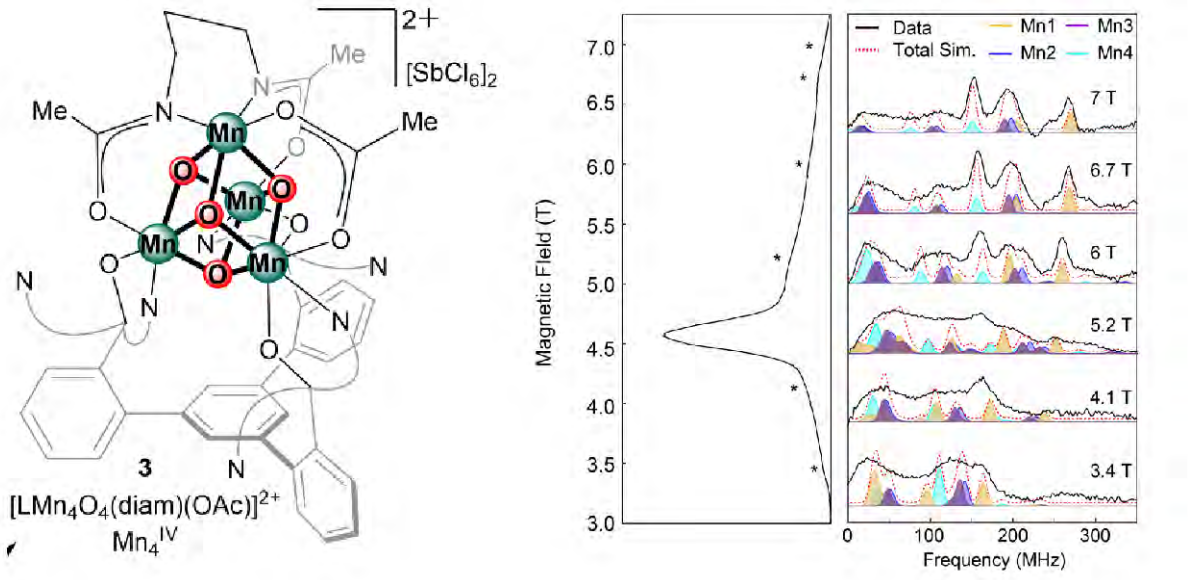

Figure 1. Left Panel: Structure of a $Mn(IV)_4O_4$ complex mimicking the S_3 state of the OEC. Right Panel: field swept 130 GHz pulse EPR of the S=3 spin state of this complex and our EDNMR detection of the ^{55}Mn spin transitions. From Lee et al., JACS submitted.

Science objectives for 2009-2010:

- Continued advancement in high field EPR studies of the S_3-state, as well as other "problematic" high spin OEC EPR signals including the integer spin S_1-state signal and the S_2-state S=5/2 "g=4.1" signal.
- Work on technological advances in support of these OEC EPR targets: development of a higher power version of our new homebuilt 263 GHz quasi-optics bridge HFEPR spectrometer and exploration of the utility of the high power THz free electron laser at UCSB for high power ENDOR studies focused on these high spin OEC EPR signals.

Cooperative Control of the Oxygen Reduction Reaction by Photosynthetic Flavodiiron Proteins

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Overall research goals: The goal of this work is to investigate the biochemical and mechanistic properties of flavodiiron enzymes (Flv) from the cyanobacteria *Synechocystis* sp. PCC 6803 that enable their function in photosynthetic electron transfer (PET). The catalytic activity of Flv's help to maintain energy balance by coupling oxidation of reduced pyridine nucleotides (NADH and NADPH) to the oxygen reduction reaction (ORR, $O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$). This auxiliary electron transport pathway is important for preventing the over-reduction of the PET chain and soluble electron acceptors and modulating the ATP/NADPH ratio to meet metabolic demands in changing conditions. Cyanobacterial Flv's are composed of a non-heme diiron binding domain, a flavin mononucleotide (FMN) binding domain, and a flavin oxidoreductase domain. The structural and functional properties of cyanobacterial Flv's are only partially defined. For example, it is not well understood how quaternary structures and cofactor arrangements of Flvs control substrate binding, reactivity and electron flow to mediate proton-coupled electron transfer (PCET) steps for catalytic activation of oxygen and reduction to water. Reaction kinetics combined with biophysical approaches are resolving these questions and determining how Flv reactivity is tuned to accomplish selective activation and reduction of oxygen. A predicted outcome of the catalytic mechanism is it will minimize stability of cofactor oxidation states that otherwise promote formation of radical oxygen species.

Significant achievements 2018-2019: Expression and purification of Flv1 and Flv3 were followed by a detailed investigation of reaction kinetics, substrate binding and quaternary structure (Figure 1). Reaction kinetic profiles together with substrate binding kinetics determined by UV-Vis showed *Synechocystis* PCC 6803 Flv1 and Flv3 uses cooperativity to catalyze ORR coupled to oxidation of NAD(P)H. Spectroscopic investigation of the reduction of Flv3 by either NAD(P)H or dithionite showed the absence of stable one-electron reduced flavin semiquinone, suggesting electron pairs are rapidly transferred from FMN to the diiron site. Chemical cross-linking mass spectrometry showed Flv3 forms homodimeric structures with inter-subunit electron transfer pathways to enable ORR. Collectively, these results demonstrate how the catalytic mechanism and unique PCET chemistry of Flv is tuned to operate under the dynamic conditions of PET.

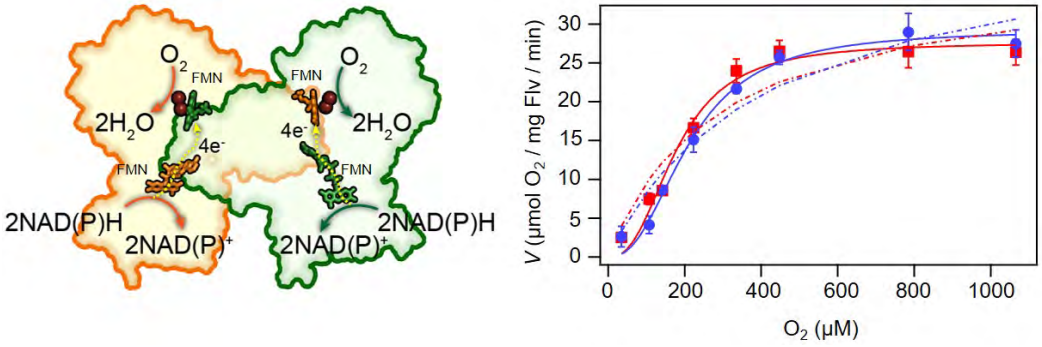

Figure 1. Left Panel: Structural model of the Flavodiiron 3 homodimer showing non-heme iron sites (brown spheres), flavins (FMN) and the proposed electron transfer pathways (yellow arrows) that transect the two subunits (green and

orange). Right Panel: The O_2 concentration dependence of Flv3 ORR kinetics with NADH (red squares) or NADPH (blue circles). Solid lines are fits to the steady-state Hill equation showing cooperativity. Dashed lines are fits to the steady-state Michaelis-Menten equation.

Science objectives for 2020-2021:

- Analyze the mechanism of Flv catalyzed ORR and the function of the cofactors in coordinating control of electron transfer and O_2 activation by biophysical and time-resolved kinetics techniques. The redox properties of the flavin and diiron cofactors will be interrogated. The potentials of the diiron site redox transitions from Fe^{II}-Fe^{II} to Fe^{II}-Fe^{III} to Fe^{III}-Fe^{III} will be investigated by electron paramagnetic spectroscopy (EPR), while the potentials of the oxidized, semiquinone and fully reduced states of the flavin cofactors will be measured by spectroelectrochemistry (SEC). Using stop-flow UV-Vis the short time scale intermediates of reduced pyridine nucleotide binding, O_2 binding, and catalytic turnover will be investigated in order to develop a mechanistic model of Flv catalyzed ORR.
- Obtain higher resolution structures by X-ray crystallography to refine electron transfer pathway models and structural features of cofactor-protein interactions that enable of Flv to catalyze ORR. Cryo-EM imaging will also be attempted as intact cyanobacterial Flv's have been challenging to crystallize.
- In addition to Flv1 and Flv3 *Synechocystis* PCC 6803 also expresses Flv2 and Flv4, which appear to have unique functions in PET control. There is very little is known about their structures, reaction kinetics and substrate binding, or mechanism. Investigation of the dimeric structure and ORR catalysis by Flv2 and Flv4 will be carried out using mass spectrometry, UV-Vis and florescence spectroscopy and membrane inlet mass spectrometry.

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Insights into Proton-Transfer Pathways During Water Oxidation in Photosystem II

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Abstract: Water oxidation by photosystem II (PSII) involves the release of O_2, electrons and protons at the oxygen-evolving complex (OEC). These processes are facilitated by a hydrogen-bonded network of amino-acid residues and waters surrounding the OEC. In this study, we generate point mutations in the cyanobacterium *Synechocystis* sp. PCC 6803 at secondary-shell amino-acid residues surrounding the OEC: D2-K317, D1-S169, CP43-R357, D1-D61 and D1-N181. The pH dependence of the O_2-evolution rate follows a bell-shaped curve in both wild-type and mutated PSII from which we can derive the *effective* acidic pK_a, which provides insights on the protonation states of the amino-acid residues participating in the proton-transfer process during the rate-determining step of water-oxidation. The presence of an additional *effective* pK_a in D1-S169A and D2-K317A PSII indicates that multiple proton-transfer pathways may function in the rate-determining step of water oxidation. We also studied the O_2-evolution rate in H_2O and D_2O with varying pL (L = H or D) to identify the amino-acid residues participating in the proton-transfer process. D2-K317A and D1-D61A PSII exhibit a significantly enhanced kinetic solvent isotope effect (KSIE), indicating that proton transfer becomes rate limiting at the optimal pH in these mutated PSII. However, the KSIE remains unchanged for D1-N181A, D1-S169A and CP43-R357K PSII. Thus, perturbing the channel defined by the D1-D61 and D2-K317 residues strongly hampers the proton-transfer mechanism, and in turn the water-oxidation reaction of PSII. Hence, our study provides a direct experimental probe to identify that the D1-D61 and D2-K317 residues participate in the proton-transfer process.

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 are 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 are determined, and studies of small molecule water analogues and anions are carried out to gain insight into the substrate (water) and chloride binding sites.

Science objectives for 2018-2019:

- 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.
- To 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.
- To study the binding of small molecule water analogues and anions 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 aim to 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: 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 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 enzymes electron flow to hydrate CO_2 to form bicarbonate. The enzymes remain poorly understood, but could be used 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. Essentially, it functions as a 'supercharger' for CO_2 by concentrating it within the cell, thereby saturating the active sites of the CO_2-fixation enzymes and increasing the efficiency of photosynthesis. The project is focuses on the **specialized NDH-1 complexes** that function directly in hydrating CO_2, but remain to be understood on the molecular level.

Significant achievements 2017-2019: Development of a **genetic system** for probing structure-function relationships of the CO_2-uptake NDH-1 complexes and working out *in vivo* **H^+-pumping assays**. After knocking out the full set of Cup genes (constitutive and inducible), an integration vector system was developed to re-introduce the Cup genes at an ectopic location under the constitutive expression of the rubisco promoter[1].

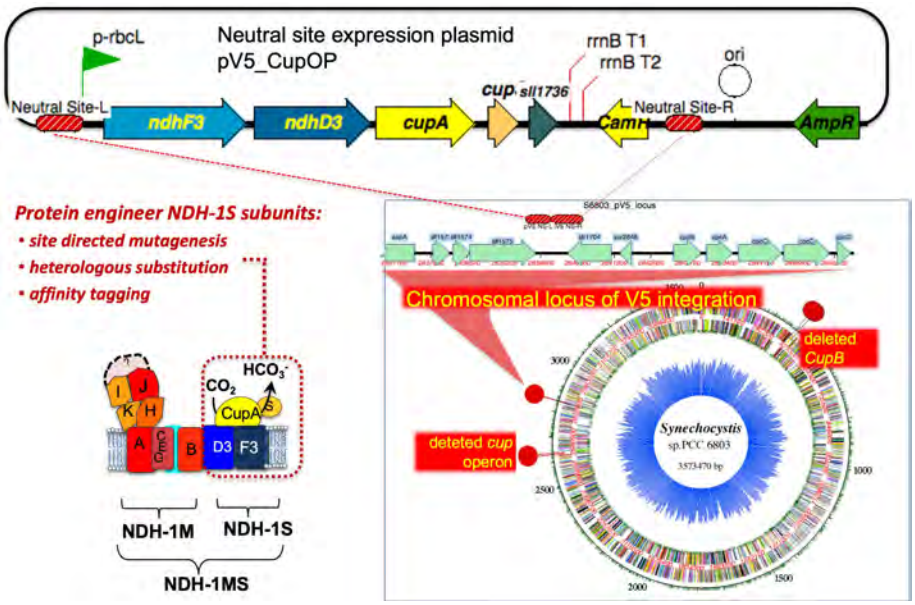

Figure 1. Engineering the CO_2 uptake proteins using genetic platform for ectopic expression. The pV5 family of plasmid (top) contains homologous sequences targeting the cargo (in this case the *cupA* operon encoding the NDH-1S module (pV5_CupOP,

This was accomplished with a chromosomal integration expression the system constitutively expression the *ndhF3-ndhD3-cupA-cupS* operon (*cupA* operon, CAO) encoding high affinity CO_2-uptake subcomplex NDH-1S of NDH-1_3 holozyme (=NDH-MS) **(Fig. 1)**[1]. CO_2-uptake affinity assays show the restoration of high affinity for CO_2-uptake due to the reintroduction of CupOp encoding NDH-$1S_3$. The assays demonstrated that the CupA complex can drive only modest uptake fluxes, albeit at high affinity, underlining the importance

of its tandem operation with the CupB-containing NDH-1MS$_4$, the complementary high flux, low affinity CO_2 hydration system. It will also be important to measure H^+-pumping to characterize site-directed mutants. We worked out physiological conditions and an instrumental set-up so this can be now observed by monitoring acridine orange fluorescence. Preliminary experiments shown in (**Fig. 2**) indicate the ability to have conditions isolating NDH-1 proton pumping (DCMU+DCCD). However, the respiratory complex, NDH-1MS$_{resp}$, also contributes to the activity and, consequently, one of the goals of the project will be to delete the *ndhF1* gene, which eliminates NDH-1MS$_{resp}$ activity.

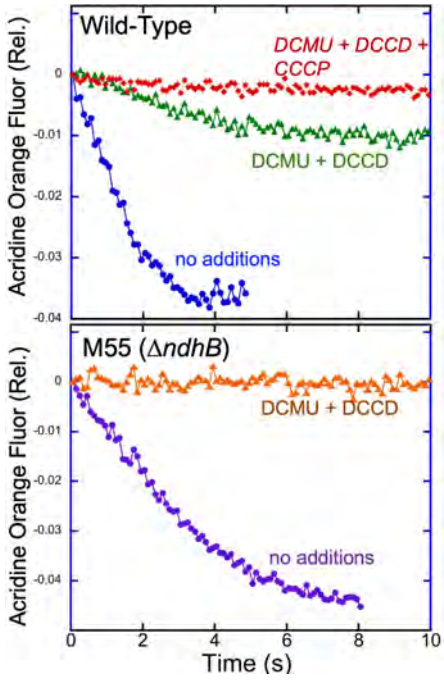

Figure 2. Light-induced measurement of proton pumping activity using acridine orange (AO) fluorescence quenching in whole *Synechocystis* cells. Wild-type (top) and cells having a deletion of the core *ndhB* gene, lacking all NHD-1 function, M55 (bottom). Dark adapted cells, incubated with acridine orange without additional treatment or with DCMU, DCCD, order CCCP as indicated. DCMU blocks PSII and all linear electron transfer associated proton pumping. DCCD blocks utilization over proton gradient by ATP synthase and enhances signal due to greater proton motive force (*pmf*). Light switched on at time zero. Assay includes valinomycin plus KCl to convert the *pmf* to mainly ΔpH.

Publications (work supported 2016-2018, DOE-BES sole financial support for our contributions):

1. Artier J, Holland SC, Miller NT, Zhang MQ, and Burnap RL (2018) *Synthetic DNA system for structure-function studies of the high affinity CO_2 uptake NDH-1$_3$ protein complex in cyanobacteria.* Biochem Biophys Acta 1859, 1108-1118
2. Zhang CC, Zhou CZ, Burnap RL, and Peng L (2018) *Carbon/nitrogen metabolic balance: lessons from cyanobacteria.* Trends in Plant Science S1360-1385(18)30213-9. doi: 10.1016/j.tplants.2018.09.008.) (*review article*)
3. Cano M, Holland SC, Artier J, Burnap RL, Ghirardi M, Morgan JA, and Yu, JP (2018) *Glycogen synthesis and metabolite overflow contribute to energy balancing in cyanobacteria.* Cell Reports 23:667-672.
4. Miller NT and Burnap RL (2018) *Navigating the fitness landscape using multiallele genome editing.* Proceedings of the National Academy of Science (USA) 115, 12547-1254 (*commentary*)

Science Objectives 2019-2021: Site-directed mutagenesis of CupA protein and the NDH-1$_3$ proton pumping subunits. Unfortunately, we have discovered that strains under inorganic carbon stress are especially prone to suppressor mutations. Some of the conditions are counterintuitive and we aim to understand this better, which should add to the overall understanding of the CCM. We will be reconstructing some of the basic strains for safety. Once resolved, 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 interact with CupA, and are included in the testing of our hypothesis that proton pumping evacuates the active site of protons to trap a deprotonated intermediate of CO_2 hydration.

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

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Overall research goals

- O*verall objective* to determine the structure and functional control of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and phospho*enol*pyruvate carboxylase (PEPC) kinetics, and the influence of carbonic anhydrase (CA) on substrate availability (HCO_3^- or CO_2) for PEPC and Rubisco, respectively.
- *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, and that CA activity limits the capacity of CO_2 reduction at high temperatures in both C_4 and C_3 plants.
- *Specific aims* 1) Determine kinetic parameters driving temperature response of Rubisco specificity; 2) Determine if specific amino acid differences between PEPC isoforms drive differences in K_m for HCO_3^- (K_{HCO3}).; and 3) Determine if CA and PEPC activity drive temperature response of C_4 photosynthesis.

Significant achievements 2018-2019

PEPC kinetics driven by variation in amino acid compositions. For C_4 photosynthesis, a specific PEPC isozyme evolved from a non-photosynthetic C_3 PEPC to power the C_4 carbon concentrating mechanism. The gene coding for this recruited C_4 PEPC is thought to have arisen from a gene duplication that allowed for the neofunctionalization of PEPC and mutations that alter the kinetic properties of PEPC. It has been hypothesized that a decrease in K_m for HCO_3^- (K_{HCO3}) has a selective advantage for maintaining high rates of C_4 photosynthesis, particularly when carbon substrate availability is low due to restricted stomatal conductance (DiMario & Cousins, 2019). We measured the K_{HCO3} of C_4 PEPC isozymes extracted from 20 species in the Poaceae family and compared this data to available PEPC peptide sequences. We identified an amino acid residue, Thr/Glu772, that was suggested to be partially responsible for the range in K_{HCO3} in observed the 20 represented C_4 grasses. This was tested by mutating the Glu772 to a threonine, E772T in the *E. esculenta* PEPC in a bacterial expression vector. Manuscript submission in October 2019.

Limitation of C_4 photosynthesis by CA and PEPC in response to temperature. CA catalyzes the first biochemical step in the C_4 pathway by hydrating CO_2 to HCO_3^- and is thought to be essential for C_4 photosynthesis but not rate limiting at ambient CO_2 partial pressure (pCO_2). The HCO_3^- is subsequently used by PEPC to initiate the CO_2 concentrating mechanism. Modeling and *in vitro* studies suggest that CA and PEPC activity may limit C_4 photosynthesis when pCO_2 is low within the leaf and under high temperatures. Therefore, deciphering how CA and PEPC limits the CO_2 concentrating mechanism has important implications for understanding factors that influence the efficiency of C_4 photosynthesis. We have characterized the influence of genetically reduced CA and PEPC activity in the C_4 plants *Zea mays* and *Setaria virids*, respectively, in response pCO_2 and temperatures. The data demonstrates that CA and PEPC increasingly limit C_4 photosynthesis at low pCO_2 and increasing temperature.

Mesophyll Conductance in C_4 plants. Mesophyll CO_2 conductance (g_m), the diffusion of CO_2 from intercellular air spaces to the first site of carboxylation in the mesophyll cells, is an important factor potentially limiting rates of photosynthesis in both C_3 and C_4 plants. In C_3 species it has been shown that g_m is influenced by diverse leaf structural and anatomical traits; however, little is known about which traits affect g_m in C_4 species. To address this knowledge gap, we used online oxygen isotope discrimination measurements to estimate g_m and microscopy techniques to measure leaf structural and anatomical traits potentially related to g_m in 18 C_4 grasses (Pathare & Cousins, 2019).

Rubisco kinetics

Breakpoints in Rubisco kinetics in response to temperature have been declared as universal but they are often not observed nor has a clear mechanism been elucidated. We measured the *in vivo* and *in vitro* 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 further suggest changes to carbon isotope discrimination for Rubisco with temperature. Therefore, we tested the temperature response of CO_2 discrimination by Rubisco, where changes in discrimination may occur due to changes in the reaction mechanism. However, Rubisco discrimination in *O. sativa* was constant with temperature, suggesting breakpoints are not associated with changes in elementary rate constants but potentially to enzyme deactivation.

Science objectives for 2019-2020

1) Publish data on the *in vivo* and *in vitro* temperature response of Rubisco kinetic parameters and carbon isotope fractionation for *O. sativa*. Submission in early 2020.
2) Publish research on genetic reduction of PEPC activity in *S. viridis*. We are currently preparing a manuscript on the temperature response of C_4 photosynthesis in these plants. Submission November 2019.
3) Publish the temperature response of C_4 photosynthesis in *Zea mays* with reduced CA activity. Submission late 2019 or early 2020.
4) Leverage parallel evolution of PEPC in the grass family to identify novel allelic variants that define key amino acid changes from a range of C_3 and C_4 species. Synthesized PEPC alleles are being expressed in *E. coli* to test specific amino acid substitutions on PEPC kinetic properties. Submission late 2019.

References to work supported by this project 2018-2019:

Published

1. Pathare V.S., **Cousins A.B.** (2019) Increased adaxial stomatal density is associated with greater mesophyll surface area exposed to intercellular air spaces and mesophyll conductance in diverse C_4 grasses. *New Phytologist* doi:10.1111/nph.16106
2. Ubierna N., Cernusak L.A., Holloway-Phillips M., Busch F.A., **Cousins A.B.**, Farquhar G.D. (2019) Critical review: incorporating the arrangement of mitochondria and chloroplasts into models of photosynthesis and carbon isotope discrimination *Photosynthesis Research* doi:10.1007/s11120-019-00635-8
3. Cano F.J., Sharwood R.E., **Cousins A.B.**, Ghannoum O. (2019) The role of leaf width and conductances to CO_2 in determining water use efficiency in C_4 grasses *New Phytologist* **223**, 1280-1295
4. DiMario, R.J., **Cousins A.B.** (2019) A single serine to alanine substitution decreases bicarbonate affinity of PEPC in C_4 *Flaveria trinervia*. *Journal of Experimental Botany* **70**, 995-1004
5. Boyd, R.D., Cavanagh, A.P., Kubien, D.S., **Cousins A.B.** (2019) Temperature response of Rubisco kinetics in *Arabidopsis thaliana*: thermal breakpoints and implications for reaction mechanisms *Journal of Experimental Botany* **70**, 231-242
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7. Ubierna N., Gandin A., **Cousins A.B.** (2018) The response of mesophyll conductance to short-term variation in CO_2 in three C_4 species. *Journal of Exp Botany* **69**,1159-1170
8. Kolbe A.R., Brutnell T.P., **Cousins A.B.**, Studer A.J. (2018) Carbonic anhydrase mutants in *Zea mays* have altered stomatal responses to environmental signals. *Plant Physiology.* **177**, 980-989

In review

1. Serrano-Romero E.A., **Cousins A.B.** (2019) Cold acclimation of mesophyll conductance, bundle-sheath conductance and leakiness in *Miscanthus* x *giganteus*. *New Phytologist*

Structure-Function of the Cytochrome b_6f Lipoprotein Complex

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I. Lipid Dependence

The photosynthetic cytochrome b_6f complex, a homo-dimer consisting of eight distinct subunits and 26 trans-membrane helices per monomer, catalyzes proton-coupled electron transfer for energy storage across the thylakoid membrane. The **2.5 Å atomic structure of the complex** from the cyanobacterium *Nostoc* sp. (Hasan and Cramer, *Structure*, 2014), **depicted below in ribbon diagram**, revealed the presence of 23 lipid binding sites per monomer (not shown). Although the crystal structure of the cytochrome b_6f complex from a plant source has not yet been solved, the identity of internal lipids present in a plant b_6f complex has been determined in the present study. The predominant lipid species are MGDG, DGDG, PG and SQDG. Despite the extensive structural analyses of b_6f -lipid interactions, the basis of the stabilization by lipids remains poorly understood. In the present study the effect of individual lipids on the structural and functional integrity of the b_6f complex, purified from *Spinacea,* is reported: (**i**) galactolipids (MGDG, DGDG and SQDG) and phospholipids (DLPG, DOPG and DOPC) provide structural stabilization to the complex to varying degrees; (**ii**) SQDG has a major role in stabilizing the dimeric complex; (**iii**) b_6f complex is stabilized by incorporation into nanodiscs or bicelles; (i**v**) removal of bound phospholipid by phospholipase A_2 inactivates the cytochrome complex, to which (**v**) the activity can be restored (S. Bhaduri *et al*., *J. Biol. Chem*, *in press*).

II. FNR Interaction with Cytochrome b_6f Complex: Thermodynamic Parameters

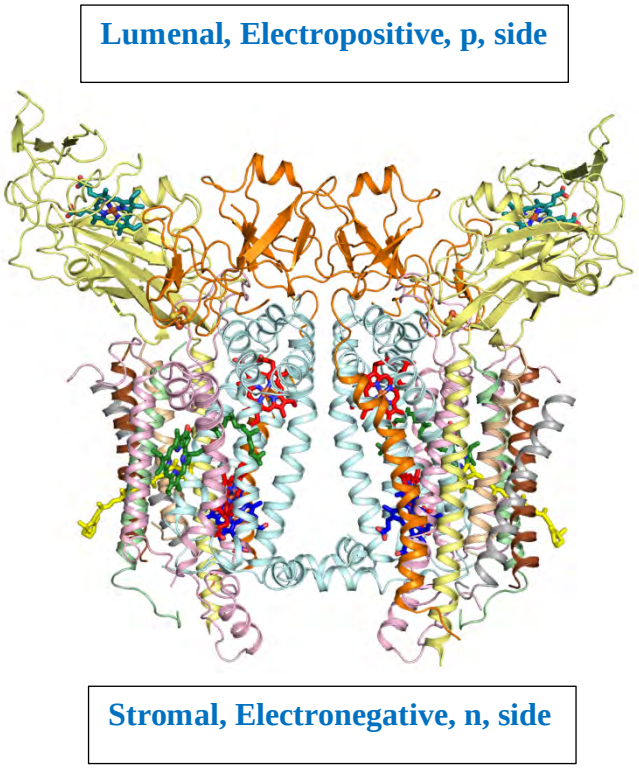
The cytochrome b_6f complex, for which a crystal structure has been obtained at 2.5 Å resolution (*Structure*, 2014) has a central role in the regulation of oxygenic photosynthetic electron transport. The photosystem I peripheral electron transfer proteins ferredoxin (Fd) and ferredoxin $NADP^+$ reductase (FNR) participate in the pathway of $NADP^+$ reduction. Cyclic electron transfer pathway provides an alternate source of electron transfer-mediated membrane energization for ATP production. The present study focuses on the physical interaction of FNR with the cytochrome b_6f complex, with relevance to the participation of FNR in a cyclic pathway. The presence of FNR in purified cytochrome b_6f complex has been documented and found to be present in sub-stoichiometric levels (Zhang *et al*. JBC, 2001). It is known that linear electron transfer does not require direct interaction of the cytochrome complex with FNR. In the present study, isothermal calorimetry (ITC) was used to detect and characterize the interaction of FNR with the dimeric cytochrome b_6f complex, derived from a plant (spinach) source, in detergent solution. A relatively weak interaction is characterized by thermodynamic parameters: K_d, 48 μM; ΔH, 23.3 kJ/mol, and ΔS, 3.3 J/mol-degK, at pH 6.5 and an ionic strength of 170 mM. These interactions were not detected at pH 8.0, implying a significant pH dependence of the FNR-b_6f interaction. Far-UV circular dichroism (CD) analysis of the structure stability of FNR via changes in secondary structure4was employed to analyze the pH dependence of the interactions and the effect of

detergents. This analysis also revealed a decrease in structure stability of FNR and less efficient interaction with the b_6f complex in the presence of the detergent UDM (S. D. Zakharov *et al*., Abstract *in press*, 64th Annual Meeting, Biophysical Society, San Diego, CA).

III. Structure-Based Control of the Rate Limitation of Photosynthetic Electron Transport (*FEBS Lett.,* **593**, 2103-2111 (2019).

The 2.5 Å structure of the cytochrome b_6f complex provides a basis for control of the rate-limiting electron transfer step of oxygenic photosynthesis associated with the plastoquinol/quinone exchange pathway. Structure change was made at a site containing two proline residues which border the intra-cytochrome pathway for plastoquinol/quinone exchange. The proline side chains confer a larger aperture for passage of plastoquinol/quinone. Change of these prolines to alanine in the cyanobacterium *Synechococcus* sp. PCC 7002 resulted in attenuation of this rate-limiting step, observed by a two-fold reduction in the rate of cell growth, O_2 evolution, and plastoquinol-mediated reduction of cytochrome *f*. This study describes modification by site-directed mutagenesis of photosynthetic energy transduction based on rational application of information in the atomic structure.

Ribbon Diagram of Structure of Cyanobacterial Cytochrome b_6f Complex (pdb 4OGQ)



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 that act as key components in these pathways. The current focus of our work is the biogenesis of cyt *c* 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 chosen model system. Its biogenesis requires two distinct maturation processes:

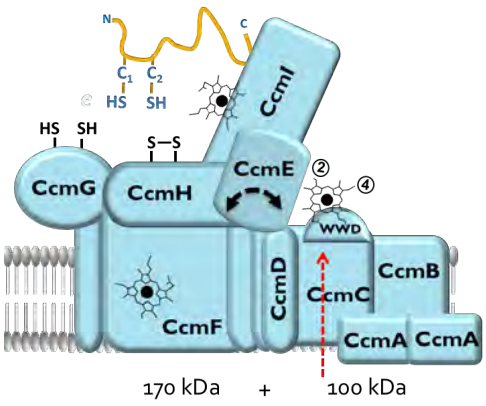
1- Covalent ligation of heme to the apocyt subunits via the cyt *c* maturation system I **(Ccm)**. Ccm is widespread among bacteria, archaea, plant mitochondria and red algae. It is composed of nine membrane proteins (CcmABCDEFGHI) that carry out the covalent ligation of heme *b* to the apocyt subunits of *cbb*$_3$-Cox. Our earlier work studied the mechanism of function of these proteins, and now we uncovered that they also form a large macromolecular entity (supercomplex).

2- Acquisition, trafficking and insertion of copper (Cu) into the active site of the catalytic subunit of *cbb*$_3$-Cox are common processes for maturation of various heme-Cu enzymes. In *Rhodobacter* species, Cu trafficking occurs via a high-affinity (*CcoA-dependent*) or a low-affinity (*CcoA-independent*) Cu acquisition pathway. Currently we are focused on the Cu importer CcoA, and on CcoG, which is the first bacterial **cupric reductase** that we discovered very recently.

Our overall goal continues to be focused on processes that are essential for *cbb*$_3$-Cox biogenesis.

Significant achievement 2018-2019:

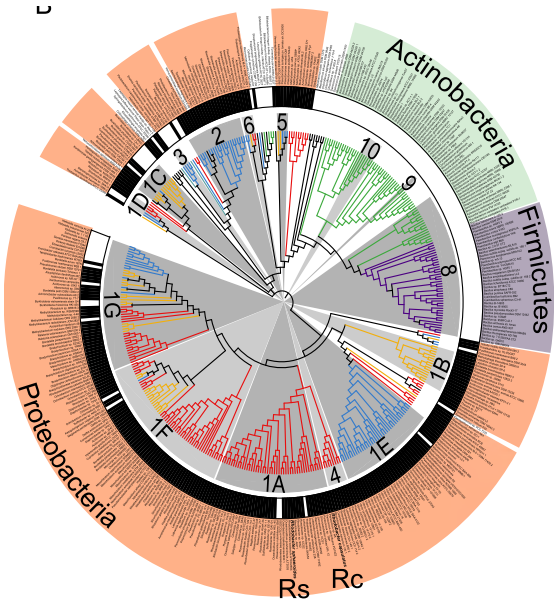
A- Ccm process: A major achievement was the finding that the Ccm components, CcmABCD transporting heme, CcmE chaperoning heme, CcmG reducing apocytochrome disulfide bond, and CcmFHI forming the covalent thioether bonds forms a membrane-integral supercomplex. Previously, these complexes and the individual components were thought to freely diffuse in the lipid bilayer, and interact transiently during their functions. We now know that at least a fraction of them are associated together to form the Ccm supercomplex, which could operate in a diffusion-independent and more efficient manner. This supercomplex is ready for purification to homogeneity, and is amenable to cryoEM approaches for structural studies as the next frontier of cytochrome *c* maturation process (*manuscript in preparation*).



The Ccm Supercomplex ~ 250-300 kDa

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 has now become the prototype of bacterial Cu importers as a comparative genomic analysis of CcoA orthologs in alpha-proteobacterial genomes showed that it is widespread among organisms. Yet, it has a highly specific role in cuproenzyme biogenesis. Remarkably, the

absence of CcoA affects only the production of *cbb*$_3$-Cox and not that of *aa*$_3$-Cox in an organism like Rhodobacter that can produce both of them. Thus, distinct copper delivery pathways provide the Cu_B atoms to the catalytic sites of highly similar Cox enzymes. Our phylogenomic studies revealed that CcoA with its conserved Cu-binding **M**xxx**M** // **H**xxx**M** motif defines a broad family of **C**co**A**-**l**ike **t**ransporters (CalT) family. Surprisingly, this family also includes the RfnT-like proteins, suggested to transport riboflavin. However, our recent findings established that this CalT subfamily also import copper. Remarkably though, they are not functionally exchangeable between species in respect to *cbb*$_3$-Cox biogenesis. This suggests that copper imported by the different CalT subgroup members convey Cu to different cuproproteins using different chaperones, illustrating the versatility of copper homeostasis pathways.



Distribution of the CalT-like copper transporters among bacteria

Science objectives for 2019-2020: The following specific aims are being pursued:

• Complete the *R. capsulatus* Ccm supercomplex manuscript.

• Continue the characterizations of the high affinity (*CcoA-dependent*) Cu delivery pathway to *cbb*$_3$-Cox. Pursue the study of the Cys mutants of CcoA, and establish the identity of the "fixed" (*i.e.*, structural) *versus* "labile" (*i.e.*, functional) disulfide bonds that these Cys residues form, and infer their role(s) in Cu binding and release during transport.

