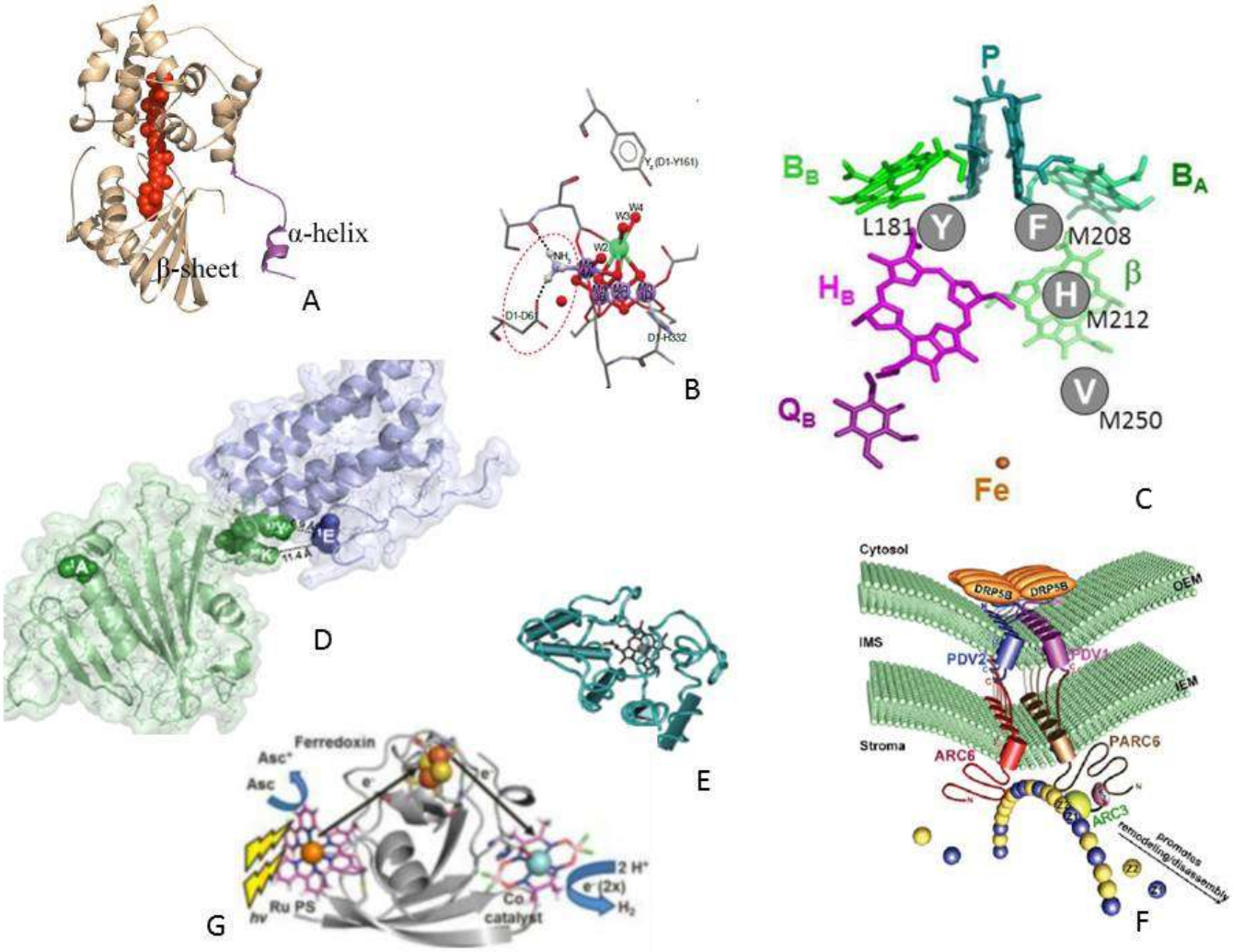
2015 Photosynthetic Systems Principal Investigators Meeting

Marriott Washingtonian Center, Gaithersburg, MD
October 19-21, 2015



U.S. DEPARTMENT OF ENERGY | Office of Science

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

Cover Art
The cover art of this abstract book is taken from the abstracts of meeting participants: **A** - **Robert Blankenship**, **B** – **R. David Britt**, **C** - **Christine Kirmaier and Phillip Laible**, **D** – **Terry Bricker**, **E** – **Fevzi Daldal**, **F** – **Katherine Osteryoung**, **G** – **Lisa Utschig**

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Foreword

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

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

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

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

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

2015 Photosynthetic Systems Principal Investigators Meeting

Gaithersburg Marriott, Washingtonian Center, Gaithersburg, MD
October 19 – 21

Agenda

Sunday, October 18

3:00 – 6:00PM *Registration*

Monday, October 19

7:30 – 8:00AM *Continental Breakfast*

8:00 – 8:30AM Overview of the Chemical Sciences, Geosciences, and Biosciences (CSGB) Division
Tanja Pietrass, Division Director, CSGB

Session I: *Self-Assembly and Self-Repair.* ***Carole Dabney-Smith****, Miami University, Moderator*

8:30 – 9:00AM Protein Targeting to the Chloroplast Thylakoid Membrane: Structure and Function of a Targeting Complex
Ralph Henry, University of Arkansas

9:00 – 9:30AM Targeting, Maturation, and Quality Control of Photosynthetic Membrane Proteins
Kentaro Inoue, University of California, Davis

9:30 – 10:00AM Molecular Genetic Dissection of Chloroplast Fe Homeostasis
Sabeeha Merchant, University of California, Los Angeles

10:00 – 10:30AM Regulation of Thylakoid Lipid Biosynthesis in Plants
Christoph Benning, Michigan State University

10:30 – 11:00AM *Coffee Break*

11:00 – 11:30AM Ubiquitin Facilitates a Quality Control Pathway that Removes Damaged Chloroplasts
Joanne Chory, The Salk Institute and University of California, San Diego

Monday, October 19

11:30 – 12:00PM	RNA Quality Control in the Chloroplast **David Stern**, Boyce Thompson Institute and Cornell University
12:00 – 12:30PM	Spatial Regulation of Chloroplast Division and Physiological Significance **Katherine Osteryoung**, Michigan State University
12:30 – 4:00PM	*Catered Lunch and Afternoon Break*

Session II: *Translational Biochemistry.* ***David Britt****, University of California, Davis, Moderator*

4:00 – 4:30PM	Photosynthetic Biomimetic Approaches for Investigating Fundamental Mechanisms in Photosynthesis **David Tiede**, Argonne National Laboratory
4:30 –5:00PM	Photosynthetic-Inspired Systems for Solar Hydrogen Production **Lisa Utschig,** Argonne National Laboratory
5:00 – 5:30PM	Photobiohybrid Solar Fuels **Paul King**, National Renewable Energy Laboratory
6:00 – 7:30PM	*Program Dinner*
7:30 – 10:00PM	Presentation of Odd-numbered Posters (Presenters and titles are listed at end of the agenda.)

Refreshments available at the hotel bar

Tuesday, October 20

7:30 – 8:00AM	*Continental Breakfast*
8:00 – 8:30AM	Update on the Photosynthetic Systems Program at DOE **Steve Herbert,** DOE BES

Tuesday, October 20

Session III: *CO_2 Reduction and Electron Transport.* ***Rebekka Wachter****, Arizona State U., Moderator*

8:30 – 9:00AM	Disruption of the CO_2-concentrating Mechanism in C_4 Plants: Implications for CO_2 Fixation and Photosynthetic Efficiency During C_4 Photosynthesis **Asaph Cousins,** Washington State University
9:00 – 9:30AM	Understanding of the Function and Regulation of the High Affinity CO_2-Concentrating Mechanism **Rob Burnap,** Oklahoma State University
9:30 – 10:00AM	The Energy Budget of Steady-State Photosynthesis **David Kramer,** Michigan State University
10:00 – 10:30AM	*Coffee Break*
10:30 – 11:00AM	The Interaction of Quinones with the Heme-Copper Cytochrome b_{03} Ubiquinol Oxidase from *E. coli* **Robert Gennis,** University of Illinois, Urbana-Champaign
11:00 – 11:30AM	Elucidating the Principles that Control Proton-Coupled Electron Transfer Reactions in the Photosynthetic Protein, Photosystem II **KV Lakshmi,** Rennsaeler Polytechnic Institute
11:30 – 12:00PM	Looking Under the Hood of Photosynthesis: How Does Your Photosystem II Measure Up? **Charles Dismukes,** Rutgers University
12:00 – 3:00PM	*Catered Lunch and Afternoon Break*
3:00 – 4:00PM	Open Forum with DOE BES Program Managers **Steve Herbert, Gail Mclean, Bob Stack**

Session IV*: Electron transport in Photosynthetic Complexes.* ***Bob Stack****, DOE BES, Moderator*

4:00 – 4:30PM	Taking Snapshots of Photosynthetic Water Oxidation: Femtosecond X-ray Diffraction and Spectroscopy Using X-ray Free Electron Lasers **Junko Yano**, Lawrence Berkeley National Lab

Tuesday, October 20

4:30 – 5:15PM The Homodimeric Type I Reaction Center of *Heliobacterium modesticaldum*
Kevin Redding, Arizona State University
John Golbeck, Pennsylvania State University

5:15 – 5:45PM Two-Dimensional Electronic Spectroscopies for Probing Coherence and Charge Separation in Photosystem II
Jennifer Ogilvie, University of Michigan

6:00 – 7:30PM *Dinner with colleagues by your own arrangement*

7:30 – 10:00PM Presentation of Even-numbered Posters
(Presenters and titles are listed at the end of the Agenda)

Refreshments available at the hotel bar

Wednesday, October 21

7:30 – 8:00AM *Continental Breakfast*

***Session V**: Light Harvesting. **Gary Brudvig**, Yale University, Moderator*

8:00 – 8:30AM High-Resolution Study of Photochemical and Nonphotochemical Processes in Biological Proteins Assembled with Photosynthetic Pigments
Ryszard Jankowiak, Kansas State University

8:30 – 9:00AM Molecular Mechanism of Action of the Cyanobacterial Orange Carotenoid Protein
Bob Blankenship, Washington University

9:00 – 9:30AM Energy Transfer and Radiationless Decay in Light-Harvesting Proteins
Warren Beck, Michigan State University

9:30 – 10:15AM Regulation of Photosynthetic Light Harvesting
Graham Fleming, Lawrence Berkeley National Lab, UC Berkeley
Kris Niyogi, Lawrence Berkeley National Lab, UC Berkeley

10:15 – 10:30AM *Coffee Break*

Wednesday, October 21

10:30 – 11:00AM Cyanobacterial Photoreceptor Systems for Regulation and Optimization of Energy Harvesting
Clark Lagarias, University of California, Davis

11:00 – 11:30AM Toward Time-Resolved Circular Dichroism Spectroscopy of Photosynthetic Proteins: Accessing Excitonic States
Sergei Savikhin, Purdue University

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

Regular Posters and Presenters

1. Photosystem II: Structure and Function
Terry Bricker, Louisiana State University

2. Ammonia Binds to the Dangler Manganese of the Photosystem II Oxygen Evolving Complex
David Britt, University of California at Davis

3. The Oxygen Isotope Effect of Photosystem II Revealed
Gary Brudvig, Yale University

4. Thylakoid Assembly and Folded Protein Transport by the Chloroplast Twin Arginine Translocation (cpTat) Pathway
Carole Dabney-Smith, Miami University

5. Membrane-Attached Electron Carriers in Photosynthesis and Respiration: Cytochrome c Maturation (Ccm-system I) in a Facultative Photosynthetic Bacterium
Fevzi Daldal, University of Pennsylvania

6. FTIR Studies of Photosynthetic Oxygen Production
Richard Debus, University of California at Riverside

7. Resolving Protein-Semiquinone Interactions byAdvanced EPR Spectroscopy: The Q_A and Q_B Sites of the Bacterial Reaction Center
Sergei Dikanov, University of Illinois at Urbana-Champaign

8. Construction and Evaluation of Well-Defined Mimics of Photosynthetic Light Harvesting Systems
Matthew Francis, University of California at Berkeley

9. Photobiological Solar Fuels Program
Maria Ghirardi, National Renewable Energy Laboratory

Regular Posters and Presenters, Continued

10. Studies of Photosynthetic Reaction Centers and Biomimetic Systems
Marilyn Gunner, City College of New York

11. Chlamydomonas Metabolism in the Light and the Dark
Arthur Grossman, Carnegie Institution for Science, Department of Plant Biology

12. Thiol-based Pathways in the Thylakoid Lumen and their Role in Photoprotections
Patrice Hamel, The Ohio University

13. Chloroplast Dynamics and Photosynthetic Efficiency
Maureen Hanson, Cornell University

14. Photobiohybrid Solar Fuels
Paul King, National Renewable Energy Laboratory

15. Controlling Electron Transfer Pathways in Photosynthetic Proteins
Christine Kirmaier, Washington University

16. Dynamics of Photosynthetic Membrane Formation, Structure, and Regulation
Krishna Niyogi, University of California at Berkeley and Lawrence Berkeley National Lab

17. Two Redoxin Proteins are Involved in the Maintenance of Photosystem Stoichiometry in the Cyanobacterium *Synechocystis* sp. PCC 6803
Himadri Pakrasi, Washington University

18. Proteins in Control of Optimal Pathways for Proton-Coupled ET
Oleg Poluetkov, Argonne National Laboratory

19. The Glucose-6-Phosphate Shunt Around the Calvin-Benson Cycle
Thomas Sharkey, Michigan State University

20. Mechanism of Protein Transport on the Twin Arginine Translocation Pathway
Steven Theg, University of California at Davis

21. Site-Selective Characterization of Plastocyanin with Infrared Spectroscopy
Megan Thielges, Indiana University

22. Structure and Function of Rubisco Activase from Higher Plants
Rebekka Wachter, Arizona State University

23. The Mn_4Ca Cluster in Photosystem II and Inorganic Complexes Studied Using In Situ and Steady State X-ray Absorption and Emission Spectroscopy
Vittal Yachandra, Lawrence Berkeley National Laboratory

Abstracts

Presented alphabetically by last name of the principal investigator

Energy Transfer and Radiationless Decay in Light-Harvesting Proteins

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Overall research goals: This project employs two-dimensional electronic spectroscopy (2DES) and transient-grating (TG) spectroscopy with optical heterodyne detection to determine the structural and physical mechanisms that control and optimize the excitation energy transfer channels involved in photosynthetic light-harvesting. The work focuses on the structurally well-characterized peridinin–chlorophyll *a* protein (PCP). The research plan compares the photophysics of peridinin in solution with that of the PCP complex in order to distinguish the effects of the protein/chromophore environment on excited-state motions and nonradiative decay mechanisms.

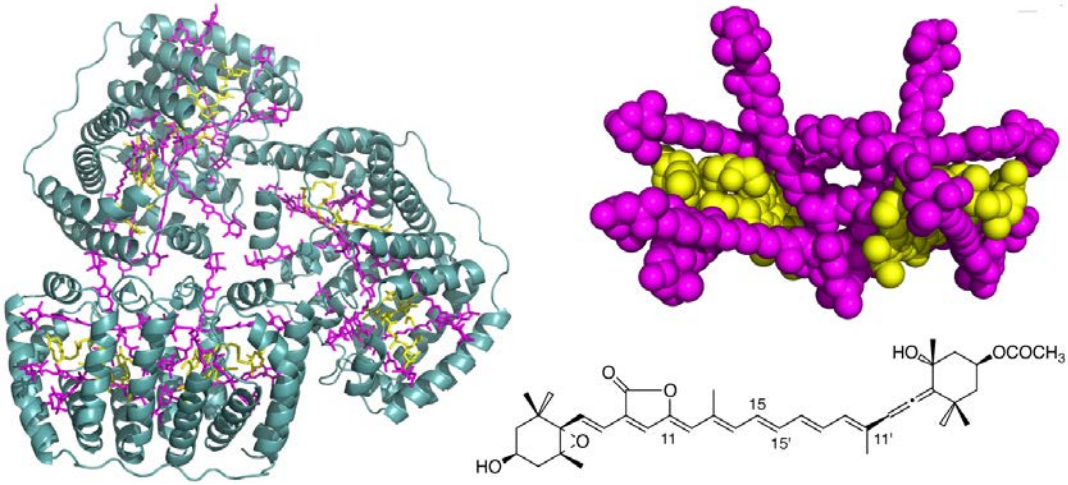

Figure 1. *Left:* Trimeric complex of the peridinin–chlorophyll *a* protein from *Amphidinium carterae* (1PPR.pdb). *Right:* Space-filling rendering of the chromophores in a single subunit: peridinin (magenta) and chlorophyll *a* (yellow). *Lower right:* structure of peridinin.

Significant achievements 2013–2015:

- Heterodyne TG signals from peridinin were obtained in a range of solvents. The results suggest that the decay of the optically prepared S_2 ($1^1B_u^+$) state and the recovery of the ground state S_0 ($1^1A_g^-$) involve formation and decay of a strong intramolecular charge-transfer (ICT) character.
- Full nonlinear optical simulations of the heterodyne TG signals from β-carotene and peridinin after optical preparation of the S_2 state were performed with the response function formalism and the multimode Brownian oscillator model. These simulations enabled us to make a direct comparison of the observed and modeled absorption and dispersion components of the third-order signal for the first time. The results of the simulations indicate that the dispersion signal definitively reports the ultrafast (<15 fs) decay of the S_2 state in carotenoids to a dark intermediate state, S_x (4). We also find that the dispersion signal allows a clear distinction to be made between

nonradiative decay of the S_1 ($2^1A_g^-$) state and conformational relaxation on the S_0 potential surface.

- A new hypothesis (1) for the structural dynamics involved in the nonradiative decay of carotenoids in light-harvesting proteins was developed. It is proposed that torsional motions are activated after an initial vibronic excursion in the S_2 state along Franck–Condon active, bond-length alternation coordinates of the conjugated polyene backbone. These motions lead to an ICT character, which may be enhanced in the S_1 state due to pyramidalization.
- We have installed and characterized a new femtosecond 2DES spectrometer that employs a high-repetition rate Yb laser, a noncollinear optical parametric amplifier (NOPA), and two channels of adaptive pulse shaping. This system permits us to obtain broadband 2DES spectra with <10 fs pulses for the first time and to exploit amplitude- and phase-shaped excitation pulses to probe excited-state vibrational dynamics.

Science objectives for 2015–2016:

- Broadband TG spectra from peridinin will be obtained in a range of solvents in order to further test the hypothesis that twisted and/or pyrimidal conformations with ICT character are formed due to ultrafast motions on the S_2 potential-energy surface.
- Broadband 2DES spectra will be recorded with the PCP complex in order to observe electronic coupling between peridinin chromophores in a given protein domain.
- With red-shifted broadband excitation, 2DES spectra will also be obtained to determine the states that mediate energy transfer from peridinin to Chl *a* in the PCP complex.
- Preparations with Chl *b* substituted for Chl *a* will be prepared for use in broadband 2DES experiments that assess the mechanism of energy transfer between peridinin and Chl in the PCP complex.