References to the works related to Ccm and Cu studies supported by this project 2018-2019

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STRUCTURE OF NDH: THE COMPLEX I-LIKE MOLECULE OF PHOTOSYNTHESIS

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Cyclic electron flow (CEF) around photosystem I (PSI) is a mechanism by which photosynthetic organisms balance levels of ATP and NADPH necessary for efficient photosynthesis. The NAD(P)H dehydrogenase-like complex (NDH) is a key component of this pathway in most oxygenic photosynthetic organisms and the last large photosynthetic membrane protein complex of unknown structure. Related to the respiratory NADH dehydrogenase complex (complex I), NDH transfers electrons originating from PSI to the plastoquinone (PQ) pool, while pumping protons across the thylakoid membrane, thereby increasing the amount of ATP produced per $NADP^+$ molecule reduced. NDH possesses 11 of the 14 core complex I subunits as well as several oxygenic photosynthesis specific (OPS) subunits, which are conserved from cyanobacteria to higher plants. However, the three complex I core subunits involved in accepting electrons from NAD(P)H are notably absent in NDH. Thus, how NDH acquires electrons and transfers them to PQ is currently unclear. Nevertheless, the OPS subunits, specifically NdhS, are proposed to enable NDH to accept electrons from ferredoxin (Fd), its electron donor. Here we report a 3.1 Å structure of the ~0.5 MDa NDH complex from the thermophilic cyanobacterium *Thermosynechoccous elongatus BP-1* (*T. elongatus*) determined by single-particle cryo-electron microscopy (cryo- EM). Our maps reveal the structure and arrangement of the principle OPS subunits in the NDH complex, as well as an unexpected cofactor near the PQ-binding site in the peripheral arm. Moreover, the location of the OPS subunits supports a role in electron transfer and defines two potential Fd-binding sites at the apex of the peripheral arm. Together, these results suggest multiple electron transfer routes could be present in NDH, which would serve to maximize PQ reduction and avoid deleterious off-target chemistry of the semiplastoquinone radical.

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Conversion of carbon dioxide (CO_2) into sugar is one of the most important metabolic pathways for the survival and propagation of nearly every organism on earth. Although confined to only a subset of species, CO_2 fixation provides the food, energy and basic building blocks for the formation of nearly every biomolecule in the world. In plants, green algae and cyanobacteria, the energy required to drive the formation of sugar from CO_2 is obtained from the sun, whose light rays excite electrons in the "green" chlorophyll molecules. These excited electrons leave the

chlorophyll molecule and start a long complex journey through the thylakoid membranes until they reach and reduce the energy storing biomolecule nicotinamide adenine dinucleotide phosphate ($NADP^+$). Furthermore, as they partake in their journey, protons are pumped across the membrane which ultimately power the formation of a second energy storing biomolecule, adenosine triphosphate (ATP). Both ATP and NADPH are required to power the conversion of CO_2 into sugar, however, the ratio produced by a single passage of electrons from chlorophyll to $NADP^+$ is not optimal for CO_2 fixation and additional ATP is required. This additional ATP is thought to be generated in part by the electron coupled proton-pumping activities of complex I-like complex of oxygenic photosynthesis - NAD(P)H Dehydrogenase-Like Complex (NDH).

FTIR Studies of Photosynthetic Oxygen Production

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Overall research goals: The project's primary aims are to (i) further delineate the networks of hydrogen bonds that make up the substrate access and proton egress pathways that link the Mn_4CaO_5 cluster with the lumen and (ii) characterize the dynamic changes in these networks that take place during the individual steps of the catalytic cycle. Both FTIR difference spectroscopy and time-resolved IR spectroscopy are being conducted with mutant Photosystem II core complexes representing residues identified crystallographically or computationally as participating in these networks or otherwise interacting with the water molecules in the Mn_4CaO_5 cluster's immediate environment, as well as altered wild-type Photosystem II core complexes.

Significant achievements 2017-2019: We have provided strong evidence that the water-derived oxygen that relocates during the catalytic step immediately prior to O-O bond formation (during the S_2 to S_3 transition) relocates from the Ca^{2+} ion instead of from the dangling Mn4 ion (*6*). Considerable evidence identifies oxo bridge O5 as one of the two substrate waters that ultimately form the O-O bond. The second substrate may be the oxygen that relocates during the S_2 to S_3 transition. This relocated oxygen has been identified in recent femtosecond crystallography experiments and denoted O6 or Ox. To distinguish whether this oxygen originates as the Mn-bound water denoted W2 or as the Ca^{2+}-bound water denoted W3, we analyzed the D-O-D bending mode of the water molecule that deprotonates/relocates to become O6/O_x during the S_2 to S_3 transition. During the report period, we showed that this D-O-D bending mode is slightly altered by the D1-V185N mutation (*3*) but is not altered by the D1-S169A mutation (*6*) (Figure 1, middle and right panels). Previously, we showed that this mode is altered substantially when Sr^{2+} is substituted for Ca^{2+} (Figure 1, left panel). Because Sr^{2+}/Ca^{2+} exchange alters this D-O-D bending mode but the D1-S169A mutation does not, we conclude that the water-derived oxygen that relocates and becomes O6/Ox originates as the Ca^{2+}-bound W3. The shift of this mode by the D1-V185N mutation suggests that W3 is replaced on Ca^{2+} by the nearby W5 water molecule. Our conclusion that O6/Ox relocates from the Ca^{2+} ion provides an important constraint for proposed mechanisms of O-O bond formation in Photosystem II.

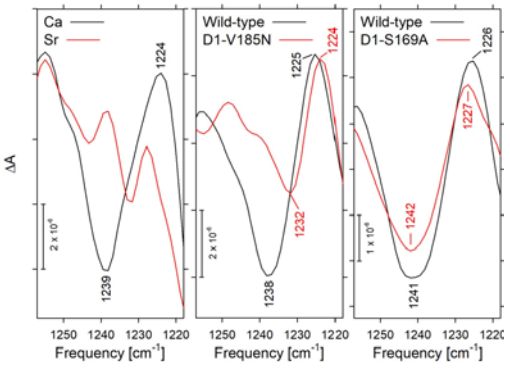

Figure 1. $D_2{}^{16}O$-$D_2{}^{18}O$ double-difference spectra of PSII core complexes containing Sr in place of Ca (left), from D1-V185N cells (middle) and from D1-S169A cells (right) in comparison to Ca-containing wild-type PSII core complexes.

During the report period, work performed in collaboration with G. W. Brudvig and coworkers at Yale University showed that D1-S169 participates in the network of hydrogen bonds that surrounds the Mn_4CaO_5 cluster and has a possible role in proton egress or substrate water delivery (*4*), that D1-N87 participates in the extensive network of hydrogen bonds that extends from Y_Z to D1-D61 and influences the Cl^- binding site that is located 13 Å away from this residue (*1*), and that D1-D61 and D2-K317 participate in a dominant protein egress pathway (*5,7*).

Our preliminary study of the D1-N298A mutation shows that this residue participates in the same extensive network of hydrogen bonds that includes many other residues such as D1-D61 and D2-K317. To test the proposal that this residue participates in a dominant proton egress pathway that functions during the S_2 to S_3 transition, the kinetics of the individual S state transitions in D1-N198A PSII core complexes were measured in collaboration with H. Dau and coworkers at the

Freie Universität of Berlin. Preliminary results show that the S_2 to S_3 transition is unaltered by the mutation, but that the S_3 to S_0 transition is slowed over 100-fold, resembling data obtained with the D1-D61A and D1-V185N mutations (Figure 2). These results suggest that D1-N298 does not participate in a dominant proton egress pathway during the S_2 to S_3 transition, but instead suggests that the substantial slowing of the S_3 to S_0 transition caused by mutations as spatially separated as D1-D61A, D1-V185N, and D1-N298A reflects the highly coupled nature of the network of hydrogen bonds that surround the Mn_4CaO_5 cluster and a global response to perturbations of this network during the S_3 to S_0 transition.

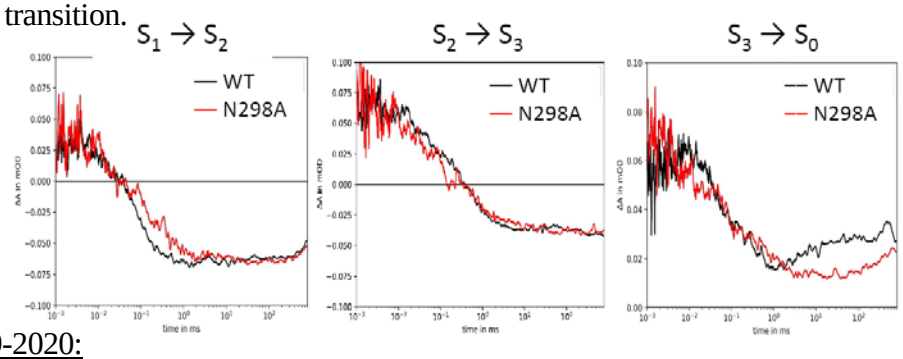

Figure 2. Absorption changes at 1400 cm^{-1} in D1-N298A PSII core complexes in comparison to wild-type during the S_1 to S_2, S_2 to S_3, and S_3 to S_0 transitions.

Science objectives for 2019-2020:

- Complete our FTIR study of the D1-N298A mutant and extend these studies to nearby residues in the same network.
- Continue conducting time-resolved IR measurements to measure the dynamics of the protein environment of the Mn_4CaO_5 cluster during the individual S state transitions. Initial work on PSII from the D1-D61A and D1-N298A will be completed and extended to additional mutations. 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.
- Continue to pursue collaborative studies of the electronic properties of the Mn_4CaO_5 cluster in its different oxidation states and with R. D. Britt and coworkers at UC Davis (e.g., *2*).

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Resolving protein-semiquinone interactions by advanced EPR spectroscopy.

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Overall research goals: Our focus is on use of modern, high-resolution EPR spectroscopy to explore the catalytic domains trapped in states with semiquinone (SQ) as an intermediate. The catalytic sites we propose to study, - the Q_A and Q_B sites of the bacterial reaction center (RC), 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 2017-2019:

a-strain effects from the H-bond nitrogen donors in ^{15}N HYSCORE spectra of semiquinones. Hydrogen bonding between semiquinone (SQ) intermediates and sidechain or backbone nitrogens in protein quinone processing sites (Q-sites) is a common motif. Previous studies on SQs from multiple protein environments have reported specific features in the ^{15}N HYSCORE spectra not reproducible by a theory based on fixed hyperfine parameters, and the source of these lineshape distortions remained unknown. Using the spectra of the SQ in the Q-sites of wild-type (nitrogen donor N_ε-Arg71) and mutant D75H (nitrogen donor N_ε-His75) cytochrome bo_3 ubiquinol oxidase from *E. coli* (cyt bo_3) we have explained the observed additional features as originating from *a*-strain of the isotropic hyperfine coupling. In 2D spectra the *a*-strain manifests in the form of the "boomerang" lineshape and low intensity lines in the opposite quadrant that allow its direct analysis. We have shown that their appearance is regulated by the relative values of the strain width Δa and parameter $\delta = |2a+T|-4\nu_{15N}$. Clearly resolved effects from the *a*-strain are only expected to appear when part of the strain broadened cross-ridge approaches or satisfies the condition $(2(a\pm\Delta a)+T) \sim 4\nu_{15N}$. The intensity and shape of the cross-features in the opposite quadrant will depend on the strain distribution function and the Δa and δ values. Fluctuations of the SQ in the protein environment, producing proton structural deviations in the O···H···N bond, are responsible for the well-resolved strain effects. Model DFT simulations of the $a(^{15}N)$ dependence on O···H bond length for N donors in the Q_A site show that variations of the order ~0.3 MHz correspond to a distance change of ~0.1 Å. This estimate suggests that fluctuations in the O···H distance of the order ~0.1 Å can be responsible for the *a*-strain distortions in the ^{15}N HYSCORE spectra of nitrogen H-bond donors. (Ref. 3)

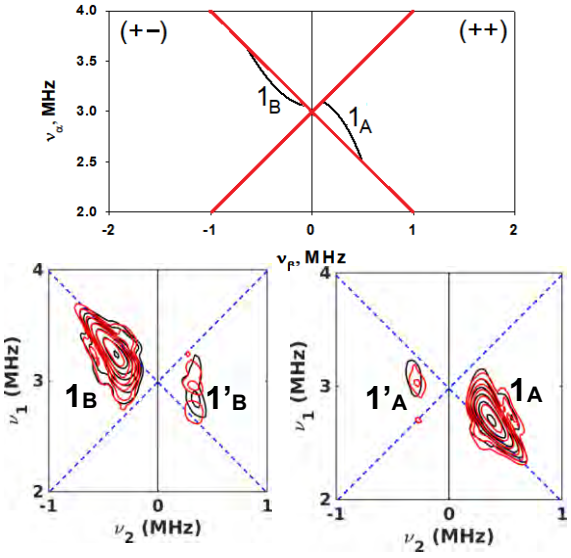
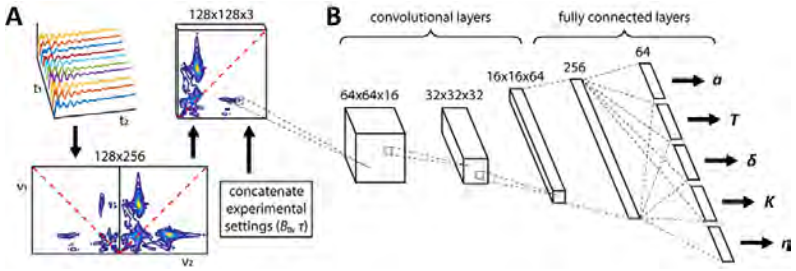


Figure. (top) The ideal theoretically predicted cross-ridge lineshapes in ^{15}N HYSCORE spectra are arcs **1A** (WT) and **1B** (D75H) extending between the points located on the $|\nu_\alpha \pm \nu_\beta| = 2\nu_{15N}$ lines. **(bottom)** Comparison of the experimental ^{15}N HYSCORE spectra possessing "boomerang" lineshape distortions of the basic cross-ridges (**1A**, **1B**) and accompanying lines of low intensity (**1'A**, **1'B**) in the opposite quadrant with the spectra calculated with a Gaussian distribution of the isotropic hyperfine coupling. The region of the spectra with the cross-features located near the $\nu_2 = 0$ line is shown. The best fit of the spectra was obtained with a = 2.4 MHz, T = 0.4 MHz, σ = 0.3 MHz for WT cyt bo_3 (left) and a = 3.3 MHz, T = 0.45 MHz, σ = 0.35 MHz for D75H cyt bo_3 (right).

Convolutional Neural Network Analysis of Two-Dimensional HYSCORE Spectra. A machine learning approach is developed for analyzing two-dimensional hyperfine sublevel correlation electron paramagnetic resonance (HYSCORE EPR) spectra with the proficiency of an expert spectroscopist, proposing an alternative paradigm to the typical trial-and-error simulation process for interpreting multi-dimensional spectra. Machine learning is demonstrated to extract the magnetic coupling parameters of interest directly from single-nuclear ^{14}N multidimensional spectra and provides the full underlying probability distributions of each parameter for the first time, allowing for error estimates free from bias.



The predicted coupling constants (a, T) and (K, η) deviate from the previous manual analyses of the experimental spectra on average by 0.11 MHz, 0.09 MHz, 0.19 MHz, and 0.09, respectively.

Thus, machine learning has demonstrated the incredible ability to extract the magnetic coupling parameters of interest directly from the multidimensional spectra by treating it as an image classification problem. In the case of HYSCORE spectroscopy, the convolutional neural network derives an entirely new algorithm for estimating the hyperfine anisotropy parameter T directly from the spectrum. Importantly, the neural network extracts the full underlying probability distributions of each parameter for the first time, allowing for error estimates free from bias. (Ref. 6)

Science objectives for 2019-2020:

- We will study the influence of mutations on SQ_A in bacterial reaction center from *Rhodobacter sphaeroides*. We will focus on the M265IS and M265IT mutations induced 80-120 mV negative shifts in the E_m of Q_A, with little perturbation of any other properties, including binding affinity.
- Previous studies showed that the 2-methoxy group is essential for simultaneous function of Q_A and Q_B, i.e. electron transfer from Q_A to Q_B works for 2-CH_3O-Q and does not work for 3-CH_3O-Q. We will characterize the interactions of the reconstituted 2- and 3-monomethxyQs with protein in the Q_A^- state by performing 2D ESEEM and ENDOR in native and ^{15}N labeled RC, and H_2O or D_2O solvent.

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Performance and Regulation of Photosystem II Energy Conversion in Oxygenic Phototrophs: From PSII Crystals to Living Cells

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Overall Research Goals: Frontiers of water oxidation catalysis by Photosystem II (PSII) (Aim 1). Method development: measuring Chl fluorescence to detect WOC cycle kinetics *in vivo* from water oxidation to CO_2 fixation (Aim 2). Regulation of PSII and photoprotection (Aim 3).

Significant Achievements 2018-2019 (September):

- Aim 1 has been addressed in publications P2, P5 and P6.
- Aim 2 has been addressed in publication P3 and submitted manuscript P9.
- Aim 3 has been addressed in publications P1 and P4 and submitted manuscripts P7 and P8.

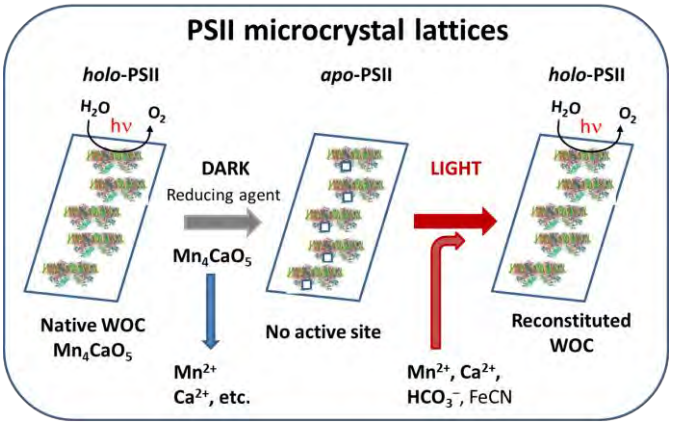
Science Objectives for 2019-2020:

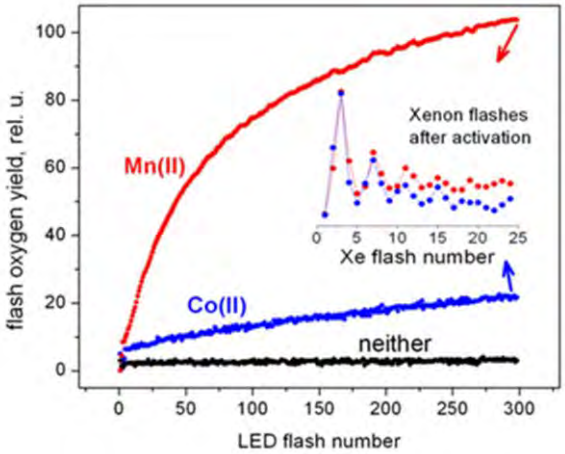
Aim 1. Despite their widespread use in crystallographic structural studies, few functional studies of PSII operation in microcrystals have been carried out. Their extreme compositional homogeneity allows the observation of PSII processes previously obscured by environmental and biological polydispersity. Our collaboration with the Fromme lab has revealed unanticipated properties of highly purified PSII centers in microcrystals (PSIIxtals). For example, in P2 we showed that PSII performance in microcrystals can be pushed far beyond what it is capable of in vivo, achieving the highest O_2 quantum yield ever observed (61%). Sustained period-4 and period-2 oscillations in O_2 yield extending over 200 flashes are now visible and show that flux from water → plastoquinone occurs through two electron acceptors at different redox potentials that are identified as the Q_AQ_B two-electron gate. The lifetimes of the S2 and S3 states in PSIIxtals are greatly extended (especially S2) by exogenous electron acceptors, an important property for all scientists working on PSIIxtals.

Aim 1. New studies of PSIIxtals with the Fromme lab are revealing that two other flux limiting reactions occur that have never been identified before in the water oxidation (Kok) cycle. These produce coherences every 2.2 and 4.7 flashes measured by both O_2 and Chl fluorescence yields that reveal coupling of the Kok cycle to PSII charge transfer steps, likely involving either backward transitions ($S_i \rightarrow S_{i-1}$) or alternative pathways. These discoveries will enable new insights into the fundamental photochemistry of PSIIxtals and WOC cycle inefficiencies.

Aim 1. All oxygenic phototrophs on the ToL exclusively use manganese at the catalytic site of water oxidation (Mn_4O_5Ca aka WOC). For the first time, we have reversibly removed and reassembled the WOC in PSIIxtals. This process, known as photoassembly, recovers > 90% activity. Use of PSIIxtals allows precise determination of the Mn oxidation states, both by reductive titration using analytical reductants and by recovery of O_2 activity using single turnover flashes.

Aim 1. For four decades, scientists have sought without success to find natural variants or replace Mn in PSII with other transition ions. Using PSIIxtals, we have produced the first "inorganic mutant" that functionally replaces manganese. The properties of cobalt-reconstituted PSII reveal the chemical principles of water oxidation catalysis. Among them, PSII turnover efficiency is shown to be a trade-off between charge separation and recombination, with Mn^{4+}/Mn^{3+} recombination kinetics appreciably slower than Co^{4+}/Co^{3+}.





Aim 2. We are working in collaboration with the ten Veldhuis lab to investigate adaptation to high CO2/DIC levels (>6000 ppm) found around point sources in the Yellowstone ecosystem, using newly developed instrumentation/methods to measure light-driven flux from water oxidation by PSII to CO2 fixation *in vivo*. We are working on drafting a manuscript describing this Chl fluorescence instrument and convenient method.

Aim 3. We are drafting a manuscript on the influence of chloride in regulation of PSII charge separation and its branching between linear electron flow and photoprotection by charge recombination.

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Using singlet-singlet annihilation to track energy transfer pathways within chlorophyll pools of cyanobacterial Photosystem I in its native environment

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University of Chicago

Cyanobacterial thylakoid membranes contain two fused antenna/reaction center complexes, Photosystem I (PSI) and Photosystem II (PSII). In the cyanobacteria *Synechocystis* sp PCC 6803, PSI monomers and trimers likely exist in dynamic equilibrium. PSI monomers contains 96 chlorophyll (Chl) molecules with a peak absorbance at 680 nm. Excitations in PSI are funneled to the P_{700} special pair Chl in reaction center on the 20-30 ps timescale. However, P_{700} is not the lowest energy chromophore in PSI. Red Chl pools with absorption at 706 nm and 714 nm are believed to increase the absorption cross section with uphill energy transfer to P_{700}. We employed femtosecond transient absorption spectroscopy to examine pathways of energy transfer within PSI in its native thylakoid membranes. We use singlet-singlet annihilation to measure the flow of excitations within and between PSI complexes before trapping by P_{700}. At low laser fluences, in the absence of annihilation, the red Chl pool at 705 nm has a monoexponential decay constant of 7 ps. When we increase the laser fluence to correspond to three and five excitations per PSI monomer, the red Chl pool decay constant changes to 5 ps and 3.5 ps, respectively. These faster decay times correspond to singlet-singlet annihilation. We are now exploring models that can explain these fluence-dependent dynamics. Using, a diffusion-limited trapping model that we have previously applied to energy transfer pathways in *Rba. sphaeroides*, we calculate a hopping time of ~13 ps for the red Chl pool. Ongoing work focuses on determining whether our observation correspond to inter- or intra-monomer dynamics and repeating these studies for thylakoids containing only PSII.

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

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Overall research goals: The research objectives are to study the photophysical processes by which photosynthetic organisms harvest light energy by: (1) tuning the excited state dynamics of chromophores by modifying the chemical linking groups between chromophores and proteins; (2) preparing arrays of metalloporphyrins within TMVcp assemblies to probe whether fluctuating environments fleetingly facilitate inter-chromophore energy transfer; (3) creating protein-based mesoscale architectures to study interassembly excited state energy transfer. By combining extended electronic structure calculations with dynamical simulations that span multiple length scales, the effects of protein dynamics and conformational disorder on the performance of natural light harvesting systems can be elucidated. Determining the key features that enable the unparalleled energy transduction efficiencies of natural photosynthetic systems is paramount to our ability to harness and mimic them in the context of photocatalytic materials and optical sensors.

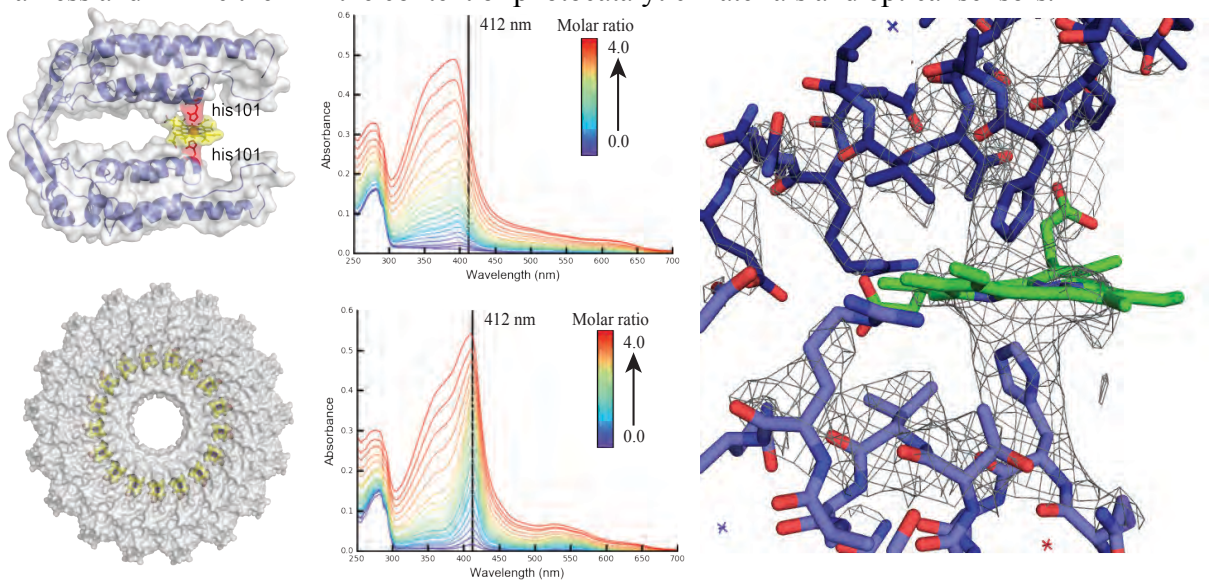

Figure 1. Left Panel: the cross-section view of designed binding pocket within a single cpTMV dimer (top) and the top-down view of the 17-heme cpTMV double disk (bottom). Middle Panel: the negative control cpTMV native protein (top) and histidine-containing protein cpTMV-101 (bottom) were incubated with hemin of different molar ratios from 0.0 to 4.0 (relative to dimer concentration) and the cofactor-protein interaction was monitored by UV/Vis spectroscopy. Right Panel: the electron density map of heme-containing cpTMV.

Significant achievements 2018-2019: Through a combination of synthetic linker and protein tailoring, we showed that the initial vibrational relaxation of excited chromophores could be slowed by up to 14-fold. This capability is essential for testing whether "hot EET" transfer mechanisms are indeed functional in photosynthesis. We have generated a new series of TMVcp disks with reduced symmetry, which will provide us with the unique opportunity to place chromophores in increasingly tailored environments that more closely resemble those in natural light harvesting systems. We have devised a strategy for asymmetrically conjugating peripheral sites in TMVcp disks using unnatural amino acids, and in preliminary studies, we have observed covalently linked pairs of disks. These structures will serve as the key platform for studying EET across the interfaces of individual light harvesting assemblies.

Science objectives for 2019-2020:

- We are building on the computational work in our *JACS* article to further our understanding of how environment affects the timescales of relaxation of dimers and arrays of sulforhodamine B (SRB) chromophores relative to uncoupled SRB chromophores.
- We devised an approach to simulate larger, multi-chromophore systems in a computationally tractable manner that will enable investigations into inter-chromophore couplings at an atomistic level and provide insights into experimental results for fully-labeled systems. We are developing a new workflow for computing accurate singlet and triplet energetics and dynamics for coupled chromophores in protein environments.
- In order to use chromophores that are more similar to those in natural systems, we have prepared a new set of synthetic bacteriochlorin analogs with tailorable linkers for protein attachment. We have also initiated new studies to generate phycocyanobilins with linkers for protein attachment and spectroscopic study.

References to work supported by this project 2018-2019:

1. Delor, M.; Dai, J.; Roberts, T. D.; Rogers, J. R.; Hamed, S. M.; Neaton, J. B.; Geissler, P. L.; Francis, M. B.; Ginsberg, N. S. "Exploiting Chromophore–Protein Interactions through Linker Engineering To Tune Photoinduced Dynamics in a Biomimetic Light-Harvesting Platform" *J. Am. Chem. Soc.* **2018**, *140*, 6278-6287.
2. Garfield, D. J.; Borys, N. J.; Hamed, S. M.; Torquato, N. A.; Tajon, C. A.; Tian, B.; Shevitski, B.; Barnard, E. S.; Doug Suh, Y.; Aloni, S.; Neaton, J. B.; Chan, E. M.; Cohen, B. E.; Schuck, P. J. "Enrichment of molecular antenna triplets amplifies upconverting nanoparticle emission" *Nature Photonics* **2018**, *12*, 402-407.
3. Hamed, S.; Bruneval, F.; Neaton, J. B. "Accurate singlet and triplet excitation energies from an *ab initio* Bethe-Salpeter equation approach with an optimally-tuned range-separated hybrid functional", *to be submitted this fall (2019).*
4. Jing, D.; Yano, J.; Yachandra, V.; Francis, M. B. "Protein-embedded porphyrin arrays templated by circularly permuted tobacco mosaic viral coat proteins", *to be submitted this fall (2019).*

Towards Structure Determination of the Photosynthetic Supercomplex to understand the Regulation of sustained Cyclic Electron Flow (CEF) in the photopsychrophile *Chlamydomonas* sp. UWO241

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Overall research goals: This abstracts reports on the structural aspects of the project, which aims to determine the structure of the supercomplex with Cryo-EM. The overall goal of this project is to describe the function of sustained CEF and assembly of the PSI supercomplex in the Antarctic psychrophile Chlamydomonas sp. UWO241 (UWO241) and the model Chlamydomonas reinhardtii acclimated to long-term salinity stress. Major objectives are: 1) determine the functional role of sustained CEF and impacts on downstream carbon metabolism in UWO241 acclimated to variable environmental stressors; 2) dissect the structure of the UWO241 PSI supercomplex through proteomic and structural studies; 3) determine whether C. reinhardtii utilizes "UWO-like" supercomplexes to support sustained CEF during long-term stress acclimation. Outcomes of this project will support research focused on meeting future energy and food needs by advancing our understanding of how extremophilic phototrophs use sustained CEF and rewired carbon metabolism to survive long-term exposure to environmental stressors, such as excessive light, low temperature, and high salinity. The work at ASU is focused on determining the high resolution structures of the PSI supercomplex in the Antarctic Chlamydomonas sp. UWO24 by single-particle cryo electron microscopy (cryo-EM). We aim to gain detailed information on the interaction of the proteins and cofactors in the supercomplex at molecular resolution. The goal is to identify the key amino acids in the formation and stabilization of the supercomplex including details on the cofactor interactions that will reveal the electron transfer pathways that drive the cyclic electron transfer in the supercomplex.

Significant achievements 2018-2019: Biophysical characterization was performed on supercomplex samples prepared under different isolation conditions in the lab of Dr. Morgan-Kiss. For the first preps, the sucrose was removed using a G50 desalting column and then directly applied the sample to an electron microscopy grid. The sample was negatively stained and imaged by EM (**Fig. 1**). The image shows a very good density of particles on the gird, but the particles have a large size distribution indicating inhomogeneity, so the sample must be significantly improved before high resolution cryo-EM is performed The negative stained image shows heterogeneous size distribution of particles which range from larger particles that could represent intact supercomplexes (red arrows) to very small particles in the size range of 30 to 40 Å that could represent detergent micelles (yellow arrows). The large number of medium size particles with a large variation of sizes indicates that the majority of the complexes have disintegrated. We considered three possible reasons for the disassembly of the complex: 1) EDTA removes divalent cations that may be needed to stabilize the supercomplex, 2) removal of sucrose may destabilize the complex, or 3) the stain (uranylaccetate) may have destabilized the supercomplex.

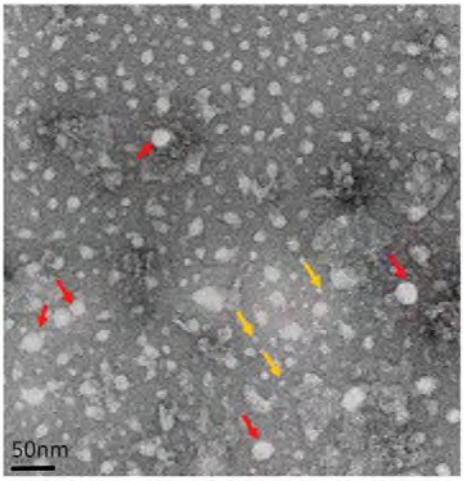

Figure 1: Electron micrograph (negative stain) of the Chlamydomonas sp. UWO241 supercomplex preparation after removal of sucrose by desalting columns. Only few supercomplexes are visible (red arrows), the image shows many particles which indicate disassembly of the supercomplex as well as detergent micelles (yellow arrows).

We also explored if the supercomplex can be further stabilized by the omission of EDTA and by

the further addition of magnesium to the density gradient centrifugation step. As sucrose leads to very high background and has to be removed before cryo-EM images can be taken, sucrose was removed from both samples by a desalting spin column before preparation of the sample grids for EM. **Figure 2** shows the results: the negative stain EM images without EDTA (without magnesium addition; **Fig. 2a**) shows many small particles indicating the supercomplex is disassembled. In contrast the prep in the presence of Mg^{2+} shows many large particles that could represent the intact supercomplex (**Fig. 2b**). While homogeneity still has to be further improved these results are extremely promising and encouraging as they show that we can stabilize the supercomplex in the presence of magnesium and in the absence of sucrose.

The teams works closely together to improve the homogeneity of the preps which includes visits from the lead researcher on the structural side at ASU. Dr. Yang traveled to Miami University to perform joint preparations with Dr. Morgan-Kiss' team to optimize sample yield and quality for Cryo-EM analysis. One of the critical points might be the shipping and freezing of the samples.

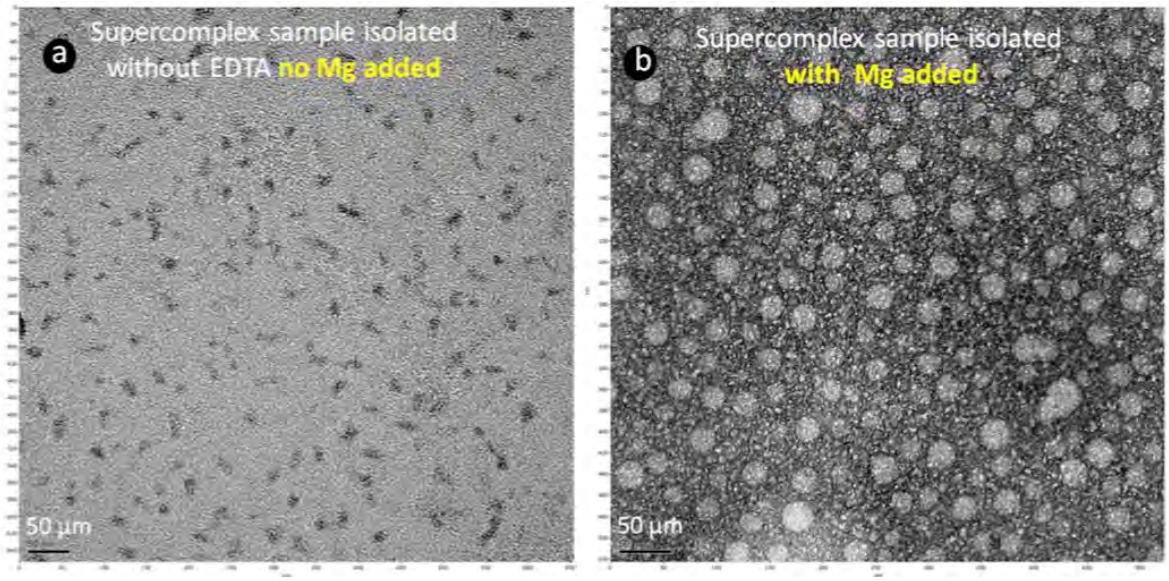

Figure 2: Negative stain electron micrographs of the supercomplexes with removal of sucrose on the EM grid. **a)** supercomplexes isolated in the absence of EDTA (no Mg^{2+} added) and **b)** isolated with Mg^{2+} added.