References to work supported by this project 2013-2015:

(1) Beck, W. F.; Bishop, M. M.; Roscioli, J. D.; Ghosh, S.; Frank, H. A. Excited state conformational dynamics in carotenoids: dark intermediates and excitation energy transfer. *Arch. Biochem. Biophys.* **2015**, *572*, 175-183. DOI: 10.1016/j.abb.2015.02.016.

(2) Bishop, M. M.; Roscioli, J. D.; Ghosh, S.; Mueller, J. J.; Shepherd, N. C.; Beck, W. F. Vibrationally coherent preparation of the transition state for photoisomerization of the cyanine dye Cy5 in water. *J. Phys. Chem. B* **2015**, 6905-6915. DOI: 10.1021/acs.jpcb.5b02391.

(3) Bishop, M. M.; Roscioli, J. D.; Ghosh, S.; Mueller, J. J.; Shepherd, N. C.; Beck, W. F. Probing hydrogen-bonding dynamics and friction in the hydration shell on the 100 fs timescale: Photon echo peak shift and vibrational coherence of the cyanine dye Cy5 tethered to Zn^{II}-substituted cytochrome *c*. *J. Phys. Chem. Lett.*, submitted.

(4) Ghosh, S.; Bishop, M. M.; Roscioli, J. D.; Mueller, J. J.; Shepherd, N. C.; LaFountain, A. M.; Frank, H. A.; Beck, W. F. Femtosecond heterodyne transient grating studies of nonradiative decay in β-carotene: Contributions from dark intermediates and double quantum coherences. *J. Phys. Chem. B*, submitted.

Regulation of Thylakoid Lipid Biosynthesis in Plants

Christoph Benning (Principal Investigator)
Kun Wang (Graduate Student); Rebecca Roston (Postdoctoral Research Associate); Hope Hersh (Undergraduate Student), Patrick Horn, (Postdoctoral Research Associate)
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Overall Research Goal: The overall goal is to gain a mechanistic understanding of the assembly and maintenance of the photosynthetic membranes in plants with a strong focus on its lipid constituents. Establishment and maintenance of the photosynthetic membrane is a dynamic process throughout the life cycle of the plant and involves finely tuned mechanisms to adjust synthesis and breakdown of polar lipids. Currently, the focus is on the turnover of the principal membrane lipid of the photosynthetic membrane, monogalactosyldiacylglycerol (MGDG). Two different processes are investigated that involve recently discovered enzymes SENSITIVE TO FREEZING2 (SFR2) and PLASTID GALACTOLIPID DEGRADATION 1 (PGD1), which catalyze the recycling of MGDG derived diacylglycerol or acyl moieties, respectively, into triacylglycerol following the exposure to abiotic stresses. The specific aims are (1) to determine the activation mechanism of SFR2, (2) to determine the function of SFR2 in lipid remodeling in response to different abiotic stresses, and (3) to determine the function of plant PGD1-like proteins in lipid remodeling and abiotic stress responses.

Significant Achievements (2014 – 2015):

1. SFR2 structure-function studies and activation mechanism. We completed a molecular model of Arabidopsis SFR2, and used site-directed mutagenesis in combination with activity assays to determine the relationship between protein structure and function (Roston, et al 2014).SFR2 has no hydrolase activity even though its active site residues are identical with those of glycosyl hydrolase family 1 proteins. We identified several structural features required for its activity. The analysis of temperature dependence of SFR2 activation in Arabidopsis in planta shows that it is activated posttranslationally between -2 to -8 ℃. At the chloroplast level, SFR2 is activated by a precise combination of pH decrease and $MgCl_2$ increase at physiologically relevant levels of KCl. Using transgenic lines producing a fluorescent *in vivo* marker, a drop in cytosolic pH following freezing *in vivo* was identified. At the tissue level, decrease of the pH by infiltration of leaves with weak organic acids also activates SFR2. Thus, it is proposed that this drop in cytosolic pH in combination with an increase in the concentration of $MgCl_2$ on the cytosolic face of the outer envelope due to release of $MgCl_2$ from the chloroplast following freezing injury leads to an activation of SFR2.
2. Function of SFR2 in lipid remodeling in response to different abiotic stresses. SFR2 is present in the chloroplast envelopes of all land plants. It is hypothesized that SFR2 plays roles in drought and salt stress tolerance in non-freezing tolerant plants. We generated tomato *sfr2* RNAi lines to determine the role of SFR2 in a cold/salt stress sensitive plant. The transgenic lines are more salt and drought sensitive. We produced active tomato SFR2 in yeast enabling us to compare its activity and activation to Arabidopsis SFR2 *in vitro*.
3. Function of plant PGD1-like (PGDL) proteins in lipid remodeling and abiotic stress responses. In initial experiments PGDL3 caused a strong lipid phenotype when produced at low levels in *E. coli* suggesting it to be a lipase. The purification protocol for recombinant PGDL3 produced in *E. coli* was optimized and the protein was obtained at near homogeneity in its active form. Development of a lipase assay for PGDL3 recombinant protein allowed characterization of the protein's activity *in vitro*. The enzyme is specific for the *sn*-1 position of monogalactosyldiacylglycerol and also acts on different phosphoglycerolipids. Using different methods, the protein was shown to be located in the chloroplast. Following transient expression of *PGDL3* in tobacco or stable over expression in Arabidopsis, a specific lipid phenotype was observed: The ratio of $16{:}1^{\Delta 3trans}$ over 16:0 fatty acids in phosphatidylglycerol was

decreased. The first fatty acid, $16{:}1^{\Delta 3trans}$, is specific to the *sn-2* position of phosphatidylglycerol found only in the thylakoid membranes. The function of this specific lipid species, although present in all chloroplasts, is not yet known. It should be noted that PGDL3 overexpressing plants were smaller and their leaves more yellow hinting at a problem in chloroplast development or photosynthesis. This will be a focus of further studies.

PGDL1 was also produced in recombinant form in *E. coli.* Lipase activity was confirmed in microsomes from the transgenic *E. coli*. The PGDL1 activity profile is similar to that of PGDL3 and it is also located in the chloroplast. This was surprising as the protein does not contain a typical stromally-targeting transit peptide. It is hypothesized that this protein, like other chloroplast envelope proteins does not need a transit peptide, and may therefore be associated with different chloroplast membranes than PGDL3, which does have a transit peptide. Transient expression of *PGDL1* in tobacco caused a similar lipid phenotype as described for PDGL3 above. In addition, PGDL1 also seems to act on phosphatidylcholine, which showed altered fatty acid composition. As phosphatidylcholine is present in chloroplast mostly in the outer envelope membrane, this difference in phenotype between PGDL1 and PGDL3 transient expression lines supports a different suborganellar location of the two enzyme isoforms, with PDGL1 being associated with the outer envelope and PGDL3 with thylakoid membranes. This hypothesis will have to be further corroborated.

PGDL2 is also present in the chloroplast confirmed by two independent methods. The lipid phenotype following transient expression of *PGDL2* in tobacco leaves was similar to that of *PGDL1*. Studying *pgdl* single and double mutants and transgenic RNAi and overexpression lines it was observed that germinating seedlings of *pgdl* lines are more sensitive to ABA. In addition, *pgdl* mutants have less triacylglycerol in their mature seeds and the fatty acid composition of seed oil in the *pgdl3* mutants is altered. It seems possible that these enzymes play a particularly important role during seedling establishment following germination as plantlets transition from heterotrophic to photoautotrophic growth, a time when chloroplasts and their photosynthetic membranes rapidly develop.

Science Objectives for (2015-2016):

1. Before the end of 2015 we plan to wrap up work on SFR2 activation by submitting work for publication.
2. We plan to conclude the analysis of SFR2 function in tomato before the end of 2015.
3. We will continue our analysis of PGDL1,2,3 proteins in Arabidopsis. We will continue to explore phenotypes of the *pgdl* mutants under different environmental conditions and at different stages of development. In particular, we will explore the hypothesis that these proteins are associated with different chloroplast suborganellar membranes where they act on specific chloroplast lipid species.
4. To better understand the PGDL overexpression lipid phenotypes, we will develop a new objective focused on chloroplast phosphatidylglycerol biosynthesis and function. The initial focus will be on identifying the phosphatidylglycerolphosphate phosphatase and novel chloroplast proteins related to changes $16{:}1^{\Delta 3trans}$ content in the respective mutants.

References to Work Supported by this Project 2014-2015:

1. Lipid trafficking in plant cells. 2014. Hurlock AK, Roston RL, Wang K, Benning C. (2014) Traffic. 15:915-32
2. Roston RL, Wang K, Kuhn LA, Benning C. 2014. Structural determinants allowing transferase activity in SENSITIVE TO FREEZING 2, classified as a family I glycosyl hydrolase. J. Biol. Chem. 289:26089-106. (on the cover)

Molecular Mechanism of Action of the Cyanobacterial Orange Carotenoid Protein

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Overall Research Goals: All photosynthetic organisms contain a light-harvesting antenna system, which expands the solar spectrum utilization and supplies excitations to reaction center complexes where photochemistry takes place and the energy is stored ultimately in reduced carbon of biomass. Photosynthetic antenna complexes are extremely diverse, both in terms of structural organization and type of pigment utilized. Equally diverse are the photoprotection mechanisms through which the excitation energy transfer from light harvesting complexes to the reaction centers is modulated to cope with fluctuating light and other environmental conditions.

This project centers on the Orange Carotenoid Protein (OCP) that is found in cyanobacteria and quenches excited states in the phycobilisome (PBS) antenna complex. The OCP is a soluble photoactive protein and the first identified photoactive protein that binds a carotenoid molecule as the pigment. The OCP is found in an inactive orange form in the dark but is converted into a red form by excess light. The red form binds to the PBS and quenches excited states of the bilin chromophores, providing photoprotection.

The goal of this research project is to give us a much-improved understanding of the photoactivation and mechanism of action of this important photoprotection process at the molecular level and also improve our understanding of its physiological roles in cyanobacterial adaptation to changing environmental conditions. A working model of OCP conformational changes is shown in Fig. 1.

Significant Achievements 2014-2015: We have carried out a variety of structural and mechanistic studies on the isolated OCP derived from both wild type and genetically modified strains as well as examining its interaction with the phycobilisome. These studies have involved ultrafast spectroscopy and mass spectrometry of the protein in both orange and red forms, as well as chemical labeling and cross linking studies. The results are being synthesized into a molecular mechanism of how the OCP functions to regulate energy transfer in cyanobacterial antennas.

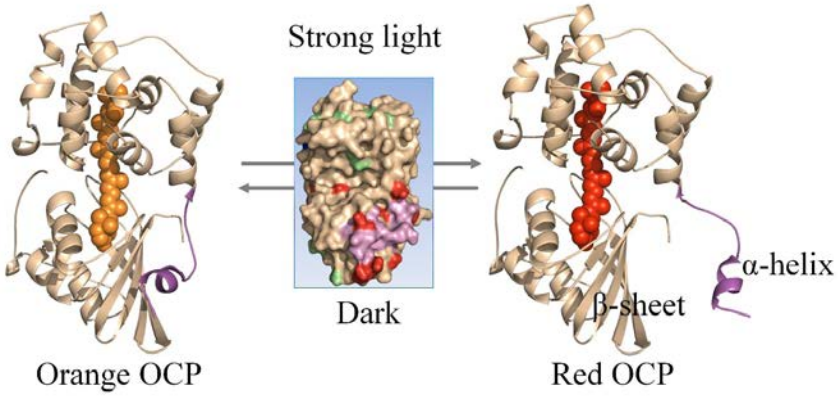

Fig. 1. Working model of OCP photoinduced conformational changes.

In the course of this research we have developed a method that makes use of isotopically labeled chemical labeling reagents to obtain quantitative information about the conformation changes induced in the OCP by the orange to red transition (Fig. 2). In this new method, isotope-coded 'heavy' and 'light' glycine ethyl ester (GEE) are used to label separately the two states of the OCP. Carbodiimide-mediated GEE coupling reaction is used to introduce modification. Two samples digested with trypsin, were mixed (1:1 ratio) and analyzed in a single LC-MS/MS experiment. The difference in labeling extent between the two states was determined by the ratio of the abundances of the 'heavy' and 'light' peptides. Conformational changes were elucidated by comparing ratios of the 'light' and 'heavy' isotopologues. A paper describing this work was recently published (1).

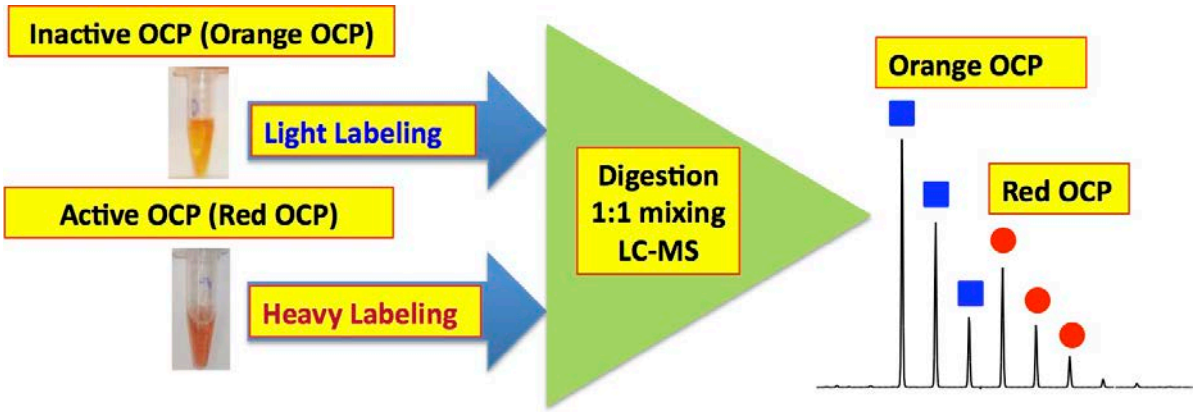

Fig. 2. Logic of the isotope-assisted quantitation of chemical labeling in the OCP protein in the orange and red forms. The orange form is labeled with isotopically light reagent, while the red form is labeled with isotopically heavy reagent. The samples are mixed, digested and analyzed using LCMS. The amplitude difference of the light and heavy peptides directly reflects the extent of labeling in the orange and red forms of OCP.

Science Objectives for 2015-2016: We are continuing our analysis of the conformational changes in the OCP that take place upon the orange to red transition and are also investigating the interaction and detachment mechanisms of the OCP with the phycobilisome. Techniques used include biochemical analysis, site-directed mutagenesis, chemical modification, mass spectrometry, ultrafast spectroscopy and neutron scattering.

References to Work Supported by This Project 2014-2015:

1. Zhang H, Liu H, Blankenship RE and Gross ML (2015) Isotope-encoded Carboxyl Group Footprinting for Mass Spectrometry-based Protein Conformational Studies. *Journal of the American Society for Mass Spectrometry*, In Press DOI: 10.1007/s13361-015-1260-5

Photosystem II: Structure and Function

Terry M. Bricker, Principal Investigator
Laurie K. Frankel, Co-PI
Manjula Mummadisetti, graduate student
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Overall research goals: The principal research objective is to study the structure and function of Photosystem II (PS II). These studies include: (1) identification of the principal sites responsible for reactive oxygen species generation in PS II, (2) determination of the structural arrangement of the extrinsic proteins of PS II in higher plants, and, (3) the use of protein crosslinking to examine components which interact with PS II *in vivo* (*Synechocystis* 6803) and *in organello* (Arabidopsis)

Significant achievements 2014-2015: Significant progress has been made: (1) Using protein crosslinking and synchrotron radiation radiolysis, the structural interaction between PsbP and PsbQ (Fig. 1) has been determined in higher plants (ref. 4). Additionally, we have determined that significant differences exist between the structure of the cyanobacterial PsbO protein and that of higher plants. (2) We have determined that the N-terminal post-translational lipid modification of CyanoQ is required for stability/assembly (ref. 1). (3) In other experiments we have determined that the PPD1 protein is an assembly factor for the lumenal domains of PS I (ref. 6) and have developed a non-detergent isolation method for the isolation of the PS I – LHC II supercomplex (ref. 3).

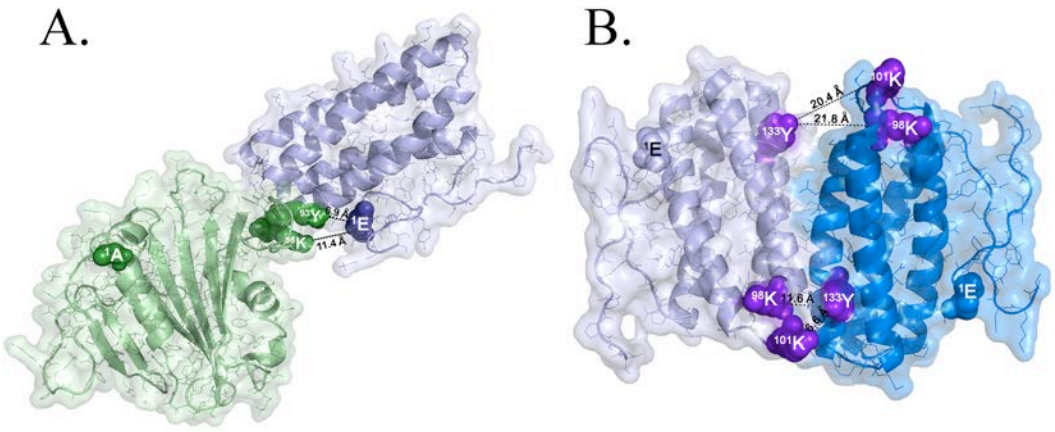

Figure 1. Interaction of PsbP and PsbQ in Higher Plant PS II. A. Proposed structural interaction between PsbP (pale-green) and PsbQ (pale-blue). The distances between the crosslinked residues are labeled. B. Proposed structure of the higher plant PsbQ-PsbQ dimer with one subunit shown in light blue and the other in blue. The distances between the inter-PsbQ crosslinked residues are labeled. These structures were proposed in Ref. 4.

Science objectives for 2015-2016:

- Protein crosslinking studies are ongoing examining the interaction of the higher plant extrinsic proteins with each other and intrinsic core subunits.
- Ongoing experiments are designed to establish the kinetics of oxidative modifications which occur during the photoinactivation of PS II, using both $H_2{}^{18}O$ to probe oxidizing-side modifications and $^{18}O_2$ to probe both reducing-side modifications and modifications produced by singlet oxygen. Additionally, under chloride-depleted conditions, mild heat treatment leads to the generation of $^{\bullet}OH$ at or near the manganese cluster. Identification of the location of

oxidative modification under these conditions should help to establish the pathway of ROS and/or dioxygen out of the photosystem.

- We have identified a number of *in vivo* and *in organello* crosslinked products involving PS II using an isotopically encoded, affinity isolatable membrane permeate crosslinker. We have also identified crosslink products within PS I, the cytochrome b_6/f complex and ATP synthase. We are currently attempting to identify these crosslinked species.

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Ammonia Binds to the Dangler Manganese of the Photosystem II Oxygen Evolving Complex

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Overall research goals: The goal of our proposed research is to better understand the catalytic mechanism of the 4Mn-Ca water oxidizing/oxygen evolving center of Photosystem II. This multimetal center carries out the four electron oxidation of two water molecules, producing bio-activated electrons and protons, with molecular oxygen released as a byproduct. Each of the four oxidation steps in the catalytic cycle is driven by photon induced electron transfer, which allows us to step through the successive intermediates of the S-state cycle using saturating laser flashes. We need to understand the electronic structure of this complex metal cluster and its ligand environment as it advances through the cycle in order to determine how this unique natural catalyst carries out this crucial chemistry.

Significant achievements 20014-2015: High-resolution X-ray structures of Photosystem II reveal several potential sub- strate binding sites at the water-oxidizing/oxygen-evolving 4MnCa cluster. Aspartate- 61 of the D1 protein hydrogen bonds with one such water (W1), which is bound to the dangler Mn4A of the oxygen-evolving complex. Comparison of pulse EPR spectra of $^{14}NH_3$ and $^{15}NH_3$ bound to wild-type Synechocystis PSII and a D1-D61A mutant lacking this H-bonding interaction demonstrates that ammonia binds as a terminal NH_3 at this dangler Mn4A site, and not as a partially deprotonated bridge between two metal centers. The implications of this finding on identifying the binding sites of the substrate and the subsequent mechanism of dioxygen formation are discussed, as will details oncovered by new ^{55}Mn ENDOR experiments on native and ammonia-bound forms (1).

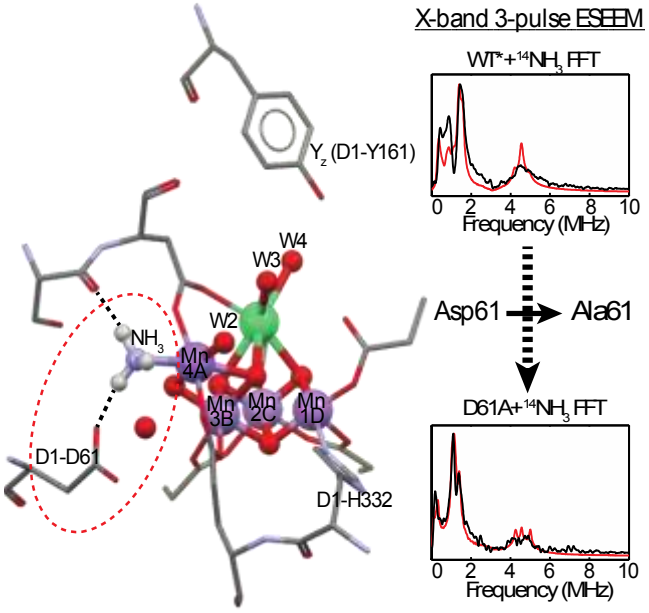

Fig 1. Left: Suggested binding mode for ammonia to the "dangler Mn" based on the change of the symmetry of ^{14}N nuclear quadrupole interaction as measured by electron spin echo envelop modulation (right) comparing WT enzyme with the D1-D61A mutant which deletes a strong H-bond to crystallographically defined W1.

References to work supported by this project 2014-2015:

1. P. H. Oyala, T. A. Stich, R. J. Debus, R. D. Britt, "Ammonia Binds to the Dangler Manganese of the Photosystem II Oxygen Evolving Complex" JACS **137**:8829-8837 (2015).

The Oxygen Isotope Effect of Photosystem II Unveiled

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Abstract: Photosystem II (PSII) catalyzes sunlight-driven oxidation of water during photosynthesis, supplying nearly all the O_2 in our biosphere. The photosynthesis ^{18}O kinetic isotope effect (KIE) is used in global Dole-effect models to explain the isotopic composition of O_2 in the atmosphere, but there is ambiguity in both the magnitude of the PSII ^{18}O KIE, and its relevance to proposed water-splitting mechanisms. We find that the ^{18}O KIE of PSII is 1.002 ± 0.001 in thylakoid membranes and PSII membrane fragments when assayed under standard laboratory conditions, which is explained because plastoquinone exchange is the rate-limiting step during steady-state turnover of PSII. When this rate limitation is removed by using high exogenous quinone concentrations (2,5-dichloro-p-benzoquinone, DCBQ) and low pH, the ^{18}O KIE of 1.022 ± 0.003 associated with water oxidation is unveiled. This value is consistent with proton-coupled electron transfer being the rate-determining step in photosynthetic water oxidation.

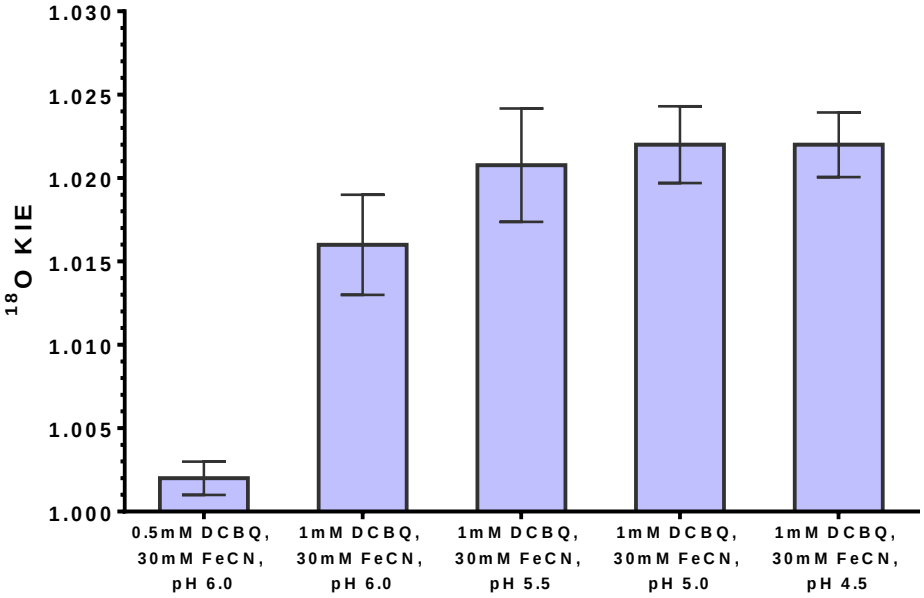

Figure 1. Measured ^{18}O KIE of PSII. The ^{18}O KIE increases as the [DCBQ]:[PSII] ratio is changed in the reaction mixture. Data represent the means of 3-5 independent experiments with standard error.

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

Science objectives for 2015-2016:

- We aim to probe the mechanism of O-O bond formation by measuring $^{16}O/^{18}O$ kinetic isotope effects (KIE) of OEC turnover. These values will be measured when OEC turnover is limiting, in selected point mutants with slow O_2-release kinetics, and in the presence of D_2O.
- We will also determine and compare the effective pK_a's and H/D KIE's of point mutants in the hydrogen-bonding network surrounding the OEC to determine bottlenecks in proton release.
- The effect of the D1-N87A point mutation on the equilibrium between the two spin isomers of S_2 will be determined. Spinach PSII typically shows both the S_2 g=2 "multiline" and g=4.1 EPR signals, while cyanobacterial PSII only shows the g=2 signal. This difference may be connected to the change from D1-A87 in plants to D1-N87 in cyanobacteria.
- Ammonia binds in two sites in the OEC: Site A directly on Mn and a second-shell site that is competitive with Cl^- and stabilizes the g=4.1 S_2 state (Site B). We will investigate the "Site B" ammonia-binding site in point mutants that prevent Cl^- binding (D2-K317A and D1-N181A).

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2. "Oxygen-Evolving Complex of Photosystem II: An Analysis of Second-Shell Residues and Hydrogen-Bonding Networks", Leslie Vogt, David J. Vinyard, Sahr Khan and Gary W. Brudvig (2015) *Curr. Opin. Chem. Biol.* **25**, 152-158.
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4. "Probing the Effect of Mutations of Asparagine 181 in the D1 Subunit of Photosystem II", Ravi Pokhrel, Richard J. Debus and Gary W. Brudvig (2015) *Biochemistry* **54**, 1663-1672.
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7. "Photosynthetic Water Oxidation: Insights from Manganese Model Chemistry", Karin J. Young, Bradley J. Brennan, Ranitendranath Tagore and Gary W. Brudvig (2015) *Accts. Chem. Res.* **48**, 567-574.
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Understanding of the function and regulation of the high affinity CO_2-concentrating mechanism

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Abstract

Cyanobacteria are considered as ideal vehicles for numerous bioengineering applications ranging from CO_2 mitigation to the production of biofuels and chemicals. The acquisition of inorganic carbon (C_i=CO_2+HCO_3^-) very often limits photosynthetic performance in natural systems, including cyanobacteria. This limitation is likely to be even more acute in engineered systems that may have even higher demands for inorganic carbon, due for example, to the release of hydrocarbons.

To overcome C_i-limitation, cyanobacteria naturally possess a complex **CO_2-concentrating mechanism** (**CCM**) that is capable of utilizing cellular energy to acquire and concentrate CO_2 and bicarbonate from the environment. However, much remains to be understood regarding the structure and regulation of the CCM. Accordingly, the overall objective is to better understand the cyanobacterial high affinity CCM and its integration with the reactions of oxygenic photosynthesis using the model organism *Synechocystis* sp. PCC6803. ***Our main questions:***

1.) How is the CCM regulated *vis-à-vi* the oxygenic photosynthetic mechanism?

2.) What is the mechanism of the fascinating redox-powered membrane-bound CO_2-hydration enzymes that are central to the cyanobacterial CCM?

Significant Achievements 2014-2015:

1. **Regulatory mechanisms of the CCM and its integration with metabolism and cyclic electron transfer (CET).** Our earlier DOE-supported work led to the identification of metabolites and transcriptional regulators that act in concert to control the transcriptional expression of the inducible high affinity CCM. These metabolites were shown to modify the DNA binding activities of LysR-type regulatory proteins and revealed a homeostatic regulatory mechanism that adjusts the CCM to changes in the demand for CO_2 based upon internal metabolic cues that are, in turn, responsive to C_i availability in the environment. More recently, we have performed an analysis of cells experiencing C_i limitation. Cells experiencing C_i limitation exhibited systematic changes associated with physiological adjustments

and a trend towards the plastoquinone and NADP pools becoming highly reduced prior to adaptation followed by a return to normal levels upon adaptation to the low C_i regime. The results are consistent with the proposed regulation of the CCM and provide new information on the nature of the Chl and NADPH fluorescence induction curves (1). These experiments are now being extended to strains obtained from NREL test hypotheses regarding the operation of photosynthesis and the CCM in model strains engineered for biofuel production.

2. **Structure/Function of CO_2-uptake (CUP) proteins of NDH-$1_{3/4}$ complexes.** The unique CO_2 uptake enzymology remains to be clarified, although it is known to involve variants of the Type-1 NAD(P)H dehydrogenase (NDH-1) complexes that have additional subunits. A mutagenesis system is being developed to probe the structure-function relationships of the NDH-$1_{3/4}$ complexes regarding their CO_2-hydration activity. This enhances the on-going effort to use synthetic techniques to manipulate the cyanobacterial CCM. Progress is being made in the direction of engineering an expression platform for the full-length *cupA* operon (*ndhF3/ndhD3/cupA/cupS*). This is being developed to enable site-directed mutagenesis for probing structure-function relationships *in vivo* and to introduce affinity tags to purify membrane complexes for *in vitro* analysis. Other parts of the work involved the heterologous expression of the putative carbonic anhydrase (CupA) subunit. However, that approach has proven difficult since the protein is highly sensitive to unfolding and aggregation once it is liberated from the stabilizing affinity tag (maltose binding protein).

3. Additionally, an optimization computer model for autotrophic cyanobacterial growth, the autotrophic replicator model (ARM), was developed (3). The present model describes autotrophic growth in terms of the allocation protein resources among core functional groups including the photosynthetic electron transport chain, light harvesting antennae, and the ribosome groups. This model will help us connect our molecular genetic and physiological analyses with the metabolomic studies of our NREL collaborators.

Scientific Objectives: 2016-2017

During the previous funding periods, our focus has been on the regulation of the inducible, high affinity CCM where we were able to decipher the key elements in its transcriptional activation. We will now turn our attention to the structure-function relationships of the most enigmatic components of the CCM: CO_2-uptake (Cup) enzymes associated with NDH-$1_{3/4}$ complexes. These are poorly understood membrane protein complexes that use redox energy from cyclic electron flow to drive CO_2-hydration by unique carbonic anhydrases. The hypothesis is that the proton pumping activity of the NDH-1 complexes is coupled to the evacuation of protons from the active site of a carbonic anhydrase moiety within the Cup protein or from the interface with the proton-

pumping subunits and the interacting Cup subunit. This is an opportune time to perform this investigation given the significance of the activity and the fact that good structural models are available for the highly conserved underlying core NDH-1 complex, yet the structure and function the Cup subunits remain obscure. We will present our efforts to develop an experimental system to investigate the structure-function relationships of the enigmatic of these NDH-$1_{3/4}$ complexes.

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Ubiquitin Facilitates a Quality Control Pathway that Removes Damaged Chloroplasts

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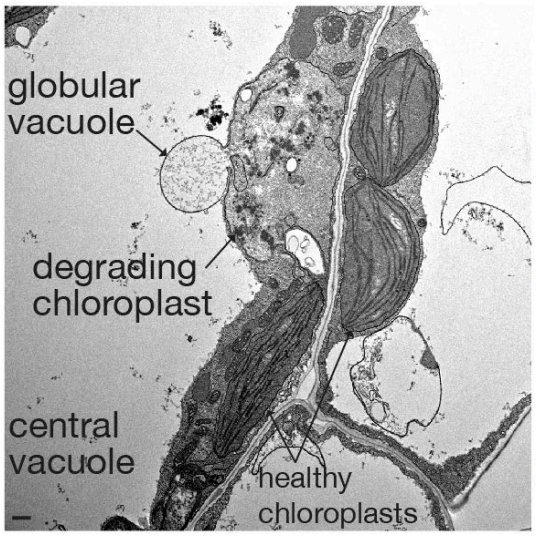
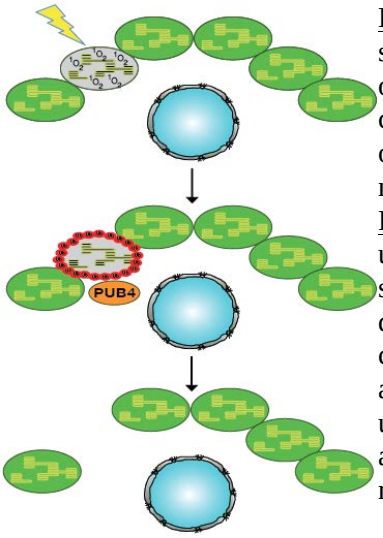
Overall research goals: Chloroplasts of higher plants contain about 3000 proteins of which more than 95% are encoded by nuclear genes. This necessitates a tight coordination of gene expression that involves two-way signaling between these spatially separated genomes. Thus, while plastid differentiation and development are largely under nuclear control, developmentally arrested or damaged plastids can regulate expression of a subset of nuclear genes via retrograde signaling pathways. Multiple retrograde signals have been proposed, but almost nothing is known of their signaling pathways. In previous years of this DOE-funded study, we performed a number of genetic screens that implicated the chloroplast-localized tetrapyrrole biosynthesis pathway as the source of both positive and stress-related retrograde signals. However, the mechanisms by which these molecules regulate nuclear gene expression or cell physiology are mostly unknown.

Very recently, we have discovered a new pathway that involves the selective degradation of singlet oxygen ($^1O_2$) stressed chloroplasts though the ubiquitination system. The major goal of our renewal proposal is to define the signal(s) and signaling pathways involved in this process. Specifically, we will: (1) Explore how $^1O_2$ accumulation leads to chloroplast protein ubiquitination and chloroplast degradation; (2) Determine what role the E3 ubiquitin ligase PUB4 plays in this chloroplast quality control pathway. The successful completion of the proposed projects should allow us to begin to engineer these crucial pathways. Since heat, cold, and high light stress share at least some common signaling elements, generation of new genotypes will ultimately influence our abilities to manipulate plant growth and development, and will aid in the understanding of the developmental control of photosynthesis.





Left panel: TEM of selective degradation of a stressed chloroplast in an otherwise healthy *fc2* mutant mesophyll cell. Right panel: Model of ubiquitin-facilitated selective chloroplast degradation. Stressed chloroplasts that accumulate $^1O_2$ are ubiquitinated by PUB4 and subsequently removed from the cell.

Significant achievements 2011-2015:

- High light stress screen: We used a reporter gene fusion to identify mutants defective in the response to high light stress, and found genes involved in both early and late phases of the response. Early on, 3 heat shock factors (HSFs) of greater than 20 in Arabidopsis play an important role (ref 5). We have identified mutations in 4 genes involved in later phases of light stress; these include: cryptochrome, two RNA processing enzymes, and a co-activator. Yeast 1-hybrid analysis identified a subset of AP2-type transcriptional regulators as part of the network.

- Heme as a positive chloroplast signal. A genetic screen identified *plastid ferrochelatase I* (*FC1*), which encodes one of the two conserved plant enzymes that produce heme, as playing a role in retrograde signaling. Characterization of this gene suggested that chloroplast-produced heme could act as a retrograde signal to coordinate plastid and nuclear gene expression (Ref 1). This was further supported by the analysis of *sigma factor* mutants that are defective in the transcription of defined sets of plastid genes. Some of these mutants triggered a retrograde signal to the nucleus to reduce photosynthesis gene expression, which could be altered by increasing heme synthesis (Ref 4). Together this work supports earlier conclusions that retrograde signals do represent biologically important signaling pathways for coordinating chloroplast biogenesis with nuclear transcription.
- Discovery of a chloroplast quality control pathway. Our analysis of the *Arabidopsis fc2* mutant suggested that chloroplast-produced $^1O_2$ can lead to the ubiquitination of chloroplast outer membrane proteins. This can subsequently lead to the selective degradation of these severely stressed chloroplasts (see figure above). A large forward genetic screen identified the E3 ubiquitin ligase PUB4 as being necessary for removal of damaged chloroplasts (Ref 6).

Science objectives for 2015-2016:

- A major research goal is to understand how chloroplast produced $^1O_2$ has the ability to lead to the ubiquitination of chloroplast proteins and the selective removal of stressed chloroplasts. As $^1O_2$ has an extremely short half-life, it is expected that second messengers are involved (see figure). In collaboration with Dr. Alan Saghatelian at the Salk Institute, we plan to use targeted and untargeted metabolite profiling to identify potential second messengers created by chloroplast $^1O_2$ damage. Candidate molecules will be tested for their ability to signal and induce ubiquitination of chloroplast proteins and/or chloroplast degradation.
- To understand how selective chloroplast degradation is triggered, it will be necessary to uncover the identities of the proteins being ubiquitinated. We plan to use a proteomic approach to identify the chloroplast proteins being modified during stress. Genetic and molecular biology tools can then be applied to understand what role these modified proteins play in this pathway.
- Our genetic screen identified the E3 ligase PUB4 as being essential for chloroplast degradation. As E3 ligases impart specificity of the ubiquitination system by binding specific protein substrates, we expect the targets of PUB4 to be directly involved in the chloroplast signaling pathway. As such, we will take several approaches to identify the protein substrate target(s) of PUB4. At the same time, we will begin the characterization of closely-related PUB family members that may also be important for removal of defective plastids.

References to work supported by this project 2011-2015:

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Disruption of the CO_2 concentrating mechanism in C_4 plants: Implications for CO_2 fixation and photosynthetic efficiency during C_4 photosynthesis.