Science objectives for 2019-2020:

Graduate student Isha Kalra will travel to ASU at the end of October to perform a joint preparation at ASU from frozen cells and cells shipped on ice to minimize the time between supercomplex isolation and Cryo-EM analysis. We will characterize new supercomplex samples by a combination of biophysical methods including DLS, SDS PAGE and mass spectrometry and verify sample homogeneity using negative stain EM. As soon as a homogenous sample prep has been achieved we will perform single particle analysis at our Titan Krios cryo-electron microscope. Dr. Yang will also optimize sample preparation including establishing preparation of Cry-EM grids to uniformly embed the supercomplex in a thin layer of vitrified ice to enable high resolution structure determination by Cryo-EM.

We anticipate that we will be able optimize the sample quality for cryo-EM studies to be able to collect the first full data sets at resolution of 3–5 Å using the cryo-EM Titan Kryos instrument at ASU in the next funding period. Dr. Yang will screen the grids and collect high resolution cryo-EM data using the Titan Krios instrument. This will allow us to extend the time of operation and collect a full first data sets of approximately 500,000 particles for image analysis at 3–5 Å.

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

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Overall research goals: During peak light intensity, around the solar meridian, downstream processes of CO_2 fixation become the limiting step in oxygenic photosynthesis, resulting in the backup of electrons on the acceptor side of Photosystem I (PS I). We have proposed two *in vivo* mechanisms involving orthogonal metabolic pathways that could take advantage of electrons locked out of these downstream processes for the productive generation of biofuels. Our research goals are as follows: (1) *In vivo* wiring of PS I to an [Fe-Fe] hydrogenase enzyme via a modified dicluster ferredoxin from *Clostridium pasteuranium* (CpFd) expressed in *Synechocccus sp*. PCC 7002; (2) Electron extraction through the A_{1A}/A_{1B} (phylloquinone) sites of PS I, by utilizing the *menB* deletion strain in *Synechocystis sp.* PCC 6803; and (3) Expressing an active and oxygen tolerant [Fe-Fe] hydrogenase enzyme in a cyanobacterium capable of being wired to PS I.

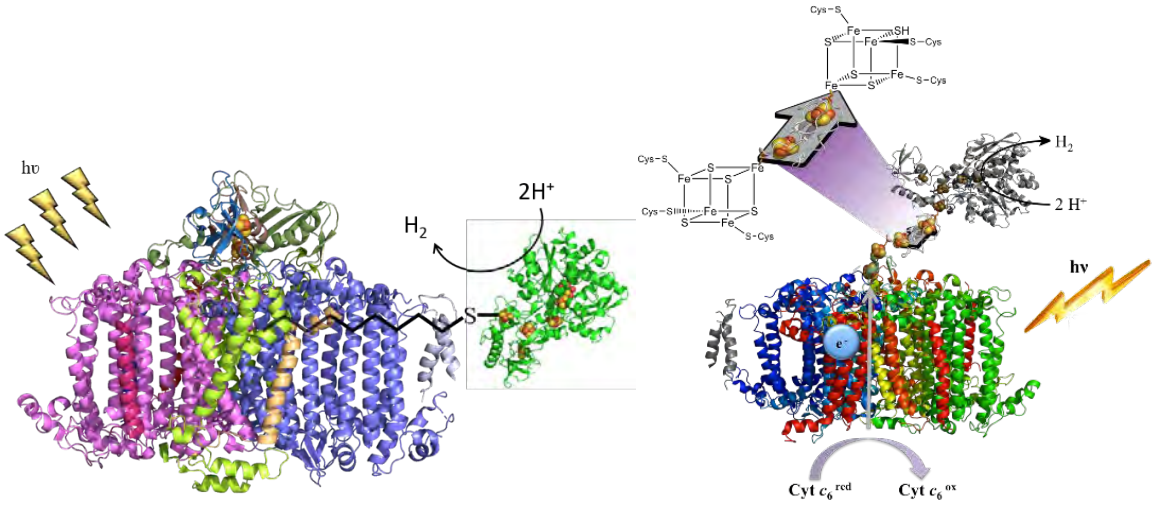

Figure 1. Depiction hydrogen generating nanoconstructs formed by PS I tethered to an [Fe-Fe] hydrogenase from *Clostridium acetobutylicum*. Left: Tethering through the A_1 (phylloquinone) site. Right: Tethering via a modified dicluster ferredoxin from *Clostridium pasteuranium*.

Significant achievements 2017-2019:

- While wiring PS I through a CpFd has been shown to generate dihydrogen *in vitro* when attached to a Pt-nanoparticle, we found that we could not generate the construct *in vivo* for two reasons: (i) The propensity of the necessary point mutation on PsaC (C14G) to rapidly form a pseudo-revertant on successive generations, and (ii) the toxicity of the *C. pasteuranium* ferredoxin variant when expressed in cyanobacteria.

- We have generated a PsaE-CpFd fusion protein to circumvent these issues. Preliminary EPR experiments show that both [4Fe-4S] clusters of the ferredoxin are able to assemble and show resonances similar to that of the native ferredoxin.
- We were successful at wiring PS I to an [Fe-Fe] hydrogenase from *C. acetobutylicum* through the A_{1A} and A_{1B} sites *in vitro*. The data obtained supports a model of electron transfer proposed previously, whereby the FeS clusters serve to 'store' the electron until the primary donor (P_{700}^{+}) can be reduced, thus defeating the recombination channel and allowing otherwise unfavorable electron transfer through the quinone wire to the catalyst.
- We have successfully expressed the oxygen-tolerant HydA protein from *C. beijerinckii* in *Synechocccus sp*. PCC 7002.

Science objectives for 2019-2020:

- The surface location of the FeS clusters in the PsaE-CpFd fusion protein indicates that the construct may be viable candidate to accept electrons from F_A/F_B. To that end, we will investigate its ability to bind to PS I as well as attempt *in vitro* wiring experiments with both a Pt-nanoparticle and an [Fe-Fe] hydrogenase from *C. acetobutylicum.*
- Our previous work provides supporting evidence that a successful wiring from A_{1A} and/or A_{1B} to an [Fe-Fe] hydrogenase is possible. To study this system *in vivo,* we will begin testing and optimizing conditions to incorporate the wiring quinone into growing cultures of the *menB* strain of *Synechocystis sp*. PCC 6803.
- Due to the successful expression of the HydA from *C. beijerinckii* in *Synechocccus sp*. PCC 7002, we are currently optimizing artificial maturation both *in vitro* and *in vivo* by addition of a synthetic H-cluster. Further, we will attempt to express this protein in the *ΔmenB* background strain of *Synechocystis sp*. PCC 6803.

References to work supported by this project 2017-2019:

1. M. Gorka, J. H. Golbeck, "Generating dihydrogen by tethering an [FeFe] hydrogenase via a molecular wire to the A_{1A}/A_{1B} Sites of Photosystem I." Photosyn Res (2019) (in review).
2. K. Walters, J. H. Golbeck, "Designing a modified Clostridial 2[4Fe-4S] ferredoxin as a redox coupler to directly link Photosystem I with a Pt nanparticle." Photosyn Res (2019) (in review).

A Novel Molecular Switch Controls Electron Flow Across the Cytochrome b_6f to Protect PSI from Photoinhibition

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Matthew C. Posewitz[b], Co-Investigator
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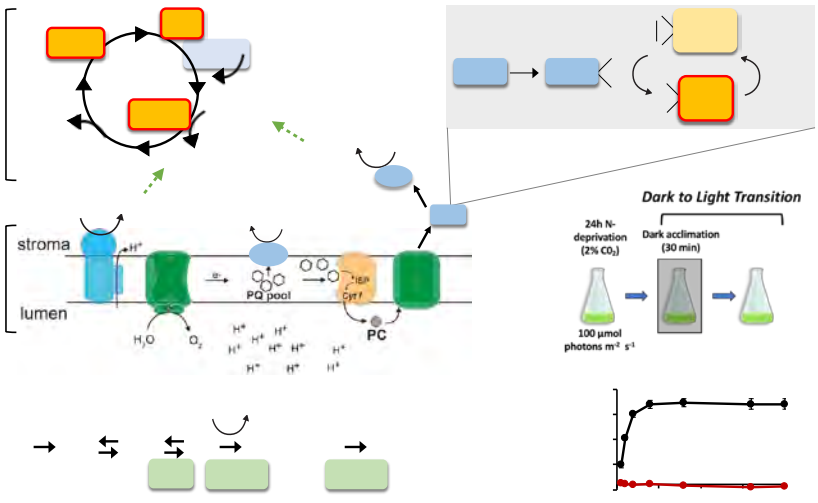
Overall Research Goals: Most forms of life on Earth cannot exist without photosynthesis; our food and atmosphere depend on it. To obtain high photosynthetic yields, light energy must be efficiently coupled to the fixation of CO_2 into organic molecules. However, photosynthetic organisms frequently experience abiotic stresses that restrict their growth and development. Under such circumstances, most absorbed solar energy cannot be used for CO_2 fixation and can cause photoproduction of toxic reactive oxygen species, damage to photosynthetic reaction centers (photosystems I and II) and a resulting decline in primary productivity. We are studying mechanisms that enable photosynthetic organisms to avoid photodamage and loss of photosynthetic function by initiating quenching mechanisms and altering electron flow, especially when there is a limitation for acceptors that are downstream of the electron transport system.

Significant achievements 2017-2019: We examined electron transport in a *sta6* mutant (unable to synthesize starch) of *Chlamydomonas reinhardtii* that was also exposed to nitrogen deprivation. In a dark to light transition, we observed a novel molecular 'switch' that caused a reversible block in photosynthetic electron transport (PET) at the cytochrome b_6f complex when reductant and ATP generated by PET markedly exceeded the capacity of downstream carbon metabolism to utilize these products. When the switch is 'on', electron flow to PSI is restricted, which prevents PSI photodamage, and the plastid alternative oxidase (PTOX) becomes fully activated, serving as an electron valve that dissipates excitation energy absorbed by PSII, thereby lessening PSII photoinhibition. Elucidating this photoprotective mechanism offers new directions for modifying photosynthesis and increasing agricultural yields to satisfy growing global food and energy needs.



A schematic showing the integration of PET with the Calvin-Benson-Bassham cycle (CBBC), redox control through ferredoxin (FDX) and thioredoxin (TRX) systems, starch metabolism, the design of the initial experiments with the *sta6* and C6 (complemented) strains, and oxygen evolution in those strains is given in **Fig. 1**.

Fig. 1: PET, starch synthesis, experimental design and loss of net O_2 evolution following N deprivation in the *sta6* mutant: **A**. Schematic of link between PET and downstream C metabolism (CBBC; upper left). CBBC utilizes ATP and NADPH (green lettering and dashed green arrows) to fix CO_2 by Rubisco (light blue box). Several CBBC enzymes are activated by redox control (highlighted in orange boxes-red frames). Gray box: Redox activation of targeted CBBC enzymes by reduction of specific disulfide bonds mediated by the FDX and TRX systems. **B**. Starch biosynthesis: Metabolites in black lettering and enzymes highlighted by green rectangles. **C**. Experimental design: Cells were cultured in high CO_2 for 24 h in medium devoid of N, acclimated for 30 min in the dark, and exposed to light. **D**. Net O_2 evolution of N-deprived C6 (complemented *sta6*, black curve) and *sta6* (red curve). N=3 ± SE.

Science objectives for 2019-2020:

- Continue to examine the role of alternative electron valves in quenching.
- Study the integration among quenching processes and the environmental factors that control those processes.
- Explore signaling processes that activate alternative electron transport.

References to work supported by this project 2018-2019:

1. Kaye, Y., Huang, W., Saroussi, S., Idoine, A., Clowez, S., Sanz-Luque, E., **Grossman**, A.R. (2019) *Chlamydomonas reinhardtii* Mitochondrial Alternative Oxidases Allows Survival in High *Light. J Biol Chem*. 294(4):1380-1395. doi: 10.1074/jbc.RA118.004667.
2. Saroussi, S., Karns, D., Thomas, D., Posewitz, M., Grossman, A.R. (2019) Alternative outlets for sustaining photosynthetic electron transport during dark to light transitions. *Proc Natl Acad Sci USA*. 201903185 DOI: 10.1073/pnas.1903185116.
3. Saroussi, S., Karns, D., Thomas, D., Posewitz, M., Grossman, A.R. (2019) Electron flow in *Chlamydomonas reinhardtii* in the absence of major energy sinks and reversible inactivation of cytochrome b_6f. In revision.
4. Sanz-Luque, E., Saroussi, S., Grossman, A.R. (2019) Metabolic Control Dependent on Polyphosphate Synthesis is Required for Nutrient Deprivation Responses. In Submission.
5. Volger, B. W., Posewitz, M. (2019) CRISPR/Cas9 disruption of glucan synthase in *Nannochloropsis gaditana* attenuates accumulation of β-1,3-glucose oligomers. Algal Research. In Submission.

Role of protons and substrate waters near the OEC of Photosystem II

Marilyn R Gunner, City College of New York, Principal Investigator
Victor S Batista, Gary W Brudvig, Yale University Co-PIs

Collaborators: Jimin Wang, Yale University; Muhamed Amin, University of Groningen

Divya K Matta, City College of New York (CUNY), Krystle M Reiss and Ke Yang (Yale)

Overall research goals: The biological oxidation of water to O_2 takes place at room temperature, at neutral pH using earth abundant elements in the Oxygen Evolving Complex (OEC) of Photosystem II (PSII), in higher plants, algae and cyanobacteria[1]. The OEC is a $CaMn_4O_5$ inorganic complex, found on the lumen (low pH) side of PSII. This reaction requires the loss of four electrons and four protons, which occurs in four steps of OEC oxidation, between the 5 S-states. We use a mixture of computational techniques including Molecular Dynamics, Monte Carlo sampling, DFT and network analysis to understand the thermodynamics and kinetics of oxygen evolution including the shifts in protonation states, changes in the OEC structure and the role of the protein in modulating the electrochemistry, anchoring the OEC, and providing pathways for proton removal.

Hydrogen bond networks near the OEC. Amino acid residues and waters form water channels around the OEC that facilitate proton release the entry of substrate water and perhaps the pathway for O_2 release. There are three established water channels namely narrow, large and broad channel.[2] Different roles have been proposed for each, which may depend on where the reaction is in the Kok cycle. Combined MD and MC analysis allow us to distinguish the role of the different channels for substrate (H_2O) and product (O_2 and H^+) transport in each S-state. Water and residue hydrogen-bond networks were compared in wild-type PSII.[2] Long range (>20 Å) highly interconnected networks are seen from the OEC to the lumen of PSII. A surprising conclusion is that all the channels are highly interconnected near the OEC. The eigen vector centrality[3] (EC) analysis is applied to the water network dynamics, showing that the large channel has highest flexibility while narrow and broad channel are more ordered. Companion experimental studies (Brudvig) studied O_2 evolution in wild-type and mutant PSII to understand the substrate water delivery upon S-state transition.[4,5]

Protonation of waters and protein in the S_1 and S_2 states. An open question is what are the protonation states of the water ligands to the OEC in the different S-states. A classical MCCE analysis of the protonation state changes of the protein and OEC suggested that the W2 ligand to Mn4 would be deprotonated when the Mn in oxidized[6] . A DFT modelling of hyperfine coupling constants (K.V. Lakshmi) strongly suggests a different model where W1 is deprotonated and D61 protonated in the S_2 state. MCCE calculations also finds with a shortened distance between these two groups ionization of W1, the proton trapped on the Asp, is preferred over ionization of W2. MCCE studies are done to analyse the protonation states of residues in the apo crystal structure[7]. The results were compared with the cluster removed S_1 state and showed similar changes in both the structures.

Analysis of structures. Amin used MC methods to sample positions for insertion of waters into the OEC in the S_3 state. DFT is then used to optimize the structure. These studies suggest the movements of ligands coupled to the water insertion seen in XFEL studies.[8] Wang has

reanalysed X-ray crystallography and cryo-EM data that suggest new buried ion binding sites near OEC (paper in revision).

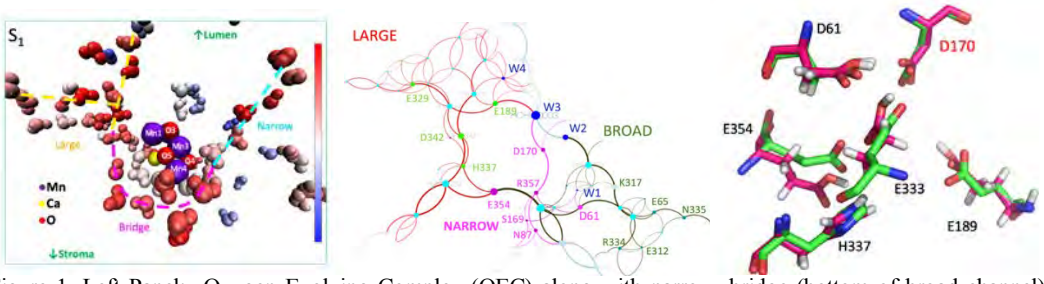


Figure 1. Left Panel: Oxygen Evolving Complex (OEC) along with narrow, bridge (bottom of broad channel) and large channel residues with water[3]. Middle Panel: Hydrogen bond network connections for the residues around OEC with water[2]. Right Panel: Protonation states changes of residues[7] in apo crystal structure (A) and cluster removed S_1 structure.

Science objectives for 2019-2020:

- Characterizing the dynamics of the pathways for substrate and product through the S-state cycle in wild-type and mutated proteins. Determine the relative free energy for transfer of H_2O, O_2 and H^+ in each path.
- Combine different computational methods to analyze the site for proton loss and water insertion through the S-state cycle.
- Analyze the order of Mn addition to the apo-protein.

We would like to acknowledge financial support from the Division of Chemical Sciences, Geosciences, and Biosciences, Office of Basic Energy Sciences, U.S. Department of Energy (DE-SC0001423).

Recent References to work supported by this project:

(1) Brudvig, G. W. Water Oxidation Chemistry of Photosystem II. *Philos Trans R Soc Lond B Biol Sci* **2008**, *363* (1494), 1211–1219. https://doi.org/10.1098/rstb.2007.2217.

(2) Kaur, D.; Cai, X.; Khaniya, U.; Zhang, Y.; Mao, J.; Mandal, M.; Gunner, M. R. Tracing the Pathways of Waters and Protons in Photosystem II and Cytochrome c Oxidase. *Inorganics* **2019**, *7* (2), 14. https://doi.org/10.3390/inorganics7020014.

(3) Reiss, K.; Morzan, U. N.; Grigas, A. T.; Batista, V. S. Water Network Dynamics next to the Oxygen-Evolving Complex of Photosystem II. *Inorganics* **2019**, *7* (3), 39. https://doi.org/10.3390/inorganics7030039.

(4) Ghosh, I.; Banerjee, G.; Kim, C. J.; Reiss, K.; Batista, V. S.; Debus, R. J.; Brudvig, G. W. D1-S169A Substitution of Photosystem II Perturbs Water Oxidation. *Biochemistry* **2019**, *58* (10), 1379–1387. https://doi.org/10.1021/acs.biochem.8b01184.

(5) Ghosh, I.; Khan, S.; Banerjee, G.; Dziarski, A.; Vinyard, D. J.; Debus, R. J.; Brudvig, G. W. Insights into Proton-Transfer Pathways during Water Oxidation in Photosystem II. *J Phys Chem B* **2019**. https://doi.org/10.1021/acs.jpcb.9b06244.

(6) Kaur, D.; Szejgis, W.; Mao, J.; Amin, M.; Reiss, K. M.; Askerka, M.; Cai, X.; Khaniya, U.; Zhang, Y.; Brudvig, G. W.; et al. Relative Stability of the S_2 Isomers of the Oxygen Evolving Complex of Photosystem II. *Photosyn. Res.* **2019**, *141* (3), 331–341. https://doi.org/10.1007/s11120-019-00637-6.

(7) Vinyard, D. J.; Badshah, S. L.; Riggio, M. R.; Kaur, D.; Fanguy, A. R.; Gunner, M. R. Photosystem II Oxygen-Evolving Complex Photoassembly Displays an Inverse H/D Solvent Isotope Effect under Chloride-Limiting Conditions. *Proc. Natl. Acad. Sci. U.S.A.* **2019**, *116* (38), 18917–18922. https://doi.org/10.1073/pnas.1910231116.

(8) Amin, M.; Kaur, D.; Yang, K. R.; Wang, J.; Mohamed, Z.; Brudvig, G. W.; Gunner, M. R.; Batista, V. Thermodynamics of the S_2 to S_3 State Transition of the Oxygen-Evolving Complex of Photosystem II. *Phys Chem Chem Phys* **2019**, *21* (37), 20840–20848. https://doi.org/10.1039/c9cp02308a.

Photophosphorylation requires thiol-disulfide transactions at the thylakoid membrane

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Overall research goals: The long-term goal of this research is to detail thiol-disulfide transactions at the thylakoid membrane, a specialized membrane involved in transducing energy via photophosphorylation. The molecular identity of the redox players mediating thiol-disulfide exchanges, their relevant targets of action *in vivo* and how thiol-disulfide chemistry in general controls photosynthesis are the experimental questions we are currently pursuing. Through previous work, we documented the requirement of catalyzed disulfide formation and reduction for the biogenesis of two photosynthetic enzymes, namely Photosystem II and the cytochrome b_6f complex. We established that dedicated trans-thylakoid pathways operate in these processes by delivering reducing and oxidizing power to cysteine-containing subunits of the photosynthetic complexes, which are localized in the lumen compartment.

Significant achievements: Cytochrome *f*, a hemoprotein (classified as a *c*-type cytochrome) and structural subunit of the b_6f complex undergoes several essential steps for conversion of apo to holoform. One step is the covalent attachment of heme to the sulfhydryl (-SH) groups of the C*XX*CH motif, which requires the heme-linking cysteines of apocytochrome *f* to be maintained in a reduced state (providing free -SH), by the operation of a disulfide – reducing pathway at the thylakoid membrane (**Fig. 1**). Two components of the disulfide reducing pathway, CCDA and CCS5, are proposed to transfer electrons, sequentially from stroma to the thylakoid lumen, to reduce disulfide bonded apocytochrome *f* in the lumen (**Fig. 1**). CCS4 is a thylakoid-bound protein with a stroma-facing C-terminal domain highly enriched in charged residues (20% of the protein sequence). The biochemical activity of CCS4 is obscure as the protein lacks any known motifs suggestive of function. In the green lineage, CCS4-like proteins display very little sequence similarity but are all characterized by a predicted membrane anchor and a hydrophilic domain whose total number of charged residues appears to be conserved. CCS4 and CCS5 were identified in *Chlamydomonas reinhardtii,* and a *ccs4*-null or *ccs5*-null mutant is partially deficient for photosynthesis and accumulates decreased levels of holocytochrome *f.* A *ccs4 ccs5* double mutant exhibits a synthetic photosynthetic defect characterized by a complete block in holocytochrome *f* assembly, which can be rescued by application of exogenous thiols. This indicates that CCS5 and CCS4 are operating in independent pathways controlling the reduction of disulfide-bonded apocytochrome *f*. We evidence that the level of the CCDA protein is partially decreased in a *ccs4* mutant but not in the *ccs5* mutant, suggesting CCS4 is required to stabilize CCDA at the thylakoid membrane. Consistently, overexpression of *CCDA* in the *ccs4* mutant rescues the photosynthetic deficiency. In two *ccs4*-null *ccs5*-null strains in which holocytochrome *f* assembly and photosynthetic growth are restored, sequence analysis of the *CCS4* gene revealed that the original non-sense allele is no longer present due to the presence of an in-frame deletion or a change to a missense mutation, enabling to by-pass the requirement for CCS5. Hence, we postulate that CCS4, in addition to its function in stabilizing CCDA, also operates in an unknown trans- thylakoid pathway distinct from the known CCDA/CCS5 disulfide – reducing pathway (**Fig. 1**). The source of reducing power and the mechanism by

which it is transduced across the thylakoid membrane via the CCS4-dependent route remain currently unknown.

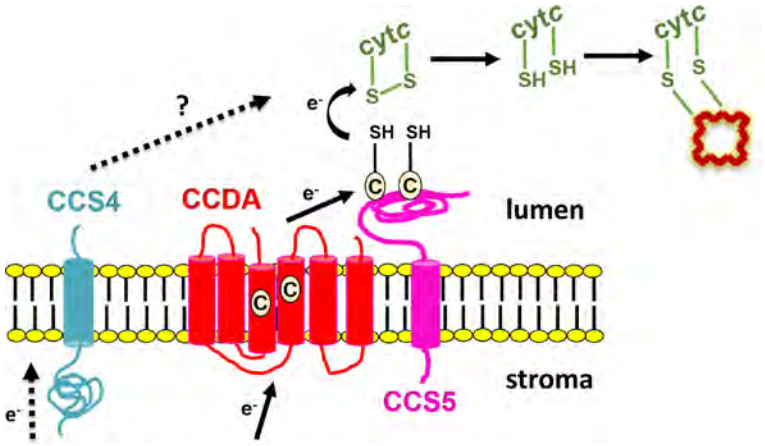

Fig.1. Trans-thylakoid disulfide-reducing pathways. *CCDA and CCS5 define one route for delivering of reducing power (via thiol-disulfide exchange involving conserved cysteines) across the thylakoid membrane. CCS4 is a component of a second pathway for provision of reductants. CCS5 is a membrane anchored lumen-facing thioredoxin-like protein, which is postulated to be reduced by CCDA. Stromal Trx-m is presumed to be the electron donor to CCDA. Operation of these pathways is required to counter the oxidation of the heme-linking cysteines of apocytochrome f. Provision of free SH in apocytochrome f CXXCH heme binding motif is essential for heme attachment.*

Science objectives for 2019-2020:

Our genetic dissection supports the operation of a CCDA-dependent and a CCDA-independent trans-thylakoid pathways, partially redundant and required for the provision of reducing equivalents to the lumen (**Fig 1**). The proposed model is that operation of these pathways is necessary to counter sulfhydryl oxidation of the heme-linking cysteines in apocytochrome *f*, a biochemical requirement for the heme ligation reaction in the lumen. While the CCDA-dependent pathway is presumed to catalyze a cascade of specific, sequentially arranged thiol-disulfide exchanges from stroma to lumen, *in vivo* biochemical proof was never substantiated. We will elaborate the biochemical activity of the trans-thylakoid reducing pathways *in vivo* using thiol-labelling a) by testing if apocytochrome *f* heme binding site is in the oxidized form when the reducing pathways are no longer operating and b) by determining the redox state of active cysteines in CCDA and CCS5 to establish the sequence of thiol-disulfide exchanges.

References to work supported by this project:

(1) Cline, S.G., Laughenbaum, I.A. and Hamel, P. (2017) CCS2, an octatricopeptide-repeat protein is required for plastid cytochrome *c* assembly in the green alga *Chlamydomonas reinhardtii Front Plant Sci.* 8:1306. doi: 10.3389/fpls.2017.01306. eCollection 2017

(2) Gabilly, S. and Hamel, P. (2017) Plastid cytochrome *c* maturation. *Front Plant Sci.* 8:1313. doi: 10.3389/fpls.2017.01313. eCollection 2017

(3) Cline, S., Gabilly, S., Subrahmanian, N. and Hamel, P. (2016) Co-factor assembly of bc_1 and b_6f complexes in *Cytochrome Complexes: Evolution, Structures, Energy Transduction, and Signaling* (Eds. T. Kallas and W. Cramer, Publisher: Springer)"

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: Experimentally the main objective is to use time resolved visible and infrared difference spectroscopies to study photosystem I (PSI) with a series of substituted benzoquinones (BQs) and naphthoquinones (NQs) incorporated into the A_1 binding site.

For time-resolved infrared spectroscopy studies the goal is to undertake work at 77 K, with microsecond time resolution. For time-resolved visible spectroscopy the objective is to undertake work at both 298 and 77 K, on timescales from femtoseconds to seconds.

Computationally the main objective is to develop QM/MM methods in order to adequately simulate *all* of the time-resolved infrared difference spectra within the context of a single molecular model. A secondary objective is to develop a more robust set of methods for modeling the bioenergetics in PSI with native and foreign quinones incorporated, at both 298 and 77 K.

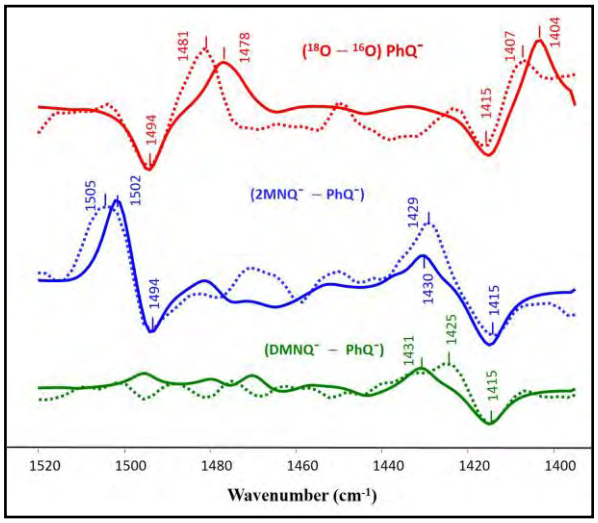

Figure 1. QM/MM calculated (^{18}O – ^{16}O) (*red*), ($2MNQ^-$ – PhQ^-) (*blue*) and ($DMNQ^-$ – PhQ^-) (*green*) IR double difference spectra (DDS). Corresponding experimental IR DDS are also shown (*dotted*). Bands of PhQ^- are negative while bands of the other semiquinones are positive. Figure is from reference 1.[1]

Significant achievements 2018-2019: We have studied PSI with several different NQs[1] and BQs[2,3] incorporated into the A_1 binding site. For the incorporated BQs we have established conditions under which they will and will not be functional in electron transfer (ET). BQ protonation is proposed to explain the loss of functionality.[2] For PSI with non-functional BQs incorporated, the P700 triplet state (^{3}P700) can be photo-generated, and we have studied ^{3}P700 in some detail, in unlabeled and fully ^{13}C labeled PSI complexes.[4] For PSI with BQs incorporated the $P700^+A_1^-$ lifetime at 77 K is longer than expected, indicating that the ET parameters (reorganization energy, driving force, etc.) for the incorporated BQs are different compared to that of the NQs.

Recently we have used femtosecond time-resolved spectroscopy to probe forward ET in native PSI at 77 K.[5] Femtosecond time-resolved studies of PSI samples mostly focus on probing antenna energy transfer processes, and little work has been done to probe the A_0^- to A_1 ET.

We have obtained ($P700^+$ – P700) FTIR difference spectra for cyanobacterial PSI samples grown under far red light (λ>740 nm). The spectra indicate that the A_{-1} pigment in these samples is changed from chlorophyll *a* to chlorophyll *f*.[6]

In the last year we have tested several molecular models in QM/MM type (ONIOM) vibrational frequency calculations.[1,7,8] We have established that one particular molecular model, and associated computational method, works particularly well in that it can predict the positions and intensities of bands in multiple spectra simultaneously (Fig. 1).[1] By comparing calculated and experimental spectra

we also showed that the orientation of the asymmetrically substituted NQs in the A_1 binding site could be determined.[1] The conformation of substituted NQ side-chains could also be established.

Science objectives for 2019-2020:

- Incorporate a wide range of substituted NQs and BQs into the A_1 binding site in PSI, and study using time resolved FTIR difference spectroscopy. This quinones incorporated will include a series of halogenated NQs and BQs, as well as NQs with acetoxy and hydroxyl substitutions.
- Undertake calculations in order to interpret FTIR difference spectra in the anion region, obtained using PSI with this wider range of NQs incorporated.
- Previous work has focused on the study of A_1^-, the quinone anion radical (Fig. 1). To identify neutral state quinone bands, PSI with a wider series of substituted NQs incorporated will be studied. With knowledge of the frequencies associated with neutral and anion state quinone bands we will assess radical induced alterations in hydrogen bonding to the incorporated quinones.
- Computational work will be undertaken in order to simulate spectra associated with neutral quinones in the A_1 binding site. This work will be compared to corresponding computational work for the same neutral NQs in the Q_A binding site in purple bacterial reaction centers. Similarly, we will undertake computational work on NQ anions in the Q_A binding site, and compare to corresponding work for the same NQ anions in the A_1 binding site.
- Develop more robust methods for modeling the bioenergetics in PSI.[9] Then, establish parameters associated with ET in PSI with different BQs incorporated. Compare these to calculated parameters obtained for PSI with NQs incorporated.
- Use femtosecond time-resolved spectroscopy at both 298 and 77 K to study PSI with a variety of NQs incorporated into the A_1 binding site. This will allow us to more fully probe the nature of the A_0^- to A_1 ET process in PSI.

References to work supported by this project 2018-2019:

1 Rohani, L., Makita, H., Levitz, A., Henary, M. & Hastings, G. Calculated vibrational properties of semiquinones in the A_1 binding site in photosystem I. *Biochim. et Biophys. Acta-Bioenergetics* **1860**, 699-707, (2019).

2 Makita, H. & Hastings, G. Photosystem I with benzoquinone analogues incorporated into the A_1 binding site. *Photosynthesis Research* **137**, 85-93, (2018).

3 Makita, H. & Hastings, G. Time-resolved step-scan FTIR difference spectroscopy for the study of photosystem I with different benzoquinones incorporated into the A_1 binding site. *Biochim. et Biophys. Acta - Bioenergetics* **1859**, 1199-1206, (2018).

4 Makita, H. & Hastings, G. Time-Resolved Step-Scan FTIR Difference Spectroscopy for the Study of the P700 Triplet State in Photosystem I. *Front. Sci. Technol. Eng. Math.* **3**, in press, (2019).

5 Hastings, G., Makita, H., Shenje, L. & Ullrich, S. Femtosecond Time-resolved Spectroscopy for the Study of Photosystem I Reaction Centers at 77 K. *Front. Sci. Technol. Eng. Math.* **3**, in press, (2019).

6 Hastings, G., Makita, H., Agarwala, N., Rohani, L., Shen, G. Z. & Bryant, D. A. Fourier transform visible and infrared difference spectroscopy for the study of P700 in photosystem I from Fischerella thermalis PCC 7521 cells grown under white light and far-red light: Evidence that the A_{-1} cofactor is chlorophyll *f*. *Biochim. et Biophys. Acta - Bioenergetics* **1860**, 452-460, (2019).

7 Rohani, L. & Hastings, G. Vibrational Frequency Calculations of Phylloquinone in the A_1 Binding Site: Layer Selection in ONIOM Methods. *Front. Sci. Technol. Eng. Math.* **2**, 130-137, (2018).

8 Rohani, L. & Hastings, G. Vibrational Properties of Quinones in the A_1 Binding Site of Photosystem I Calculated Using a Three-Layer ONIOM Method. *Front. Sci. Technol. Eng. Math.* **3**, in press, (2019).

9 Santabarbara, S., Casazza, A. P. & Hastings, G. Modelling electron transfer in photosystem I: limits and perspectives. *Physiologia plantarum* **166**, 73-87, (2019).