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Overall research goals

- The *overall objective* of this proposal is to determine how changes in the capacity of the C_3 and C_4 cycles and bundle sheath cell wall properties determine the photosynthetic efficiency of the CO_2 concentrating mechanism in C_4 plants in response to changes in light and temperatures.
- Our *central hypothesis* is that the efficiency of the CO_2 concentrating mechanism in C_4 plants is optimized through balancing the activity of the C_3 and C_4 cycles with bundle sheath properties.
- Our *specific aims* are 1) Determine the mechanisms that disrupt the metabolic coordination of C_4 photosynthesis in response to changes in leaf temperature and 2) Determine how changes in the capacity of the C_3 and C_4 cycle control the photosynthetic efficiency of C_4 photosynthesis in response to changes in leaf temperature and light availability.

Significant achievements 2012-2013

***Temperature response in* Zea mays *and* Setaria viridis.** We compared measured and modeled C_4 photosynthesis and CO_2 isotope exchange to constrain the rate limiting steps of C_4 photosynthesis and the CO_2 concentrating mechanism between 10 and 40 °C. This more accurately determined the rate limiting steps of C_4 photosynthesis and the CO_2 concentrating in response to temperature. Measurements have been conducted, data analyzed and we are currently preparing for publication (Ubierna et al., *In prep*).

***Biochemical temperature response in* S. viridis.** Phosphoenolpyruvate carboxylase (PEPC), carbonic anhydrase (CA) and Rubisco potentially catalyze rate-limiting steps of C_4 photosynthesis in response to temperature. However, there are few reports of the temperature response of these enzymes from C_4 plants. We measured with membrane inlet mass spectrometer the *in vitro* temperature responses of CA, PEPC, and Rubisco from the C_4 model plant *S. viridis* (Boyd et al., 2015). These parameters were incorporated into theoretical models of C_4 photosynthesis to constrain the rate limiting steps of C_4 photosynthesis and the CO_2 concentrating mechanism in response to temperature (Gandin et al., *In prep*).

Carbonic anhydrase and C_4 photosynthesis. The importance of CA for C_4 photosynthesis is unclear but potentially limits rates of photosynthesis. Therefore, measurements of leaf gas exchange were made in *Z. mays* mutants with <2% of wild-type CA activity. Net CO_2 assimilation under sub-ambient CO_2 concentration was lower in the CA mutant; however, at ambient and above CO_2 there was no effect of on photosynthesis. This suggests that CA in *Z. mays* is essential for maximum of CO_2 assimilation only under limited CO_2 availability (Studer et al., 2014).

Growth light and leaf structure The goal of this research was to determine how growth under limited light affects leaf structure and C_4 photosynthesis. We grew *Miscanthus* × *giganteus* under two light treatments and three nitrogen levels to determine how these conditions influence leaf development and photosynthetic efficiency (Ma et al., *In review*). Additionally, we published a paper on the influence of growth light conditions on the efficiency of the single-cell C_4 system (Stutz et a., 2014).

Antisense reduction in PEPC and Rubisco We synthesized eight RNAi constructs using Gateway technology to target genetic reduction in PEPC and Rubisco content in *S. viridis*. We have genotyped and phenotyped two knockdown lines for both PEPC and Rubisco. These plants are currently being used to study how these changes influences the temperature response of C_4 photosynthesis.

Science objectives for 2015-2016

RNAi reduction in PEPC and Rubisco The RNAi plants have been generated and are being screened for low PEPC or Rubisco activity. These plants will be used to determine how changes in the relative capacity of the

C_3 and C_4 cycles influence the efficiency of C_4 photosynthesis in response to temperature. Unfortunately, these plants took much longer to generate than we anticipated but we are now conducting the experiments.

References for work supported by this project 2014-2015:

1. Boyd RA, Gandin A, **Cousins AB** (2015) Temperature response of C_4 photosynthesis: Biochemical analysis of Rubisco, Phosphoenolpyruvate Carboxylase and Carbonic Anhydrase in *Setaria viridis*. *Plant Physiology* doi: http://dx.doi.org/10.1104/pp.15.00586 **Entirely support by current BES support.*

2. Gandin A, Koteeva N, Voznesenskaya E, Edwards G, **Cousins AB** (2014) Temperature response of the photosynthetic machinery in the C_3-C_4 intermediate *Salsola divaricate*. *Plant Cell and Environment* 37:2601-2612 **Collaborative project: all gas exchange, isotope analysis, biochemistry and growth experiments funded by current BES support.*

3. Sun W, Ubierna N, Ma JY, Walker B, Kramer D, **Cousins AB** (2014) The coordination of C_4 photosynthesis and the CO_2 concentrating mechanism in *Zea mays* and *Miscanthus* × *giganteus* in response to transient changes in light quality. *Plant Physiology* 164:1283-92 **Research and data generation during previous BES grant; however, writing, data analysis and manuscript preparation during current funding.*

4. Stutz SS, Edwards GE, **Cousins AB** (2014) Single-cell C_4 photosynthesis: efficiency and acclimation of *Bienertia sinuspersici* to growth under low light *New Phytologist* 202:220-32 **Research and data generation during previous BES grant; however, writing, data analysis and manuscript preparation during current BES support.*

5. Walker B, Strand D, Kramer DM, **Cousins AB** (2014) The Response of Cyclic Electron Flow around Photosystem I to Changes in Photorespiration and Nitrate Assimilation. *Plant Physiology* 165:453-462 **Collaborative project: supported by both NSF and current BES funds.*

6. Studer A, Gandin A, Kolbe A, **Cousins AB,** Brutnell T. (2014) Carbonic anhydrase does not limit photosynthesis in *Zea mays* under current atmospheric CO_2 conditions. *Plant Physiology* 165:608-617 **Collaborative project: mutants from collaborators but all gas exchange, isotope analysis and growth experiments funded by current BES support.*

7. Koteyeva, N, Voznesenskaya E, **Cousins AB,** Edwards GE(2014) Differentiation of C_4 photosynthesis along a leaf developmental gradient in two Cleome species having different forms of Kranz anatomy *Journal of Experimental Botany* 65:3525-3541 **Collaborative project: all gas exchange experiments funded by current BES support*

8. von Caemmerer S, Edwards GE, Koteyeva N, **Cousins AB** (2014) Single Cell C_4 photosynthesis in aquatic and terrestrial plants: a gas exchange perspective *Journal of Aquatic Botany* 118:71–80 **Review article: PI supported by current BES funding.*

9. von Caemmerer S, Ghannoum O, **Cousins AB** (2014) Carbon isotope discrimination as a tool to explore C_4 photosynthesis. *Journal of Experimental Botany* 65:3459-3470 **Review article: PI supported by current BES funding.*

10. Kromdijk J, Ubierna N, **Cousins AB**, Griffiths H. (2014) Bundle sheath leakiness in C_4 photosynthesis: a careful balancing act between CO_2 concentration and assimilation. *Journal of Experimental Botany* 65:3443-3457 **Review article: PI supported by current BES funding.*

11. Cousins AB, Johnson M, Leakey ADB (2014) Photosynthesis and the environment. *Photosynthesis Research* 119:1–2 **Co-editor for a special issue organized by PI and co-authors*

Under review

1. MA J, Sun W, Koteyeva, K, Voznesenskay E, Stutz SS, Gandin A, Smith-Moritz AM, Heazlewood JL, **Cousins AB** Influence of Light and Nitrogen on the Photosynthetic Efficiency in the C_4 Plant *Miscanthus* × *giganteus Photosynthesis Research *Research and data generation during previous BES grant; however, writing, data analysis and manuscript preparation during current BES support.*

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

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

Significant achievements 2013-2015: Proteins destined for the thylakoid lumen of chloroplasts are nuclear-encoded, synthesized in the cytoplasm, and must cross three membranes *en route* to their final destination. The chloroplast Twin Arginine Translocation (cpTat) system facilitates transport of about half of all proteins that cross the thylakoid membrane in chloroplasts. Known mechanistic features of the cpTat system are drastically different from other known translocation systems, notably in its formation of a transient complex composed of thylakoid proteins cpTatC, Hcf106, and Tha4 to transport fully folded proteins utilizing only the protonmotive force (pmf) for energy. cpTat and Hcf106 form a receptor complex, which binds the signal sequence on the precursor and Tha4 is thought to play a role in generating the point of passage for the precursor during the transport process. However, key details such as the structure and composition of the translocation pore are still unknown. We hypothesize that the receptor complex protein Hcf106, acts as a nucleator for Tha4 assembly in the presence of precursor and a pmf. Yet, little is known about cpTatC-Hcf106 interactions or precursor mature domain-Hcf106 interactions, which are expected if Hcf106 serves as part of the receptor and a nucleator for Tha4 assembly. First, we present interaction studies of Hcf106 and mature domain of the precursor via cysteine disulfide bond formation (**Fig. 1**). A cysteine (Cys) placed in the mature domain of the precursor to the 17 kDa subunit of the oxygen evolving complex (pOE17J99C or PsbQK99C) was able to interact with Cys placed in the N terminus of Hcf106 as well as regions throughout the amphipathic helix. By comparison, a Cys in the same location on the precursor predominantly interacts with the C-proximal portion of the APH of Tha4. Second, we have been able to map interactions between cpTatC and Hcf106. cpTatC has six transmembrane domains (TM1-M6) connected by three stromal loops (S1-S3) and three lumen loops (L1-L3). Our data show that Cys placed in the TMD of Hcf106 preferentially interacts with Cys placed in TM5 of cpTatC. Likewise Cys placed in the APH of Hcf106 preferentially interacts with the stromal loops (S1~S2>S3) of cpTatC (**Fig. 2**). We have therefore established that we can use *in vitro* expressed

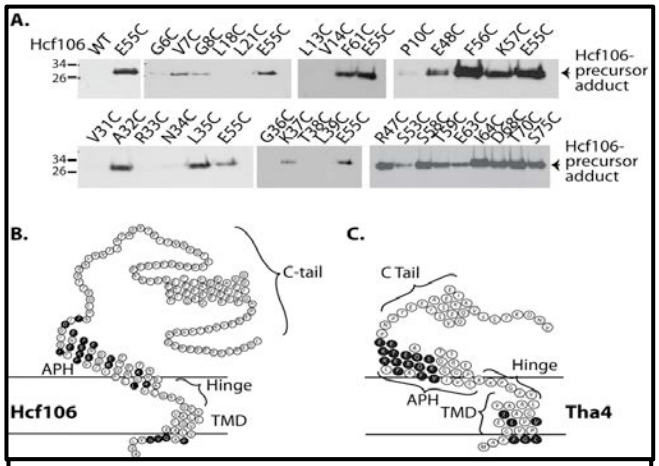

Figure 1. Analysis of precursor-Hcf106 interaction. ***A***, Hcf106 interacts directly with His-tagged precursor, pOE17K99C (prePsbQK99C). The His-tagged precursor was used to affinity precipitate Hcf106 with the indicated Cys substitution. ***B***, A schematic of Hcf106 summarizing Cys substitutions able to form disulfide bonds with the precursor (black filled circles). ***C***, For comparison, a schematic of Tha4 summarizing Cys substitutions that interact with precursor (filled circles) adapted from (Pal et al. 2013).

exogenous forms of cpTat proteins in native thylakoid to query the organization of the cpTat receptor and translocase.

Science objectives for 2016-2017:

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

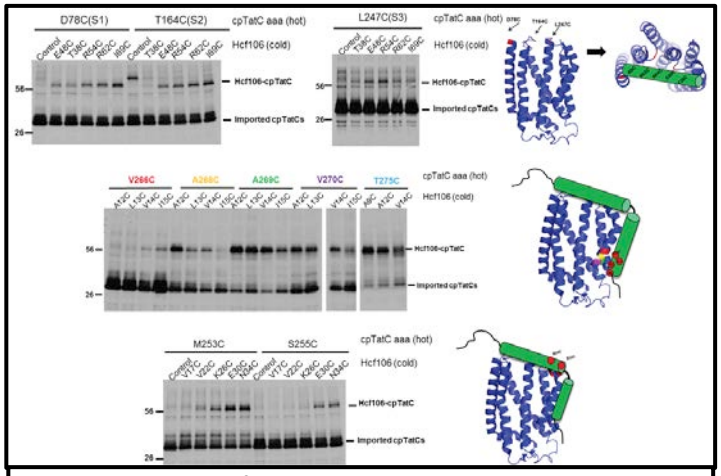

Figure 2. Mapping Hcf106 interactions with cpTatC. [^{3}H]-pre-cpTatC (hot) with the indicated Cys substitution (X_nC) was imported into isolated chloroplasts, which were subject to osmotic lysis and the thylakoids harvested. Unlabeled Hcf106 (cold) was integrated into the isolated thylakoids and the oxidant CuP was added to promote disulfide bond formation. *Upper panel,* The APH of Hcf106 can interact with the stromal loop regions of cpTatC. *Middle panel,* the N-proximal portion of the transmembrane domain of Hcf106 preferentially interacts with the lumen proximal portion of TM5 on cpTatC. *Lower panel,* the N-proximal portion of the APH of Hcf106 preferentially interacts with the stromal-proximal portion of TM5 on cpTatC. Taken together, Hcf106 TMD sits along TM5 on cpTatC and the APH stretches across the stromal loops. Cartoon representations of the interactions between Hcf106 (green) and cpTatC (blue) are depicted to the right of each panel.

References to work supported by this project 2013-2015:

1. D. Pal, K. Fite, C. Dabney-Smith. (2013) Direct interaction between a precursor mature domain and transport component Tha4 during twin arginine transport of chloroplasts, Plant Physiology, **161**:990–1001.

   Zhang, L., Liu, L., Maltsev, S., Lorigan, G.A., Dabney-Smith, C. (2013) Solid-State NMR Investigations of Peptide-Lipid Interactions of the Transmembrane Domain of A Plant-Derived Protein, Hcf106. Chemistry and Physics of Lipids **175-176:**123-30 doi:10.1016/j.chemphyslip.2013.09.002
2. Zhang, L., Liu, L., Maltsev, S., Lorigan, G.A., and Dabney-Smith, C. (2014) Investigating the interaction between amphipathic helix of Hcf106 peptides and the phospholipid bilayer by solid-state NMR spectroscopy. BBA: Biomembranes 1838(1):413-8. doi: 10.1016/j.bbamem.2013.10.007 .
3. Dabney-Smith, C., & Storm, A. (2014). Protein Routing Processes in the Thylakoid. In S. M. Theg & F.-A. Wollman, *Advances in Plant Biology* (Vol. 5, pp. 271–289). New York, NY: Springer New York. doi:10.1007/978-1-4939-1136-3_10.
4. Q. Ma, K. Fite, and C. Dabney-Smith. (2015) Cysteine scanning and cross-linking study reveals the oligomeric property of Hcf106 in the chloroplast Tat system. PLoS One. Revisions submitted.
5. Q. Ma and C. Dabney-Smith. Organization of the receptor complex of the Twin Arginine Transport pathway in thylakoids of chloroplasts. Molecular Biology of the Cell. In review.

Membrane-attached Electron Carriers in Photosynthesis and Respiration: Cytochrome *c* maturation (Ccm-system I) in a facultative photosynthetic bacterium

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Overall research goals: Our long term aim is to contribute to a comprehensive characterization of major energy transducing pathways like Photosynthesis and Respiration. We investigate the molecular nature, mechanism of function and biogenesis (maturation and assembly) of cytochromes (cyts) and cyt complexes. Cyts are ubiquitous electron transfer proteins that act as key components in energy production. They are matured by the Cyt *c* maturation (Ccm)-system I found in bacteria, archaea, mitochondria of plants and red algae. We use mainly phototrophic bacteria (of *Rhodobacter species*), which are excellent models for these studies. Ccm is composed of nine membrane-associated proteins (CcmABCDEFGHI), which carry out the covalent ligation of heme *b* to the conserved heme-binding site (CXXCH motif) of apocyts. They form three different functional units, performing apocyt (1) and heme (2) delivery to the central ligation (3) module. Our current focus is on the interactions between the Ccm components and the Ccm substrates (heme and apocyts *c*). We employ protein biochemistry, kinetics and spectroscopic approaches. Our goal is to define the molecular basis of substrate recognition (heme and apocyts *c*) by the Ccm apparatus, which is now emerging.

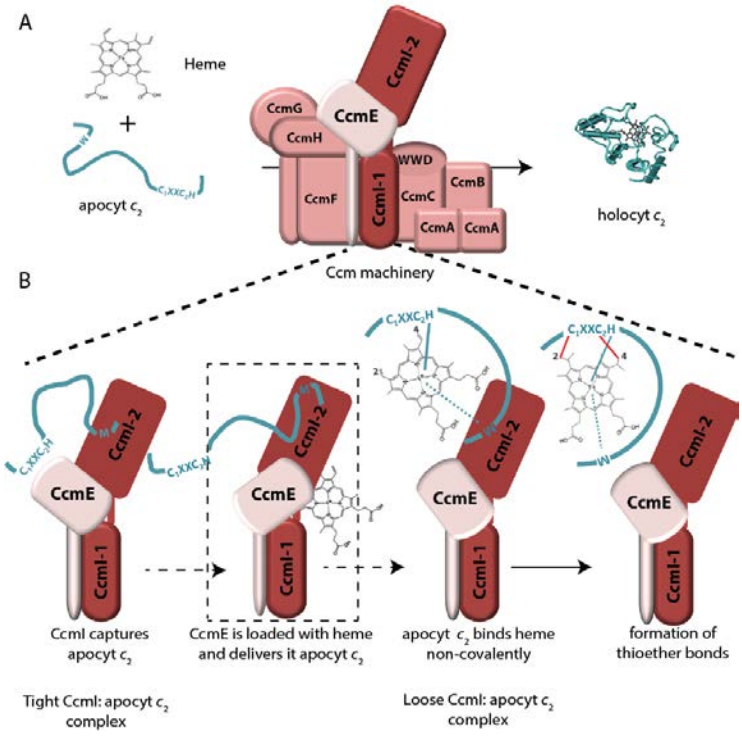

Figure. CcmI-apocyt *c* interactions in the presence and absence of heme during Ccm. **A.** shows the "Ccm machine" and its two substrates, apocyt c and heme, forming holocyt *c*. **B**. depicts the interactions between CcmI and a class I apocyt c in the presence and absence of heme. The periplasmic CcmI-2 domain of CcmI binds tightly the C-terminal helix of apocyt *c*, bringing its heme binding site close to CcmE. Upon availability of heme (via CcmE), the apocyt *c* binds heme noncovalently to form a *b*-type cyt intermediate that interacts less tightly with CcmI-2. Thioether bonds formation between heme and apocyt *c*, via currently unknown steps is catalyzed by the remaining components of Ccm, to yield holocyt *c*.

Significant achievement 2014-2015: We are interested in specific interactions between the apocyt *c* chaperone CcmI, the heme chaperone CcmE and the apocyt *c* substrates: ***a***- Using a multi-prone approach, including co-purification assays, bio-layer interferometry and CD spectroscopy, we showed that the apocyt *c* chaperone CcmI in the absence of heme recognizes apocyts *c* from different classes with

remarkably different affinity constants (nM K_d for class I and μM K_d for class II apocyts *c*). Consistent with this finding, we showed that the class I, and class II apocyts *c* have different structures. ***b***- We found that, the presence of heme induces conformational changes in class I apocyts *c*, and this decreases their affinity towards CcmI. These findings suggest that CcmI holds the class I apocyts *c* tightly until their non-covalent heme-containing *b*-type cytochrome-like intermediate is formed prior to covalent heme ligation (**Figure**). ***c***- Using a similar approach, we also established that the heme chaperone CcmE is able to differentiate between different classes of apocyt *c*, by recognizing them with different affinities. ***d***- Our previous data showed that both CcmI and CcmH from the CcmFHI core complex form together with CcmE and apocyt *c* a multi subunit complex, and that apoCcmE interacts with the CcmABCD components to receive heme (*i.e.*, to become holoCcmE). Based on these findings, we developed a model proposing that CcmABCD together with CcmFHI-CcmE-apocyt *c* complexes might form a large macromolecular supercomplex ("Ccm machine").

Science objectives for 2015-2017: The following specific aims are being pursued:

- Elucidation of how the Ccm components recognize and interact with different types of apocyt *c* substrates, expanding current studies to class III multiheme *c*-type cyts, such as a pentaheme cyt *c* (*e.g., R. capsulatus* DorC) and compare with classes I and II apocyts.
- Initiate and pursue actively at atomic scale the molecular nature of the interactions between the apocyt *c* chaperone CcmI and class I and class II apocyts *c* (*e.g.*, apocyts c_2 and *c*', respectively, and related peptides) using isotope labeling and solution NMR.
- Investigation of the sequence of thioreduction events that involve CcmG, CcmH and apocyt *c* during Ccm, in order to define the precise role of CcmH as parts of the heme ligation complex and the thioredox reactions.

References to the works directly related to Ccm studies supported by this project 2014-2015

• Andreia F. Verissimo and Fevzi Daldal. (2014) Cytochrome *c* biogenesis System I: an intricate process catalyzed by a maturase supercomplex? *B.B.A-Bioenergetics* **1837**, 989-998.

• Andreia F. Verissimo, Namita P. Shroff and Fevzi Daldal. (2015). During Cytochrome *c* Maturation CcmI Chaperones the Class I Apocytochromes until the Formation of Their *b*-Type Cytochrome Intermediates. *J.Biol.Chem.* **290**, 16989-17003.

• Bahia Khalfaoui-Hassani*, Andreia F. Verissimo*, Namita P. Shroff, Seda Ekici, Petru-Iulian Trasnea, Marcel Utz, Hans-Georg Koch and Fevzi Daldal (2015). Biogenesis of Cytochrome *c* complexes: from Insertion of Redox Cofactors to Assembly of Different Subunits. In: *Cytochrome Complexes: Evolution, Structures, Energy Transduction and Signaling.* Edited by Cramer W and Kallas T, *in press* (* both authors contributed equally).

Our ongoing studies related to other aspects of the biogenesis of cyt *c* complexes, in particular, those on the discovery and ongoing studies of the novel bacterial copper importer CcoA, which is a Major Facilitator Superfamily type transporter, are not listed.

FTIR Studies of Photosynthetic Oxygen Production

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

Significant achievements 2014-2015: FTIR difference spectroscopy is a powerful tool for studying the structural changes of H-bonded H_2O molecules (*1*). The O–H stretching mode of H_2O molecules having relatively weak H-bonds can be monitored near 3600 cm^{-1}, the D-O-D bending mode can be monitored near 1210 cm^{-1}, and highly polarizable networks of hydrogen bonds can be monitored as broad features between 3000 and 2000 cm^{-1}. We have been exploring the consequences of substituting Sr for Ca and mutating D1-V185. Of all the cations that can competitively replace Ca in PSII, only Sr is capable of supporting O_2 evolution. The substitution of Sr for Ca slows the S state transitions and alters the positions of some of the water molecules located near the Mn_4CaO_5 cluster. The substitution of Sr for Ca has little effect on the broad feature between 3000 and 2000 cm^{-1} (Figure 1) or the features near 3600 cm^{-1} in any of the S_{n+1}-*minus*-S_n spectra. These observations imply that the highly polarizable networks of hydrogen bonds whose polarizability or protonation state increases during the individual S state transitions do not include the Ca ion and that the H_2O molecules that deprotonate or have their H-bond strengths increased during the S state transitions do not interact with the Ca ion. We reached a similar conclusion regarding D1-N181 from examination of the mutants D1-N181S and D1-N181A in collaboration with G. W. Brudvig and coworkers at Yale (*2*). The substitution of Sr for Ca slightly perturbs the $D_2{}^{16}O$-*minus*-$D_2{}^{18}O$ double difference spectrum of the S_1 to S_2 transition (Figure 3). Although the amplitudes of several features are increased or decreased by Sr, none of the major features is eliminated, implying that the H_2O molecules whose H-O-H bending mode changes during the S_1 to S_2 transition do not interact directly with the Ca ion.

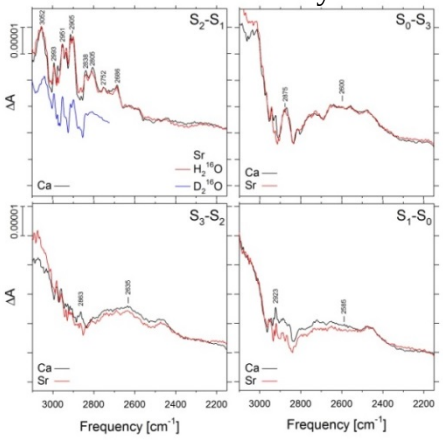

Figure 1. The FTIR difference spectra of PSII core complexes containing Ca (black) and Sr (red) between 3100 and 2150 cm^{-1} in response to four successive flash illuminations applied at 0°C.

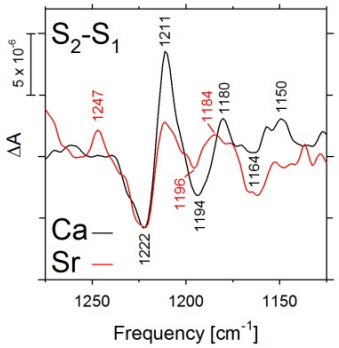

Figure 2. The S_2-*minus*-S_1 $D_2{}^{16}O$-*minus*-$D_2{}^{18}O$ double difference spectra of PSII core complexes containing Ca (black) or Sr (red) in the D-O-D bending region.

The residue D1-V185 contacts numerous water molecules located between tyrosine Y_Z and D1-D61, the initial residue in a dominant proton egress pathway linking the Mn_4CaO_5 cluster with the lumen. The mutation D1-V185N decreases the efficiency of O_2 formation and dramatically decreases the rate of O_2 release, features in common with mutations of D1-D61. These features have been attributed to mutation-induced alterations to the H-bond network that facilitates proton egress. To determine if the water molecules perturbed by the D1-V185N mutation can be identified spectroscopically, we are examining the FTIR properties of D1-V185N PSII core complexes in collaboration with R. L. Burnap and coworkers at Oklahoma State University. In common with mutations of residues interacting with H-bond networks near the Mn_4CaO_5 cluster (*1*), the D1-V185N mutation alters features in the symmetric and asymmetric carboxylate stretching regions of the FTIR difference spectra, but perturbs the protonated carboxylic acid carbonyl stretching region differently than any other mutant yet examined. Instead of eliminating or decreasing the amplitudes of features in this region, the D1-V185 mutation shifts features by 5-9 cm^{-1}. These shifts imply that the mutation alters the environments of the carboxylate groups whose pK_a values change in response to the S_1 to S_2 and S_2 to S_3 transitions.

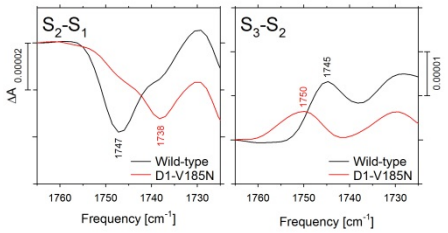

Figure 3. The protonated carboxylic acid ν(C=O) regions of the FTIR difference spectra of wild-type (black) and D1-V185N (red) PSII core complexes.

In collaborative investigation of D1-D61A PSII core complexes, conducted with R. D. Britt and coworkers at UC Davis, it was determined that ammonia, a substrate water analogue, coordinates to the dangling Mn_{A4} ion of the Mn_4CaO_5 cluster in place of W1, eliminating W1 as one of the two substrate H_2O molecules (*3*).

Science objectives for 2015-2016:

- Complete FTIR studies on Sr-substituted samples and the mutant D1-V185N and extend these studies to other mutations at this site such as D1-V185A and D1-V185S.
- Begin static FTIR measurements of proton release by mutants such as D1-D61A and D1-V185N
- Initiate time-resolved IR measurements of proton release by mutants such as D1-D61A and D1-V185N.

References to work supported by this project 2014-2015:

1. Debus, R. J. (2015) "FTIR Studies of Metal Ligands, Networks of Hydrogen Bonds, and Water Molecules near the Active Site Mn_4CaO_5 Cluster in Photosystem II, *Biochimica et Biophysica Acta 1847*, 19-34.
2. Pokhrel, R., Debus, R. J., and Brudvig, G. W. (2015) "Probing the Effect of Mutations of Asparagine 181 in the D1 Subunit of Photosystem II, *Biochemistry 54*, 1663-1672.
3. Oyala, P. H., Stich, T. A., Debus, R. J., and Britt, R. D. (2015) "Ammonia Binds to the Dangler Manganese of the Photosystem II Oxygen-Evolving Complex," *J. Am. Chem. Soc. 137*, 8829-8837.

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

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

Significant achievements 2014-2015:

Nuclear tensors of nitrogen H-bond donors to SQ_A or SQ_B. Our previous studies of SQ_A and SQ_B by X-band 2D ESEEM revealed interactions with His-M219 N_δ and Ala-M260 peptide nitrogen (N_p) and His-L190 N_δ and Gly-L225 N_p H-bonded to the Q_A and Q_B carbonyls, respectively, based on both estimated quadrupole coupling constant of nuclear quadrupole interaction (*nqi*) and a comparison of the experimental and calculated isotropic hyperfine (*hfi*) couplings. Continuing this work, multifrequency pulsed EPR and DFT calculations were used with the aim to determine complete *hfi* and *nqi* tensors, i.e. principal vales and principal directions in the g-tensor coordinate system, for the nitrogens indicated above. The *hfi* and *nqi* tensors for His-M219 N_δ and Ala-M260 N_p were evaluated through simultaneous simulation of the Q-band ^{15}N Davies ENDOR, X- and Q-band 2D $^{14,15}N$ ESEEM, and X-band ^{14}N three-pulse ESEEM spectra. Estimates of the tensors for His-L190 N_δ and of Gly-L225 N_p were obtained from simulations of 1D and 2D $^{14,15}N$ X-band and three-pulse ^{14}N S-band ESEEM spectra. The agreement between our ESEEM data and DFT calculations of the nitrogen *hfi* and *nqi* tensors justifies their use in theoretical investigations for an understanding of the relationship between tensor characteristics and the geometry and strength of H-bonds. By applying the Townes-Dailey model to the SQs, we find the *nqi* asymmetry parameter η to be a sensitive probe of the His N_δ–SQ hydrogen bond strength. This is supported by a strong correlation observed between η and the isotropic coupling $a(^{14}N)$ (Figure), and is consistent with our own and previous SQ-His model calculations. The empirical relationship between $a(^{14}N)$ and η will provide an important structural characterization tool in future studies of SQ-binding proteins.

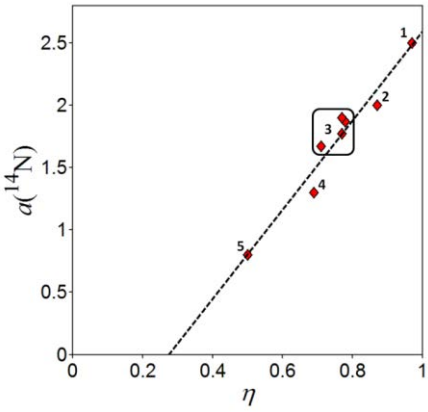

Figure. Correlation between $a(^{14}N)$ and η for His N_δ H-bonded with SQ: 1. Q_A *Rb. sphaeroides,* 2. Q_A *Rp. viridis*, 3. Q_A PSII, 4. Q_B *Rb. sphaeroides,* 5. Q_D NarGHI .

Hydrogen bond network around the Q_B site semiquinone. By utilizing a combined pulsed EPR and DFT approach, a full characterization of the hydrogen bond network around the Q_B site semiquinone (SQ_B) was determined. The development of such a technique is crucial toward an understanding of protein-bound semiquinones on the structural level, as (i) membrane protein crystallography typically results in low resolution structures, and (ii) obtaining protein crystals in the semiquinone form is rarely feasible. The SQ_B hydrogen bond network was investigated with Q-band (~34 GHz) ^1H ENDOR and X-band (~9.7 GHz) HYSCORE on fully deuterated reactions centers from *Rhodobacter sphaeroides* in an H_2O solvent.

Three protons were detected in HYSCORE and ENDOR spectra, one with an anisotropic tensor component, T = 4.6 MHz, assigned to the histidine $N_\delta H$ of His-L190, and two others with similar anisotropic constants T = 3.2 and 3.0 MHz assigned to the peptide N_pH of Gly-L225 and Ile-L224, respectively. The principal values of the ^1H hyperfine tensors and their orientations with respect to the SQ_B g-tensor reference frame are obtained by least-squares fitting of the simulations to the orientation selective Q-band ^1H ENDOR spectra. The Euler angles describing the series of rotations that bring the hyperfine tensors into the SQ_B g-tensor reference frame show the locations of the hydrogen bonded protons with respect to the semiquinone. These Euler angles are found to be in agreement with our geometry optimized DFT model of SQ_B providing the foundation for future joint pulsed EPR and DFT semiquinone structural determinations in other proteins.

Science objectives for 2015-2016:

- ^{13}C couplings provide insight into the SQ binding to the protein. We will apply a biochemical approach for selective ^{13}C labeling of ring carbons in the SQ_H. The quinones biochemically labeled in bo_3 enzyme will also be used in studies of bacterial reaction center and bc_1 complex.
- We will investigate the influence of H subunit removal on the interaction between the primary semiquinone and the LM protein environment, using a combination of site-specific isotope labelling techniques, pulsed EPR, and DFT calculations.

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Looking Under the Hood of Photosynthesis: How Does Your Photosystem II Measure Up?

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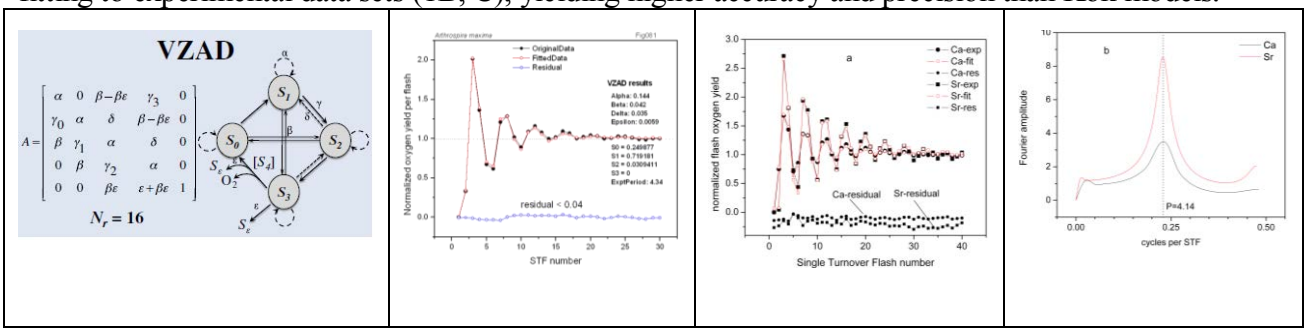
Overall research goals: Oxygenic photosynthesis is the dominate lifeform on Earth and sustains most non-photosynthetic life. It is powered by redox and protonic energy produced within the photosystem II (PSII) reaction center and its integral water oxidizing complex (WOC). PSII-WOCs from only a few model organisms have been investigated thus far, as isolated complexes, in sufficient detail to compare intrinsic performance and regulation. Our goals are to extend this knowledge more broadly to include PSII-WOCs from phylogenetically diverse aquatic phototrophs and using native (intact) cells where regulation can be investigated. This knowledge is needed for understanding the physico-chemical principles of operation of native PSII-WOCs, and for contributing to advances in artificial photosynthesis.

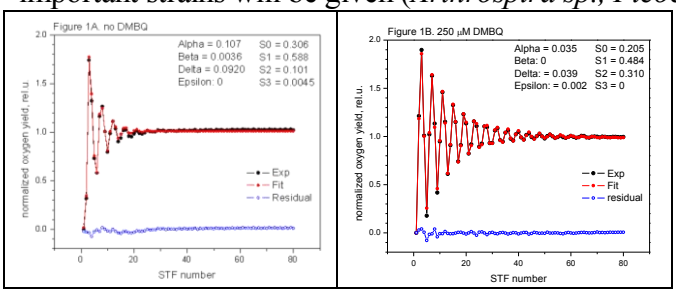
Significant achievements 2013-2015: We have made progress in understanding the kinetic and energy efficiency metrics of PSII-WOCs: 1) in native cells from six phylogenetically diverse species of aquatic phototrophs including biotechnologically important strains; 2) natural isoforms and targeted mutants of the D1 reaction center subunit of a cyanobacterial PSII; 3) a WOC inorganic mutant: strontium replacement for calcium in *T. elongates*; 4) new mathematical solutions to the water oxidation cycle (Kok-Joliot cycle); and 5) fluorescence method development for measuring turnover kinetics of intermediates (S states) in whole cells.

We have developed data acquisition and simulation tools (STEAMM) to observe and interpret the physical meaning of flash-induced oscillations of O_2 yield and Chl variable fluorescence yield (Fv/Fm) initiated by charge separation in PSII[1]. These tools allow both model-dependent (Kok & VZAD models, 1A) and model-independent (Fourier transformation, 1D) descriptions of kinetic performance and efficiency as a function of flash rate/light intensity. The new VZAD algorithm (1A) enables solutions to Markov models using fewer adjustable parameters and uses least-squares fitting to experimental data sets (1B, C), yielding higher accuracy and precision than Kok models.



These tools reveal that the PSII photochemical quantum yield (Fv/Fm) is comprised of two contributions: quenching of primary charge separation by recombination (photoprotection), and energy conversion (S state dependent water oxidation). The relative contributions vary with PSII phylogeny, the PSII-D1 isoform that is expressed, and the rates of clearing of photoproducts (PQH_2 pool and luminal protons), resulting in different efficiencies of light-to-chemical energy conversion *in vivo*[4]. Our experiments show: 1) two differentially expressed PSII-D_1 subunits are designed *either* to achieve photoprotection at high light by charge recombination at the cost of decreased efficiency of WOC operation, *or* more efficient water oxidation at low light flux by long-lived intermediates (S states) at the cost of greater photoinhibition[2]; 2) Nine mutants of these D_1 isoforms confirm a

continuous trade-off of these two properties[3]; 3) removal of the kinetic chokepoint limiting PSII turnover rate in cells -between the PQ pool and cyt-b_6f complex - by addition of membrane-permeable benzoquinone derivatives, allows assessment of the upper limit to water oxidation yield and PSII turnover rate independent of downstream regulation (see 2A,2B). This method enables quantification of the extent of regulation of water oxidation for any cell type, as well as which S states are the sources of regulation and photoinhibition. Applications to biotechnologically important strains will be given (*Arthrospira sp., Picochlorum sp.* (2A,2B)[5], *Nannochloropsis sp.*);



4) Strontium substitution for calcium in the WOC inorganic core (Mn_4CaO_5) has been widely studied previously in isolated PSII complexes. Here we show novel results demonstrating that Sr significantly *increases* the efficiency of water oxidation at low light flux in native cells (*T. elongates,* see 1C, 1D) as a result of slowing charge recombination reactions. Important insights into the chemical mechanism of water oxidation have been learned.