Protein Targeting to the Chloroplast Thylakoid Membrane: Structure and Function of a Targeting Complex

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Overall research goals: The overall research goal is to elucidate the mechanistic basis of how light harvesting complex (LHC) proteins are vectorially targeted to and inserted into the thylakoid membrane. Our central hypothesis is that vectorial targeting of LHCs 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 for transfer to Alb3. Using this working hypothesis, the following specific aims were developed in this award period towards reaching the overall research goal.

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.

Significant achievements 2017-2019:

As part of aims 1 and 2, we fluorescently labeled cpSRP43 and cpSRP54 and isolated the FRET-labeled heterdimeric cpSRP complex and the cpSRP-LHC transit complex. We used single molecule FRET to identify changes in structure of the cpSRP heterodimer upon interaction with the LHC substrate as the transit complex is formed. The structural changes measured by single molecule FRET were supported by all-atom molecular dynamics (MD) simulations of the cpSRP43 and cpSRP54 proteins.

The Alb3-Cterm tail has been reported to be intrinsically disordered. We identified a site in the N-terminal portion of the Alb3-Cterm tail that exhibits secondary structure. Single molecule FRET and CD experiments showed that this secondary structure can be disturbed by chemical denaturant, temperature and site-directed mutation. The existence of secondary structure was also supported by all atom MD simulations and verified that the observed smFRET distances were in agreement with those expected from the secondary structure.

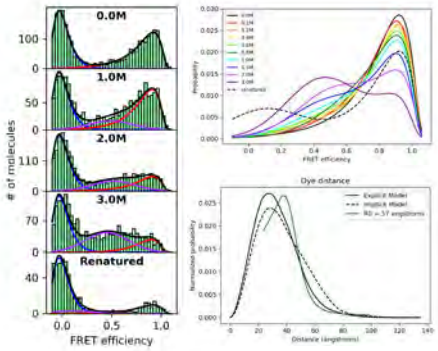

Figure 1 – left: smFRET histograms of Alb3 C-term as a function of denaturant concentration, top right: overlay of multiple fitted curves of smFRET histograms of Alb3 C-term as a function of denaturant concentration. Bottom right: comparison of experimental smFRET curves (green, converted to interdye distance) and MD simulations using explicit water (solid black) and implicit (dashed black) water.

As part of Aim 3, we hypothesized that the secondary structure in the Lab3 C-term plays an important role in the interaction with cpSRP43, and subsequently the insertion of LHC into the thylakoid membrane. To test this hypothesis, we designed several site-specific mutants of Alb3 C-term that were hypothesized to disturb the secondary structure and measured the changes in their CD structure. We also designed site-specific mutants of Alb3 C-term to fluorescently label and measure the smFRET histograms in the absence and presence of cpSRP43.

Science objectives for 2019-2020:

- We will express and purify the designed smFRET mutants and measure the smFRET histograms in the absence and presence of cpSRP43 to determine changes in secondary structure in Alb3 C-term upon binding cpSRP43. Several such mutants have been designed to more specifically localize the regions in Alb3 C-term that are affected by interaction with cpSRP43.
- In order to relate the observed Alb3 C-term secondary structure to function, we will perform functional assays such as pull-down assays to measure if the changes in secondary structure affect the efficiency of binding to cpSRP43
- There is debate in the literature as to the domains on cpSRP43 that interact with Alb3 C-term (Ankyrin repeat region vs CD3). In order to identify these interaction regions more specifically, we will label both cpSRP43 in different domains and Alb3 C-term, isolate their complex and measure the single molecule FRET histograms to determine interdomain distances. These data will be compared to all atom MD simulations to propose structural models for the Alb3 Cterm-cpSPR43 complex.
- The ^{1}H-^{15}N HSQC 2D-NMR spectrum of Alb3-Cterm has been measured. We also plan to measure changes in this NMR spectrum upon adding cpSRP43 and the CD3-deleted mutant of cpSRP43 to determine if Alb3 C-term interacts with the CD3 domain of cpSRP43 or the Ankyrin repeat region, and to more specifically identify which residues on Alb3 C-term are involved in the interaction.

MSU-DOE Plant Research Laboratory: Subproject B, Integrating Energy Supply and Demand in the Biological Solar Panel

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Overall research goals: This multi-investigator project (Subproject B) in the Plant Research Laboratory focuses on understanding photosynthesis as set of highly integrated processes, including photosynthetic membrane homeostasis, assimilation of CO_2 by rubisco with competing photorespiratory reactions, export of reduced carbon intermediates from the Calvin-Benson cycle, and final partitioning of photosynthetic products to various metabolic sinks. Our research team works collaboratively across disciplines to answer fundamental photosynthesis-related questions that cannot be easily addressed by any one laboratory alone. Here, we highlight recent progress in understanding how photosynthetic organisms sense and respond to shifts in carbon partitioning, and how alterations in sink demand influence the activity and composition of the photosynthetic apparatus. The overall goal is to leverage powerful genetic tools in Arabidopsis to understand how the partitioning of photoassimilates is regulated during transitions from growth to defense.

Significant achievements: 2017-2019

- In targeting the JAZ family of transcriptional repressors in Arabidopsis, we constructed a series of constitutive jasmonate response mutants with increased levels of resource partitioning to defense compounds. These higher-order *jaz* mutants were used to study the relationship between carbon resource management, growth, and photosynthesis.
- Comparisons of growth and defense phenotypes in wild-type plants to a *jaz* quintuple mutant (*jazQ*, lacking *JAZ1/3/4/9/10*) and a *jaz* decuple mutant (*jazD*, lacking *JAZ1/2/3/4/5/6/7/9/10/13*) showed that increasing levels of defense are associated with graded reduction in growth rate and seed yield. Strong partitioning of primary metabolites to defense in *jazD* leaves was also associated with increased rates of respiration, decreased abundance of photosynthesis proteins, and symptoms of carbon starvation.
- Many phenotypes of *jaz* mutants can be attributed to changes in chloroplast metabolic pathways that fuel defense, including amino acid metabolism, nitrogen and sulfur acquisition, transport processes, and photosynthetic metabolism. Consistent with this view, MYC2/3/4 transcription factors were identified as negative regulators of photosynthesis; mutations in *MYC* genes resulted in increased photosynthesis and growth.
- Depletion of the remaining pool of JAZ repressors in *jazD*, using either chemical or genetic approaches, resulted in near complete loss of seed production and, under some conditions, lethality. This hyperactivation of jasmonate response was associated with rapid chlorosis and loss of photosynthetic capacity.
- *jazD* was used as a parent for a genetic suppressor screen to identify "uncoupled" mutants that grow and defend well at the same time. Among 13 independent suppressor of *jazD* (*sjd*) mutants identified, nine had mutations in the *PHYB* gene encoding the red light receptor, phytochrome B. One non-*phyB sjd* mutant exhibiting partial uncoupling of growth-defense tradeoffs was sequenced to identify the underlying genetic lesion.

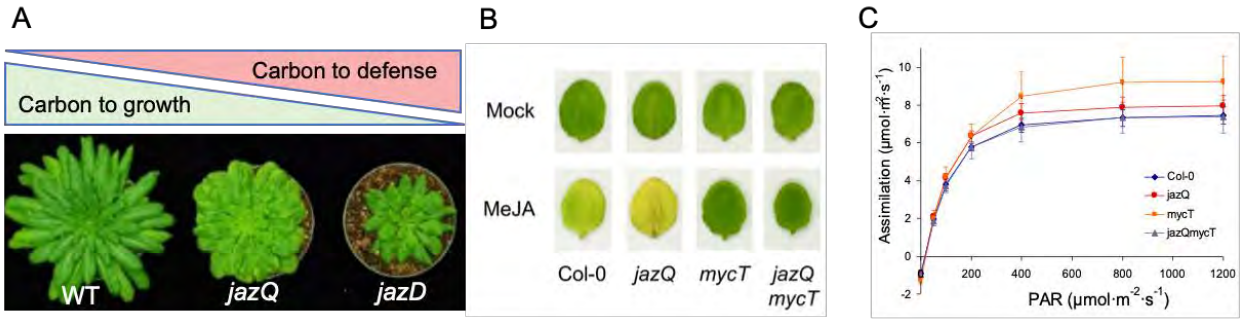

Figure 1. (A) Genetic "tuning" of photoassimilate partitioning to growth and defense processes through mutation of JAZ repressors in Arabidopsis. WT, wild-type; *jazQ*, *jaz* quintuple mutant; *jazD*, *jaz* decuple mutant. (B) MYC factors promote chlorophyll degradation in response to jasmonate. Leaves of the indicated genotype were treated with methyl jasmonate (MeJA) and photographed four later. *mycT* is a triple mutant lacking *MYC2/3/4*. (C) Light response curve of CO_2 assimilation rate in various genotypes. *mycT* plants have modestly increased photosynthetic capacity.

Specific objectives for 2019-2020:

1. Investigate the mechanisms by which altered metabolic sink demand for defense compounds modulates photosynthetic processes.

2. Investigate the hypothesis that strong partitioning of photoassimilates to defense activates carbon starvation-response pathways.

3. Characterize additional *sjd* suppressor mutants to understand the genetic and physiological basis of growth-defense tradeoffs.

4. Use jasmonate hypersensitive mutants of Arabidopsis as tools to understand the process by which stress triggers chlorophyll loss and disassembly of thylakoid membranes.

References to work supported by this project 2017-2019:

1. 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.
2. Guo Q, Major IT, Howe GA. 2018. Resolution of growth-defense conflict: mechanistic insights from jasmonate signaling. *Current Opinion in Plant Biology* 44: 72-81.
3. Guo Q, Yoshida Y, Major IT, Wang K, Sugimoto K, Kapali G, Havko NE, Benning C, Howe GA. 2018. JAZ repressors of metabolic defense promote growth and reproductive fitness in Arabidopsis. *Proceedings of the National Academy of Sciences USA* 115: E10768-E10777.
4. Weraduwage S.M., Campos M.L., Yoshida Y., Major I., Kim Y-S., Kim S-J., Renna L., Anozie F.C., Brandizzi F., Thomashow M.F., Howe G.A., Sharkey T.D. (2018) Molecular Mechanisms Determining Leaf Architecture. In: Adams III W. W., Terashima I. (eds), The Leaf: A Platform for Performing Photosynthesis. Advances in Photosynthesis and Respiration (Including Bioenergy and Related Processes), vol 44. Springer, Dordrecht

MSU-DOE Plant Research Laboratory Subproject C: Characterizing and Engineering Subcellular and Cellular Modules for Photosynthetic Productivity

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Overall research goals: Our overarching goal is to gain 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. We have focused on the structure, function, and interconnectivity of two cyanobacterial modules: light harvesting and the carboxysome. These modules are prominent components of cyanobacterial photosynthesis and possess features broadly useful for bioengineering applications. Co-PI Kerfeld will present results and objectives related to the carboxysome. The full list of SubProject C publications for 2018-2019 is given below

Significant achievements 2018-2019:

- We have identified and characterized a cyanobacterial rubisco activase homolog that is localized to the carboxysome, and causes an ATP-dependent coalescence of rubisco (1).
- We have bioinformatically identified new families of carboxysome shell proteins and experimentally shown that shell protein paralogs can form hetero-oligomers, increasing the range of permeability properties of carboxysome shell proteins (2).
- In collaboration with Dr. Karen Davies of LBNL, we have determined the structure of a synthetic carboxysome shell (3).
- We have identified a novel protein family that associates with the carboxysome shell and which is involved in the subcellular positioning of this compartment through a Brownian-Ratchet mechanism (12)

Science objectives for 2019-2020:

- Identify the mechanism of ATP transit across the carboxysome shell
- Learn the primary function of Activase-Like protein of cyanobacteria
- Develop methods for the direct measurements of gas and metabolite permeability across carboxysome shells
- Begin to characterize the mechanism of electron transfer between the shell and the lumen in a model system.

References to work supported by this project 2018-2019:

1. Lechno-Yossef, S., Rohnke, B.A., Belza, A.C.O., Melnicki, M.R., Montgomery, B.L., and Kerfeld, C.A. Cyanobacterial Carboxysomes Contain a Unique Rubisco-Activase-Like protein. New Phytologist, in press.

2. Sommer, M. Sutter, M., Gupta, S., Turmo, A., Burton, R.L., Saechao, C., Chan, L-J.G., Petzold, C.J., Ralston, C.Y., and Kerfeld, C.A. Heterohexamers Formed by CcmK3 and CcmK4 Increase the Complexity of Beta-Carboxysome Shells. Plant Physiology, in press. doi: 10.1104/pp.18.01190.
3. Sutter, M., Laughlin, T.G., Sloan, N.B., Serwas, D., Davies, K.M. and Kerfeld, C. A. Structure of a Synthetic beta-carboxysome shell. Plant Physiology, in press.
4. Greber, B.J., Sutter, M and Kerfeld, C.A. The plasticity of molecular interactions governs bacterial microcompartment shell assembly. Structure 7(5):749-763, 2019.
5. Ferlez, B., Sutter, M., Kerfeld, C.A. Glycyl radical enzyme-associated microcompartments: redox replete bacterial organelles. mBio 10: e02327-18, 2019.
6. Dominguez-Martin, M.A., Polivka, T., Sutter, M., Ferlez, B., Lechno-Yossef, S., Montgomery, B.L. and Kerfeld, C.A. Structural and spectroscopic characterization of HCP2. Biochmica Biophysica Acta 1860:414-424, 2019.
7. Dominguez-Martin, M.A. and Kerfeld, C.A. Engineering the Orange Carotenoid Protein for Applications in Synthetic Biology. Current Opinion in Structural Biology 57:110-117, 2019.
8. Gupta, S., Dominguez-Martin, M.A., Bao, H., Sutter, M., Feng, Pawlowski, E.G., Feng, J., Chan, L-J.G., Petzold, C.J., Ralston, C.Y., Kerfeld, C.A. Molecular mechanism of structural rearrangements during photoregulation of OCP and binding of FRP. Journal of Biological Chemistry, 294:8848-8860, 2019.
9. Sutter, M., McGuire, S., Ferlez, B. and Kerfeld, C.A. Structural Characterization of a Synthetic Tandem-domain Bacterial Microcompartment Shell Protein Capable of Forming Icosahedral Shell Assemblies. ACS Synthetic Biology 8:668-674, 2019.
10. Abramson, B.W., et al. Redirecting carbon to bioproduction via a growth arrest switch in a sucrose-secreting cyanobacterium. Algal Research 33: 248-255, 2018.
11. Rohnke, B.A., Singh, S.P., Pattanaik, B., and Montgomery, B.L. RcaE-Dependent Regulation of Carboxysome Structural Proteins has a Central Role in Environmental Determination of Carboxysome Morphology and Abundance in Fremyella diplosiphon. mSphere, 3(1): e00617-17, 2018.
12. MacCready, J.S., et al. "Protein Gradients on the Nucleoid Position the Carbon-fixing Organelles of Cyanobacteria." eLife 7:e39723, 2018.
13. Plegaria, J.S and Kerfeld, C.A. Engineering Nanoreactors Using Bacterial Microcompartment Architectures. Current Opinion in Biotechnology 51:1-7, 2018.

Infrastructure support:

Hagen, A.R., Plegaria, J.S., Ferlez, B., Sloan, N., Aussignargues, C. Burton, R. and Kerfeld, C.A. In vitro assembly of diverse bacterial microcompartment shell architectures. NanoLetters 18(11): 7030-7037, 2018.

Visualizing Structural Dynamics in Photosynthetic Reaction Centers on the femtosecond to Seconds Time Scale

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Overall research goals: The research objectives for this project are to visualize and understand the light triggered structural changes in photosynthetic protein complexes, especially during charge separation in photosynthetic reaction centers. To enable such studies, we will develop methodologies using femtosecond X-ray pulses to collect time resolved structural pump-probe data. The main questions we aim to answer are: 1) How are charge-separated states sufficiently stabilized to allow the desired chemistry to take place, before the back reaction of the ion pair takes place, and specifically, what are the details of the geometric structural changes upon formation of charge separated states in photosynthetic reaction center complexes? 2) How does the protein environment facilitate and direct electron transfer along a specific branch of co-factors within the highly symmetric reaction centers? We will utilize crystals of type I (Photosystem I, PSI) and type II (purple bacterial reaction center, PBRC) reaction centers, and in addition to wild type systems, we will also investigate structural effects in specific mutants of the PBRC.

Significant achievements 2018-2019: We worked on improvements in the procedure to generate large amounts of uniformly sized microcrystals of PSI and obtained conditions that allowed to us to conduct serial femtosecond X-ray diffraction experiments on photosystem I under different illumination conditions. The room temperature dark state structure of PSI was refined to 2.75 Å resolution. This is the highest resolution room temperature structure of PSI available up to now, and the structural model obtained allows us to track differences between the previously published cryogenic structures and the new data. One important feature seen in the new data is an expansion of the entire PSI complex along the plane of the membrane. Although the expansion is relatively small (in the range of 0.5 -1 Å), there is a distinct effect on cofactor-cofactor distances. They are elongated in the range of 0.2-0.5 Å compared to the cryogenic structures (Fig. 1). As electron and excitation transfer rates are highly distance dependent, these slightly elongated distances have a non-negligible impact on the expected electron and excitation transfer kinetics at room temperature. It was also found that the

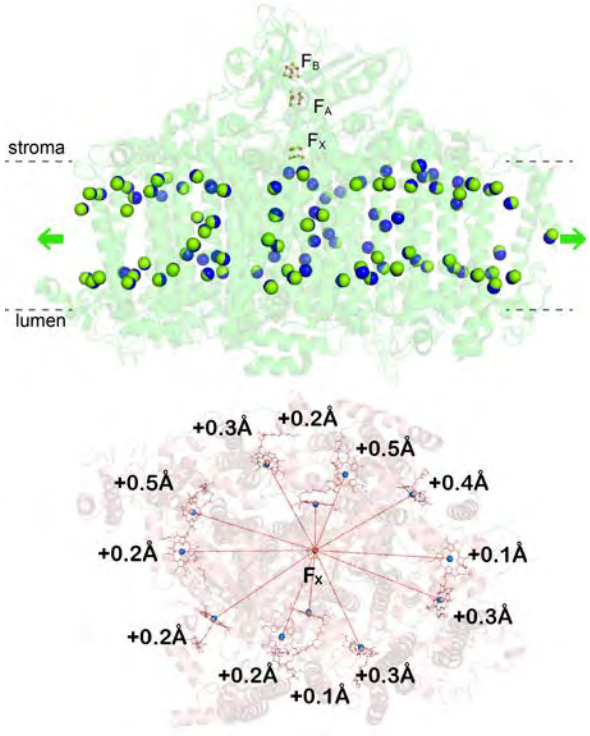

Figure. 1: Comparison of Chl positions for RT (green) and cryo (blue spheres) structures of PSI, view along the membrane (top) and from the stromal side (bottom). Change in Chl-F_X distance between cryo and RT structures are given (bottom).

environment of one of the two phylloquinones in the electron transfer chain of the reaction center is slightly modified compared to the cryogenic structure.

Science objectives for 2019-2020:

- In order to detect small scale structural changes, higher resolution structural data are key. We are working on improving the PSI crystal quality and the measurement conditions to obtain high quality room temperature diffraction data.
- We conducted initial light-dark difference measurements on PSI crystals at 100 ns after illuminating the sample with a ns laser pulse and analysis of that data will be continued. The preliminary data is encouraging, but we need better quality data before we can come to conclusions. We will expand these studies to improve the available resolution of the structural data and cover a number of different delay times in order to follow structural changes induced/correlated with charge separation in PSI.
- We will start preparation and characterization of microcrystals from wt PBRC from *R. capsulatus*. Optimum illumination conditions for these crystals will be established using ultrafast UV-Vis spectroscopy on crystal suspensions in collaboration with the Kirmaier/Holten/Laible groups. Subsequently these will be used for time resolved laser pump X-ray probe measurements to follow structural changes correlated with charge separation in a type II RC and will allow comparison with results obtained for PSI. In a next step (in collaboration with the Kirmaier/Holten/Laible groups), we plan to extend our work to selected mutants in PBRC that specifically tune the branching ratio between the A and B branch in the RT and we will prepare for time resolved structural studies of these by optimization of crystallization and illumination conditions.

References to work supported by this project 2018-2019:

1. N.K. Sauter, J. Kern, J. Yano, J. Holton, "Towards the spatial resolution of metalloprotein charge states by detailied modelling of XFEL crystallographic diffraction." Under review.
2. S. Keable, P. Simon, J. Kern et al., "Room Temperature Structure of Photosystem I." In preparation.

Modulation of State Transition by Flavodiiron Activity in *Synechocystis* PCC 6803

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Overall research goals: The research objectives of this work are to define and understand the mechanisms in photosynthetic microbes that coordinate peripheral redox reactions and electron flow to the light harvesting processes that control excitation energy distribution among reaction centers. Coordination of these processes involves state transition that is hypothesized to function in concert with electron flow regulation to enable energy balancing. The factors that regulate state transitions in cyanobacteria involve complex molecular interactions such as the redox poise of the plastoquinone (PQ) pool that is tightly coupled to peripheral pathway electron flow. In contrast to inhibitor-based studies, knockouts of specific peripheral reaction pathway(s) enzymes put the cells in a dynamic environment subject to an altered PQ pool redox state. Whether the function of these components contributes to modification of the excitation energy distribution and state transition has not been defined. An example of this coordination was studied in *Synechocystis* PCC 6803 by modification of flavodiiron-dependent oxygen reduction in strains with deletions of the structural genes encoding Flavodiiron 1 (flv1) and Flavodiiron 3 (flv3). The state transition kinetics and fluorescence properties of these strains were measured in cells cultured under different light-growth regimes towards developing a fundamental model of photon and electron flow coordination in photosynthetic microbes.

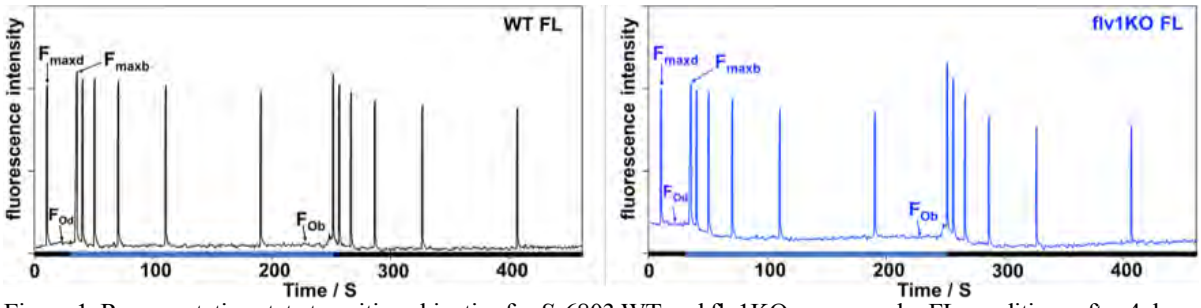

Figure 1. Representative state transitions kinetics for *S.* 6803 WT and flv1KO grown under FL conditions after 4 days. Dark-adapted cells poised in state II (initial black-shaded x-axis) were subject blue light (460 nm, 90 µM photon m^{-2} s^{-1}) to induce state I (blue-shaded x-axis). Saturating pulses (@150 µs x 60,000 µM photon m^{-2} s^{-1}) were used to probe fluorescence maximum in state I (F_{maxb}=F_{max} under blue light) and state II (F_{maxd}=F_{max} in the dark). F_{Ob} is measured state I fluorescence minimum and F_{Od} state II fluorescence minimum.

Significant achievements 2017-2019: We generated both flv1 or flv3 knock-out strains (flv1KO and flv3KO, respectively) and measured pigment compositions, 77K fluorescence emission spectra, and state transition kinetics. Both strains were found to have higher phycocyanin (PC) to chlorophyll (chl) ratios than wild-type (WT) under fluctuating light (FL) conditions. The ratio of variable fluorescence values for state II and state I were used as an indicator of plastoquinone (PQ) pool changes induced under state transition (Figure 1). The following trends were observed; under GL, flv1KO ≈ flv3KO > WT; and under FL, flv1KO ≈ WT > flv3KO. Under GL, the trend was consistent with the 77K fluorescence changes, however, the ratio could not explain the observed 77K fluorescence changes under FL (Figure 2). With addition of various photosynthetic inhibitors, we were able to synchronize the PQ pool redox changes induced by state transition, however, these treatments did not lead to equivalent 77K fluorescence changes. This result indicates other factors contribute to the regulation

of state transition in absence of Flv's. Along these lines, transient fluorescence measurements and sucrose gradient were used to determine increased PSI monomeric content in the flvKO strains. In summary, the photon flux distribution by PBS into PSII and PSI is modulated by the activity of peripheral enzymes through redox control and PSI oligomeric state changes.

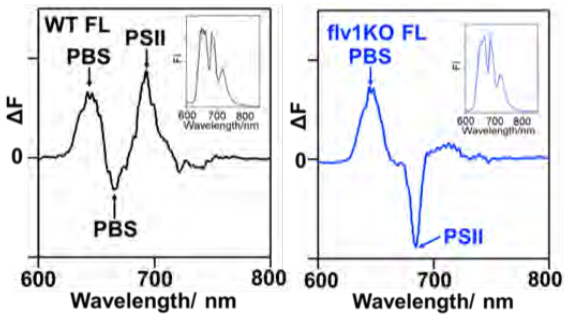

Figure 2. 77K fluorescence difference spectra (state I minus state II) for *S.* 6803 WT and flv1KO grown under FL. Inset, state I and state II spectra (state I, solid line; state II, dash line).

Science objectives for 2019-2021:

- Directly measure the redox PQ pool in flvKO strains to determine the extent that PQ poise is altered by the loss of Flv1 and Flv3, and to what extent that PQ redox state is a component of state transition regulation.
- *Synechococcus* PCC 7002 flvKO strains have been generated to enable single-cell florescence imaging in collaboration with Dr. J. Cameron at CU-Boulder with the objective of visualizing the sub-cellular localization dynamics and molecular interactions of Flv proteins under differential light regimes.
- The observed increase in orange cartenoid protein (OCP) content in isolated PSI trimers by mass spectroscopy, and increased PBS fluorescence during state transition, indicate excitation energy that is funneled into reaction centers is lower in the flvKOs than WT. We want to determine whether this involves non-photochemical quenching (NPQ) by OCP or whether this is related to changes in PBS mobility due to changes in PSI oligomeric state.

References to work supported by this project 2017-2019:

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Mechanistic understanding of ion fluxes in photosynthetic thylakoid membranes

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Overall research goals: The overarching research objective is to understand structural and functional implications of architectural dynamics in plant photosynthetic thylakoid membranes. In particular, we aim to identify key players that control thylakoid membrane dynamics by: (1) studying the role of thylakoid ion transporter/channels, (2) examine how reversible phosphorylation of the membrane bending CURT1 proteins change the thylakoid ultrastructure, and (3) identify how alterations in thylakoid stacked membranes control the lateral distribution of the cytochrome b_6f complex. The results of our studies are expected to help give a better understanding of how plants modulate photosynthetic energy conversion by structural alterations that can define new strategies for developing bio-hybrid systems and artificial photosynthetic devices as detailed in the next section.

Significant achievements 2018-2019: After the generation of higher-order thylakoid ion channel/transporter loss-of-function mutants (*vccn1, clce, kea3*), the functional and compositional analysis of these mutants was completed. The experimental results were complemented by an extended and refined computer model that was recently published (Davis et al. 2017 Phil. Trans. R. Soc. B 372, 20160381) describing photosynthetic light reactions. Combination of functional results collected on our mutants with the computer model leads to an in-depth mechanistic understanding of light-induced ion fluxes, membrane energization, and photoprotection in thylakoid membranes (Fig. 1, manuscript in preparation). Furthermore, electron microscopic studies on intact leaf tissues and computer image analysis of TEM images was further optimized and refined (methods manuscript in preparation).

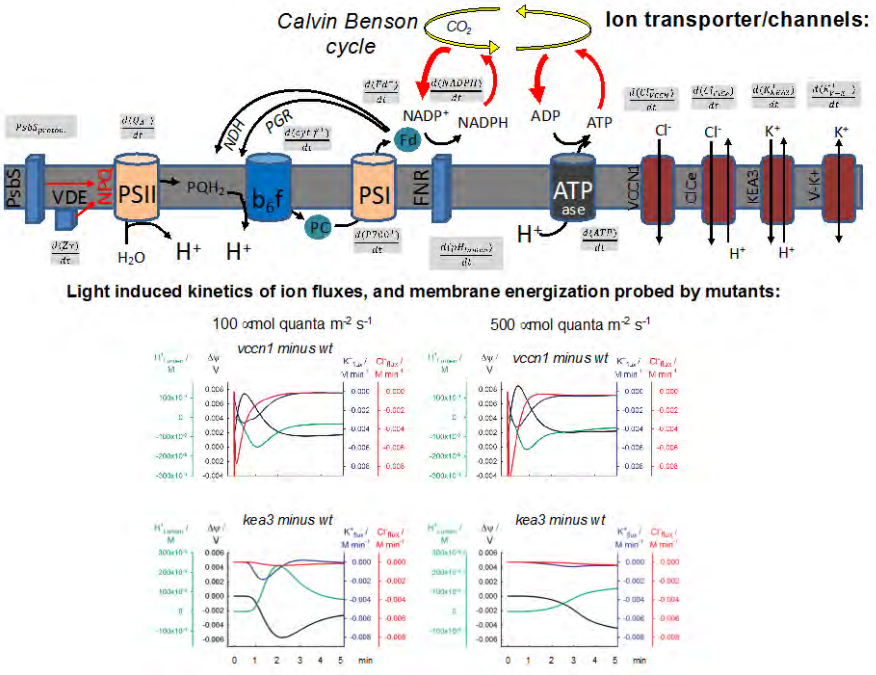

Figure 1. Upper Panel: Scheme of the computer model describing light harvesting, electron transport, proton pumping, ATP synthesis, NPQ, ion fluxes, and ATP/NADPH consumption in the Calvin-Benson cycle. Lower Panel: Results showing kinetics of ion fluxes and membrane energization in VCCN1 and KEA3 knock out mutants for two different light intensities.

In our sub-project on the light-induced cytochrome b_6f redistribution between stacked and unstacked thylakoid domains, we could identify significant accumulation of this complex in untacked regions in illuminated samples. This redistribution seems phosphorylation dependent since it is impacted in thylakoid kinase/phosphatase mutants.

Science objectives for 2019-2020:

- Collect ultrastructural (electron microscopic) data from leaves on ion transporter/channel mutants for dark and light adapted states by using our developed sample preparation and image analysis pipeline. Examine which ion transporter/channels cause architectural thylakoid dynamics by facilitating osmotic swelling.
- Further analyze the cytochrome b_6f redistribution by extending our mutant studies to other low abundant chloroplast proteins involved in regulation of energy conversion. Also, we aim to complement our biochemical sub-localization studies with immunocytochemical electron microscopic studies (immuno-gold labeling) on cytochrome b_6f subunits.
- Start to examine the role of CURT1 protein phosphorylation in the thylakoid membrane ultrastructure by employing phosphomimetic mutants provided by our collaborator in Copenhagen.

References to work supported by this project 2018-2019:

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Controlling Electron Transfer Pathways in Photosynthetic Reaction Centers

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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 house two symmetry-related sets of A- and B-branch cofactors that are functionally asymmetric (Fig. 1A). In RCs from photosynthetic bacteria, only the A cofactors are used for light-induced ET. Differences in the free energies of the A- versus B-side charge-separated states, and in electronic couplings between the cofactors, underlie differences in time constants for both forward ET and charge recombination processes on the two ET pathways (Fig. 1B).

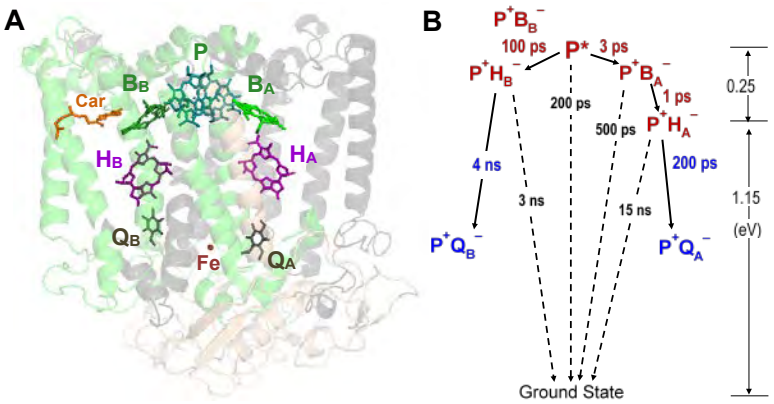

Fig. 1. *(A)* R. sphaeroides *RC (pdb:1PCR). (B) Model free energy diagram and time constants for primary (red) and secondary (blue) processes: forward ET (solid) and charge recombination (dashed).*

The goal of this project is to engineer an RC that performs transmembrane charge separation efficiently and exclusively via the B-side cofactors. Such an RC, and equally the steps toward it, will provide a fundamental understanding of how ET between the A-side cofactors is optimized and ET between the B-side cofactors is inhibited in the native RC. We have developed semi-directed molecular evolution approaches in which saturation mutagenesis gives rise to all possible amino acid substitutions at a given site. Here, streamlined mutagenesis and RC isolation are coupled with time-resolved spectroscopic screening and photochemical analysis spanning <1 ps to >10 s. Our work initially focused on RCs from the purple photosynthetic bacterium *Rhodobacter (R.) capsulatus*, while recent work has focused on RCs from *R. sphaeroides*. Differences in ET found in analogous mutants from the two species inform on both the roles of specific cofactors and specific protein-cofactor interactions. Combinations of mutations are employed to manipulate the free energies of the charge-separated states, seeking to minimize ET from the excited primary electron donor (P*) to the A-side cofactors and maximize ET from P* to the B-side bacteriochlorophyll (B_B) or bacteriopheophytin (H_B) or both (Fig. 1B, red). Another goal is to enhance secondary ET from H_B to quinone (Q_B) and slow charge recombination of $P^+H_B^-$, thereby obtaining insight into these B-side processes (Fig. 1B, blue). The principles realized will afford detailed insight into RC function and aid the design of biomimetic systems that are competent in light-induced charge separation.

Significant achievements 9/2017 – 9/2019: Studies have focused on enhancing both the primary and secondary ET processes on the B side as well as further detuning A-side ET.

- Single residues dictate the type of cofactor bound within the protein complex and significantly impact B-branch ET.
  - Two sets of *R. capsulatus* mutants were generated at HisM180 (the ligand to B_B) in RCs carrying different background mutations. Out of 40 possible mutant RCs, 33 were isolated and fully characterized for pigment content and ET functionality by ultrafast spectroscopy. Substitutions of Asp, Ser, Pro, Glu, Asn, Gly, Cys, Lys and Thr largely resulted in retention of a bacteriochlorophyll in the B_B site but Leu or Ile RCs largely harbor the Mg-free bacteriopheophytin (Φ_B). A substantial mixture of these two RC types occurred when Ala, Asp, Val, Met and, in one set, Arg are substituted at M180. Insights were gleaned into the widely observed multi-exponential decay of the excited primary electron donor. This work is now published.
  - Two sets of *R. sphaeroides* mutants were generated at LeuL185, which is located over once face of B_B, in RCs carrying different background mutations. LeuL185 is symmetry related to LeuM214, which, when replaced with His, leads to replacement of H_A with a bacteriochlorophyll (β_A). Some substitutions at L185 led to the retention of H_B and some to incorporation of a bacteriochlorophyll (β_B). Some variants afford a very high yield of ET to H_B, including a significant fraction utilizing $P^+B_B^-$ as an intermediate. This two-step mechanism is analogous to the $P^* \rightarrow P^+B_A^- \rightarrow P^+H_A^-$ primary ET process on the A side of WT RCs. Some mutants also showed a notable slowing of $P^+H_B^-$ charge recombination. Both of these effects are major accomplishments toward a high yield of transmembrane charge separation using only the B-side cofactors. A paper describing a subset of these mutants is under review.
- Strategic placement of tryptophan residues increases the rate of $P^+H_B^- \rightarrow P^+Q_B^-$ ET. This step is a bottleneck in B-side ET as the native rate is ~$(4\ ns)^{-1}$, compared to $(200\ ps)^{-1}$ for $P^+H_A^- \rightarrow P^+Q_A^-$ ET (Fig. 1B, blue). Rational and irrational combinations of Trp substitutions – both on and off the axis between H_B and Q_B cofactors, increasing π-electron density in the area – are the subject of a paper that is being readied for publication.
- Collaborated in 2D electronic spectroscopy measurements with Dr. Jennifer Ogilvie, furnishing mutants and insights into experimental designs and data analysis strategies.