Science objectives for 2015-2016:

- Apply our quantitative modelling software (STEAMM) across the full solar intensity profile to deduce how the kinetic turnover efficiencies change and differ among various PSIIs.
- Identify the molecular mechanisms for the PSII inefficiency parameters.
- Physico-chemical explanation for the Sr^{2+}/Ca^{2+} replacement effect on PSII kinetic performance.
- Development of fluorescence induction "long-pulse" experiments to determine S-state lifetimes and turnover rate of PSII in native cells via direct, rapid survey across many clades.
- Investigation of the role of non-heme iron as a key regulator of electron flow through PSII.
- Characterization of *Picochlorum sp.* and other novel microalgae as potential crop species for biotechnology.
- Expressing cyanobacterial D1 isoforms in a higher plant for application to biomass production.

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Construction and Evaluation of Well-Defined Mimics of Photosynthetic Light Harvesting Systems

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Overall research goals: This program seeks to understand and model the fundamental underpinnings of natural photosynthetic systems through the construction of well-defined and tailorable experimental systems. The central approach involves the attachment of a variety of synthetic chromophores to specific sites on self-assembled protein scaffolds through conformationally constrained linkages. Using this approach, the distances and angles between the individual chromophores in the arrays can be varied systematically. A key advantage of the approach is its ability to compare isolated chromophores on protein surfaces to those that are present in extended arrays. This can allow the effects of the protein environment to be studied separately from electronic coupling behavior. New time-resolved spectroscopy techniques are being applied to study exciton transfer in these systems, as are new computational methods to model the effects of conformational fluctuations on the systems. It is anticipated that the models and approaches developed through this program will inform the design of new artificial systems, and elucidate some of the features responsible for the high quantum efficiencies exhibited in natural photosynthesis.

Significant achievements in FY 2015:

- Three self-assembling protein scaffolds have been developed for the construction of pigment arrays with different geometries and multiplicities. These are (1) a new C_2-symmetric double disk structure based on the tobacco mosaic virus coat protein, (2) a trimeric assembly with deep surface grooves based on the thermostable MTH1491 protein, and (3) an extended rod structure based on a salmonella needle protein assembly. In each case, chemistry has been developed to attach a variety of chromophores to different positions on the protein surfaces.
- Ginsberg, Francis, and Geissler showed that hydrated nanoscale protein cavities are a promising new way to mimic the tight protein pockets for chromophores in natural light harvesting systems. Time-resolved spectroscopic data showed that interior pigments experienced hindered chromophore motion to allow for more prolonged and efficient energy transfer (publication 1).
- Francis has developed new method for the purification of protein assemblies labelled with different numbers of chromophores. This new tool will be essential for the future preparation of well-defined models of light harvesting systems (publication 2).
- Francis has completed a systematic study of the ability of conformationally constrained linkers to direct the orientations and restrict the motions of protein-bound chromophores.
- The electronic structures of unique chromophore monomers and dimers have been studied using GW/BSE and TDDFT. Additionally, Neaton has completed the first study benchmarking the GW/BSE approach against higher-level quantum chemistry methods (publication 3).
- Using computer simulations, Geissler helped to rationalize the complex dynamics observed (by Ginsberg and Francis) in a protein-chromophore complex by ultrafast spectroscopy. Specifically, they found that solvent dynamics inside a protein complex vary strongly with location in ways that mirror experimental measurements.
- Using theory and simulation, Geissler has explored several fundamental issues of driven nonequilibrium dynamics in mesoscale systems, with general implications for energy transfer kinetics in protein-chromophore systems driven by irradiation (publications 5-7). This work has

shown that the thermodynamic efficiency in such systems can be a strongly fluctuating quantity with statistics that have interesting and general features. Dynamical phase transitions have also been found to occur in such systems even with very simple underlying kinetic rules.

Science objectives for FY 2015-2016:

- Work will continue to test the hypothesis that conformational constraints can lead to substantial efficiency improvements to energy transfer systems.
- Using a combination of synthetic chemistry and computational modelling, the display angles of chromophores will be tuned and optimized to achieve ideal transfer efficiencies.
- Isolated chromophores on protein surfaces will be compared to to fully-substituted chromophore rings that have the potential for electronic coupling. This is anticipated to provide a unique new opportunity to explore the effects of the protein environment on energy transfer.
- A new attachment strategy is being developed to allow the generation of chromophore arrays that are fully embedded within the protein structure. Once prepared, amino acid mutations will be used to explore the effects of the protein environment on their energy transfer properties.
- We will continue to prepare additional protein assemblies that allow chromophore attachment in different locations. This will allow the distances between the pigments to be changed systematically. In combination with our synthetic efforts for the preparation of new dye molecules, an unprecedented library of synthetic light harvesting systems will soon be available.
- Neaton will be continuing his work on understanding the excited states of chromophore monomers and dimers to explain measurements of strongly absorbing excited states on chromophores synthesized by Francis and transient absorption spectra collected by Ginsberg. He will also work with Geissler to benchmark low-level calculations of chromophore excited-state energies, which will provide critical input into reduced models of excited-state evolution in chromophore environments.
- Geissler has already made progress in developing molecular dynamics simulations that characterize energy gap fluctuations in a model protein-chromophore complex. In the next year, this study will determine whether statistics of these fluctuations are consistent with the simple quantum mechanical models often assumed for such systems.
- Geissler will explore the implications of our findings on the statistics of thermodynamic efficiency out of equilibrium, specifically what they suggest about the ability of natural systems to switch between different modes of dynamical behavior in energy harvesting.

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The Interaction of Quinones with the Heme-Copper Cytochrome bo_3 Ubiquinol Oxidase from *Escherichia coli*

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Overall research Goals: The research goals are focused on defining the mechanism of the heme-copper quinol oxidases. These enzymes are variants of the predominant family of proton-pumping respiratory oxygen reductases. Whereas most of these enzymes utilize cytochrome c as substrate, the quinol oxidases use either menaquinol or ubiquinol as substrates. These enzymes are found exclusively in microorganisms and they are members of the A-family of heme-copper oxygen reductases. As such, the quinol oxidases are related to the mitochondrial aa_3-type cytochrome c oxidase. The quinol oxidases, however, lack the binding site for cytochrome c as well as the Cu_A redox center. The best studied quinol oxidases are the cytochrome bo_3 ubiquinol oxidase from *E. coli* and the cytochrome aa_3-600 menaquinol oxidase from *Bacillus subtilis*. These two enzymes are close homologues but utilize different substrates- ubiquinol vs menaquinol, and contain different heme prothetic groups- heme B and heme O vs Heme A. Our primary tools in the past few years have been site-directed mutagenesis and pulsed EPR spectroscopy of the semiquinone species that are stabilized at the high affinity quinone binding sites of these enzymes. The EPR spectroscopy is performed as part of a collaboration with Dr. Sergei Dikanov, who is also funded by the Office of Basic Energy Sciences.

Significant Achievements 2014-2015: We have used pulsed EPR to define the hydrogen bonding to the proteins that stabilize the semiquinone species of both the ubiquinol oxidase and the menaquinol oxidase. The residues that are observed define the high affinity quinone binding site or Q_H site. The enzymes isolated in the detergent dodecyl maltoside contain one equivalent of quinone bound to this site. Because the quinone appears to be tightly associated with the protein, this quinone has been viewed as a cofactor component of these enzymes. A second binding site has been postulated to exist where the quinone can rapidly bind and dissociate during catalytic turnover. This is referred to as the low affinity quinone binding site or Q_L site.

One aspect of our studies revealed significant differences in the interactions of the quinones bound at the Q_H sites of the two enzymes. In particular, we found that R71 in the *E. coli* ubiquinol oxidase forms a strong hydrogen bond to the ubisemiquione and that mutants at this site eliminate enzyme catalytic function. In contrast, the equivalent R70 residue in the menaquinol oxidase has a weaker interaction with the menasemiquinone species and the equivalent mutations that are lethal for the ubiquinol oxidase does not substantially reduce the catalytic function of the menaquinol oxidase.
We have made substantial progress in the past year to address the question of whether the quinol oxidases have one or two quinone binding sites. It has been thought that there are two sites, one high affinity site which binds a non-exchanging quinone that functions as a cofactor, and a second low affinity site which is in rapid equilibrium with the pool of quinone species in the

membrane bilayer. Although the residues defining the Q_H site are well defined, particularly from our EPR studies with Dr. Dikanov, efforts to locate the Q_L site have not been successful. We have carried out an exhaustive search for the Q_L site of cytochrome bo_3, following claims of locating this site based on a new X-ray structure and also claims based on bioinformatics studies. Despite examining the properties of many mutants, we have failed to locate this site and have no evidence that either of the suggested locations is correct. We have been forced to consider that the low affinity Q_L site may not exist, leaving us with a one-site model. Supporting this model, we have now shown that several mutations at the Q_H site show behavior expected for perturbations at the exchangeable Q_L site. Furthermore, we have demonstrated that the quinone that is isolated bound to the Q_H site actually can exchange with exogenously added quinones. Hence, we now favor a model of a single quinone binding site for the quinol oxidases.

Science objectives for 2015-2016: Our priority next year will be to test the one-site model which we are now proposing for the quinol oxidases. Enzyme from which UQ_8 has been removed by treatment with Triton X-100 will be titrated with UQ_1 to determine if there is one or if there are two binding sites. It may be possible to do this experiment using saturation-transfer NMR. Alternatively, we can engineer the enzyme so we can place a fluorescent probe near the binding site and use fluorescence quenching to monitor binding of the quinone. In addition, we will characterize mutants in the Q_H site of the cytochrome aa_3-600 menaquinol oxidase from *B. subtilis* to determine whether these mutants also show characteristics of a perturbation to the exchangeable quinone binding site. In addition, pulse EPR experiments will characterize the semiquinone forms of both menaquinone and ubiquinone in the binding sites of the two quinol oxidases.

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Photobiological Solar Fuels Program

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Overall research goals: The overarching goal of the Photobiology project under the Photobiological and Photobiohybrid Solar Fuels NREL Program is to investigate the regulation of photosynthetic reductant partitioning between H_2 evolution, biomass and other storage molecules that accumulate under different stress conditions. The objective is to understand the interplay between stress sensing, signal transduction, and transcriptional regulation of specific genes that determine the fate of photosynthetic reductant. This, in turn, will allow one to manipulate and change the metabolic state of the cells under controlled conditions. We are particularly interested in the mechanism of the interaction between different signal/sensor, and sensor/transducer molecules, and the complex networks between different signal and the expression of specific genes at the mechanistic level. This project consists of two subtasks that address, respectively, (a) factors required for the regulation of photosynthetic reductant partitioning between CO_2 fixation and H_2 production under anaerobic conditions; and (b) factors required for regulation of reductant and carbon partitioning between biomass and carbon storage molecules under N and other stresses.

Significant achievements 2014-2015:

Subtask 1: **Regulation of photosynthetic reductant partitioning between CO_2 fixation and H_2 production under anaerobic conditions**. We identified a mutant attenuated in H_2 production as interrupted in the 3' UTR of a prolyl 4-hydroxylase (P4H) gene. Characterization of the mutant shows decreased rates of H_2 photoproduction, decreased accumulation of fermentative H_2, and decreased levels of acetate, formate, and ethanol production. Complementation with the wildtype version of the P4H gene restores H_2 and fermentative metabolites production. Characterization also shows decreased levels of accumulated starch under aerobic conditions, although not fully restored upon complementation. Due to the use of two different strains of Chlamydomonas for the initial random mutagenesis library (strain 4C-) and the performance of genetic crosses used to confirm segregation of the mutation (strain R3+), all data were repeated with each of the strains and the phenotype is being confirmed. Our results up to date suggest that the mutant phenotype may be due to decreased starch substrate available for H_2 production, which may result from the hydroxylation of one or more starch synthesis enzymes. P4Hs hydroxylate structural or transcription factor proteins under aerobic conditions, requiring O_2 as a substrate. The O_2-dependence of P4Hs may play a role in *C. reinhardtii's* ability to respond to varied O_2 levels, thus facilitate the regulation of photosynthetic reductant during transitions between aerobiosis and anaerobiosis.

Subtask 2: Regulation of carbon partitioning in Synechocystis mutants altered in carbon storage. A Synechocystis 6803 mutant that was engineered to be (1) unable to synthesize glycogen, a major carbon storage compound, and (2) able to grow on xylose, a previously unusable carbon source for this cyanobacterium was employed for the study of carbon partitioning. Under either N starvation and/or mixotrophic xylose utilization conditions, carbon flux was redirected to the synthesis and excretion of acetate, in addition to pyruvate and alpha ketoglutarate (AKG) that were previously identified. We hypothesized that acetate was

synthesized via a pathway that bypassed pyruvate dehydrogenase, and found a functional phosphoketolase pathway in this model cyanobacteria. This pathway carried one third of carbon flux in the central carbon metabolism under mixotrophic conditions with xylose, and is hypothesized to help balance carbon and energy metabolism. In addition, we found that energy charge (ATP levels) was higher in the glycogen synthesis mutant than in the WT in the light, and was lower in the dark, indicating that glycogen synthesis serves as energy buffer. Multiple changes in the light reaction and in carbon metabolism have been observed in this mutant that indicate adaptation to high energy charge.

Science objectives for 2015-2016:

- **Subtask 1**: Concluding work will finalize a manuscript in-progress, which will be submitted for publidation. Additional work will use pull-down protein assays to identify proteins hydroxylated by this prolyl 4-hydroxylase, and confirmation of interactions using biochemical assays, which will result in a second publication. The overall project will be transitioned to a different and more integrated approach with the Photobiohybrid Component of the Program, and the prolyl 4-hydroxylase work will not be included in it.
- **Subtask 2**: The hypothesis that energy balance (both ATP and NADPH charges) is maintained by multiple mechanisms in the light reaction and carbon metabolism will be further tested by measurement of these metabolites and photosynthetic parameters in mutants affecting various carbon storage pathways as well as alternative electron flow pathways such as Flv1/3.

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- *Review*: Maria L. Ghirardi, Paul W. King, david W. Mulder, Carrie Eckert, Alexandra Dubini, Pin-Ching Maness and Jianping Yu. Hydrogen production by water biophotolysis. In: Microbial bioenergy: Hydrogen Production (eds. D. Zannoni and R. de Philippis), Advances in Photosynthesis and Respiration 38, 101-135.
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Chlamydomonas Metabolism in the Light and Dark: The Role of FDX5

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Overall research goals: We have little information that provides insights into the functions and regulatory features that uniquely govern metabolism in photosynthetic organisms in the light relative to the dark. Filling in this landscape is critical if we are to understand the energetics of day/night metabolism, and then ultimately use photosynthetic organisms (particularly algae) for the efficient generation of bio-products. The focus of our work is to explore the energetics of photosynthetic organisms in the light and dark, and elucidate the different electron carriers, supramolecular complexes and alternative electron transport pathways required for respiratory and fermentative dark metabolism, and how these pathways interface with photosynthetic processes, which dominate during the day.

Significant achievements 2014-2015: Photosynthetic microorganisms typically have multiple isoforms of the electron transfer protein ferredoxin, although we know little about their exact functions. We discovered that a *Chlamydomonas reinhardtii* mutant null for the ferredoxin-5 gene (*FDX5*) completely ceased growth in the dark, with both photosynthetic and respiratory functions severely compromised. Growth in the light was essentially unaffected. Furthermore, analyses of this mutant demonstrated an increased accumulation of triacylglycerol in the dark, with thylakoid membranes becoming severely disorganized concomitant with a marked decrease in the ratio of monogalactosyldiacylglycerol (MGDG) to digalactosyldiacylglycerol (DGDG), major lipids in photosynthetic membranes. An image of the membranes from the dark grown wild-type cells and the *fdx5* mutant are show in **Figure 1**. FDX5 was shown to physically interact with the fatty acid desaturases CrΔ4FAD and CrFAD6, likely donating electrons for the formation of the desaturated fatty acids that stabilize MGDG. **Our results clearly establish that specific electron mediators sustain dark metabolism, with little impact on daytime growth, likely reflecting the tailoring of electron carriers to unique intracellular metabolic circuits under these two very distinct redox conditions.**

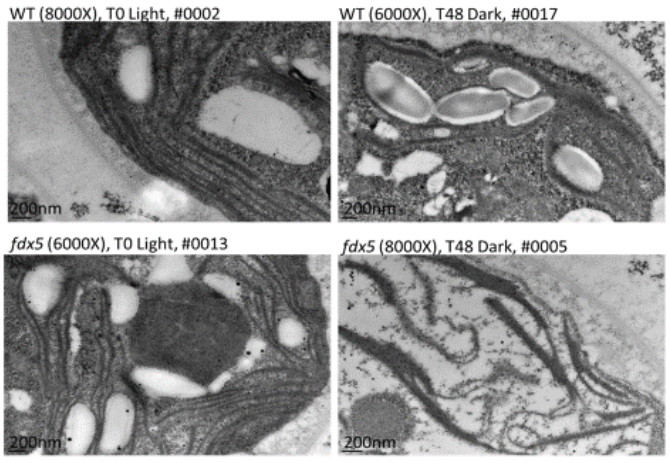

Figure 1. Comparison of thylakoid membranes in wild-type *C. reinhardtii* cells (upper right and left) and the *fdx5* mutant (lower left and right), both in the light (upper and lower left) and dark (upper and lower right). Note the membrane aberrations in the *fdx5* mutant after 48 h in the dark (lower right).

The concept that FDX5 is an electron carrier required for dark metabolism and growth was tested in a number of ways. FDX1 was proposed to assume at least some of the roles of FDX5 in the light as it

associates with at least one of the FDX5-interacting desaturases. Therefore in an attempt to rescue the *fdx5* mutant in the dark, we performed random mutagenesis of FDX1, introduced it into the *fdx5* strain, and screened for transformants able to restore the dark growth phenotype. The premise was that the mutated FDX1 would only be able to rescue the phenotype if its midpoint potential was made positive enough to be able to accept electrons from NADH/NAD(P)H (and therefore be able to donate electrons to the key FDX5-driven dark reactions). We now know that wild-type FDX1 has a midpoint potential of about -400 mV (it will not readily accept electrons from NADH/NAD(P)H) (Terauchi et al., 2009), while FDX5 has a midpoint potential of about -215 mV (readily accepts electrons from NADH/NAD(P)H) (D'Adamo/Posewitz et al., unpublished data). Specific transformants of *fdx5* harboring the randomly mutated *fdx1* were able to grow in the dark (but generally the growth is slower than that of wild-type cells and the rescued strains are bleached to some extent); the lesions and midpoint potentials of the mutant *fdx1* proteins in these transformants are presently being analyzed.