Main science objectives for 2019-2020:

- Mine *R. sphaeroides* mutants carrying substitutions at L185 near H_B for altered photochemistry.
- Generate additional mutants to further improve efficiency of ET from H_B to Q_B.
- Develop a suite of complementary assays for Q_B occupancy.
- Seek photocompetent, phenotypic revertant strains that grow via B-side cofactors only.

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

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Overall research goals:

Subproject A focuses on one of the grand challenges in basic energy science, understanding how the components of natural photosynthesis are integrated into living organisms. We have 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:

A major focus of Subproject A has been on developing and deploying core *enabling technologies* (instrumentation, methods and analytical tools) to collect, process and interpret high throughput and detailed photosynthetic phenotyping data (e.g. *1, 2, 3*), connections between such phenotypes and genetic components (*4*) and modeling of functional connections among photosynthetic processes (*5*). Most recently, we have extended these platforms to allow measurements of fluorescence changes of *in vivo* nanosensors, allowing us to simultaneously probe photosynthesis and key metabolic intermediates.

We mined data from both phenotyping platforms to bio-prospect for "new" photosynthetic behaviors, leading us to propose new mechanistic models for what limits photosynthesis in the real world (*6*), and in particular genotypes that can usefully take advantage of sudden increased in light intensity to perform productive photochemistry, as well as genetic determinants of photosynthetic responses to a range of environmental conditions (e.g. *4, 7, 8, 9*). Analyses of a wide range of data resulted in a model that appears to explain much of the observed co-regulatory behaviors of the light reactions and metabolic processes in the vascular plants studied thus far (e.g. *5, 6*), which posits that key steps in feedback and feedforward regulation of photosynthesis involve the thylakoid protonmotive force (*pmf*) as key intermediate in both energy transduction and controlling light capture and electron flow. Strikingly, we demonstrated a (previously unreported) mechanism for sensitization of photosynthesis to photodamage (*5*) in which high extents of pmf accelerate recombination reactions within PSII, resulting in $^1O_2$ production and photodamage, substantially limiting photosynthesis even in wild type plants under field-like fluctuating light and likely driving the evolution of key photosynthetic complexes (*10*). We also find strong connections between the light reactions and key downstream metabolic and physiological processes. As examples, we find interactions between electron and proton transport reactions and the capacity for triose phosphate use (TPU) involving feedback through the ATP synthase (*11*), between the activation of CEF and the impaired photorespiration (*12*), stimulation of the glucose-6-phosphate shunt (*13*), production of H_2O_2 (*14*) and ATP deficits, directly linking metabolic demands and the energy output of the light reactions. We used synthetic biology approaches in cyanobaceria to explore the relationship between energy capture and sink strength, and found that engineered sucrose export pathways (*15, 16*) and alternative energy sinks can increase photosynthetic efficiency and even replace natural cellular electron sinks in protecting against photodamage.

Finally, we tested for mechanistic connections between photosynthetic responses and the thylakoid lipid environment (*17*), which is intimately associated with the photosynthetic apparatus, and shows extremely high diversity across genotypes and large responses to environmental conditions. Using mutation approaches, we showed that loss of MGDG-specific lipase PGD1 impacted the chloroplast ultrastructure, key photosynthetic partial reactions and the production of reactive oxygen species (ROS) (*17*). In a second approach, we used high throughput phenotyping in genetic diversity panels to discover novel relationships between specific alterations of the lipidome and photosynthetic parameters under different stress conditions.

Science objectives for 2019-2020:

We are focusing on testing the limits of our models for regulation of photosynthesis in diverse organisms and environments, in particular cyanobacteria in which the established models appear to fail. We are employing new *in vivo* nanosensors, with new spectroscopic methods and genetic diversity panels and spectroscopic assays to test specific mechanisms that enable high productivity photosynthesis. We are following up on large-scale screens of lipid mutants and natural variants to explore the mechanisms to maintain higher photosynthetic efficiency.

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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. The major mechanism for these photoprotective mechanisms in higher plants and green algae involve the thylakoid protonmotive force (*pmf*) (*1*). 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; and 2) how CEF is regulated at the level of the key enzyme, the NDH complex.

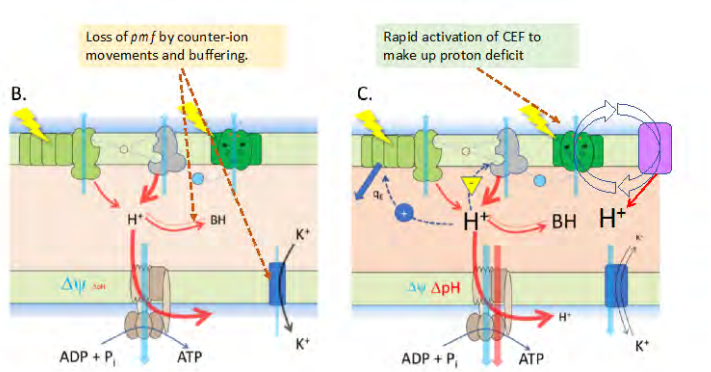

Figure 1. Schematic illustrating our hypothesis that rapid induction of qE requires activation of CEF to make up deficit in free protons caused by buffering during dissipation of Δψ.

Significant achievements 2017-2019:

Work in the current funding period has focused on elucidating the mechanisms of regulation of the various CEF pathways, and how these contribute to precise balancing of ATP/NADPH output with demands. In the following, we highlight specific advances most relevant to the proposed work.

1. NDH is a reversible proton pump enables highly efficient ATP production by CEF. The NDH complex shows strong homology to the bacterial or mitochondrial respiratory type I NADH:quinone reductases (Complex I) that pump up to two protons across the energetic membrane for each electron transferred to quinone. Our work (*2*) is the first to show that NDH is also a proton pump, and pumps protons at a highly efficient ratio of 2 H^+/e^-, effectively doubling the efficiency of ATP production by NDH-CEF compared to other pathways, allowing for more efficient balancing of the ATP/NADPH budget with relatively low turnover rates. We also show that NDH-CEF likely has a higher capacity for ATP production than previously thought, potentially resolving a long-standing question whether its activity can account for energy balancing in photosynthesis.

2. CEF through the b_6f-complex. One proposed route for CEF involves a supercomplex containing FNR and cytochrome b_6f complex and PGRL1, by directing electrons from ferredoxin, to the heme c_i of the cytochrome b_6f complex and then to the PQ reductase (Q_i) site of *b_6f*. Our results

(*3*) demonstrated that, even in thylakoids with active CEF, electron transfer from Fd or NADPH to cytochrome b_H was exceedingly slow and only observed under anaerobic conditions.

3. Evidence that both NDH and FQR CEF pathways are directly regulated by stromal ATP. A major open question is: how is CEF regulated? We show that both NDH and FQR are inhibited by ATP at physiological conditions, leading us to propose a straight-forward model for regulation of CEF in plant chloroplasts in which CEF is activated under conditions when stromal ATP low, but is downregulated as ATP levels build up (*4*). The differences in K_i values suggest a two-tiered regulatory system, where the highly efficient proton pumping NDH is activated with moderate decreases in ATP, while the (presumably non-proton pumping, but less constrained) FQR being activated under more severe ATP depletion. ATP inhibition of CEF can explain, in a very straightforward way, much of the known phenomenology of CEF and the regulation of the photosynthetic energy budget.

4. Evidence that plants have evolved very rapid NPQ and photosynthetic control mechanisms that involve CEF. To assess the diversity of NPQ phenotypes in a wide range of plant species and conditions, we mined the PhotosynQ.org field phenotyping data set (www.photosynq.org), consisting of over 1.5M data sets on a range of photosynthetic parameters. Using newly-developed bioinformatics tools, we found species and conditions that showed very rapid initiation and decay of NPQ (presumably q_E) could be observed. More detailed analyses led us to propose that CEF is needed to supply free lumen protons lost during to buffering pools during the dissipation of $\Delta\psi$, which is required to form ΔpH.

Science objectives for 2019-2020:

- Confirm that ATP also inhibits the NDH complex in cyanobacteria and use this platform to determine the structural bases for regulation of NDH complex by ATP;
- Test our model for CEF regulation using in vivo ATP nanosensors
- Test if increased CEF is mechanistically or genetically linked to rapid q_E by testing for co-segregation of photosynthetic properties in genetic diversity panels.

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Photoreceptors and photosynthesis, past and future: it's all about bilin

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Overall research goals: Our current project, ***"LIGHT SENSING AND HARVESTING IN CYANOBACTERIAL PHOTORECEPTORS,"*** leverages cyanobacterial cyanobacteriochrome (CBCR) photoreceptors distantly related to phytochromes to address fundamental questions applicable to all photoproteins. We use phylogenetic analysis, *in vitro* spectroscopic characterization of recombinantly expressed CBCRs, protein engineering, and other structure/function techniques to learn how CBCRs have answered these questions.

Significant achievements, 2017-2019: The Lagarias lab continues to examine CBCR evolution, diversification, biological function, and spectral diversity **[1-3, 6].** Former co-PI Delmar S. Larsen has also published several studies on primary and secondary photochemical processes in different CBCR and phytochrome lineages **[3-5, 7-9].** All three PI's joined forces to determine solution structures of the model CBCR NpR6012g4 from *Nostoc punctiforme* in a study of the photochemical consequences of structural heterogeneity **[3]**.

In work now being prepared for publication, we **completed a large-scale survey** of over 70 CBCRs with DOE support, providing a sweeping overview of CBCR diversity. We have **identified an early-diverging CBCR lineage sister to all other CBCRs**. We have **converted NpR6012g4 into a photochemically inert red fluorescent protein**. We have also **identified a far-red-absorbing CBCR lineage with altered chromophore specificity** and determined the atomic-resolution structure for one such photosensor.

Science objectives (2019-2021): We will be focusing on completing and publishing current work, on **exploring evolution of CBCR color sensors into broadband sensors of light intensity,** and on using protein engineering to examine the **evolution of phytochromes into CBCRs** and to regulate **pH-gated photoconversion in CBCRs.**

Subsequent research goals (2020 and beyond): These studies illuminate the interplay between protein and bilin in determining the spectral and photochemical properties of the holoprotein photoreceptor. However, bilins have functions outside the phytochrome superfamily, such as their use in light-harvesting phycobiliproteins of cyanobacteria and rhodophyte algae. Remarkably, we have shown that **bilin is essential for phototrophic growth in *Chlamydomonas* [10-11].** We are restructuring our studies to examine this new role for bilins in photosynthetic organisms lacking both phytochromes and phycobiliproteins. We have shown that the **bilin biosynthesis pathway is ubiquitous in photosynthetic eukaryotes [12].** Moreover, we have very recently demonstrated that **bilins are essential for the maintenance of the photosynthetic apparatus** in light-grown *Chlamydomonas.* Future studies will assess whether there is a general requirement for bilins in oxygenic phototrophs.

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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.[1] 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.

Significant Achievements 2018-19. (A) Binding of ammonia as a substrate analog in the oxygen-evolving complex of photosystem II (PSII): The light-driven four-electron water oxidation reaction occurs at the tetranuclear manganese-calcium-oxo (Mn_4Ca-oxo) catalytic cluster in the oxygen-evolving complex (OEC) of PSII.[1] The mechanism of the water oxidation reaction has been the subject of intense interest and the OEC has been studied extensively by structural, spectroscopic, biochemical and computational methods. The recent 3.8-1.9 Å resolution X-ray crystal structures and single-crystal EXAFS studies provide a model for the catalytic Mn_4Ca-oxo cluster. However, the structure of the OEC, the participation of the protein environment in substrate activation and the mechanism of charge transfer at the bound water molecules have been elusive. In previous studies, we had unambiguously resolved the individual spectroscopic signatures of the amino acids and water molecules that are ligated to the Mn_4Ca-oxo cluster in the S_2 state of the OEC of PSII.[2-3] These experiments provided a handle to monitor the catalysis of the substrate water molecules in the solar water oxidation reaction. There have been previous efforts to interrogate the binding sites of the substrate water molecules 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. We have developed high-resolution two-dimensional (2D) hyperfine sublevel correlation spectroscopy methods[2-8] that provide direct 'snapshots' of the binding of NH_3 and 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 S_2 state of the OEC of PSII. **(B) Comparison of the mechanism of PCET at the Y_Z and Y_D residue of PSII**: 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. Our recent studies are focused on understanding the mechanism of PCET at the Y_D and Y_Z residues of PSII using both computational and experimental methods. (a) We have conducted quantum mechanical (all-QM) calculations that have allowed us to investigate the alignment of the energy levels of the Y_Z and Y_D orbitals relative to other components of the whole system (Figure 1).[9] We find that there is a difference in the energy levels of the frontier orbitals of Y_Z and Y_D within the overall electronic structure of each protein model. Moreover, the H-bonding network within the protein matrix determines the protonation state of the respective conjugate base, D1-His190 and D2-His189, which in turn tunes the energy levels of the frontier orbitals of Y_Z and Y_D. (b) We are currently investigating the role of the respective conjugate base residues, D1-His190 and D2-His189, in the PCET reactions of Y_Z and Y_D by

probing the effect of site-directed mutagenesis on the PCET intermediates. 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 in the site-directed mutants of PSII.[10, 11] These studies provide direct 'snapshots' of the functional PCET intermediates and, for the first time, makes it possible to detail the mechanism of PCET in biological solar energy transduction.

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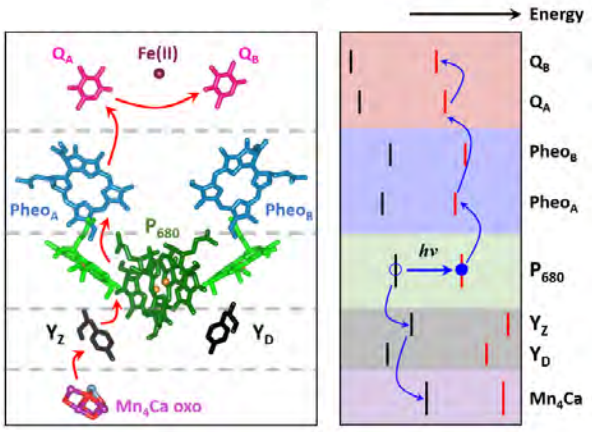

Figure 1. (a) The electron transfer cofactors of PSII as observed in the 1.9 Å resolution X-ray crystal structure[1] with the primary electron transfer pathway shown by red arrows. (b) An energy-level diagram illustrating the alignment of the HOMO and LUMO states of the cofactors. Blue arrows depict the electron and hole transport following charge separation at the primary donor, P_{680}.

Science Objectives for 2019-20. (i) We have determined the electronic structure and substrate activation in the S_2 state of the OEC. We are extending these studies to include the determination of the binding and protonation states of the substrate waters in the S_3-state intermediate of the solar water oxidation reaction. (ii) We are investigating the mechanism of light-induced PCET at the redox-active tyrosine residues of PSII.

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2. V. Kalendra, G. Banerjee, I. Ghosh, K. Yang, V. S. Batista, G. W. Brudvig% and K. V. Lakshmi% "Mechanism of Binding of Ammonia as a Substrate Analog in the Oxygen-evolving Complex of Photosystem II" (2019) Manuscript in preparation.
3. V. Kalendra, G. Banerjee, I. Ghosh, G. W. Brudvig% and K. V. Lakshmi% "Binding of Substrate Water in the S_2 to S_3 State Transition of Photosystem II" (2019) Manuscript in Preparation.
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Characterizing Rubisco by Phylogeny-Informed Mutagenesis

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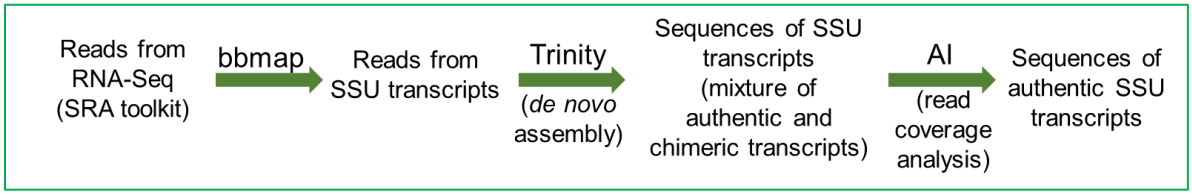
Overall research goals: The research objectives are to study the evolution of the main carbon-fixing enzyme, Rubisco, in higher plants by: (1) obtaining more sequences from publicly available RNA-Seq data; (2) predicting ancestral Rubisco sequences using phylogenetics; (3) mutagenesis of target residues predicted to be present in ancestral enzymes as well as important for kinetics based on other studies; (4) producing and characterizing transgenic plants with modified Rubisco enzymes. Mutations that increase as well as those that decrease Rubisco activity will provide new insights into the function of Rubisco. Transgenic plants containing Rubisco enzymes with favorable properties may be informative for future engineering of crop plants. .

Significant achievements 2017-2019: Phylogenetic analyses of Rubisco large subunits (LSU) indicated the importance of small subunits (SSU) during the Rubisco evolution in higher plants. Since relatively few sequences of Rubisco small subunits are available, we have developed an efficient protocol for next-generation sequencing and wrote a Python script to automate subsequent rapid *de novo* assembly of Rubisco subunit genes from RNA-Seq data (Figure 1). We have also adapted the recently developed *E. coli* Rubisco expression system to produce tobacco Rubisco with different SSUs (Figure 2). We found that tobacco Rubisco enzymes produced in *E. coli* have different kinetic properties depending on the identity of the SSU (Figure 3).

Figure 1. Flowchart of assembling SSU transcript sequences from RNA-Seq data.



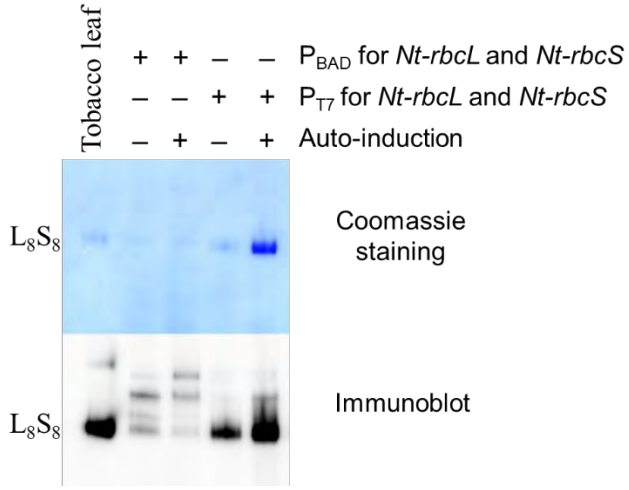


Figure 2. The Native PAGE analysis of BL21* (DE3) *E. coli* soluble extracts expressing tobacco Rubisco subunits along with seven additional plant chaperones.

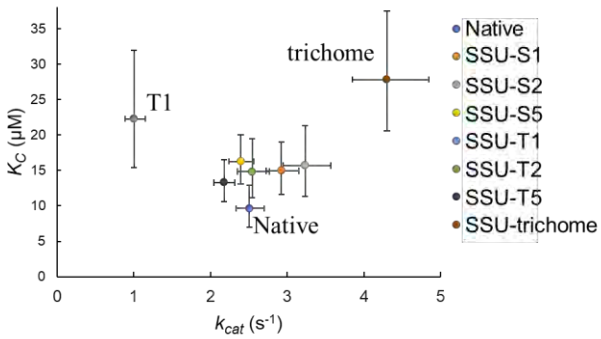


Figure 3. The kinetics of tobacco Rubisco expressed in *E. coli* with different small subunits compared to the native tobacco Rubisco in the absence of O_2. The Michaelis-Menten constants for CO_2 (K_C) and turnover numbers (k_{cat}) were obtained from nonlinear regression with the error bars showing 95% confidence intervals.

Science objectives for 2019-2021:

- A high-resolution phylogenetic tree is necessary to predict ancestral Rubisco sequences. The challenge is to obtain enough sequences for SSU genes. Since RNA-Seq data are available for a large number of plants, SSU sequences can be assembled from these data. In addition to automating the tools already available for processing and assembling the transcripts from RNA-Seq data, we are exploring approaches to distinguish chimeric transcripts from authentic ones. We will then predict ancestral Rubisco sequences from phylogenetic analyses.
- We will produce predicted ancestral Rubisco enzymes using the *E. coli* expression system that we have adapted for tobacco Rubisco. We will also test the mutations that were reported in other studies to be potentially important for the enzymatic properties of Rubisco. We hope to understand how Rubisco enzymes in higher plants were adapted to different environmental conditions such as higher atmospheric CO_2 levels. This would allow us to identify modifications necessary to optimize the enzyme for enhanced carbon fixation.
- We will introduce Rubisco enzymes with favorable kinetic properties into tobacco plants using chloroplast transformation technology to test their effects on plant growth and physiology. It is preferable to use a tobacco line lacking the native SSUs for transformation. We are using CRISPR-Cas9 to knock out all major SSUs in a tobacco line that possesses cyanobacterial Rubisco and does not require native SSUs.

Reference to work supported by this project 2018-2019:

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Molecular Mechanism of Action of the Cyanobacterial Orange Carotenoid Protein

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Overall research goals: The research objectives are to use an optimal mass spectrometry (MS) and associated chemistry to study the structural basis that dictates the cyanobacterial orange carotenoid protein (OCP) involved Non–Photochemical Quenching (NPQ) regulatory circuit by: (1) developing native electrospray mass spectrometry capable of probing biological samples in their native conformational states in the absence/presence of binding partners; (2) protein footprinting at residue level; (3) testing whether a 3rd state of OCP exists after active OCP is recruited to PBS–core. Realization of the full potential embodied in MS requires improved abilities to prepare and stabilize OCP or Phycobilisome–OCP (PBS–OCP) complex in their biological relevant states. Extending studies of using a suite of advanced MS techniques to the level of capturing quasi–stable state(s) of OCP in their functional state should reveal a much improved understanding of the dynamic nature of the photoprotection at the molecular level, perhaps eventually lending ideas for biomimicry molecular apparatus design.

Significant achievements 2016–2019: Combined native MS, collisional activation, and ion mobility analysis indicated that the N–terminal domain (NTD) of OCP releases its bound carotenoid without forming any intermediates and the CTD (C–terminal domain) is resistant to unfolding upon collisional energy ramping. The unfolding intermediates observed in inactive, intact OCP suggest that it is the N–terminal extension (NTE) and the loop connecting NTD and CTD that contribute to the stepwise unfolding. Monomeric OCP dominates at low protein concentration, with an observable population of dimeric OCP. The ratio of dimer and monomer, however, increases proportionally with protein concentration. Two distinct OCP–PBS quenched states with lifetimes 0.09 ns and 0.21 ns were observed using Anti–Brownian Electrokinetic (ABEL) trap.

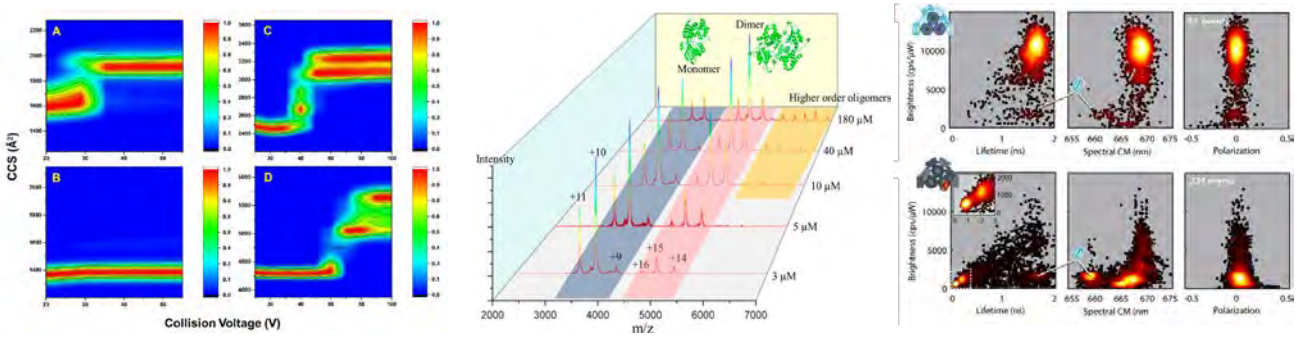

Figure 1. Right Panel: The collisional unfolding heat map for intact OCP and two functional domains of OCP. (A) N–terminal domain (NTD) and (B) C–terminal domain (CTD). (C) Intact OCP monomer. (D) Intact OCP dimer. Central Panel: Native mass spectra of OCP diluted to concentration of 180, 40, 10, 5, and 3 μM. Structures of monomeric and dimeric OCP are shown (PDB ID: 5UI2). Right Panel: Photophysical states of unquenched and OCP–quenched PBS. Scatter heatmap of the photophysical state observed for the unquenched PBS (upper panel) and quenched CB–PBS (lower panel). Lifetime (ns) and Spectral (nm) and Polarization are shown.

Science objectives for 2019-2010:

- We previously reported that light illumination of inactive, orange OCP (OCP^O) in the presence of Cu^{2+}, a biological redox active metal ion, can result in it being locked in its active, red state (OCP^R). We further reported that upon addition of ascorbic acid, a ubiquitous reductant in photosynthetic organisms, the conversion of OCP^O to OCP^R (+ Cu^{2+}) takes place spontaneously, indicative of a locked OCP^R–Cu^+ complex, which is consistent with the addition of Cu^+ alone

experiment. We are developing native MS and Hydrogen/Deuterium Exchange MS (HDX) to detect and characterize Cu^{+}–OCP complex and examine relationships of redox active components and OCP functional cycle.

- It is known that there are two states of OCP: OCP^{O} (first) and OCP^{R} (second). Molecular details on *in vitro* photoactivation of OCP (i.e., $OCP^{O} \rightarrow OCP^{R}$) were also collected over the past 10 years. The dynamic binding properties of OCP^{R} to the PBS–core and post–binding events of OCP^{R} on the PBS–core, however, remain unclear. The project must be transitioned from its initial focus on the characterization of two states of OCP to the more dynamic, post–binding events of OCP and PBS as well using mass spectrometry and molecular spectrophotometry. Using site–directed mutagenesis and bioinformatics analysis, we recently identified a mutant OCP that binds to PBS upon photoactivation, but fails to quench the PBS excitation energy. These results indicated that OCP binding to PBS is not sufficient for its quenching function.
- Our previously published work demonstrated the remarkable capability of using protein footprinting (reversible or irreversible) MS to track protein conformational dynamics. We currently develop residue level footprinting MS to characterize OCP–PBS–core complex. This will be accomplished by comparing three protein/protein complexes in two parallel experiments (OCP^{WT} vs OCP^{mutant}). We aim to test if there is a translocation of 3′ECN towards the center of PBS–core to facilitate excitation energy quenching. The challenge here is to find a footprinter that is compatible with the biochemistry of stabilized OCP–PBS–core complex.

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5. Lu, Y., Liu, H., Saer, R.G., *Li, V.L., Zhang, H., Shi, L., Goodson, C., Gross, M.L., and Blankenship, R.E. (2017) ″A Molecular Mechanism for Non-Photochemical Quenching in Cyanobacteria.″ *Biochemistry* 56(22):2812-2823 *Undergraduate researcher
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Analysis of the effects of flavodiiron 1 and 3 on the function of Photosystem I and photo-driven electron flux in *Synechocystis* sp. PCC 6803

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Overall research goals: Photosynthetic microorganisms have evolved complex metabolic networks and biochemical pathways that balance energy generation with utilization. In order to better understand and manipulate energy flow within these systems, investigations into how peripheral redox reactions integrate with photosynthetic electron transport (PET) to create pathways that affect specific and selective electron transfer events are required. We aim to establish 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 enzyme function and reaction kinetics, and interrogating the mechanisms that control dynamics within PET. One objective is to generate a fundamental and physical basis for the role of the electron transfer pathway involving flavodiiron proteins, including their coupling with and effect on the Photosystem I (PSI) reaction center. These investigations are providing insights into how and why certain pathways are favoured under changing conditions experienced during photosynthetic growth.

Significant achievements 2018-2019: To identify and define the mechanisms that maintain a balance between electron flow and photon capture, the effects of the loss of flavodiiron proteins (Flv) on PSI reaction center photochemistry and oligomeric structure were studied in response to changes in light conditions. In a flv1 knockout mutant, PSI exhibits a decreased proportion of trimers to monomers in continuous moderate light. These changes occurred to a greater extent in fluctuating light for both flv1 or flv3 knockout mutants. As evidenced by time-resolved optical spectroscopic investigations, electron flux was altered through PSI in the absence of Flv1 or Flv3, resulting in a lesser degree of oxidized P700 primary donor during steady-state illumination (Fig. 1). These effects indicate a significant PSI acceptor side limitation. In addition to oligomeric changes, mass spectrometry indicates alterations to specific PSI subunits. EPR studies were used to identify an altered distribution of electron density in photoexcited PSI centers that vary for each strain after exposure to increasing light stress conditions.

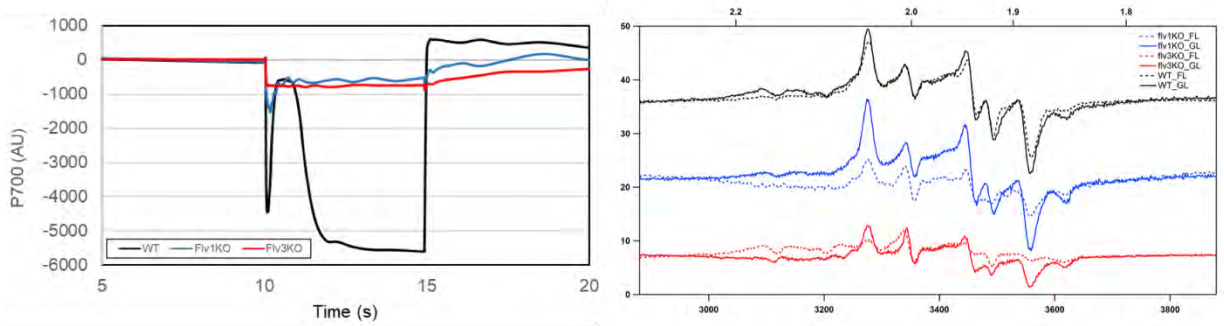

Figure 1. Left Panel: P700 kinetic traces (700 nm) of WT, flv1 KO and flv3 KO strains after exposure to fluctuating light 35 µE (5 min)/500 µE (30 s). Right Panel: EPR difference spectra (light minus dark) of thylakoid membranes isolated from WT, flv1 KO and flv3 KO grown under continuous moderate light (solid lines) or fluctuating light (dashed lines).

Science objectives for 2019-2020:

- Determine the extent of PSI subunit changes resulting after exposure to increasing light stress in both PSI trimeric and monomeric fractions to enable insights into the mechanism of PSI oligomeric changes.
- Previous work demonstrates an altered distribution of electrons within PSI as a result of the lack of either Flv1 or Flv3 mediated pathways as well as the duration and type of light exposure. Studying the generation and evolution of this altered distribution will lead to a more thorough understanding of adaptive mechanisms employed for coping with excess electron and light stress.
- Interactions between PSI and other PET components, such as light harvesting complexes and redox partners, will be probed using cryo-EM imaging techniques combined with mass spectrometry and spectroscopy to decipher the longer range effects of the loss of the Flv pathway on electron transfer and light energy distribution.

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EVIDENCE OF INTRAMOLECULAR STRUCTURAL STABILIZATION IN LIGHT ACTIVATED STATE OF ORANGE CAROTENOID PROTEIN

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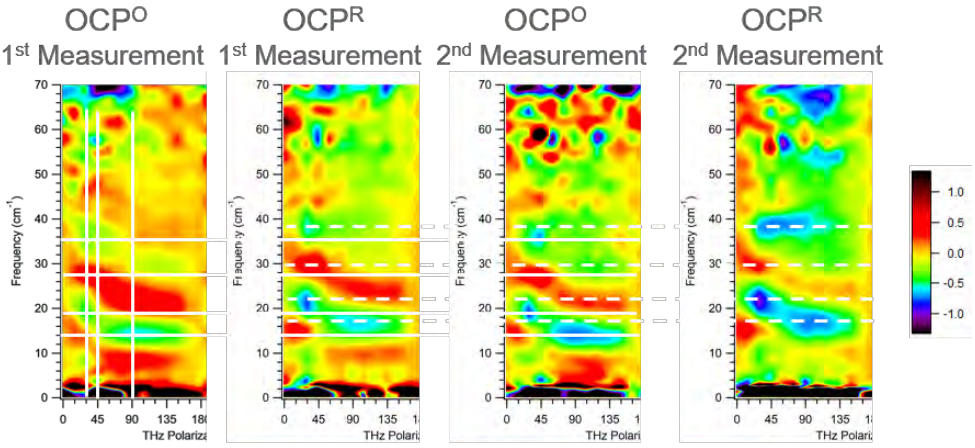
Orange carotenoid protein (OCP) controls efficiency of the light harvesting antenna, the phycobilisome (PBS), in diverse cyanobacteria and prevents oxidative damage. It is the only known photoactive protein that uses a carotenoid, canthaxanthin, as its chromophore. The structure of OCP consists of two globular domains, connected by an unstructured loop, that forms a hydrophobic pocket for the carotenoid. In low light, canthaxanthin bound OCP is inactive and appears orange. Illumination by strong light results in an active state that interacts with the PBS to induce fluorescence quenching, a red appearance and conformational changes that include a 12Å shift by canthaxanthin into the N-terminal domain. Terahertz (THz) dynamical transition measurements and anisotropic terahertz microscopy are used to measure the intramolecular structural dynamics in the inactive and active states, which can be induced by photoexcitation or chaotropic salts. The measurements indicate that the active state has a decrease in structural flexibility, which may be related to enhanced interactions with the PBS.



Angle	OCP^O	OCP^R
90°	14 cm^{-1}	17 cm^{-1}
30°	19 cm^{-1}	22 cm^{-1}
45°	28.5 cm^{-1}	30 cm^{-1}
45°	36 cm^{-1}	39 cm^{-1}

***Anisotropic Terahertz Microscopy Measurements**: Surface plots of OCP crystals in darkness (OCP^o) and following illumination (OCP^R). Solid white vertical lines indicate THz polarization angles where resonance shifts are observed between OCP^O and OCP^R at frequencies indicated by solid (OCP^o) and dashed (OCP^R) horizontal lines.*

Regulation of sustained Cyclic Electron Flow (CEF) in the photopsychrophile *Chlamydomonas* sp. UWO241

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Overall research goals: The goal of this project is to describe the function of sustained CEF and assembly of the PSI supercomplex in the Antarctic psychrophile *Chlamydomonas* sp. UWO241 (UWO241) and the model *Chlamydomonas reinhardtii* acclimated to long-term salinity stress. Major objectives are: 1) determine the functional role of sustained CEF and impacts on downstream carbon metabolism in UWO241 acclimated to variable environmental stressors; 2) dissect the structure of the UWO241 PSI supercomplex through proteomic and structural studies; 3) determine whether *C. reinhardtii* utilizes "UWO-like" supercomplexes to support sustained CEF during long-term stress acclimation. Outcomes of this project will support research focused on meeting future energy and food needs by advancing our understanding of how extremophilic phototrophs use sustained CEF and rewired carbon metabolism to survive long-term exposure to environmental stressors, such as excessive light, low/high temperature, and high salinity.

Significant achievements 2018-2019: During our first year of the project, we have made progress on all three objectives.

- Whole cell proteomic and metabolomic analyses of UWO241 acclimated to a long-term low temperature (8°C)/high salinity (HS, 700 mM NaCl) exhibited significant changes in carbon metabolism. Proteomic analyses by Co-PI Wang shows that key enzymes within the Calvin Benson Bassham (CBB) Cycle are upregulated under HS, including RubisCO and several enzymes important for RuBP regeneration. In addition, two key enzymes for the synthesis of starch and the secondary metabolite, chorismate, were also upregulated in the proteomes of HS cells (Fig. 1). Metabolic analyses performed by our collaborators M. Cvetkovska and N Hüner (Western University, CA), showed high accumulation of glycerol and the compatible solutes, sucrose and proline in HS-grown cells, as well as significant shifts in amino acid metabolism. Last, sustained CEF rates was also associated with upregulation of chloroplastic ATP synthase subunits, suggesting that sustained CEF in UWO241 supplies additional ATP. Taken together, the proteome and metabolome studies thus far suggest rewiring of carbon metabolism in UWO241 as a major acclimatory strategy for robust growth and photosynthesis under permanent environmental stress conditions. The proteomes from this study have been deposited in the MassIVE repository with the identifier MSV000084382 and we have a manuscript under submission [1].

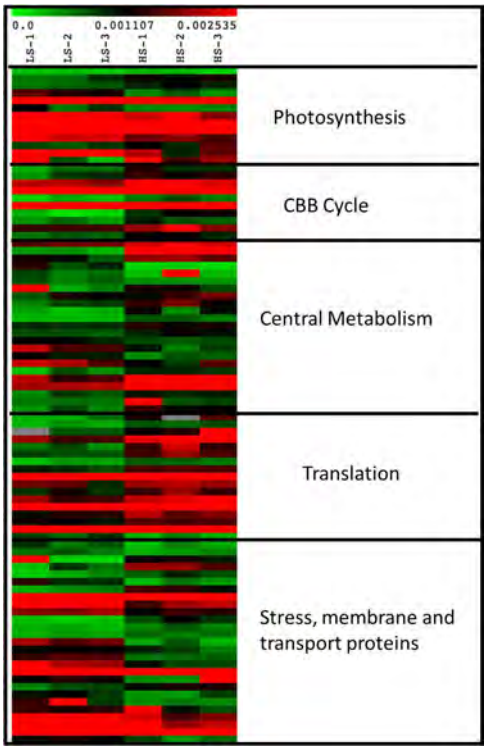

Fig 1: Heat map of differentially regulated proteins in UWO241 under low (LS) and high salinity (HS). The normalized spectral abundance factor (NSAF) values are plotted for each replicate in the two conditions (n=3).

- We discovered that a bestrophin-like anion channel protein is differentially expressed in cells of UWO241 grown under variable iron availability [2]. A search in the UWO241 transcriptome revealed two BEST1 homologues (contig 864 and 973),

one of which possesses putative chloroplast transit peptide and cleavage sites (Fig. 2). Modeling of the UWO241 BEST1 protein suggests that the UWO241 BEST channel forms a pentamer, with the Cl- entryway and exit located on the stromal and luminal sites, respectively, of the thylakoid membrane [1]. The UWO BEST protein likely functions to provide ionic homeostasis during high accumulation of transthylakoid pH.

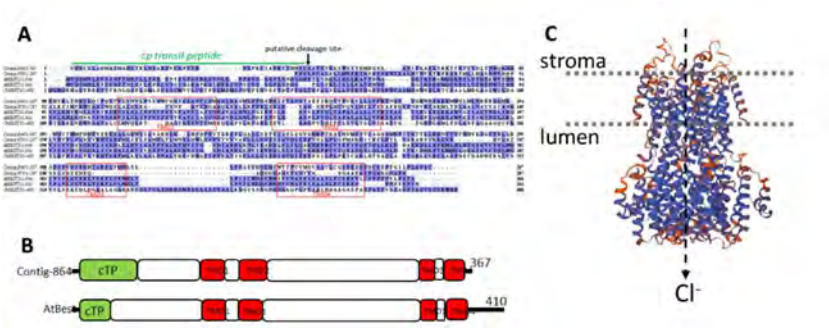

Fig 2 A. Protein alignment of BEST proteins. B. Protein schematics for putative C. sp. UWO BEST protein (contig 367) and *A. thaliana* Best1 (ref). Green and orange blocks indicate putative chloroplastic transit peptides and trans-membrane domains, respectively. C. Predicted tertiary structure of UWO241 BEST channel generated by SwissModel (https://swissmodel.expasy.org/) using *Klebsiella pneumoniae* Best complex as a template

- We have optimized isolation and describe the proteomic composition of the PSI supercomplex UWO241 (SC). Under long term stress conditions, virtually all of PSI appears to be associated with the SC. Furthermore, both the SC and PSI complexes of UWO241 appear to have unique organization and/or composition, as revealed by a lack of typical long wavelength PSI chlorophyll a fluorescence emission at 77K and the absence of the majority of the LHCI proteins, with the exception of Lhca3 and Lhca 5 [1]. Moreover, growth under variable iron, a condition which is typically associated with major remodeling of PSI, had no effect on the LHCI content of UWO241 [2]. Surprisingly, despite the lack of LHCI proteins in the proteomes of either whole cells or isolated complexes, we can detect expression of many Lhca homologues in the transcriptome.
- Unlike UWO241, the model *C. reinhardtii* is highly sensitive to salinity. Over the past year, we have challenged cultures of *C. reinhardtii* grown in minimal medium containing 100 mM NaCl. After 1400 generations, we have a high-salt evolved strain which grows robustly under photoautotrophic conditions. Preliminary growth physiology shows that relative to the wild-type strain, the evolved strain exhibits high growth rates and biomass accumulation under control and high salinity growth conditions.

Science objectives for 2019-2020:

Objective 1: We are growing UWO241 under other long-term stress conditions and assessing: (i) CEF, (ii) supercomplex formation, (iii) expression of a suite of photosynthetic and metabolic genes identified in ref [1], and (iv) tolerance to photoinhibition. ***Objective 2:*** We plan to isolate supercomplexes from UWO241 grown under various conditions and analyze the proteomes and phosophoproteomes. We will collaborate with Co-PI Fromme on supercomplex structural studies. ***Objective 3:*** We are preparing a manuscript which compares CEF, capacity for state transitions, and protein composition of supercomplexes in *C. reinhardtii* vs. psychrophilic strains grown under low and high salinity. We will analyze CEF, supercomplex formation and the whole cell proteomes of the *C. reinhardtii* high salt evolved strain (with Co-PI Wang). We will work with Co-PI Zhang to identify gene targets to test CEF and supercomplex formation in *C. reinhardtii* mutants.

References to work supported by this project 2018-2019 (**Co-PI and PI** of this project; *graduate students)

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Defining the Structure and Reactivity of the HoxEFU Enzyme Complex from *Synechocystis* sp. PCC 6803

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Overall research goals: Adapting to rapidly changing photosynthetic electron and photon flux is a key challenge that photosynthetic organisms must overcome and requires complex enzyme mechanisms for transforming light into chemical bond energy through redox catalysis. Here, cyanobacterial HOX is used as a model system to develop an understanding for how the reaction kinetics and proton-coupled electron transfer (PCET) mechanisms of peripheral redox enzymes function under the dynamic flux of photosynthetic energy transduction (PET). HOX is a bidirectional [NiFe]-hydrogenase pentameric complex that couples photosynthetic electron transfer to the overall $2H^+ + 2e^- \rightleftarrows H_2$ catalytic reaction. HOX consists of a hydrogen (H_2) activating HoxYH [NiFe]-hydrogenase, which forms a complex with HoxEFU that mediates reactions with soluble electron carriers like NAD(P)H and ferredoxin (Fdx). In this project, we aim to understand the reactivity and PCET mechanism of HOX using biochemical and kinetic approaches along with spectroscopic characterization of the cofactor rich enzyme complex. We are particularly interested in determining how the cofactors of the functional HoxEFU diaphorase module are tuned through molecular and protein-protein interactions to coordinate efficient electron-transfer between different substrates and redox partners of PET. Through these studies, we hope to reveal new fundamental knowledge for how multiple electron-transfer reactions are carefully orchestrated across complex enzyme landscapes to achieve efficient energy conversion.

Significant achievements 2018-2019: A heterologous expression and purification method was developed to isolate HoxEFU, enabling the biochemical and kinetic studies in reactions with natural substrates. The purified HoxEFU catalyzed the oxidation of NAD(P)H coupled to reduction of methylene blue, and catalyzed Fdx1-dependent reduction of $NAD(P)^+$. The k_{cat} range for this reaction was 0.6-4 s^{-1} with μM binding affinity for reduced Fdx1, and mM affinity for $NAD(P)^+$. Both Fdx1-dependent reductions of NAD^+ and $NADP^+$ were cooperative. HoxEFU also catalyzed the flavodoxin (Fld)-dependent reduction of $NAD(P)^+$, and Fdx2-dependent oxidation of NADH. Mass spectrometry based mapping of the Fdx1 binding site indicates that Fdx1 binds at an interface between HoxE and HoxF, adjacent to the FeS clusters located in both

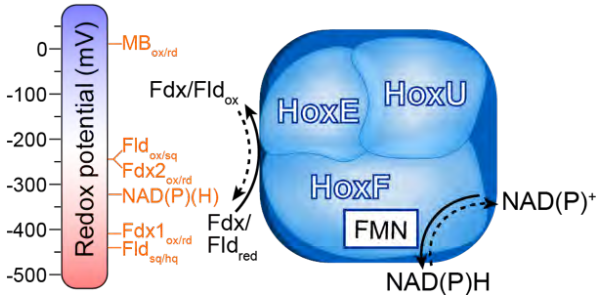

Figure 1. Summary model of *S.* 6803 HoxEFU diaphorase module and observed catalytic activity with different redox partners. HoxEFU catalyzes diaphorase activity either accepting electrons from lower-potential donors Fdx1 and Fld_{red}, or donating electrons to higher-potential acceptors Fdx2, Fld, or methylene blue (MB).

subunits. Negative stain imaging of HoxEFU indicated formation of higher ordered oligomeric structures. EPR spectroscopy also revealed spectral signatures of the reduced FeS clusters in HoxEFU when reduced with either sodium dithionite of NAD(P)H. Overall, the collective results on the reactivity, spectroscopic properties, and structure of HoxEFU implicate a function in managing peripheral electron flow from photosynthetic electron transfer and supports a better understanding of how ubiquitous components may be used to allocate energy flow into desirable outcomes.

Science objectives for 2020-2021:

- Produce new insights on molecular interactions and cofactor tuning that contribute toward highly orchestrated management of electron and proton flux by photosynthesis for transforming light energy into chemical bond energy. Refine the 3D structure of HoxEFU using Cryo-EM techniques to address functional properties associated with higher-ordered structures.
- Define the kinetics of HoxEFU using other physiological electron donors/acceptors such as cytochrome, quinone, and other ferredoxins, which are hypothesized to coordinate electron transfer through peripheral photosynthetic pathways.
- Map possible electron-transfer pathways and free-energy landscape of HoxEFU by EPR spectroscopy and examine additional intracellular redox partners such as NDH1 and PSI by measuring PSI/HoxEFU activity through light-driven reactions.
- Determine how interaction of HoxEFU with HoxYH influences kinetics, reactivity, structure, and oligomeric state, along with the how enzyme complex interacts with redox partners of PET. Identify how these properties further influence photosynthetic pathways.

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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 qH and qI types downregulate 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 2017-2019: We developed snapshot transient absorption (TA) spectroscopy as an approach to directly measure appearance of zeaxanthin radical cations ($Zea^{\bullet+}$) and Zea S_1 excited states as signatures of charge-transfer (CT) and excitation energy transfer (EET) quenching mechanisms, respectively. We performed ultrafast TA measurements on live cells of the microalga *Nannochloropsis oceanica* and successfully observed the formation of $Zea^{\bullet+}$ and the transient population of the Zea S_1 state after Chl *a* excitation, suggesting that both CT and EET mechanisms contribute to NPQ in this alga. Neither TA signal was observed in mutants lacking LHCX1 or Zea. We have used super-resolution structured illumination microscopy (SIM) to investigate the sub-organellar structures of chloroplasts in live cells of *Arabidopsis* and *Chlamydomonas*. We established the capability to perform single-particle cryo-EM analysis of photosynthetic complexes and revealed a unique organization of the PSI supercomplex from the moss *Physcomitrella patens*. Through forward genetics, we discovered a new sustained mode of energy dissipation, called qH, that occurs in wild-type plants under stress conditions such as cold and high light. qH involves at least three proteins: the chloroplastic lipocalin (LCNP) that is required for quenching to occur, the suppressor of quenching 1 (SOQ1) that negatively regulates LCNP activity through direct or indirect biochemical interaction, and a stromal protein called recovery of qH (ROQH1). We identified a putative E3 ubiquitin ligase complex and a homolog of the CONSTANS transcription factor as upstream regulators of light-regulated *LHCSR* and *PSBS* gene expression in *Chlamydomonas*. We developed a pigment-scale model of excitation transfer and showed that qE decreases the excitation diffusion length in grana membranes.

Science objectives for 2019-2020:

- Use snapshot TA spectroscopy to measure timescales of CT and EET quenching in thylakoids from mutants affecting PsbS and xanthophylls.
- Elucidate the biophysical mechanism of qH using TA spectroscopy.

- Perform structure-function analysis of LHCX1 in *Nannochloropsis* using knock-in mutations.
- Investigate functions of LHCX2 and LHCX3 in *Nannochloropsis*.
- Determine the location of PsbS in photosystem II using single-particle cryo-EM analysis.
- Reconstitute qE in proteoliposomes containing oriented LHCII, zeaxanthin, and PsbS.

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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) and remaining open questions about the bacterial reaction center (BRC). We propose a synergistic series of experiments on wild-type and mutant RCs and simpler dyad systems aimed at addressing the following questions:

1) What is the electronic structure of the PSII RC and the BRC?
2) What are the charge separation pathways in the PSII RC?
3) Do electronic-vibrational resonances enhance energy transfer and charge separation in the PSII RC and the BRC?

Significant achievements (2018-2019):

In the past year we have performed several new spectroscopic measurements aimed at addressing the key open questions about the PSII RC. These include broadband 2D electronic spectroscopy (2DES) as well as two dimensional electronic vibrational spectroscopy (2DEV). In addition we have developed a PSII RC-inspired theoretical model for understanding two dimensional electronic Stark spectroscopy (2DESS) which shows high sensitivity to charge transfer states. To better understand the electronic structure and interactions between the Qx and Qy transitions in photosynthetic systems we also performed a comparative 2DES study of bacteriochlorophyll a and chlorophyll a[1].

Although the PSII RC pigments have complete spectral overlap in the Q_y region, the visible-near-IR region contains a wealth of better-separated transitions, including the Q_x transitions and charge-separated product absorptions at ~460nm and ~800nm. 2D excitation spanning Q_y with broadband probing enables the use of these other transitions to identify the role of each pigment in the excitonic structure and charge separation of the PSII RC. We have studied the D1D2-RC by broadband 2DES with a continuum probe to correlate changes in the Q_x region with excitation of the Q_y transitions. The resulting Q_y–Q_x cross-peaks provide a sensitive measure of the electronic interactions throughout the PSII RC network and complement one-color 2D studies in which such interactions are often obscured by energy transfer and excited state absorption signals. We will use this data to refine our excitonic model of the PSII RC and test proposed charge separation mechanisms.

To uncover the charge-transfer states involved in the charge-separation in the PSII RC we developed 2D electronic Stark spectroscopy (2DESS) and transient-grating Stark spectroscopy (TGSS). We expect these methods to be widely applicable to studies of charge separation in other systems. We have collected high quality 2DESS data on the PSII RC and have developed simulations of 2DESS spectra for a PSII RC-inspired dimer model, showing that the 2DESS method is sensitive to the presence of charge transfer states. We are expanding this model to include additional pigments and a more realistic spectral density to model our 2DESS data of the PSII RC.

Bacteriochlorophyll a (Bchl a) and chlorophyll a (Chl a) play important roles as light-absorbers in photosynthetic antennae and participate in the initial charge-separation steps in photosynthetic reaction centers. Despite decades of study, questions remain about the interplay of electronic and vibrational states within the Q-band and the effect of vibronic coupling on the photoexcited dynamics. We performed polarized 2DES measurements on penta-coordinated Bchl a and Chl a, interpreting the findings in the context of state-of-the-art time-dependent density functional theory (TD-DFT)

calculations and vibrational mode analysis for the spectral shape[1]. We found that the Q-band of Bchl a is comprised of two independent bands, that are assigned following the Gouterman model to Q_x and Q_y states, which are predicted to bear orthogonal transition dipole moments (TDM). We measured the angle to be ~75°, a finding that is confirmed by *ab initio* calculations. The internal conversion between the higher Q_x to the lower Q_y band rate is found to be 11 ps^{-1}. In contrast to Bchl a, the Q-band of Chl a contains three distinct spectral lines with different polarizations. *Ab initio* calculations trace these features back to a spectral overlap between two electronic transitions and their vibrational replicas. The smaller energy gap and possible contributions from vibronic coupling result in a fast internal conversion rate in Chl a of 38-50 ps^{-1}.

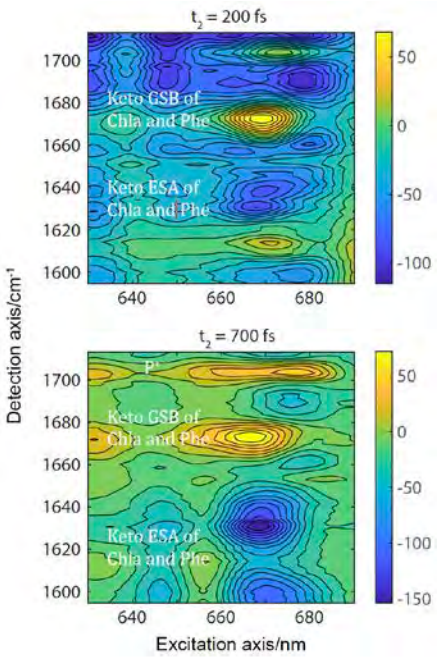

Figure 1: Two-dimensional electronic vibrational spectroscopy (2DEV) of the D1D2 PSII RC at 77K, correlating Q_y excitonic states with pigment vibrational modes as a function of waiting time t_2. The t_2 dependence reveals signatures of charge separation.

The combination of infrared and visible or UV pulses enables a direct mapping between, in the case of molecular systems, vibrational and electronic degrees of freedom. Both typically exhibit inhomogeneous broadening and spectral overlap of distinct transitions. By correlating excited electronic frequencies with detected IR frequencies, we will be able to use the chemical specificity of vibrational bands to help assign the structural nature of electronic transitions even in the presence of environmentally induced spectral heterogeneity. Using the dynamical aspects of 2D spectroscopy by varying the waiting time, we will be able to follow excited electronic state dynamics by viewing the transient influence over well-defined vibrational probes. Combining 2D electronic excitation with a mid-IR probe was first demonstrated by the Fleming group in a method they call 2D electronic-vibrational spectroscopy (2DEV). They have used to follow energy transfer processes between Chl a and Chl b in light-harvesting complex II. The previous implementations of 2DEV are based on our design of pulse-shaper-based pump-probe geometry 2DES. We have recently implemented this approach to 2DEV[2] and have obtained our first data on the PSII RC, shown in Figure 2.

<u>Science objectives for 2019-2020:</u>

- Complete PSII RC-inspired and BRC-inspired 2DESS simulations. Collect 2DESS data for the BRC and use simulations to test and refine excitonic and charge separation models of both systems.
- Compare signatures of coherence in 2DES studies of dyads and site-directed RC mutants.
- Use broadband 2DES in the Qx region and 2DEV to test PSII RC excitonic models
- Combine broadband 2DES and 2DEV data to help elucidate the charge separation mechanism of the PSII RC.

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A novel chlorophyll protein complex in the repair cycle of Photosystem II

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In oxygenic photosynthetic organisms, photosystem II (PSII) catalyzes light–driven oxidation of water. PSII undergoes frequent damage due to its demanding photochemistry. It must undergo a repair and reassembly process following photodamage, many facets of which remain unknown. We have discovered a novel PSII subcomplex that lacks five key PSII core reaction center polypeptides: D1, D2, PsbE, PsbF, and PsbI [1]. This pigment–protein complex does contain the PSII core antenna proteins CP47 and CP43, as well as most of their associated low–molecular–mass subunits, and the assembly factor Psb27. Immunoblotting, mass spectrometry, and ultrafast spectroscopic results support the absence of a functional reaction center in this complex, which we call the 'no reaction center' complex (NRC). Analytical ultracentrifugation and clear native PAGE analysis show that NRC is a stable pigment–protein complex and not a mixture of free CP47 and CP43 proteins. NRC appears in higher abundance in cells exposed to high light and impaired protein synthesis, and genetic deletion of PsbO on the PSII lumenal side results in an increased NRC population, indicative that NRC forms in response to photodamage as part of the PSII repair process. Our finding challenges the current model of the PSII repair cycle and implies an alternative PSII repair strategy. Formation of this complex may maximize PSII repair economy by preserving intact PSII core antennas in a single complex available for PSII reassembly, minimizing the risk of randomly diluting multiple recycling components in the thylakoid membrane following a photodamage event.

This study was supported by the Chemical Sciences, Geosciences, and Biosciences Division, Office of Basic Energy Sciences, Office of Science, U.S. Department of Energy (DOE) (Grant DE-FG02-99ER20350 to H.B.P.); the Photosynthetic Antenna Research Center, an Energy Frontier Research Center funded by the U.S. DOE, Office of Basic Energy Sciences (Grant DE-SC 0001035 to H.B.P., R.E.B., and M.L.G.); NIH Grant P41GM103422 to M.L.G. and NIH Grant GM030498 to T.M.L. V.M.J was supported by a training grant T32 EB014855 from the National Institute of Biomedical Imaging and Bioengineering, NIH.

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Spin-Correlated Radical Pairs as Quantum Sensors for Resolving Structure-Function Relationship in Photosynthetic Proteins

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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 2017-2019: *Regulation of Electron Transfer in Type I Reaction Center Proteins.* While in Type II photosynthetic proteins, ET takes place along only one branch of cofactors, the bidirectional nature of the ET in PSI is well-accepted these days. Our current efforts are focused on the way how protein regulates ET dynamics in PSI, what determines that ET proceed along one or another branch. To address this question, we are using spin-quantum phenomena as sensors of the charge separation processes. Upon light-induced charge separation in RCs, two unpaired spins in the form of a radical-ion pair are created in initially entangled or correlated state with relatively long coherence time allowing them to be detected by time-resolved EPR (TR-EPR). These entangled states were originally termed as spin-correlated radical pairs (SCRP). A single electron spin can be considered as a classical qubit in quantum information science. SCRP is thus an entangled two spin qubit system and is of great interest in quantum information and quantum sensing applications. The exceptional sensitivity of entangled quantum spin

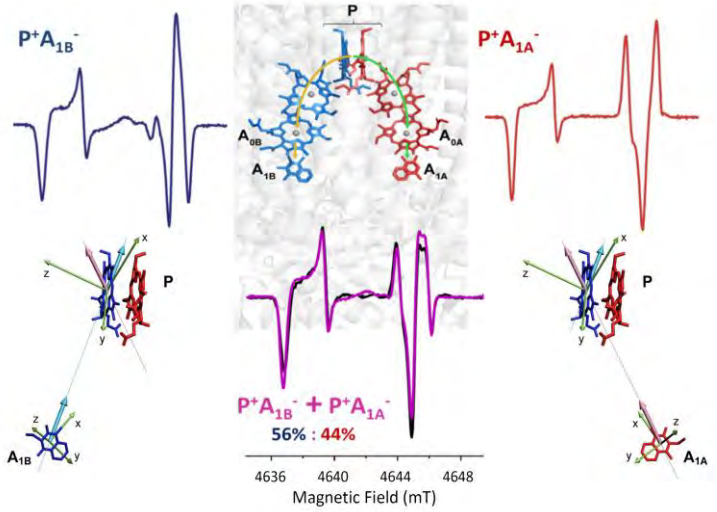

Figure. 130 GHz TR-EPR spectra of PSI complexes from fully deuterated cyanobacterium and schematic structure of ET pathways in PSI. SCRP spectra and structure of RP for PSI were only A-branch is detectable (blue), only B-branch is detectable (red), both A- and B-branch are detectable (purple).

states to weak magnetic interactions, structure, and local environments was used to monitor the directionality of ET in PSI. By photochemical reduction and biochemical modification of PSI we created samples where the radical pair(s) from (1) only A-branch, (2) only B-branch, or (3) both A- and B-branches are detectable. These PSI samples were used to analyse the asymmetry of electron transfer as a function of temperature, freezing condition, and temperature cycling. The temperature dependency agrees with a dynamic model in which the conformational states of the protein regulate the directionality of electron transfer. High spectral resolution afforded by high frequency (130 GHz) EPR, combined with extra resolution afforded by deuterated proteins, provides new mechanistic insight via structural and environmental sensitivity of the entangled electron spins of photogenerated radical pairs.

Science objectives for 2019-2020:

- SCRPs will be used as quantum sensors to locate and characterize the conformational changes that are responsible for ET regulation in PSI. Namely, we will apply time-resolved ENDOR/HYSCORE technique to check protein environment around primary donor and quinone acceptors before and after light excitation for samples were only A-branch or only B-branch is active.
- Examine time-resolved EPR and TA data to clarify the influence of spin-quantum effect on ET-dynamics in PSI.
- Use 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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Electron Flow, O_2 Reduction and Cellular Redox Balancing: Crosstalk Between Chloroplasts and Mitochondria

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Overall Research Goals: When light energy inputs exceed the capacity of photosynthetic organisms to fix inorganic carbon, they can activate a myriad of alternative electron transport pathways that reduce the damaging effects of excess excitation. Several of these pathways catalyze the reduction of O_2 to H_2O and elicit the production of signaling molecules such as reactive oxygen species (ROS) and reduced thioredoxins. A more informed understanding of how the cell prioritizes and activates alternative electron circuits, which can involve distinct enzymes in different cellular compartments, will improve our understanding of photosynthetic processes in the natural environment, where nutrients are often limiting to growth and cells experience dynamic temperature and light conditions. Our results will also establish links between excitation pressure/ROS and novel regulatory circuits that preserve photosynthetic activity and that suggest strategies to potentially enhance photosynthetic yields in the field.

Significant achievements 2017-2019: We have leveraged a *Chlamydomonas reinhardtii* mutant library to isolate several strains that are unable to grow under high light (HL, 1000 µmole m^{-2} s^{-1} PAR), a condition that supports robust growth of the parental strain. Currently, we are focusing on mutants in genes putatively involved in redox communication between chloroplasts and mitochondria, and that serve to sustain cell viability in HL. Mutants of recent interest that are associated with chloroplast-mitochondria communication are defective for genes encoding *a*) the malate-oxaloacetate (MAL-OAA) transporter (OMT) and *b*) the triose phosphate–phosphate transporters (TPTs). These transport systems facilitate the oxidation of NADPH in chloroplasts and the reduction of NAD in mitochondria (**Fig. 1**). HL sensitivity of these mutants suggests that under conditions of excess excitation, mitochondrial respiration and chloroplast photosynthesis are coupled, which helps protect cells from hyper-reduction of the photosynthetic electron transport chain and diminishes ROS production. The expression of genes encoding these shuttles were elevated in HL, with the triose-P transporter, TPT3, exhibiting the most pronounced increase.

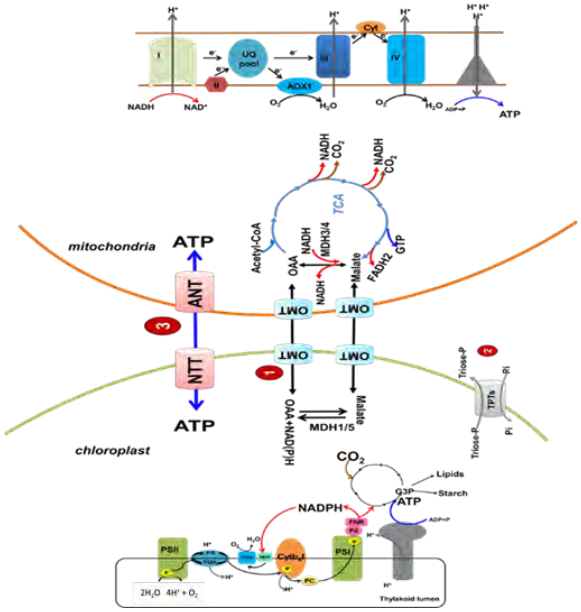

Fig. 1. Chloroplast-mitochondria crosstalk: 1. MAL-OAA shuttle (OMT); 2. TPT shuttle; 3. chloroplast ATP antiporters and mitochondrial ATP carriers (ANT).

TPT3 is on the chloroplast envelope based on localization using a TPT3-VENUS fusion protein (**Fig. 2**). The *tpt3* mutant suffers a complete loss of oxygen evolution and dies when exposed to HL. Complementation of *tpt3* is sufficient to partially rescue the HL phenotype, suggesting that TPT3 plays a critical role in regulating photosynthetic electron transport under HL photoautotrophic conditions. Additionally, transcripts for the malate dehydrogenases MDH4

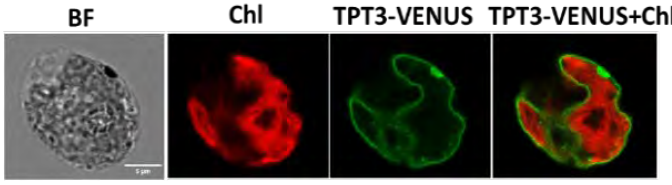

Fig. 2. Cellular localization of TPT3 in *C. reinhardtii*. Bright field (BF), chlorophyll autofluorescence (Chl) in red; Venus fluorescence (VENUS) in green; and merged chlorophyll and Venus signals.

and MDH5 also strongly increase in cells exposed to HL. Overall, these results suggest that the MDH-OAA shuttle plays a critical role in the intracelluar distribution of reductant.

Science objectives for 2019-2020:

- Conduct comprehensive membrane inlet mass spectrometery (MIMS) and genetic experiments to test the hypothesis that trios-phosphate and malate-oxaloacete shuttles are the most prominent redox shuttles used to generate NADH in mitochondria in HL.
- Examine the roles of the different MDH enzymes (five in *C. reinhardtii*) in this process.
- Conduct metabolite analysis to test the hypothesis that the *tpt3* and *omt* mutants accumulate metabolites that are normally exported to mitochondria.

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The Type I Homodimeric Reaction Center in *Heliobacterium modesticaldum*

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Overall research goals: The research objectives are to (1) genetically test our models of light-driven cyclic electron flow pathways within heliobacterial cells by deleting genes for each protein implicated in the pathways and assessing the activities of the mutants; (2) measure the *in vivo* abundance of each ferredoxin by mass spectrometry and the affinity of each for the HbRC by isothermal calorimetry; (3) investigate key structural features of the electron transfer chain within the HbRC by a combination of site-directed mutagenesis and X-ray crystallography, including identification of the quinone-binding site; (4) explore the reduction of quinone to quinol in a newly-created proteoliposome system using biochemical techniques and probe reduction of bound quinone to a semiquinone in a mutant lacking the F_X cluster with time-resolved optical and EPR (electron paramagnetic resonance) spectroscopy; (5) elucidate the electronic structure of the primary electron donor and acceptor using advanced pulsed EPR techniques and quantum mechanical computational chemistry, as well as determine the mechanism of primary charge separation using a combination of site-directed mutagenesis and ultra-fast pump-probe spectroscopy; (6) determine the requirements for assembling an active HbRC by isolating heliobacterial mutants unable to do so and by co-expressing heliobacterial proteins in *Rhodobacter sphaeroides* cells that synthesize BChl *g* and the HbRC subunits.

Significant achievements 2017-2019: We have accomplished the following in the last 2 years.

- Determined the structure of the heliobacterial reaction center (HbRC) from *Heliobacterium modesticaldum* to 2.2 Å resolution. Based on comparisons of the structure with other RCs, we have proposed an evolutionary trajectory for how the extant RCs evolved from a simple homodimeric ancestor that performed Type II RC photochemistry, albeit poorly.
- Determined the midpoint potentials of the 4 low-molecular mass bacterial ferredoxins: PshB1, PshB2, HM1_2505, and FdxB. We have also performed initial proteomic studies on these proteins in heliobacterial cells grown under a variety of conditions.
- Created a genetic system for *H. modesticaldum* that allows us to express genes on replicating plasmids, as well as edit the chromosome by manipulating the endogenous CRISPR/Cas system.
- Used this genetic system to delete genes encoding several proteins: the PshA and PshX subunits of the HbRC, and the PshB1 and PshB2 ferredoxins.
- Complemented the *ΔpshA* mutant with genes expressing oligohistidine-tagged versions of PshA from a replicating plasmid. Using this system, we have created the first HbRC site-directed mutants. Results will be presented on mutations targeting the A_0 primary electron acceptor chlorophyll and the F_X terminal iron-sulfur cluster.
- Demonstrated that illumination of heliobacterial membranes results in reduction of menaquinone (MQ), the pool quinone of this organism, to menaquinol. We have found that MQ photoreduction is inhibited by triazine derivatives such as terbutryn and terbuthylazine, which are potent inhibitors of Type II RCs and bind at the Q_B site, blocking reduction of the mobile quinone.

- Created a proteoliposome system in which purified HbRC is reconstituted into artificial vesicles that are subsequently decorated with hexahistidine-tagged heliobacterial cytochrome c_{553} (the endogenous donor) by virtue of a Ni(II):nitrilotriacetate-linked diacylglycerol incorporated into the membrane. We have shown that membrane-attached cyt c_{553} is capable of reducing P_{800}^{+}, the oxidized primary donor of the HbRC, at rates much faster than the same protein when not attached to the membrane. Initial studies of MQ photoreduction have been performed with this system, demonstrating that the HbRC is capable of reducing MQ without any other proteins beyond the membrane-attached cyt c_{553}.
- Characterized the properties of HbRC lacking the PshX subunit by purifying it from the *ΔpshX* mutant strain. Initiated X-ray crystallography experiments, producing a low-resolution structure.
- Elucidated the pathway of synthesis of bacteriochlorophyll (BChl) *g*, the main pigment of heliobacteria, and engineered *R. sphaeroides* cells to synthesize this pigment.

Science objectives for 2019-2020:

- We will delete genes encoding Complex I and Complex III to test the hypothesis that heliobacteria are capable of operating two different cyclic electron flow pathways.
- We will determine the association constants of the four ferredoxins with the HbRC and carry out affinity studies to determine their primary metabolic targets.
- We will test hypotheses using the site-directed mutants and advanced spectroscopic techniques, including pulsed EPR and pump-probe optical spectroscopy, as well as X-ray crystallography.
- We will test the ability of engineered *R. sphaeroides* cells to assemble active HbRC.

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Revealing excitonic structure and charge transfer in photosynthetic proteins by time-resolved circular dichroism spectroscopy

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Overall research goals: (1) To provide new insight onto the structure and energy transfer dynamics in the Fenna Matthews Olson (FMO) complex using the newly developed time-resolved nanosecond circular dichroism (TRCD) spectrometer and Quantum Mechanical/ Molecular Mechanical (QM/MM) modeling; (2) to demonstrate that the TRCD technique can provide a valuable information on the structure and function of other photosynthetic pigment-complexes and (3) to develop a femtosecond TRCD spectrometer, apply it to studies of the energy transfer FMO and primary charge separation in the photosystem I complex.