Additionally, we hypothesized that we would also be able to rescue the *fdx5* mutant for dark growth if we were able to transfer electrons to FDX1 in the dark. This was attempted by bubbling *fdx5* cells in the dark (in a medium containing acetate) with a gas mixture containing low O_2 (0.5%) plus H_2 (if the hydrogenase is working under these conditions the H_2 would donate electrons to FDX1). Indeed, the *fdx5* mutant was able to grow in the dark under these conditions, although again the growth was slower than that of wild-type cells and it also exhibited reduced chlorophyll levels.

Finally, we have isolated additional mutants that do not grow in the dark. One mutant is in the *ACK1* (acetate kinase) gene. This finding suggests that under some dark conditions the cells need ACK1 (but not ACK2) to utilize acetate.

Science objectives for 2015-2016

- Examine interactions of FDX5 and FDX1 with other electron acceptors and begin to probe the functions of the various other FDXs in dark and light metabolism (we already have some additional mutants in the other ferredoxin isozymes).
- Analyze other key hubs of metabolism and determine if enzymes central to these hubs interact with other proteins forming macromolecular complexes (which might streamline metabolic pathways and their regulation); at this point we are beginning to explore protein-protein interactions of alcohol dehydrogenase (ADH1), pyruvate formate lyase (PFL1) and pyruvate ferredoxin oxido-reductase (PFR1).
- Optimize growth of the *fdx5* mutant in the dark in the presence of H_2 and explore the oxyhydrogen reaction; attempt to elucidate its mechanism and determine whether it causes a 'tailoring' of the thylakoid electron transport system, especially photosystem I.

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Studies of Photosynthetic Reaction Centers and Biomimetic Systems

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Overall research goals: The goal is to establish a research program for fundamental studies of natural photosynthetic proteins and biomemetic systems, incorporating Classical Electrostatics with Monte Carlo (MCCE) simulations for calculations of pK_as and electrochemical midpoints (Gunner, CCNY); quantum mechanics and quantum mechanics/molecular mechanics (QM/MM) analysis (Batista, Yale Univ, USA); and large-scale molecular dynamics (MD) of photosynthetic reaction centers (Bruce, Brock Univ, Canada). Our efforts have been focused on the analysis of the structure and reactions of the Oxygen Evolving Complex (OEC) of Photosystem II (PSII) and the proton transport pathways from the OEC and to the quinones.

Significant achievements 2013-2015

- Recently, femtosecond X-ray diffraction provided the first high-resolution structural model of the OEC that is free of radiation-induced manganese reduction (Protein Data Bank entries 4UB6 and 4UB8). We assessed the accuracy of this structure using combined QM/MM, extended X-ray absorption fine structure (EXAFS) and electron density maps analysis. This structure does not match the EXAFS and QM/MM data for the S_1 state. We attributed this to uncertainty about the positions of oxygen atoms next to the heavy manganese centers that remain partially unresolved, even at 1.95 Å resolution. In addition, the photosystem II crystals may contain significant amounts of the S_0 state, because of extensive dark adaptation prior to data collection. *(ref. 8)*

- Having reliable structures of the OEC in S_1 and S_2 states allowed us to explore binding of ammonia to the OEC. Ammonia binds directly to the OEC upon formation of the S_2 intermediate, as evidenced by EPR spectroscopy but does not inhibit oxygen evolution, showing it does not compete with substrate water binding. We explored the binding mode of ammonia with QM/MM methods and simulations of EXAFS spectra. We find that NH_3 binds as an additional terminal ligand to the dangling Mn4, instead of exchanging with a terminal water. Since water and ammonia are electronic and structural analogues, these findings suggest that water binds analogously during the $S_2 \rightarrow S_3$ transition, leading to rearrangement of ligands in a carrousel around Mn4. *(ref. 9)*

- We have used a new method for tracking the changes in the OEC structure through QM/MM simulations combined with the experimental electron density maps analysis for the S_1 to S_2 transition. This transition does not involve the transfer of a proton to the lumen and occurs at cryogenic temperatures. Therefore, it is thought to involve only Mn oxidation without significant structural change. We analysed structural changes as revealed by QM/MM methods and the isomorphous difference Fourier method applied to low-resolution serial femtosecond X-ray diffraction data to show the main structural change in the OEC is in the position of the dangling Mn and its coordination environment. This procedure shows how valuable structural information can be extracted from low-resolution electron density maps. *(ref. 4)*

- The protonation changes through the S state cycle were investigated using structures of the DFT optimized OEC docked into the whole PSII using the MCCE classical electrostatic – Monte Carlo method. Two possible S_2 ($Mn(III)_1Mn(IV)_3$) states are found depending on the OEC geometry. If O5 is closer to Mn4, S_2 has Mn4(IV) with a proton lost from a terminal water (W1) trapped by the nearby D1-D61. If O5 is closer to Mn1 S_2 has Mn1(IV), with partial deprotonation of D1-H337 and D1-E329. In S_3, the OEC is $Mn(IV)_4$ with W2 deprotonated. The S_1/S_2 and S_2/S_3 E_ms range from +0.7 to +1.3 V, enabling oxidation by P_{680}^+, the primary electron donor in PSII. *(ref. 7)*

- Prior to each new S state Y_Z is oxidized by P_{680}^+, leaving a positive $H190^+Y_Z^\bullet$ complex as the Tyr proton is transferred to the His. The proton release on formation of $H190^+$ in S_0 to S_3 states was calculated with MCCE. In each S state the proton donor is the group that is poised to become deprotonated when the OEC is oxidized, which is likely to be H337, D61 and E329. Thus, the proton loss from the protein prepares the system for the transition to the new S state. *(in preparation).*

Science objectives for 2015-2017:

- The affects of ammonia and Cl^- on the protonation states of the OEC and surrounding residues, the S state transitions and the proton transfer pathways will be studied by combined QM/MM and MCCE calculations. The binding of ammonia directly to the OEC and to secondary sites near the OEC will be explored.
- The pathways and barriers for proton release for various S states will be investigated using community network analysis and MCCE pathway and energetic analysis based on the MD trajectories.
- Structure of the S_3 state will be investigated using QM/MM combined with EXAFS analysis as well as the analysis of experimental electron density maps
- Possible mechanisms of O-O bond formation in the S_4 state will be investigated by locating intermediates and transition states using the QM/MM approach.
- The effects of oxidation of Y_Z and proton trapping on H190 on the protonation states of the OEC and surrounding residues will be explored to determine the source of the lag phase for electron transfer from the OEC to Y_Z.

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Thiol-based pathways in the thylakoid lumen and their role in photoprotection

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Overall research goals: Thiol oxidation into disulfides and the reverse reaction, *i.e.* disulfide reduction to free thiols are under the control of dedicated catalysts *in vivo*, which are required for the biogenesis of all energy-transducing membrane systems. This research aims to elaborate the catalysis of thiol-disulfide chemistry in the thylakoid lumen of the chloroplast. Our long-term objectives are to discover the molecular identity of the enzymes defining the thiol-metabolizing pathways in the lumen, their relevant targets of action and to understand how they control or regulate photosynthesis.

Preliminary data: In a former NSF-funded project, we discovered that both disulfide bond formation and disulfide bond reduction in the thylakoid lumen are catalyzed reactions under the control of dedicated components, whose activities are essential for the biogenesis of the photosynthetic complexes (Fig. 1). Two disulfide-reducing pathways control plastid cytochrome *c* assembly through the reduction of a disulfide at the C*XX*CH motif on apocytochrome *f*, a biochemical step necessary for heme attachment onto the apoprotein. One pathway is defined by the CCDA thiol-disulfide transporter and the CCS5/HCF164 thioredoxin-like protein (1) while the other pathway is dependent on CCS4/HCF153, a thylakoid membrane protein whose activity remains to be deciphered (2). Operation of theses pathways is required for the assembly of cytochrome b_6f complex. The disulfide bond forming pathway is defined by LTO1, a membrane protein functionally equivalent to the bacterial DsbAB catalysts and necessary for PSII assembly through sulfhydryl oxidation of the PsbO subunit in the oxygen evolving complex (3)

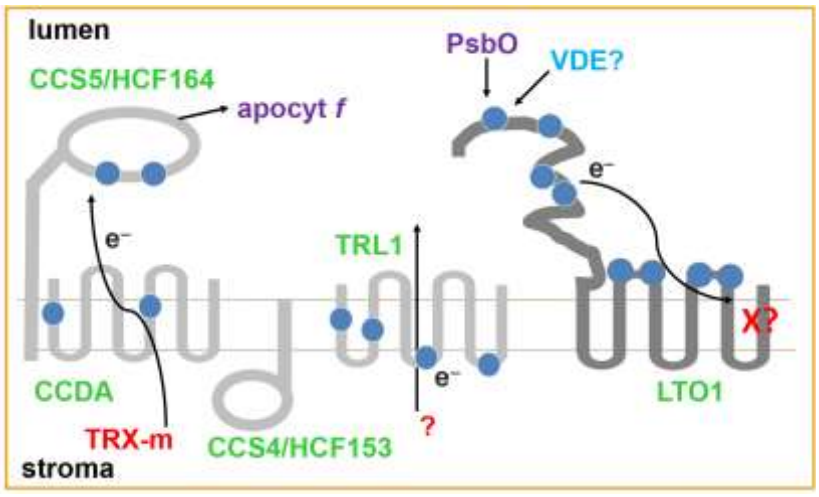

Fig.1*. *Trans-thylakoid thiol-metabolizing pathways. *Disulfiide-reducing (CCDA, CCS5/HCF164, CCS4/HCF15 and TRL1, in light gray) and disulfide-forming pathways (LTO1, in dark gray) and some of their known (purple) or suspected targets (light blue) are represented. Cysteines are in blue. Electrons (e^-) routes are indicated by arrows. CCS5/HCF164 is a membrane anchored lumen-facing thioredoxin-like protein. LTO1 contains a VKOR-like membrane domain and a lumen-facing thioredoxin-like domain. The final electron acceptor of the LTO1-dependent pathway is not known (X?). The reductant transported by TRL1 is not known but GSH is a candidate substrate.*

Science objectives for 2015-2018:

We will continue to explore catalyzed thiol-disulfide chemistry in the lumen compartment with a particular focus on photoprotective responses mediated by VDE and TRL1.

Violaxanthin de-epoxidase (VDE), a key enzyme required to dissipate excess light energy in the form of heat contains six disulfide bonds. Preliminary results indicate the disulfide bond forming cysteines are critical for VDE enzymatic activity and under the control of the disulfide bond forming catalyst LTO1. We will assess the importance of disulfide bonds for VDE activity *in vivo* using an *Arabidopsis* VDE-deficient mutant expressing disulfide bond less VDE variants *Arabidopsis* and *in vitro* by testing if LTO1 can catalysed the oxidative folding of VDE.

TRL1 (**T**ransporter of **R**eductant in the **L**umen) is a thylakoid membrane protein defining a novel thiol-based reducing pathway required for protection against light-generated reactive oxygen species. Preliminary findings support the involvement of TRL1 in the transport of a reductant from stroma to lumen. This will be experimentally tested *in vitro* using everted vesicles from a bacterial strain expressing *Arabidopsis* TRL1 and assessing the importance of key residues for the transport of reductant *in vitro*.

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Chloroplast Dynamics and Photosynthetic Efficiency

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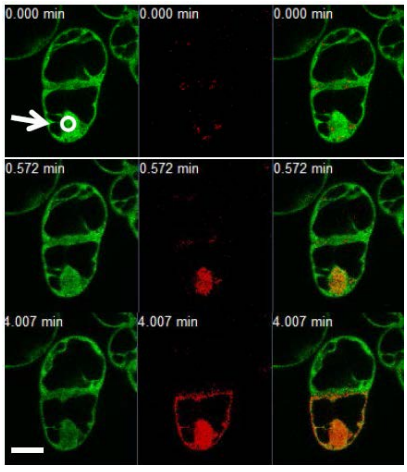
Overall research goals: Chloroplasts move within leaf cells to optimize light levels, maximizing their interception of light under limiting light conditions (accumulation) and minimizing interception under excess light conditions (avoidance). Our project explored the roles of the actin cytoskeleton and two protein families in positioning of chloroplasts. We investigated the hypothesis that chloroplast move to maximize solar energy utilization and to promote acquisition of CO_2 and efficient exchange of substrates with other compartments within the plant cell. We examined the role of the plant myosin XI and 14-3-3 protein families in chloroplast movement. Plants were studied in which one or more of the genes were silenced or mutated, localization and interactions of proteins were probes by fluorescent protein technology.

Significant achievements 2013-2015:

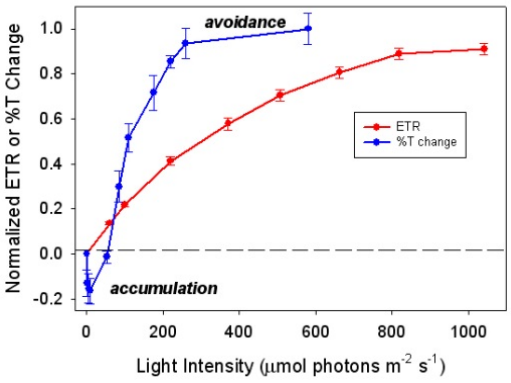
Myosin XI Family: A 42 amino-acid region of Arabidopsis myosin XI, the "PAL" domain, is homologous to the yeast myo2p tail region known to be essential for vacuole and mitochondrial inheritance. We found that 7 YFP::PAL sub-domain fusions decorate Golgi and 6 localize to mitochondria; only XI-F PAL labels chloroplast envelopes. Simultaneous transient expression of the PAL sub-domains of myosin XI-H, XI-I, and XI-K resulted in inhibition of movement of mitochondria and Golgi, likely due to a dominant negative effect (1).

Stromule Protein Flow: A paper appeared from a Canadian group (Schattat et al. 2012, *Plant Cell* 24:1465-77) that claimed our prior DOE-funded work demonstrating flow of proteins from one plastid to another was incorrect. We obtained the Arabidopsis line containing the photoconvertible GFP from the Canadian group to determine why they failed to observe transfer of protein from one plastid to another. We could demonstrate movement of both the photoconvertible GFP and standard GFP between plastids, provided that low laser power was used to perform the photoconversion (2). We believe that the use of high levels of irradiation for photoconversion by the Canadian group resulted in photodamage that prevented them from observing movement of fluorescent protein

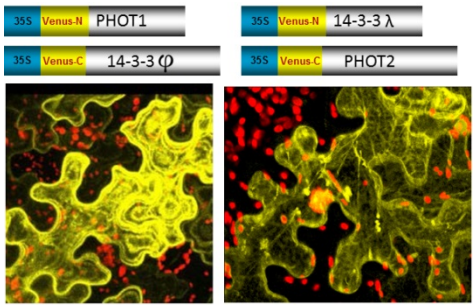
Method Development: First, we provided a methods paper that describes how to label plastids with fluorescent proteins to study dynamic morphology and movement (3). Second, while training a visiting student in GFP photobleaching, Dr. Sattarzadeh accidentally discovered that GFP itself could be photoconverted from green to red (**Fig. 1**). This observation was further investigated by further analysis of multiple cell types as well as *in vitro*. This finding will allow the use of existing transgenic lines expressing standard forms of GFP (plant or animal) to be used in photoconversion experiments to follow protein and organelle dynamics (4).



Chloroplast movement. We have obtained data indicating that the high fluence avoidance response should not be attributed entirely to photoprotective processes. First, we have observed that the transition from accumulation to avoidance occurs at light intensities far below those that saturate photosynthesis, typically closer to the light compensation intensity than to I_k, the intensity at which saturation of photosynthesis occurs (**Fig 2**). Second, the published light intensities required to induce significant photodamage in plants with impaired chloroplast movement are always 1500 umol photons m^{-2} s^{-1} or more. Further experiments allowed us to support the hypothesis that the function of chloroplast movement to the anticlinal walls during avoidance is to decrease the mesophyll resistance to CO_2 diffusion.



Protein Interactions. Bimolecular Fluorescence Complementation (BiFC) assays were implemented to observe interactions between 14-3-3 proteins and other proteins known to be involved in chloroplast movement. 16 different combinations were tested, and five 14-3-3 proteins are positive in BiFC tests for interactions with proteins that are part of the signaling pathway for induction of responses of chloroplast to high or low light. For example, the YFP signals (**Fig. 3**) indicate interactions between 14-3-3 lambda and PHOT2 and 14-3-3 phi and PHOT1.



Science objectives 2015 Completion of publications concerning chloroplast movement and 14-3-3 proteins. (Project on no-cost extension).

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(1) Sattarzadeh A, Schmelzer E, Hanson MR. 2013. Arabidopsis myosin XI sub-domains homologous to the yeast myo2p organelle inheritance sub-domain target subcellular structures in plant cells. Front Plant Sci. 4:407. doi: 10.3389/fpls.2013.00407.

(2) Hanson MR, Sattarzadeh A. 2013. Trafficking of proteins through plastid stromules. Plant Cell. 25:2774-82. doi: 10.1105/tpc.113.112870.

(3) Hanson MR, Sattarzadeh A. 2014. Fluorescent labeling and confocal microscopic imaging of chloroplasts and non-green plastids. Methods Mol Biol. 1132:125-43. doi: 10.1007/978-1-62703-995-6_7.

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(5) Ort D, Merchant SS, and 23 additional authors including Hanson MR. 2015. Redesigning photosynthesis to sustainably meet global food and bioenergy demand. Proc Natl Acad Sci U S A. 2015 Jul 14;112(28):8529-36. doi: 10.1073/pnas.1424031112. A Banbury Conference on Photosynthesis report organized by the first two authors.

(6) Hanson MR. 2015. Reactive oxygen species signal chloroplasts to extend themselves. Proc Natl Acad Sci U S A. 2015 112:9799-800. doi: 10.1073/pnas.1512645112. A commentary on stromule functions.

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

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Overall research goals: The research objectives are to understand protein interactions and structural changes in LHCP targeting/insertion components that provide the necessary order and timing of events in the chloroplast for efficient thylakoid accumulation of light harvesting complexes: In this context, our original aims were to (1) Identify Alb3 insertase-induced structural changes in the chloroplast signal recognition particle (cpSRP) that promotes transfer of LHC from cpSRP to the insertase; (2) Use a non-hydrolysable GTP analogue (GMP-PNP) to examine association of radiolabeled LHCP with a thylakoid-bound targeting complex comtaining the insertase, cpSRP and its receptor; (3) Determine whether LHC binding to cpSRP prevents GTP hydrolysis by cpSRP and its receptor in the absence of the insertase.