While the FMO complex is one of the most widely studied pigment-protein complexes, there is still a debate about the energies of the individual pigments in this system – more than 10 different empirical Hamiltonians have been proposed to model its properties. The TRCD signals associated with the level-to-level energy transfer in antenna complexes with strongly coupled pigments are extremely sensitive to variations in Hamiltonian, and this makes TRCD technique especially powerful for studies of the FMO complex, where each subunit contains 8 closely spaced bacteriochlorophyll *a* (BChl *a*) pigments. In the case of PS I RC, the primary electron donor is believed to be a special pair of strongly coupled pigments. That opens an avenue to directly excite the special pair using CD-modulated pump, which is impossible in the conventional absorption spectroscopy due to spectral overlap with numerous antenna pigments.

Significant achievements 2018-2019:

(1) The nanosecond TRCD signals (ΔA_{CD}) have been measured and analyzed in terms of existing Hamiltonians. Unlike relatively featureless ordinary absorption changes ΔA_{OD}, highly structured ΔA_{CD} signals could not be well reproduced with previously published empirical Hamiltonians. For example, Fig. 1 shows the 55 μs component of the measured decay associated difference spectrum (DADS) (black) modeled with Brixner Hamiltonian (red, Brixner et al., Nature, 434: 625) and Kell Hamiltonian (blue: Kell et al., J. Phys. Chem. A 120: 6146, 2016). Both Hamiltonians reproduce the ΔA_{OD} relatively well but result in poor fit to ΔA_{CD}.

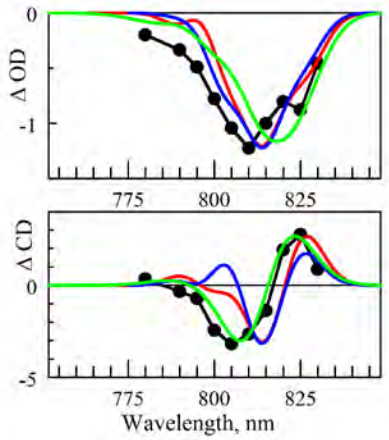

Fig.1. The measured 55 μs DADS component (black) modeled with empirical Hamiltonians (Brixner: red, Kell: blue) and our new Hamiltonian obtained from first principles (green).

(2) To address the observed discrepancies and facilitate analysis and interpretation of the data we performed quantum-mechanical modeling of the FMO complex. We utilized polarizable QM/MM methodology developed by co-PI (LV Slipchenko, J. Phys. Chem. A 114: 8824, 2010) for modeling of absorption and CD spectra of the wild type FMO complex. While this modeling reveals quantitatively accurate shapes of absorption and CD spectra, it is intriguing that the computed electronic Hamiltonian matches well the experimental Hamiltonians for the energetically low-lying pigments (BChl *a* 3, 4, 7, 1, 6) but

differs for the higher-energy sites 2, 5, 8, whose energies cannot be determined from the conventional absorption experiments unambiguously. In addition, the model reveals large thermal fluctuations in spectral positions of some pigments – while transition energies fluctuate within ~10 nm for the lower energy pigments 3 and 4, the transition energy distributions are about twice larger for pigments 2 and 8. Our first principles-based Hamiltonian is capable to reproduce the experimental ΔA_{CD} signals with much better accuracy than the earlier empirical Hamiltonians (Fig. 1, green curve). Using the newly developed Hamiltonian and experimental TRCD data, we now have a detailed triplet energy transfer dynamics in the FMO complex (papers in preparation on both experiment and theory). We have also performed QM/MM modeling of triplet state couplings and energies in the FMO complex that is crucial for correct data interpretation. Moreover, it was found that due to protein-pigment interactions the triplet site energies are significantly lowered (below singlet oxygen level), which explains the absence of experimentally detectable triplet energy transfer to molecular oxygen and high photostability of the complex (paper in preparation).
(3) The FMO growing and purification facility has been established in PI's lab to ensure that work will be not affected due to the retirement of collaborator R.E. Blankenship this summer.
(4) A number of PS I reaction center mutants were obtained and characterized by means of absorption pump-probe spectroscopy in preparation for the future TRCD experiments aimed at direct resolution of the initial charge separation in PS I RC.
(5) The development of femtosecond TRCD spectrometer continued, it was inferred that the commercial piezo-elestic CD modulators exhibit slight (undocumented) assymmetry in R/L modulation causing "false" TRCD signals. Workaround was found and is being implemented.

Science objectives for 2019-2020:

- Perform nanosecond TRCD experiments on FMO mutants along with respective QM/MM modeling to specifically address the elusive energy landscape of the high-energy pigments and to develop theoretical approach for predictive modeling of mutation effects in pigment-protein complexes.
- Finalize the femtosecond TRCD setup and apply it to study wild type FMO complex; analyze its applicability to the studies of charge separation in PS I.

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Identifying the energy transfer dynamics of light-harvesting complexes in membrane nanodiscs

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Overall research goals: The research goal of this project is to measure the timescales of energy transfer in light-harvesting complexes within a near-physiological membrane environment, including how these timescales are influenced by protein-protein interactions. We use ultrafast transient absorption spectroscopy to explore the energy transfer dynamics within and between LH2 from purple bacteria and LHCII from green plants. We embed these light-harvesting complexes within membrane nanodiscs to construct, piecewise, the membrane protein network responsible for light harvesting. Overall, we explore how interactions within this network give rise to efficient and regulated energy flow.

Significant achievements 2017-2019: We embedded LH2 from *Rhodobacter sphaeroides* in nanodiscs, and compared the rates of energy transfer in the membrane nanodisc and in detergent, the typical experimental environment. We discovered that the rate of one energy transfer step is 30% faster (Figure), likely due to a tilt of the pigments from lateral membrane pressure.

Although many species of purple bacteria co-exist, they contain structural and spectral variants of LH2, which further vary with growth conditions. We compared energy transfer rates in three LH2 variants, LH2 from *Rhodobacter sphaeroides* and both LH2 and low-light LH2 (LH3) from *Phaeospirillum molischianum*. While the effect of structural differences between these light-harvesting complexes is consistent with simple models, the effect of the spectral changes is smaller than predicted, suggesting the presence of dark states that mediate energy transfer and maintain similar dynamics at the level of individual LH2.

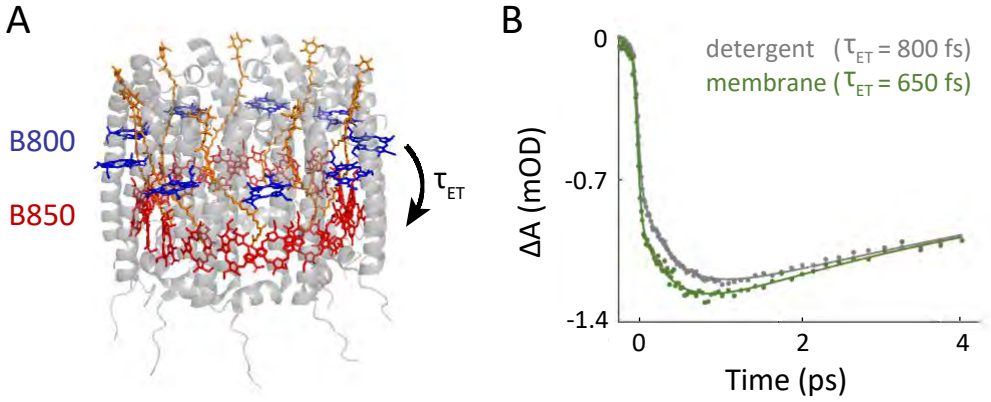

Figure. Energy transfer in LH2. (A) Structural model with the B800 (blue) and B850 (red) rings comprised of bacteriochlorophyll. The timescale of energy transfer (τ_{ET}) between the rings increases by 30% in the membrane environment as measured by (B) transient absorption traces.

Spectroscopic characterization of LHCII, in particular 2D electronic spectroscopy, was previously limited to the two lowest-energy excited states of chlorophyll, which encompasses only 20% of the absorbed solar spectrum. Our ultrabroadband setup offers a three-fold increase in bandwidth over those used previously. We observed previously unidentified, higher-energy pathways in LHCII including ultrafast energy redistribution among the carotenoid S_2 states and dissipative chlorophyll to carotenoid energy transfer.

We embedded LHCII in nanodiscs and determined that the amplitude of the dissipative chlorophyll to carotenoid energy transfer pathway increases by 14% in the membrane as compared to detergent. We identified two carotenoid-chlorophyll clusters perturbed by the membrane, and thus are likely dissipative sites. We further found that the observed dissipative energy transfer pathway is independent of carotenoid composition, and thus may be only one of multiple mechanisms *in vivo*.

Science objectives for 2019-2020:

- LH2 and a low-light analogue of LH2, LH3, have been incorporated within a membrane nanodisc and preliminary structural (AFM and cryoEM) and spectroscopic (transient absorption) characterization have been performed. Further analysis of this data will explore the role of their association in protein-to-protein energy transfer.
- LH1-RC, the site of charge separation in purple bacteria, will be incorporated within a membrane. Measurements on this membrane-protein system will show how the energy transfer dynamics of the larger ring structure of LH1 are affected by the membrane, as well as how the charge transfer dynamics of the embedded RC are protected by the surrounding protein.

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Lipid Chaperoning of a Thylakoid Membrane Signal Peptidase Whose Stability is Modified by the Protonmotive Force

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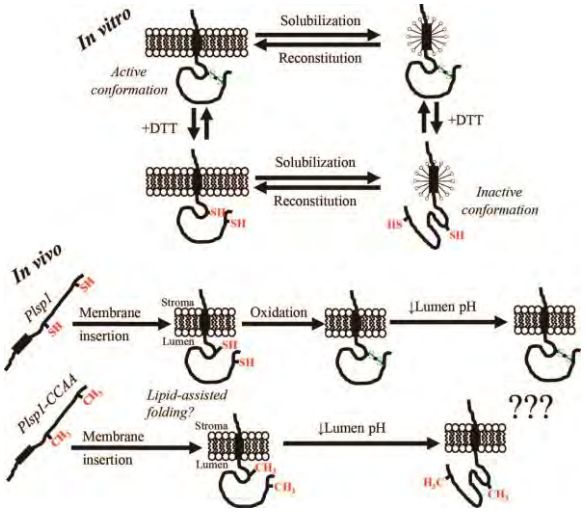
Overall research goals: The primary research goal is to understand the factors that influence the structure and function of thylakoid membrane and lumen proteins in addition to the underlying molecular mechanisms. To this end, a specific thylakoid membrane protein called Plastidic type I signal peptidase 1 (Plsp1) has thus far been used as a tool to investigate the effect of disulfide bonds, membrane lipids, and the protonmotive force (pmf) on lumen-resident proteins and protein domains. Achieving these goals will deepen our understanding of structure-function relationships in photosynthetic membranes, will provide a clearer picture of how the components of the native environment of thylakoid proteins interact, and will provide new insights into the effects of a pmf on the structure and function of proteins in energy-transducing membranes.

Significant achievements 2017-2019:
Plsp1, a single-pass integral thylakoid membrane protein, cleaves the signal sequences from lumen-targeted proteins (Shipman-Roston et al. 2010). When assayed *in vitro* in detergent micelles, this processing activity requires an intramolecular disulfide bond between a conserved Cys pair (Midorikawa et al. 2014). Tryptophan fluorescence analysis and a protease susceptibility assay suggest the abolition of activity is due to an inactivating conformational change in Plsp1. Interestingly, this disulphide bond is not required for Plsp1 activity *in vivo*, as shown by a genetic complementation assay using Plsp1 constructs lacking one or both Cys residues. Further experiments, including processing activity assays after reconstitution of Plsp1 into liposomes, revealed that: (1) thylakoid membrane lipids prevent the inactivating conformational change caused by disulfide bond reduction and (2) the membrane environment facilitates the transition of Plsp1 from the inactive into the active conformation. This suggests that membrane lipids have chaperoning activity towards Plsp1. Interestingly, this chaperoning activity is less effective at pH 5.6 than at pH 8.0, mimicking a typical pH difference between energized and non-energized thylakoids.



In vitro* and *in vivo* models of Plsp1 folding and stability.** ***(In vitro) Plsp1 is solubilized from membranes in an active form due to an intramolecular disulfide bond. Reduction of this disulfide bond causes an inactivating conformational change in detergent micelles that is prevented by association with a lipid bilayer. Reconstitution of Plsp1 into a bilayer causes refolding back into the active state. **(*In vivo*)** Plsp1 is targeted to thylakoids in a reduced form. Once the catalytic domain traverses the membrane, folding into the catalytically active form is assisted by bilayer lipids. In the case of the wild-type protein, oxidation to form the disulfide bond then takes place and stabilizes the structure. Structural stabilization by the disulfide bond allows Plsp1 to remain optimally active during fluctuations in the lumen pH.

Science objectives for 2019-2020:

- In our existing genetic complementation data, there is no discernible phenotype between plants complemented by wild-type Plsp1 or Plsp1 lacking one or both Cys residues. This may be due to the fact that the current genetic constructs are constitutively overexpressed, leading to a scenario in which mutant Plsp1 variants with reduced activity are sufficient to yield a wild type phenotype. We are currently testing whether Plsp1 is functional *in vivo* without a disulfide bond if expression is controlled by the native promoter. If this is the case, we will grow plants under a variety of conditions, including constant light exposure and fluctuating light, with the aim of revealing a

mutant phenotype of plants expressing the mutant Plsp1 variants.

- Results of experiments with proteoliposomes indicate that Plsp1 has significantly lower activity at pH 5.6 than at pH 8.0. These experiments do not provide direct evidence of an effect of pH on Plsp1 stability and are limited to two pH values. We plan to use differential scanning fluorimetry (DSF) to investigate the conformational stability of Plsp1 with or without the disulfide bond and under a wide range of pH conditions. This will provide a more quantitative understanding of the effect of disulfide bond formation and, at the least, the pH component of the protonmotive force on Plsp1 stability.
- Though we have evidence for an inactivating conformational change in Plsp1 caused by disulfide bond reduction, we do not know the structural basis for this inactivation. To this end, we are currently working to obtain a high quantity of catalytically-active Plsp1 with the goal of determining the crystal structure of the oxidized and reduced forms of Plsp1. Current efforts are focused on identifying and optimizing conditions to renature the catalytic domain of Plsp1, purified from inclusion bodies, in an active form. The major challenge thus far is obtaining the renatured oxidized form of Plsp1 at a quantity and purity that is sufficient for crystallization. Aside from providing structural insight into how disulfide bond reduction diminishes Plsp1 activity, determining the structure of Plsp1 in both redox states will provide insight into precisely how lipids act to chaperone the folding of Plsp1.
- The structure and stability of many membrane proteins and complexes are influenced by lipids. In some cases, this is due to a specific lipid species. Our current data do not indicate the chaperoning on Plsp1 folding is due to a specific lipid molecule. We plan to investigate lipid specificity toward Plsp1 by reconstituting it into proteoliposomes of varying lipid compositions. This will allow us to test whether a specific lipid species is chaperoning Plsp1 folding and can allow us to hypothesize about the underlying mechanism.

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Shipman-Roston RL, Ruppel NJ, Damoc C, Phinney BS, Inoue K (2010) The significance of protein maturation by plastidic type I signal peptidase 1 for thylakoid development in Arabidopsis chloroplasts. Plant Physiol 152 (3):1297-1308. doi:10.1104/pp.109.151977

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Mechanisms for tuning protein electron transfer investigated via site-specific linear and two-dimensional infrared spectroscopy

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Overall research goals: The proposed experiments aim to advance our understanding of protein electron transfer (ET), specifically addressing (1) how the intramolecular interactions and electrostatics of the inner and outer coordination sphere of the Cu site of blue copper proteins (BCPs) contribute to tuning ET, (2) how the formation and nature of the complexes with ET partners affect the Cu site in BCPs and their redox properties, and (3) how protein and/or solvent dynamics contribute to reorganization energy of ET. Toward addressing these questions, we have been developing site-specific linear and two-dimensional (2D) infrared (IR) spectroscopy as an approach toward high spatial and temporal investigation of proteins.

Significant achievements 2018-2019: One of the thrusts of our group has been to develop carbon deuterium (C-D) bonds as virtually non-pertubative vibrational reporter groups of specific local sites in proteins. We have applied C-D probes toward understanding how the protein environment, and moreover complexation with ET partners, tunes the Cu redox center of the blue copper protein plastocyanin (Pc). Previously, C-D probes were incorporated as probes at the axial Met97 ligand to the Cu site and used to show that binding to the redox partner cytochrome *f* (cyt *f*) perturbs the ligand's interaction with the metal. Building on this work, we have recently incorporated C-D probes at a second Cu ligand, Cys89. Like at Met97, the C-D vibrations reveal sensitivity of the Cys89 ligand to change in metal center and to binding cyt *f* (Figure 1).

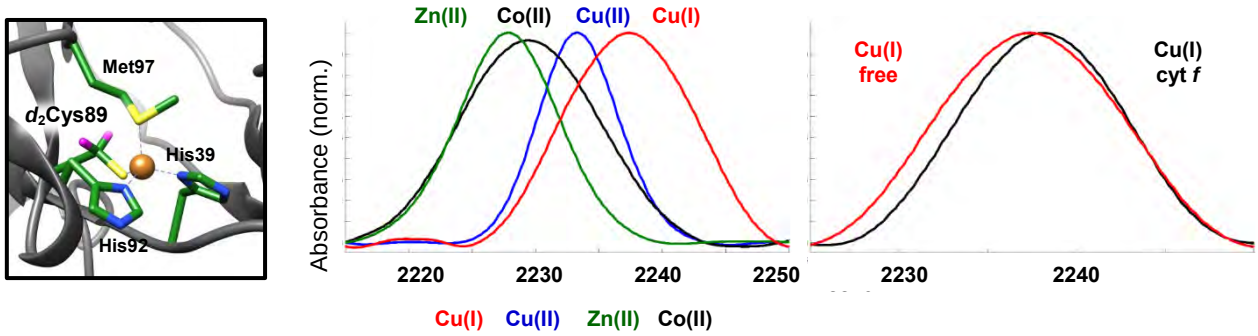

Figure 1. Left: Structural models of the Cu site in Pc showing location of C-D probe (magenta) of ligand d_2Cys89. Middle: IR spectra of d_2Cys89 of reduced (red), oxidized (blue), Zn(II)-substituted (green), and Co(II)-substituted (black) Pc. Right: IR spectra of d_2Cys89 Pc in the absence (red) and presence (black) of cyt *f*.

A second goal of our project is to develop site 2D IR spectroscopy and apply the approach to understanding the complex formed between Pc and cyt *f*. We introduced cyano groups as probes at three locations along the binding surface of Pc (Figure 2). 2D IR spectroscopy revealed increase in frequency inhomogeneity all probes upon binding to cyt *f* that indicates the surface of Pc experiences greater heterogeneity upon interaction with its binding partner. Surprisingly, the

increased inhomogeneity was mostly associated with states that interconvert on the 1-2 ps timescale, not with states that interconvert slowly, as would be expected for strong interactions formed within a tightly engaged surface. Moreover, for all probes the magnitude of the inhomogeneity increase associated with rapid dynamics was about three times greater than that associated with slow dynamics. This implies that the interface of the proteins remains highly mobile in the complex and thus supports that a large population of the ensemble of the Pc-cyt *f* complex exists as the loosely bound encounter complex.

Science objectives for 2019-2021: We plan to use the established C-D probe at Cys89 to evaluate how the strength of the Cu-Cys89 interaction depends on the axial Cu-Met97 interaction, and the consequences to the midpoint potential. The C-D probes will be used to investigate constructs with the Met97 ligand substituted by residues expected to interact more strongly (Glu) and not interact (Leu) with the Cu ion. A second goal is to investigate how disruptions in the outer sphere interactions and alterations to the polarity of residues surrounding the Cu ion affect the interactions of the Cu ion with Cys89 and Met97 and whether such a mechanism mediates changes to the midpoint potential. Finally, we plan to extend our study of the complex of Pc and cyt *f* via site-specific 2D IR spectroscopy to investigate the role electrostatic interactions by characterizing the complex at varying ionic strength.

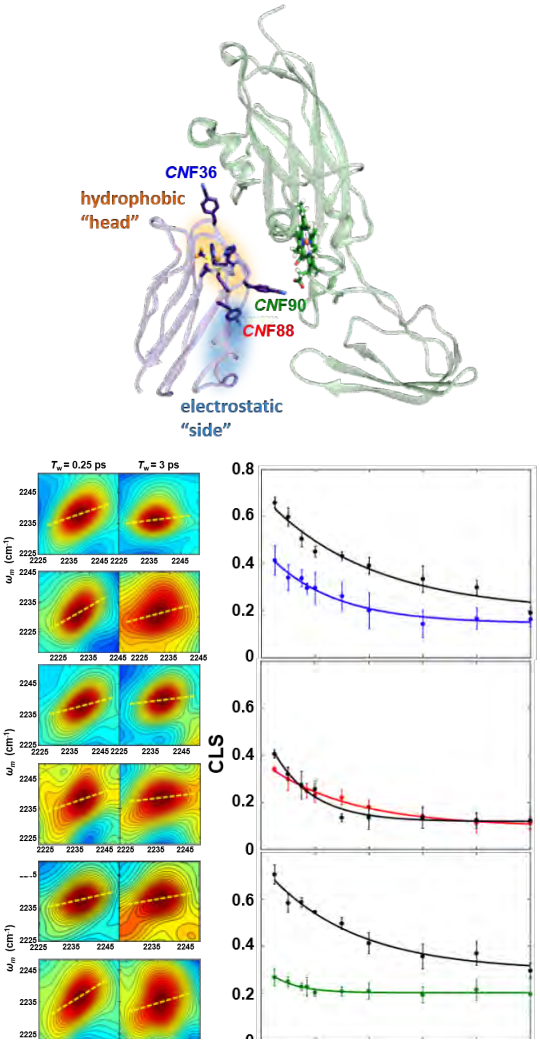

Figure 2. Top: Structural model of the Pc-cyt *f* complex (PDB 1TU2) highlighting introduced *CN*F probes. Bottom left: Representative 2D IR spectra at T_w of 0.25 ps and 3 ps for free Pc (top panels) and the complex with cyt *f* (bottom panels) for *CN*F36 (top), *CN*F88 (center), and *CN*F90 (bottom). Bottom right: Normalized frequency-frequency correlation functions (points) and fits (lines) for free Pc (colored) and the complex with cyt *f* (black) for *CN*F36 (top, blue), *CN*F88 (center, red), and *CN*F90 (bottom, green).

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Tracking Sites for Multiscale Photosynthetic Function Using X-ray Scattering and Electron Microscopy Imaging Techniques

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Overall research goals: This program addresses resolution of fundamental mechanisms controlling solar energy conversion in photosynthesis by investigating photosynthetic function across multiple length scales, ranging from local protein-cofactor interactions to long-range photosynthetic function in intact thylakoid membranes. Key questions that are investigated include the identification of mechanisms for electron transfer in multi-cofactor photosynthetic and biohybrid redox chains, mechanisms for protein-protein recognition in photosynthetic light-harvesting and electron transfer assemblies, and visualization of sites on photosynthetic and biohybrid protein surfaces where accumulated reducing equivalents are accessible for subsequent energy conversion chemistry. The program highlights the development of advanced synchrotron-based X-ray scattering and spectroscopy, correlated with multi-scale imaging approaches.

Significant achievements 2017-2019: *Implicating the Cardiolipin Binding Surface on Bacterial Reaction Centers as the Nucleating Site for Photosynthetic Supercomplex Assembly.* Recent work on the structures of reaction center-light harvesting 1 core complexes, RC-LH1, in purple bacteria highlight the intriguing feature that these proteins are responsible for both controlling the cascade of photochemical reactions at local cofactor sites, but also contain information on molecular recognition and nucleating sites for tiered self-assembly of photosynthetic core complexes, ultimately leading to photosynthetic membrane and sub-cellular structures. Since other proteins are not required for RC-LH1 assembly, we hypothesize that detergent-isolated RC and LH1 proteins contain the information needed to trigger core complex assembly. In this report, we demonstrate temperature-dependent dimerization of the detergent-solubilized RC from *Rhodobacter sphaeroides* using wide angle X-ray scattering, WAXS. Structure modeling analyses, Figure 1, identify the cardiolipin binding domain as the site for temperature-triggered RC dimer assembly, providing the first experimental detection of function for this RC-lipid cofactor. The results are discussed in terms of the proposed role for the RC in nucleating core complex assembly and suggest opportunities to probe mechanisms for supercomplex assembly by investigating the physical behavior of

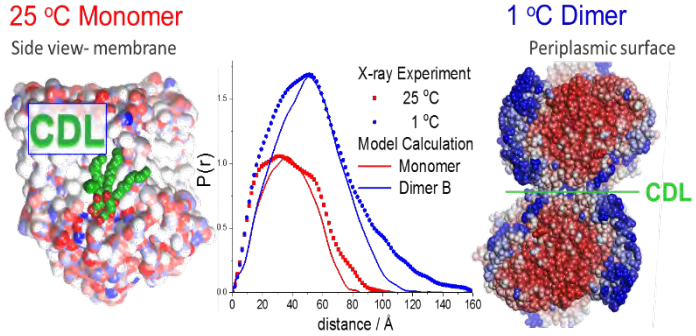

Figure 1. Modelling of RC X-ray scattering and pair distribution function (PDF) analysis shows that temperature dependent dimerization is triggered by the cardiolipin binding surface.

these proteins in detergent-isolated states. Further, these studies demonstrate opportunities to use wide angle X-ray scattering combined with coordinate model analysis to identify structures of self-assembled photosynthetic protein-protein complexes in solution.

Imaging Sites for PSI Reductive Photochemistry in Isolated Proteins and Membrane Systems. We have initiated a project to image sites for PSI photo-reductive chemistry using a combination of high-resolution scanning transmission electron microscopy (HR-STEM) and wide-angle X-ray scattering and pair distribution function scattering (PDF) analyses. The project will investigate how sites for chemical reductive activity map onto the flavodoxin (Fd) binding site, and spatially resolve PSI photosynthetic function. Crystal structures show that the Fd binding sites have a separation of 12 nm in the PSI trimer, Figure 2A. Initial STEM measurements of cyanobacterial thylakoids labelled with anionic mercaptosuccinic acid capped Pt NPs so evidence for Pt NP tri-clusters having a NP spacing with a shorter 6 nm separation, Figure 2B, that maps onto a prossible alternative site for Pt-NP docking.

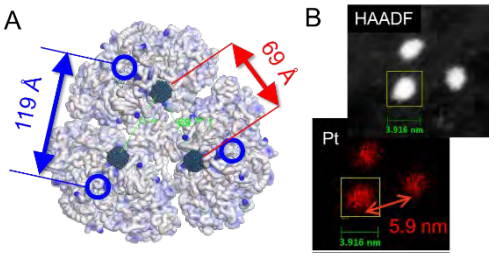

Figure 2. A. PSI trimer X-ray structure. The blue circles mark the position of flavodoxin binding. The black dots show an alternative site for Pt NP binding. **B.** STEM and STEM-EELs imaging of a Pt NP tri-cluster in cyanobacterial thylakoids.

Science objectives for 2019-2020:

- STEM and cryo-STEM will used to compare sites for anionic Pt nanoparticle binding and Pt photo-precipitation and the comparison to the crystallographically determined sites for flavodoxin binding.
- 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 use reciprocal space techniques to identify locations on the PSI surface where accumulated reducing equivalents are accessible for secondary electron.
- 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.

Selected references to work supported by this project 2018-2019:

1. D. M. Tiede, Utschig, L. M., N. Ponomarenko, "Implicating the Cardiolipin Binding Surface on Bacterial Reaction Centers as the Nucleating Site for Photosynthetic Supercomplex Assembly, *Biochemistry*, **2019**, submitted.

2. N.S. Ponomarenko, O. Kokhan, P.R. Pokkuluri, K.L. Mulfort, D.M. Tiede, "Examination of Abiotic Cofactor Assembly in Photosynthetic Biomimetics – Site-specific Stereoselective Conjugation of bis(2,2′-Bipyridyl)Ruthenium(II) to Multi-Cofactor Proteins Demonstrated by Circular Dichroism Spectroscopy" *Photosyn. Res.*, 2019, Special issue: "Photosynthetic-inspired biohybrid and biomimetic systems", submitted.

3. N. Ponomarenko*, J. Niklas, P. R. Pokkuluri, O. G. Poluektov, D. M. Tiede*. "EPR Characterization of the Triheme Cytochrome from *Geobacter sulfurreducens*." *Biochemistry,* **2018**, *57*, 1722-1732. DOI: 10.1021/acs.biochem.7b00917.

4. J.M. Dantas, M.R. Ferreira, T. Cararino, O. Kokhan, P.R.Pokkuluri, C.A. Salgueiro. Molecular interactions between *Geobacter sulfurreducens* triheme cytochromes and the redox active analogue for humic substances. *Biochim Biophys. Acta*., **2018**, 1859, 619-630. DOI: 10.1016/j.bbabio.2018.05.00

Photosynthetic Biohybrid Systems for Studying Mechanisms of Solar Energy Conversion

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Overall research goals: Natural photosynthetic energy research is aimed at resolving fundamental mechanisms of photochemical energy conversion in photosynthetic proteins. This information will enable the design of sustainable photosynthetic-inspired systems for solar energy conversion and solar fuels production. Solar energy conversion occurs in integral membrane reaction center (RC) proteins and involves light-induced rapid, sequential electron transfer steps that result in the formation of a long-lived charge separated state. In this manner, light energy is converted into chemical energy that is subsequently used to drive the chemical reactions required for photosynthesis. This program addresses outstanding questions in two important features of Nature's photosynthetic design: (1) the coupling of light-driven RC charge separation to secondary reaction sequences that involve native small electron shuttles, charge-accumulating redox protein cofactors, and synthetic catalysts, and (2) long range photosynthetic electron transfer in supercomplexes and membranes. This work leverages our team's capabilities in bioinorganic and synthetic chemistries for hybrid assembly of Nature's photosynthetic protein networks with synthetic photosensitizers and abiotic catalysts to enable spectroscopic characterization and generate a deeper, more mechanistic understanding of the chemistry that controls light-driven ET pathways, directionality and charge accumulation in photosynthetic systems.

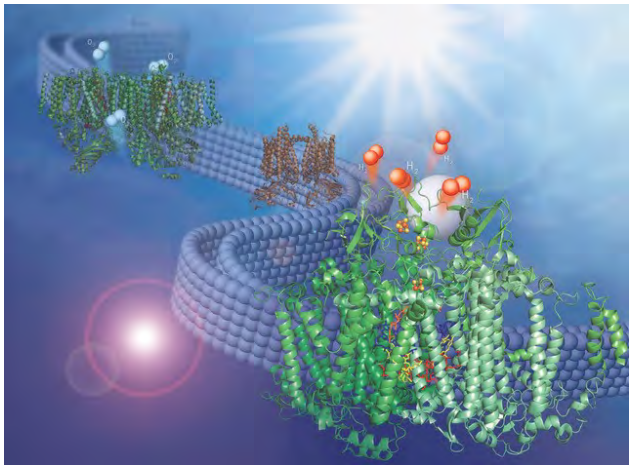

Figure 1 Schematic representation of abiotic catalyst self-assembly with the stromal end of PSI. Electrons originating from light-driven oxidation of water by PSII are transferred through the thylakoid membrane to PSI catalyst sites for light-driven generation of H_2.

Significant achievements 2017-2019:

Z-scheme solar water splitting via self-assembly of Photosystem I-catalyst hybrids in thylakoid membranes. In recent work, we utilize the native photosynthetic Z-scheme electron transport chain to drive hydrogen production from thylakoid membranes by directional electron transport to abiotic catalysts bound at the stromal end of Photosystem I. Pt-nanoparticle and first-row transition metal molecular catalysts readily self-assemble with Photosystem I in spinach and cyanobacterial membranes. By self-assembling catalysts within the photosynthetic membrane, the system is self-sustaining as electrons that originate from the light-driven oxidation of water are transferred through Nature's inherent, highly tuned electron transport chain to the abiotic sites for hydrogen generation.(Figure 1) Thus, the self-assembly of abiotic catalysts with photosynthetic membranes demonstrates a tenable method for accomplishing solar overall water splitting to generate H_2, a renewable and clean fuel. This work provides the basis for future studies implementing *in vivo* approaches to generate living photosynthetic systems as a sustainable energy source.

Interprotein electron transfer biohybrid system for photocatalytic H_2 production. An inherent interprotein ET reaction in the photosynthetic pathway has been reengineered to make it photocatalytic for H_2 production. The native electron shuttle protein Fd is used as a scaffold for binding of a ruthenium photosensitizer and H_2 catalytic function is imparted to its partner protein, FNR, by attachment of cobaloxime molecules. We find that this 2-protein biohybrid system readily produced H_2 in aqueous solutions via light-induced interprotein ET reactions, providing insight about using photosynthetic charge accumulation as a method for fuel generation.

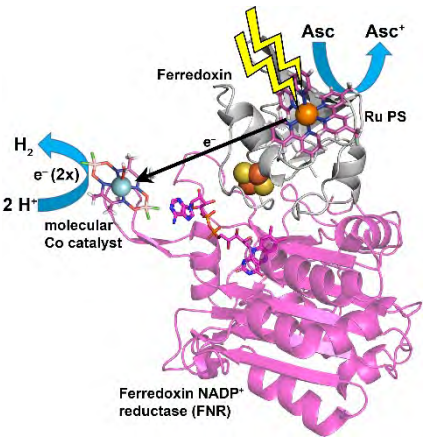

Figure 2 A new interprotein hybrid system for H_2 photocatalysis with RuPS bound to Fd and cobaloxime catalyst bound to FNR**.**

Science objectives for 2018-2020:

- Develop new experimental strategies for exploring RC-driven reduction of FNR via the electron shuttle proteins Fd and Fld with unique isotopically-labeled protein constructs.
- Investigate the structural and electronic factors that underpin photoinduced ET, charge accumulation, and catalysis in biohybrid architectures.
- Examine PSI-driven charge accumulation via one and two electron shuttle proteins and small molecule electron acceptors.
- Using a unique combination of isotopically labeled cyanobacterial membranes and isolated proteins, develop EPR methods to characterize the coupling of RC primary photochemistry to secondary reaction sequences in supercomplexes and thylakoid membranes.

References to work supported by this project 2017-2019:

1. S. R. Soltau, J. Niklas, P.D. Dahlberg, K. L. Mulfort, O. G. Poluektov, L. M. Utschig, "Charge Separation Related to Photocatalytic H_2 Production from a Ru-Apoflavodoxin-Ni Biohybrid", *ACS Energy Lett.,* **2017**, 2, 230-237.
2. J. S. Plegaria, M. Sutter, B. Ferlez, C. Aussignargues, J. Niklas, O. G. Poluektov, C. Fromwiller, M. TerAvest, L.M. Utschig, D. M. Tiede, C. A. Kerfeld, "Structural and Functional Characterization of a Short-Chain Flavodoxin Associated with a Non-Canonical PDU Bacterial Microcompartment", *Biochemistry*, **2017**, 56, 5679-5690. DOI: 10.1021/acs.biochem.7b00682.
3. L. M. Utschig, S. R. Soltau, K. L. Mulfort, J. Niklas, O. G. Poluektov, "Z-scheme solar water splittiing via self-assembly of photosystem I-catalyst hybrids in thylakoid membranes," *Chem. Sci.*, **2018**, 9, 8504-8512. DOI: 10.1039/c8sc02841a.
4. O. G. Poluektov, J. Niklas, L. M. Utschig, "Spin-correlated radical pairs as quantum sensors of bidirectional ET mechanisms in Photosystem I," *J. Phys. Chem. B*, **2019**, 123, 7536−7544. DOI: 10.1021/acsjpcb.9b06636.
5. U. Brahamachari, R. Pokkuluri, D. M. Tiede, J. Niklas, O. G. Poluektov, K. L. Mulfort, L. M. Utschig, "Interprotein electron transfer biohybrid systems for photocatalytic H_2 production," *Photosyn. Res.*, **2019**, submitted.

Assembly and Repair of the Photosystem II Reaction Center

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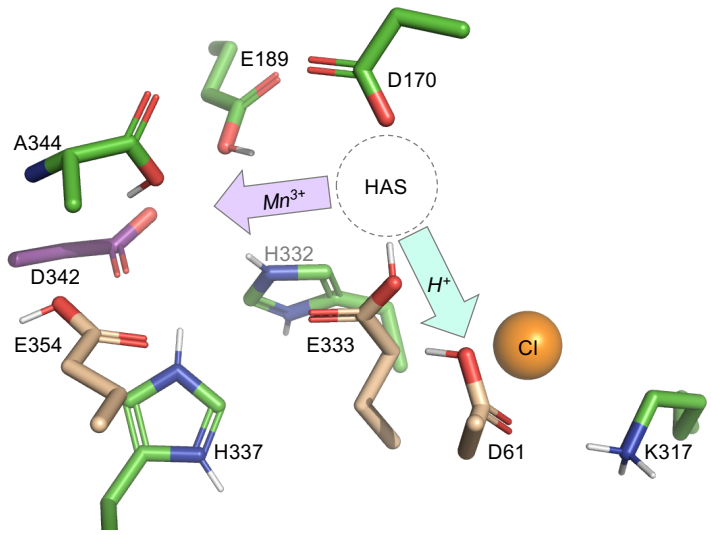
Overall research goals:

- We aim to identify and characterize protein-protein interactions that facilitate transport of early PSII intermediates from the plasma membrane to the thylakoid membrane in cyanobacteria.
- We are studying the dependence of soluble proteins, pH, and chloride on photo-assembly of the Mn_4CaO_5 oxygen-evolving complex (OEC).
- We are using mass spectrometry and numerical simulations to quantify when and how PSII centers are targeted for repair.

Significant achievements 2017-2019:

The metals for the OEC are assembled within the PSII protein environment via a series of binding events and photochemically induced oxidation events, but the full mechanism is unknown. Using spinach PSII membranes, we observed that the efficiency of OEC photo-assembly is highly dependent on protons and chloride. Under chloride limiting conditions, the kinetics of photo-assembly show a significant lag phase that exhibits an inverse H/D solvent isotope effect. In collaboration with Marilyn Gunner (City College of New York), we analyzed the protonation states of the apo-OEC protein environment using classical multi-conformer continuum electrostatics (MCCE). Combining experiments and simulations led to a model in which protons are lost from amino acids that will serve as OEC ligands as metals are bound. Chloride and D_2O increase the proton affinities of key amino acid residues. These residues tune the binding affinity of $Mn^{2+/3+}$ and facilitate the deprotonation of water to form a proposed μ-hydroxo bridged $Mn^{2+}Mn^{3+}$ intermediate.



A working model for the early steps of OEC photo-assembly. The high affinity site (HAS) represents the location where Mn^{2+} binds during photo-assembly. To make room for the second Mn^{2+} to bind, oxidized Mn^{3+} migrates deeper into the apo site (purple arrow). Water must be deprotonated for μ-hydroxo bridge formation, and the proton(s) are shuttled out of the site (cyan arrow). Residues D1-E333 and D1-61 facilitate these two processes. When chloride is removed, the pK_a values of CP43-E354, D1-E333, and D1-D61 (tan sticks) decrease and the pK_a value of D1-D342 (purple sticks) increases.

Science objectives for 2019-2020:

- A proteomics study will be completed to compare the protein compositions of plasma membranes, thylakoid membranes, and PratA-defined membranes from *Synechocystis* sp. PCC 6803. This mass spectrometry-based approach will reveal new targets for proteins that stabilize early PSII assembly intermediates.
- Kinetic studies of OEC photo-assembly will be extended to cyanobacteria core complexes and spinach PSII membranes containing controlled compositions of extrinsic subunits.
- The turnover of old and young populations of PSII reaction centers will be simultaneously tracked using stable isotope labeling and mass spectrometry. The resulting kinetics will be used to build a numerical model for the energy economy of the PSII repair cycle.

Reference:

David J. Vinyard, Syed Lal Badshah, M. Rita Riggio, Divya Kaur, Annaliesa R. Fanguy, and M. R. Gunner, "Photosystem II oxygen-evolving complex photo-assembly displays an inverse H/D solvent isotope effect under chloride-limiting conditions." *Proceedings of the National Academy of Sciences, USA*, **2019**, *116*, 18917-18922.

Single Photon Studies of Quantum Efficiency of Photosynthetic Light Harvesting

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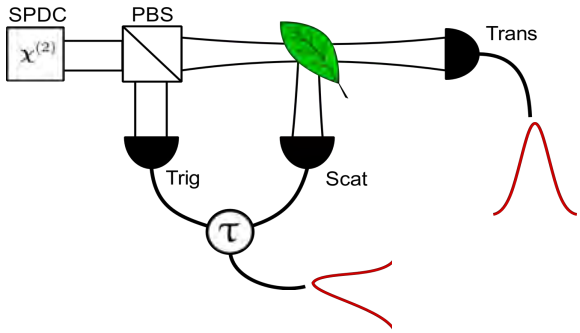
Overall research goals:
Our long-term goal is to generate a complete and consistent microscopic understanding of the dynamical processes underlying the transduction of energy from sunlight to electron-hole pairs in photosynthetic systems that takes into account the absorption of single photons from the ultraweak radiation field of sunlight. The current project is the first stage of this research program, namely the development of experimental and theoretical tools to probe and characterize the absorption of single photons by light harvesting systems from single photon states prepared with controllable quantum light sources.



Illuminating the sample with a heralded single photon states (non-classical light pulses that contain one and only one photon) guarantees that any fluorescence from an FMO complex originated from a single exciton. The time delay between the pulse's arrival time and the detection of any subsequent fluorescence contains information about the (in)efficiencies in the transport of a single excitation across the light-harvesting complex.

Figure 1. Schematic of experimental setup for a source of correlated pairs of single photons (SPDC), of which one is incident on the sample and either transmitted or scattered, while the other 'heralds' the absorption event and triggers the timing of a coincidence counter. Trans, Trig and Scat denote the detectors for transmitted, herald, and fluorescent photons, respectively.

Significant achievements 2018-2019:

- We developed the theory for scattering/absorption of states of light with exactly one photon from a light harvesting system with analysis of probabilities of transmission, fluorescence and absorption. This involves analysis of the input and output photon flux, in addition to the probability of excitation and excitonic energy transport in the light harvesting complex. Our theory includes the effects of phonons, i.e., vibrations of the chromophores and their protein environment. The basic theory employs a density matrix approach and produces expected values of excitation probabilities for different excitonic states as well as photon fluxes for transmission and fluorescence. We have also developed a theory for calculation of individual quantum trajectories based on detection and counting of the fluorescent photons.

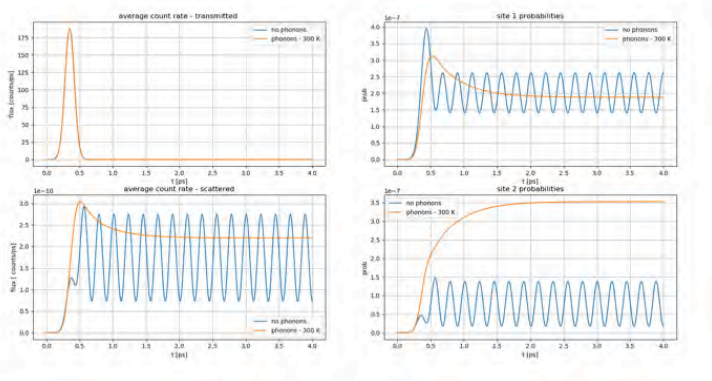


Figure 2. Average photon fluxes, i.e., counting rates, for transmitted and scattered photons (left panels) and corresponding excitation probabilities of two chromophores (right panels) resulting from incidence of a single photon on a light harvesting dimer as a function of time (ps) at T=300K. The effect of exciton-vibration coupling is to severely dampen the oscillations in both photon counting rates and chromophore excitation.

- We used the density matrix calculations to study the relationship between excitation of chromophores and transmission and fluorescence in models of small light harvesting complexes, with and without the excitonic coupling to vibrational modes. The transmitted and fluorescent photon fluxes correspond to expected average photon counting rates. Figure 2 above shows an example calculation for a light harvesting dimer.

- We have made calculations of the quantum trajectories for interaction of single photons with chromophore arrays without coupling to phonons, characterizing the statistics of emitted and transmitted photons. The temporal distributions of the emitted photons show oscillations reflecting the oscillatory behaviour of the excited electronic states after excitation. This is validated by Fourier transforming the temporal distributions and comparing with the excitonic energies.

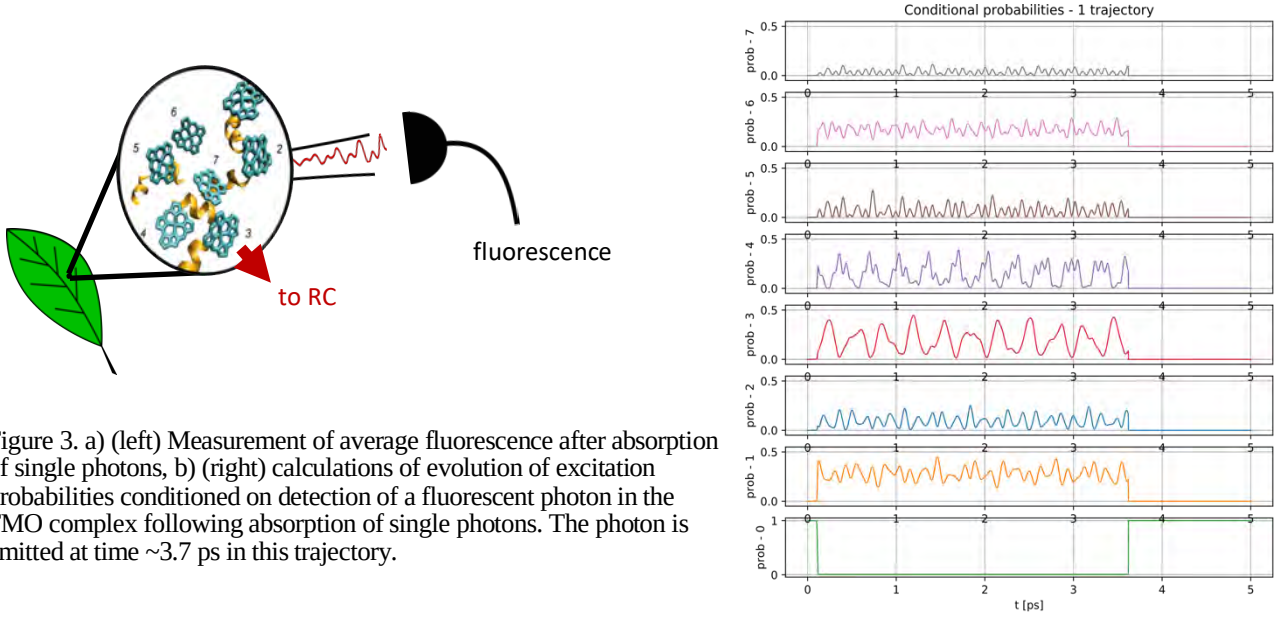


Figure 3. a) (left) Measurement of average fluorescence after absorption of single photons, b) (right) calculations of evolution of excitation probabilities conditioned on detection of a fluorescent photon in the FMO complex following absorption of single photons. The photon is emitted at time ~3.7 ps in this trajectory.

- We have made progress in building the correlated incidence/fluorescence photon counting experiment in Figure 1. We are still seeking to hire an experimental postdoc for this.

<u>Science objectives for 2019-2020:</u>

- Hire an experimental postdoc for the photon counting experiments. Complete building of experimental setup and carry out first counting experiments to establish correlation statistics between incident and fluorescent photons.

- Undertake realistic theoretical simulations of the expected incidence/fluorescence photon fluxes and excitation probabilities with additional channels to account for loss. Analyze the energy redistribution budget following interaction of a single photon with the light harvesting system.

- Develop theoretical approach to incorporate exciton-photon coupling in the photon counting trajectories and use calculations based on this to probe the energy transfer to phonons during and subsequent to absorption of single photons.

Signal Transduction Pathways of Chloroplast Quality Control

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Background: In eukaryotes, photosynthesis take place in chloroplast organelles that contain about 3000 proteins. Although chloroplasts contain their own small genomes, more than 95% of these proteins are encoded in the nucleus, translated in the cytoplasm, and imported into chloroplasts. Thus, each chloroplast must be in constant communication with the nucleus and the rest of the cell to regulate its own proteome, to maintain efficient photosynthesis, and to avoid accumulation of reactive oxygen species (ROS). Although ROS are the inherent by-product of photosynthesis, they can lead to photoinhibition or cellular degradation. One form of communication, called retrograde signaling, allows undeveloped or damaged chloroplasts to regulate the expression of nuclear genes encoding photosynthesis- and stress-related proteins. In a previous DOE-funded project, we have investigated these signals and found that the tetrapyrrole pathway (hemes and chlorophylls) in the chloroplast is one source[4,5]. Then, expanding on that work, we identified a new layer of communication that allows severely damaged chloroplasts to initiate their own degradation. Such a chloroplast quality control mechanism may be important for a cell to maintain a healthy population of chloroplasts (Figure 1[1-3]).

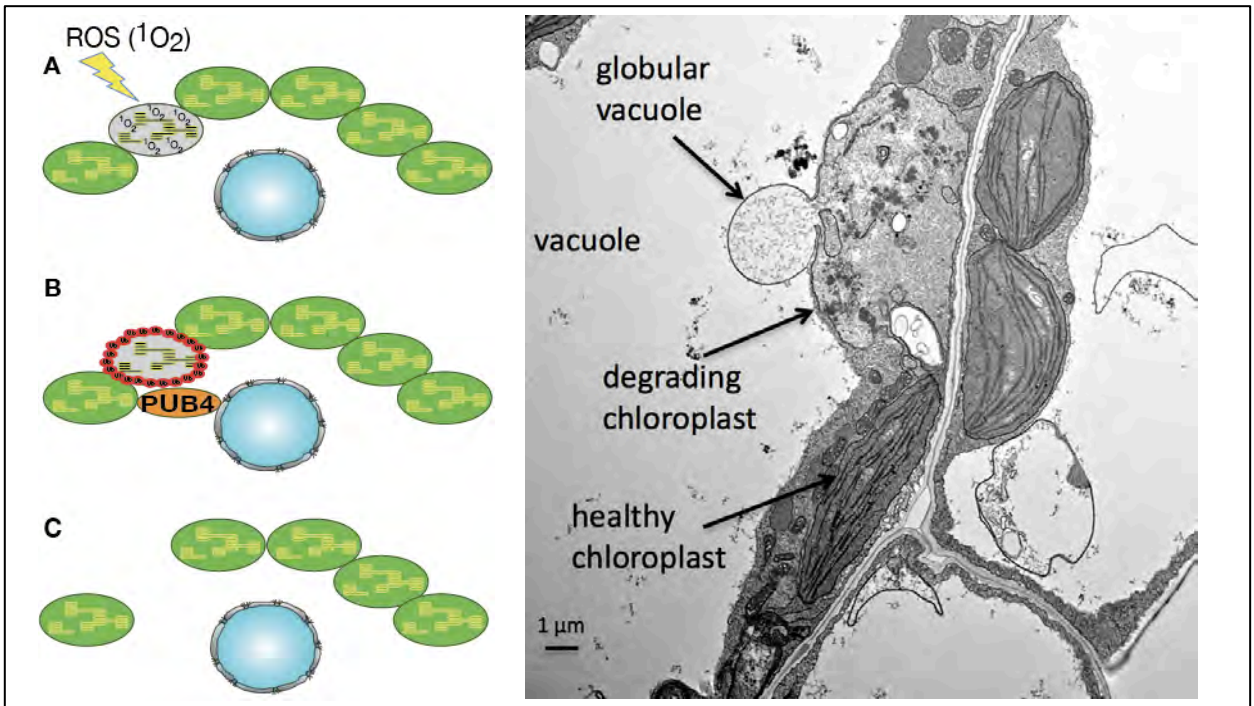

Figure 1: Left panel: Model for selective chloroplast degradation. A) During photosynthesis stress, singlet oxygen $^1O_2$ accumulates in individual chloroplasts. B) This leads to the ubiquitination of unidentified chloroplast membrane proteins. Although the mechanism is unknown, it involves the cytoplasmic 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 project 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 pathway and the generation of new genotypes will ultimately influence our abilities to manipulate plant growth and development. Furthermore, this work will aid in our understanding of the developmental control of photosynthesis, the chloroplast assembly/disassembly pathways, and how plants acclimate to stressful environments.

Significant achievements: By studying *plastid ferrochelatase 2* (*fc2*) mutants of *Arabidopsis* we have shown that a chloroplast 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 ($^1O_2$). How the accumulation of $^1O_2$ leads to signalling or chloroplast degradation is not known. To further understand this novel pathway, we used a forward genetic screen to identify suppressors of chloroplast degradation in the *fc2* mutant. We have identified 23 mutants (representing 17 loci) defective in $^1O_2$ signalling and chloroplast degradation. For twelve mutants (representing eight loci), we have successfully mapped the causative mutations and identified the genes and proteins affected. These proteins have roles in chloroplast protein import, chloroplast gene expression, tetrapyrrole metabolism, and the cellular ubiquitination machinery. The later role was inferred from the mutant *fts29*, which carries a mutation in the gene encoding Plant U-box 4 (PUB4), an E3 ubiquitin ligase. In addition, we observed that chloroplasts become ubiquitinated after $^1O_2$-stress, suggesting that such ubiquitination may be a mechanism to "mark" individually damaged chloroplasts for degradation or for repair during stressful conditions.

Science objectives for 2019-2020:

- Our genetic screen to identify genes involved $^1O_2$ signaling and chloroplast degradation has identified three proteins putatively involved in chloroplast gene expression. We are currently testing this hypothesis and investigating if chloroplast gene expression plays a specific role in $^1O_2$ signaling and chloroplast degradation.
- Our studies show that chloroplast proteins in *fc2* mutants become ubiquitinated prior to chloroplast degradation. To understand the role of this ubiquitination, we are using several strategies. In one case, we used a proteomic approach to identify possible ubiquitination targets. One promising candidate protein was identified with this method and we are using genetic and biochemical experiments to test its function in chloroplast stress signaling and to investigate what purpose its ubiquitination may play.
- Genetic analysis of the *fc2* mutant has been extremely valuable in uncovering an important pathway that involves $^1O_2$ 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 current DOE support:

1. J. D. Woodson 2019. Chloroplast stress signals: regulation of cellular degradation and chloroplast turnover. *Curr Opin Plant Biol*, 52:30-37.

References to work supported by previous DOE support:

2. J. D. Woodson 2016. Chloroplast quality control – Balancing energy production and stress. *New Phytologist*. 212:36-41.
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Electronic Structure of the Mn Cluster in Photosystem II Using an XFEL

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Research goals: The overall goal of this proposal is to investigate the molecular and electronic structure and dynamics of PS II and the OEC, during the light-driven process, and understand the mechanism by which water is oxidized to dioxygen at the metal site. We hope to answer 1) where is the O-O bond formation site (bridging or terminal ligands or exogenous water to Mn/Ca), 2) what is the sequence of events during the reaction, including the substrate water pathway and release of protons and O_2 from the site, 3) how do the ligands and protein structurally and electronically modulate this process, and 4) whether the O-O bond formation is initiated by a nucleophilic attack involving a high-valent Mn(V), by an oxo-oxyl coupling, or by some other mechanism. We further plan to study the question of assembly of the OEC; a unique chaperon-free assembly of the metal-cluster occurs seamlessly every 20 minutes under high-light conditions, and this is integral to the PS II repair mechanism. We are also applying the new tools that we develop using synchrotrons and XFELs for addressing similar chemical questions in energy conversion in inorganic systems.

Significant achievements 2018-2019: *Mn L-edge XAS:* X-ray absorption spectroscopy (XAS) at the L-edge of 3d transition metals provides unique information on the local metal charge and spin states by directly probing 3d-derived molecular orbitals through 2p-3d transitions. However, this soft X-ray technique has been rarely used at synchrotron facilities for studies of metalloenzymes, due to the difficulties of X-ray damage and background signal from light elements that can dominate the metal signal. We used fs X-ray pulses from a XFEL with a novel X-ray fluorescence-yield spectrometer to overcome these difficulties. L-edge XAS of inorganic high-valent Mn complexes (Mn ~ 6-15 mM) show with no visible effects of radiation damage. Using this method, we measured the first L-edge XAS of the Mn_4CaO_5 cluster in PS II (Mn < 1 mM) at room temperature

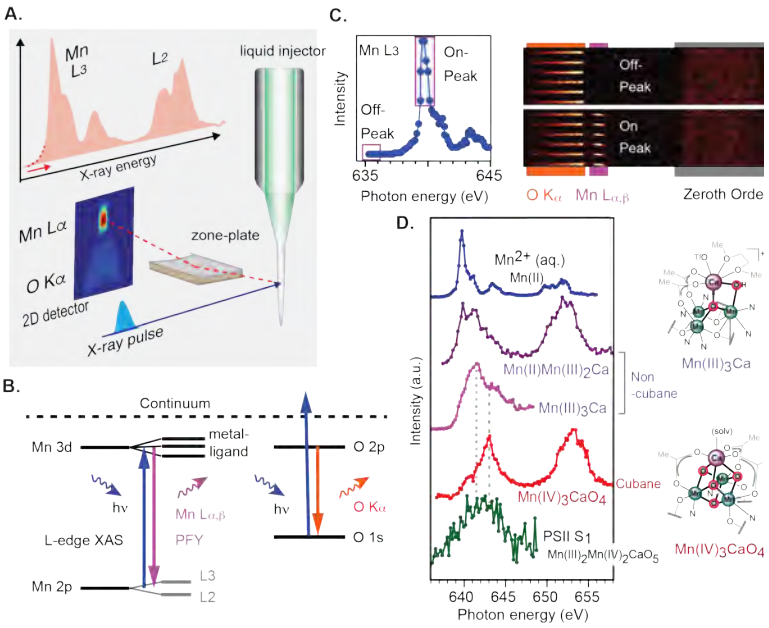

Fig. 1: A. Mn L-edge XAS setup with the zone plate showing energy discrimination between Mn L- and O K-fluorescence. **B.** Energy level diagram for Mn L-XAS (2p→3d). Spin-orbit interactions in the Mn 2p shell split XAS into L_3 and L_2 edges. The concurrent O Kα fluorescence (2p→1s) from 1s ionization of O in the sample is also shown. **C.** Example of a Mn L_3-edge PFY (partial fluorescence yield)-XAS of a 500 mM Mn^{2+}_{aq} sample, obtained by integrating Mn Lα,β fluorescence intensity on the CCD image (right) as a function of the incident photon energy. CCD images averaged over the "On-Peak" and "Off-Peak" data points are also shown. **D.** XAS data of inorganic Mn complexes with variable oxidation states and molecular structures, and data from PS II (S_1) collected with the zone plate spectrometer.

New Tools. We demonstrated that X-ray absorption spectroscopy (XAS), both Mn XANES and EXAFS, of solutions with mM Mn is possible using a new method for normalization based on solvent scattering that is compatible with highly variable fs pulses from an XFEL. XANES and EXAFS spectra of such dilute samples are in good agreement with data collected at synchrotron sources using traditional scanning protocols.

Science objectives for 2019-2020: 1) We continue to improve the method of collecting 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 complex. 2) We will use room temperature EXAFS and XANES methods that we developed 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. 3) We are also continuing our efforts with theory for understanding the spectra, and developing non-linear X-ray spectroscopy methods for dilute transition metal solutions.

References to work supported by this project 2018-2019:

Dissertation

1. Young, I. D. (2018) Tracking the Mechanism of Water Oxidation in Photosystem II Using X-ray Free Electron Laser Diffraction. Ph. D. Dissertation, University of California, Berkeley, CA, U.S.A.

Reviews

1. Bergmann, U., Yachandra, V. K., Yano, J. (2019) How Ultra-Fast X-ray Pulses Can Reveal Hidden Secrets of Photosynthesis, in *The Biochemist*, 24-29, Published by the Biochemical Society, Portland Press, London, U.K.
2. Alonso-Mori, R., Yano, J. (2018) X-ray Spectroscopy with XFELs. In: X-ray Free Electron Lasers. (Boutet, S., Fromme, P., Hunter, M. (eds)), 377-399, Springer, Cham, Switzerland.
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4. Chatterjee, R., *et al.* (2019) XANES and EXAFS of Dilute Solutions of Transition Metals at XFELs, *J. Synchrotron. Rad.* **26**, 1716-1724.
5. Chatterjee, R., *et al.* (2019) Structural Isomers of the S_2 State in Photosystem II: Do They Exist at Room Temperature and Are They Important for Function? *Physiol. Plant*., **166**, 60-72.
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10. Hussein, R., *et al.* (2018) Optimizing Crystal Size of Photosystem II by Macroseeding: Towards Neutron Protein Crystallography, *Crystal Growth & Design,* **18** 85-94.
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13. Kubin, M., *et al.* (2018) Cr L-Edge X-Ray Absorption Spectroscopy of $Cr^{III}(Acac)_3$ in Solution with Measured and Calculated Absolute Absorption Cross Sections. *J. Phys. Chem. B.,* **122**, 7375-7384.
14. Kubin, M., *et al.* (2018) Probing the Oxidation State of Transition Metal Complexes: A Case Study on How Charge and Spin Densities Determine Mn L-Edge X-ray Absorption Energies. *Chem. Sci.* **9**, 6813-6829.
15. Kubin, M., *et al.* (2018) X-ray-Induced Sample Damage at the Mn L-Edge: A Case Study for Soft X-ray Spectroscopy of Transition Metal Complexes in Solution. *Phys. Chem. Chem. Phys.,* **20**, 16817-16827.

Structures of the Intermediates of Kok's Photosynthetic Water Oxidation Clock

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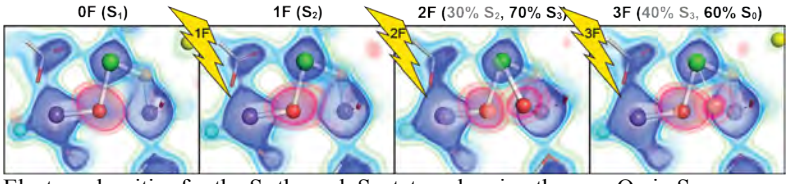
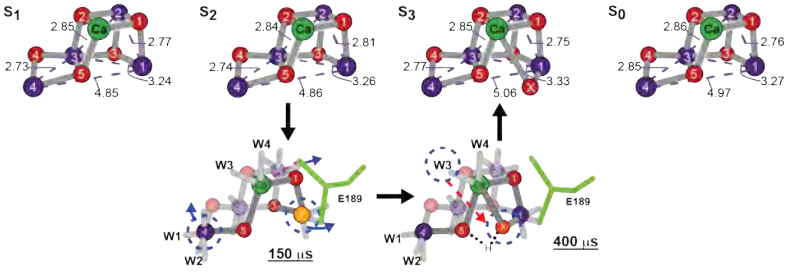
Research goals: The overall goal of this proposal is to investigate the molecular and electronic structure and dynamics of PS II and the OEC, during the light-driven process, and understand the mechanism by which water is oxidized to dioxygen at the metal site. We hope to answer 1) where is the O-O bond formation site (bridging or terminal ligands or exogenous water to Mn/Ca), 2) what is the sequence of events during the reaction, including the substrate water pathway and release of protons and O_2 from the site, 3) how do the ligands and protein structurally and electronically modulate this process, and 4) whether the O-O bond formation is initiated by a nucleophilic attack involving a high-valent Mn(V), by an oxo-oxyl coupling, or by some other mechanism. We further plan to study the question of assembly of the OEC; a unique chaperon-free assembly of the metal-cluster occurs seamlessly every 20 minutes under high-light conditions, and this is integral to the PS II repair mechanism. We are also applying the new tools that we develop using synchrotrons and XFELs for addressing similar chemical questions in the field of energy conversion in inorganic systems.

Significant achievements 2018-2019:

We 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. We significantly improved the data collection efficiency and sample handling for the simultaneous XRD/XES study by introducing the *Drop on Tape* method. Using new crystallization protocols that allow us to obtain high quality diffraction images and accompanying XES data from crystals that confirm turnover of PS II and provide real-time feedback about the integrity of PS II samples, we determined the structures of PS II in all the stable intermediate states of the Kok cycle, the S_1, S_2, S_3, and S_0 states, (~2.0 Å) and at two time-points between S_2 and S_3 at ~2.3 Å, at room temperature. The results show the insertion of an oxo bridge Ox that forms between Ca and Mn(1) in the S_3 state, and the possible involvement of Ca in substrate delivery to the catalytic site. Distinct structural changes occur in the S_3 state and these can be followed in the time-point structures between S_2 and S_3 states. These studies have now been refined with higher resolution structures for 6 time-points between S_2 and S_3 states.



Electron densities for the S_1 through S_0 states, showing the new Ox in S_3



Structures of the Mn_4Ca cluster in all stable states and at time-points 150 and 400 µs between S_2 and S_3.

Science objectives for 2019-2020:

We plan to collect simultaneous XRD/XES at a resolution of ~2 Å and XES to 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. We also started studies on Sr-PS II, and flash-induced photo-assembly of the Mn cluster.

References to work supported by this project 2018-2019:

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Regulation of sustained Cyclic Electron Flow (CEF)
in the photopsychrophile *Chlamydomonas* sp. UWO241

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Overall research goals: The goal of this project is to describe the function of sustained CEF and assembly of a PSI supercomplex in the Antarctic *Chlamydomonas* sp. UWO241 (UWO241) and the model *Chlamydomonas reinhardtii* acclimated to long-term salinity stress. Major objectives are: 1) determine the functional role of sustained CEF in UWO241; 2) dissect the structure of the UWO241 PSI supercomplex; 3) study CEF and PSI supercomplex assembly in WT and mutant strains of *C. reinhardtii*. Outcomes of this research project will support research focused on meeting future energy and food needs by advancing understanding how extremophilic phototrophs use sustained CEF to survive long-term exposure to environmental stressors, including low temperatures and high salinity.

Significant achievements 2018-2019:

- ***The Zhang Laboratory (Lab) optimized a Clark-type oxygen electrode and measured oxygen evolution rates of UWO241 grown under control and high salt conditions.*** The oxygen evolution rates provide information about the growth rates and PSII efficiencies in UWO241 grown under different conditions. The cultures grown under control versus high salt conditions did not show significantly differences in photosynthetic oxygen evolution rates or respiration rates, indicating robust growth and normal PSII function of UWO241 grown under high salt conditions. This result is consistent with the growth results measured by both the Morgan-Kiss and Zhang Labs using cell counting, chlorophyll content measurements, and OD measurements.
- ***The Zhang Lab also measured PSII efficiencies of the UWO241 cultures grown under control versus high salt conditions using an IDEA spectrophotometer*** designed by the Kramer Lab at the Michigan State University. We visited the Kramer Lab, learned and tested the device, brought the device back, and set it up in the Zhang Lab. We measured PSII quantum efficiency and linear electron flow (LEF) using chlorophyll fluorescence with the IDEA spectrophotometer.
- ***We also measured the fraction of absorbed light by PSII (fraction$_{PSII}$) in cultures grown in control and high salt conditions using 77K chlorophyll fluorescence measurement.*** The fraction$_{PSII}$ was used to calculate LEF. LEF was similar in both control and high salt grown UWO241 cultures which is consistent with the oxygen evolution results.
- ***We optimized a dark interval relaxation kinetics (DIRK) method for UWO241 by using the IDEA spectrophotometer to estimate CEF without inhibitors***. This method monitors electrochromic shift (ECS) to estimate transthylakoid proton motive force (pmf), proton conductance, and proton fluxes rates through ATP synthase to make ATP in UWO241. The cultures grown under high shalt conditions showed significantly higher light-driven *pmf*, lower proton conductance, increased proton flux rates than cultures grown under control conditions. These results indicate increased proton translocation through ATP synthase and higher ATP production rates in UWO241 cultures grown in high salt conditions as compared to control conditions. We plotted the proton flux rates vs LEF to estimate the contribution of CEF to total pmf; higher ratios of proton flux rates over LEF indicates increased CEF contribution to pmf, thus increased CEF rates. Our results indicated that UWO241 cultures grown in high salt conditions showed higher CEF than the cultures grown under control conditions. These results confirmed the CEF measurements by the Morgan-Kiss Lab using P700 reduction rates. From the spectroscopic measurements mentioned above, we showed that UWO241 cultures grown under high salt conditions had higher CEF, pmf, NPQ and proton flux rates as compared to cultures

grown under control conditions. It indicates that UWO241 cultures grown under high salt conditions employ high CEF to increase pmf for photoprotection and ATP production. We are preparing a manuscript about high CEF in UWO241 with PI Morgan-Kiss, graduate student Isha Kalra, Co-PI Wang, and others [1].

Science objectives for 2019-2020:

- ***Determine whether high CEF is associated with growth under various stressful conditions in UWO241 (Morgan-Kiss, Zhang) and physiological consequences of sustained CEF (Morgan-Kiss, Zhang)***. We will optimize the IDEA spectrophotometer further to develop alternative ways to measure CEF in UWO241 and *C. reinhardtii*, e.g. P700 oxidation and reduction rates, pmf with PSII inhibitors (e.g. DCMU). We will measure CEF and other photosynthetic parameters in UWO241 grown under other different conditions, e.g. low temperatures, high light.
- ***Compare CEF rates and PSI supercomplexes in C. reinhardtii WT and mutant strains exposed to short- and long-term stress (Zhang, Morgan-Kiss)***. We will employ WT *C. reinhardtii* under short and long-term stresses to compare CEF rates and the formation of PSI supercomplex. We will grow WT *C. reinhardtii* cells under short- and long-term stresses, measure CEF rates and other photosynthetic parameters using the IDEA spectrophotometer and the Clark-type oxygen electrode. The Lab of PI Morgan-Kiss has preliminary data to show that WT *C. reinhardtii* grown under long-term high salt stress have increased CEF and formed UWO-like PSI supercomplexes. We will confirm the high CEF in WT *C. reinhardtii* cultures grown under long-term high salt stress and will also test if the high CEF could be induced in WT *C. reinhardtii* grown under long-term heat stress. After we confirm these stress conditions, we will continue to investigate *C. reinhardtii* mutants deficient in CEF under selective long-term or short-term conditions to identify components that are important for the induction of CEF in *C. reinhardtii*.
- ***Investigate the role of PsbP and FtsH in supercomplex stabilization (Zhang)***. To investigate the role of PsbP and FtsH in supercomplex stabilization, we will functionally express UWO-*PsbP1A* and UWO-*PsbP1B* in a *C. reinhardtii PsbP1* knockout mutant, and UWO-*FTSH* in a *C. reinhardtii FTSY2* knockout mutant, by using Moclo vector system. The complemented strains will be monitored for i) PSI supercomplex formation, ii) CEF rates; and iii) other photosynthetic parameters, to investigate if UWO-PsbP and FTSH function in CEF in *C. reinhardtii*.

References to work supported by this project 2018-2019 (**Co-PI and PI** of this project; *graduate students; # Zhang lab mentees)

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