Significant achievements 2013-2015: Recent studies utilizing a combination of structural modeling, small-angle X-ray scattering (SAXS), isothermal calorimetry (ITC), and single molecule FRET (smFRET) along with assays that reconstitute LHCP targeting events have demonstrated that cpSRP43 binding to cpSRP54 raises the affinity of cpSRP43 for LHCP to promote formation of a cpSRP-LHCP targeting complex. Moreover, while cpSRP54 alone shows no ability to interact with LHCP targeting substrates, amino acids in cpSRP54 predicted from structural modeling to interact with hydrophobic signal sequences were found critical to form cpSRP-LHCP targeting complex. The loss of LHCP targeting complex formation using cpSRP54 M-domain point mutants was partially reversed using LHCPΔTM3, which lacks the third transmembrane domain. These data suggest that interaction between the cpSRP54 M-domain and TM3 of LHCP is required for cpSRP-LHCP targeting complex formation and that this interaction is modulated by cpSRP43 binding to cpSRP54. Additionally, studies to understand the role of GTP hydrolysis by cpSRP/cpFtsY at the membrane demonstrate that GTP hydrolysis takes place after LHCP release from cpSRP to Alb3 where GTP hydrolysis serves to maintain availability of the Alb3 insertase through recycling of soluble cpSRP targeting components.

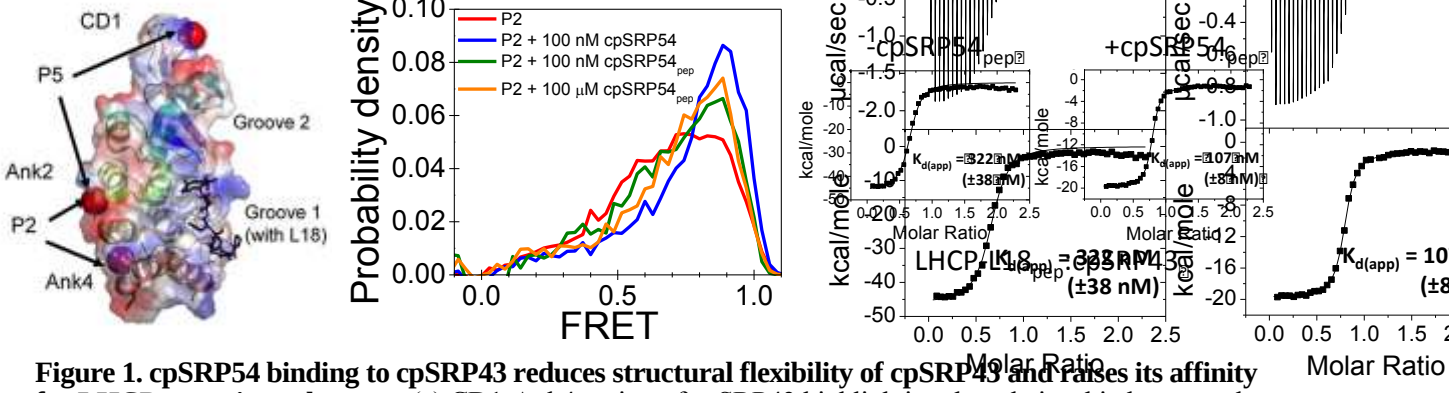

Figure 1. cpSRP54 binding to cpSRP43 reduces structural flexibility of cpSRP43 and raises its affinity for LHCP targeting substrate. (a) CD1-Ank4 region of cpSRP43 highlighting the relationship between the FRET labels in P2 and P5 with groove 1 to which the LHCP-L18 motif binds. (b) smFRET histograms for the FRET pair P2 in the absence (red) and presence of different concentrations of cpSRP54pep (green, 100 nM; orange 100 □M) and 100 nM cpSRP54 (blue). (c) ITC data showing the binding of cpSRP43 to the LHCP-derived L18 peptide in the absence (left) and presence (right) of cpSRP54pep.

Science objectives for 2015/2016:

- An original goal in our structural studies was to identify high-resolution structural changes in cpSRP43 that result at the membrane upon interaction with the Alb3 C-terminus. We now know that a likely

change in cpSRP43 upon Alb3 interaction will be re-structuring of groove 1 in cpSRP43 (see Fig 1) to enable release of LHCP from cpSRP43. We have recently overcome a considerable hurdle in our NMR-based structural studies to examine cpSRP43-Alb3 C-term structure, which is the production of both proteins in a form that is stable for days at room temperature over the time required for NMR data collection. We will continue to move forward on this front, which will reveal changes to the LHCP binding site (groove 1) and also reveal amino acids in both proteins that form the binding interface.

- Our current data (and previous publications from DOE support) indicate that protein interactions along the LHCP targeting pathway are used to regulate the affinity of cpSRP43 for the Alb3 insertase. For example, cpSRP43 alone binds to thylakoid Alb3 with nM affinity, but neither cpSRP heterodimer nor the LHCP-cpSRP targeting complex bind efficiently to Alb3. In this context, it is anticipated that additional cpSRP43 structural changes take place when cpSRP-LHCP targeting complex binds the cpSRP receptor (cpFtsY) at the membrane, an interaction that can be stabilized in the presence of non-hydrolysable GTP. In this context, we will work to develop liposomes that enable formation of a stabilized cpSRP-cpFtsY complex in order to then examine cpSRP43 structural changes at the membrane with smFRET constructs of cpSRP43 developed with our current funding. These studies are expected to reveal gross structural changes in cpSRP43 at the membrane that are required for high-affinity binding to Alb3.

References to work supported by this project during 2013-2015:

1. Gao F, AD Kight, R Henderson, S Jayanthi, P Patel, M Murchison, P Sharma, RL Goforth, TKS Kumar, RL Henry, and CD Heyes (2015) Regulation of Structural Dynamics within a Signal Recognition Particle Promotes Binding of Protein Targeting Substrates. *J Biol Chem* 290(25):15462-74. PMID: 25918165.
2. Henderson R, F Gao, S Jayanthi, AD Kight, RL Goforth, CD Heyes, RL Henry, and Thallapuranam Krishnaswamy Suresh Kumar (2015) Intrinsically Disordered Segments Drive Domain Reorganization in the 54kDa Subunit of the Chloroplast Signal Recognition Particle. (Submitted).

Targeting, Maturation and Quality Control of Photosynthetic Membrane Proteins

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Overall research goals: The research objectives are to advance our knowledge about delveopment, assembly and maintenance of the photosynthetic membrane in higher plant chloroplasts, by: (1) defining the mechanisms of protein targeting within the chloroplast, (2) elucidating the significance of oxidative protein folding in the thylakoid lumen, and (3) obtaining mechanistic insights into assembly and quality control of photosynthetic machinery. These goals have been developed based on our findings about plastidic type I signal peptidase 1 (Plsp1) in the model plant Arabidopsis, supported by DOE since 2008. Our results have revealed that Plsp1 is located in both the envelope and thylakoid membranes and its disribution appears to follow the development of thylakoids from the envelope, and that Plsp1 is required for proper assembly of photosynthetic machinery. The outcomes of the research will uncover the link between assembly and functionality of photosynthetic machinery. The long-term goals are to enhance fundamental understanding of the dynamics of natural photosynthetic systems, and to help develop technology that enables efficient capture and utilization of solar energy.

Significant achievements 2013-2015: Theare are two key achievements. The first is about *in vitro* characterization of the signal peptidase activity of Plsp1. We have demonstrated that recombinant Plsp1 catalyzes hydrolysis of a peptide bond between the C terminus of a signal peptide and the N terminus of a mature domain within precursors of various thylakoid proteins as well as the envelope protein Toc75. These data together with the results of previous genetic studies establish that Plsp1 is the main thylakoidal processing peptidase. Quite interestingly, Plsp1 homologs from angiosperms, but not from others, carry two Cys residues (Cys166 and Cys286) away from the catalytic site. Our results showed that they are redox active and disulfide-formation between them is needed for the peptidase activity *in vitro* (Fig. 1). This makes Plsp1 as a new member of redox-dependent enzymes in the thylakoid lumen.

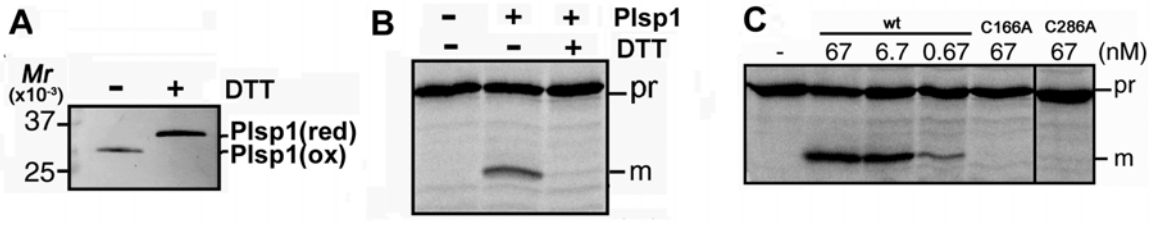

Figure 1. **A**. His-tagged Plsp1 produced by *E. coli* was treated without (-) or with (+) 10 mM DTT, separated by non-reducing SDS-PAGE, and analyzed by immunoblotting with the anti-His tag antibody. The oxidized form (ox) migrates faster than the reduced form (red) due to its compact folding. **B**. The recombinant proteins prepared in panel A were incubated with a radiolabeled Plsp1 substrate (PsbQ precursor, pr), and the reaction products were separated by SDS-PAGE and visualized by autoradiography. Oxidized Plsp1 converted the substrate to mature PsbQ (m) efficiently, while reduced Plsp1 showed much less processing activity. **C**. Recombinant Plsp1 wihout (wt) or with Ala substitution for conserved Cys166 (C166A) or Cys286 (C286) at the concentration indicated at right was incubated with the radiolabeled PsbQ precursor and the reaction products were analyzed as in panel B. The substitution of either Cys residue within Plsp1 decreases the processing activity by >99%.

The second achievement is about the mechanism of Plsp1 targeting. Results of two *in vitro* assays, one using intact chloroplasts and another using chloroplast membranes, have shown that i) Plsp1 can insert into the membrane in an incorrect orentation; ii) this mis-sorting is prevented by the stromal chaperone Cpn60 and the cpSec1 machine (Fig. 2). This finding adds to our understanding of the importance of stromal components in photosynthetic membrane assembly.

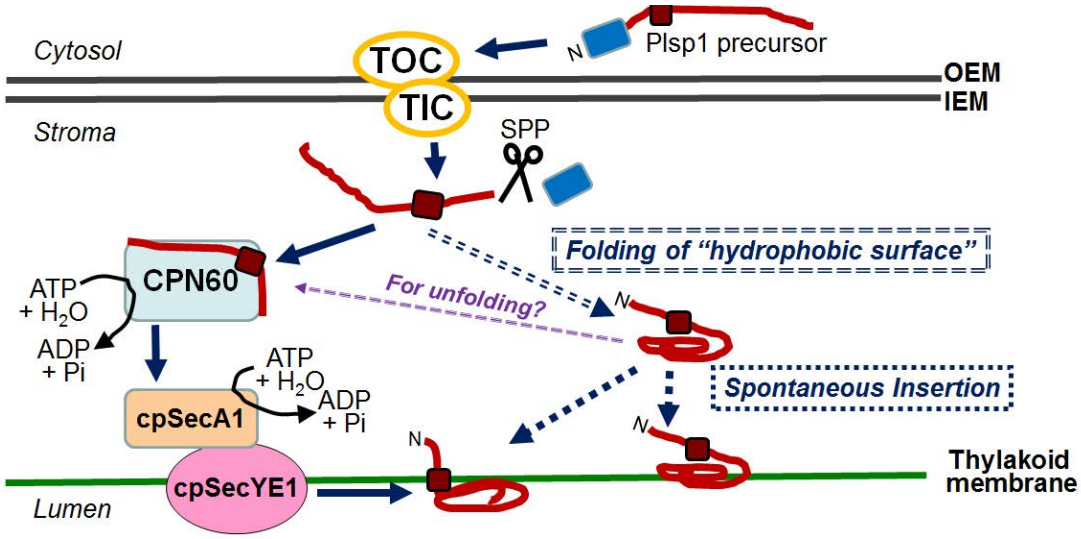

Figure 2. Plsp1 is synthesized in the cytosolic ribosome with an N-terminal extension (blue box). This extension acts as an import signal which directs the protein to the general import via TOC/TIC at the outer and inner envelope membranes (OEM and IEM), respectively. Mature Plsp1 carries a signle transmembrane domain (brown box). Its correct topology is N-stroma and C-lumen. The C-terminal portion contains the catalytic site and is predicted to form a β-sheet which presents a hydrophobic surface that associates with the membrane from the *trans*-side. Our transport data suggest that (i) the β-sheet can fold spontaneously and insert into the membrane from the *cis*-side (dotted arrows at right, including "spontaneous insertion"), and (ii) this mis-targeting is prevented by association with CPN60 in the stroma (solid arrows at left), keeping Plsp1 in an unfolded state and ensuring its proper transport by the cpSec1 machinery comprising the cpSecA1 ATPase motor and the cpSecYE1 channel.

Science objectives for 2015-2016:

- To identify amino acid residues or domains within Plsp1 and/or molecular components required for targeting of Plsp1 to each of the two membranes using *in vitro* assays.
- To define molecular bases for interaction of Plsp1 and Cpn60.
- To test if and how oxidative folding plays a role in the Plsp1's function *in vivo* by using the genetic complementation assay.
- To characterize the 400-kD complex that incorporates non-mature PsbO. PsbO is a 33-kD subunit of OEC and is a Plsp1 substrate. When the thylakoid-transfer signal could not be removed by mutation around the processing site, the non-mature PsbO protein was found in the 400-kD complex both *in vitro* and *in vivo*. Interestingly, DTT-treatment dissociates non-mature PsbO from the complex, suggesting that its assembly depends on either inter- or intra-molecular disulfide formation. We will test its biological significance by identifying its components.

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2. T. Midorikawa, J.K. Endow, J. Dufour, J. Zhu, and K. Inoue, "Plastidic Type I Signal Peptidase 1 is a Redox-Dependent Thylakoidal Processing Peptidase." The Plant Journal **80**: 592-603 (2014).
3. J.Y. Gou, K. Li, K. Wu, X. Wang, H. Lin, D. Cantu, C. Uauy, A. Dobon-Alonso, T. Midorikawa, K. Inoue K, J. Sánchez, D. Fu, A. Blechl, E. Wallington, T. Fahima, M. Meeta, I. Epstein, and J. Dubcovsky, "Wheat Stripe Rust Resistance Protein WKS1 Reduces the Ability of the Thylakoid-Associated Ascorbate Peroxidase to Detoxify Reactive Oxygen Species." The Plant Cell **27**: 1755-1770 (2015).
4. J.K. Endow, R. Singhal, D.E. Fernandez, and K. Inoue, "Chaperone-Assisted Post-Translational Transport of Plastidic Type I Signal Peptidase 1.", under revision (2015).
5. L. Klasek and K. Inoue, "Dual Protein Localization to the Envelope and Thylakoid Membranes within the Chloroplast.", under revision (2015).

High-Resolution Study of Photochemical and Nonphotochemical Processes in Biological Proteins Assembled with Photosynthetic Pigments

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Overall research goals:

Fundamental research in photosynthesis where high-resolution spectroscopic techniques, as well as excitonic calculations and modelling studies, are used to provide more insight into the relationship between excitonic structure, function, and dynamics. The following photosynthetic complexes (PCs) and issues are of interest to this project: ***i)*** B800-B850 LH2 antenna from *Alc. vinosum;* in this case the key goal is to provide more insight into an unusual splitting of the B800 band and hole-burning mechanisms (i.e., a possible proton transfer); ***ii)*** bacterial reaction centers (bRCs); here we focus on the upper exciton level P_{Y+} of the BChl *a* dimer via modelling of the linear absorption and frequency dependent (resonant) transient $P^{+}Q_{A}^{-}$ hole-burned (HB) spectra; ***iii)*** chlorosome/baseplate complexes to shed more light on the excitonic structure and excitation energy transfer (EET) dynamics of baseplate chromophores; ***iv)*** water soluble chlorophyll proteins (WSCP) to explain the nature of photochemical HB focusing on possible electron exchanges between Chl *a* cofactors and protein; and ***v)*** FMO complexes; the key objective is to provide the Hamiltonians that can simultaneously describes all linear and nonlinear optical spectra. To further advance the description of high-resolution spectroscopies, we continue developing improved algorithms to describe simultaneously various types of optical spectra at different temperatures and develop improved models to describe resonant HB/FLN spectra (and information they provide) in excitonically coupled systems. Finally, we aim to show that HB/FLN spectra can be extracted from the low-temperature two-dimensional electronic spectroscopy (2DES) frequency maps.

Significant achievements 2014-2015:

- Low-T techniques such as HB and FLN are commonly used to extract the spectral density for a given electronic transition from experimental data. We showed that the lineshape function formula reported in.[J. M. Hayes et al., J. Phys. Chem. 98, 7337 (1994)], in the mean-phonon approximation and frequently applied to analyzing HB data, contained inconsistencies in notation, which led to incorrect expressions for moderate and strong electron-phonon (el-ph) coupling strengths. A corrected lineshape function $L(\omega)$ was given in [2]. Although the corrected lineshape function could be used in modeling studies of optical spectra, we suggested that it is better to calculate the lineshape function numerically, without introducing the mean-phonon approximation. New theoretical fits of the P870 and P960 absorption bands and frequency-dependent resonant HB spectra of *Rb. sphaeroides* and *Rps. viridis* RCs were provided as examples to demonstrate the importance of correct lineshape expressions. The new fits lead to different el-ph coupling strengths and frequency of the special pair marker mode, ω_{sp}, for *Rb. sphaeroides*, which are currently being used in calculations of various low-T optical spectra obtained for bRCs and their mutants (manuscript in preparation).

- Two-dimensional electronic spectroscopy (2DES) at cryogenic- and room-T reveals excitation energy relaxation and transport, as well as vibrational dynamics, in molecular systems. These phenomena are related to the spectral densities of nuclear degrees of freedom, which are directly accessible by means of HB and FLN approaches at low-T (few K). We showed that 2DES can reveal more details about the fluctuating environment than the 1D approaches due to peak extension into the extra dimension. By studying the spectral line shape of monomers and dimeric aggregates at low-T, we demonstrated that 2DES spectra have the potential to reveal the fluctuation spectral densities for different electronic states, the interstate correlation of static disorder and, finally, the time scales of spectral diffusion with high resolution [4].

- We offered a new insight into the assignment of emission spectra reported on PSII-core complex (PSII-cc) from different organisms [5]. Previously reported spectra were compared with data obtained at different

saturation levels of the lowest energy state(s) of spinach and *T. elongatus* PSII-cc via HB in order to provide more insight into emission from bleached and/or photodamaged complexes. We showed that typical low-T emission spectra of PSII-cc (with closed RCs), in addition to the 695 nm fluorescence band assigned to the *intact* CP47 complex [M. Reppert et al., J Phys Chem B 114, 11884 (2010)], can be contributed to by several emission bands, depending on sample quality. We suggested that recently reported emission of single PSII-cc complexes from *T. elongatus* may not represent intact complexes, while those obtained for *T. elongatus* presented in [5] most likely represent *intact* PSII-cc, since they are nearly indistinguishable from emission spectra obtained for various PSII-membrane fragments.

- In the photosynthetic green sulfur bacterium *C. tepidum*, the baseplate mediates EET from the light harvesting chlorosome to the FMO complex and subsequently towards the reaction center (RC). Literature data suggest that the baseplate is a 2D lattice of BChl *a*–CsmA dimers. However, recently it has been proposed, using 2DES at 77 K, that at least four excitonically coupled BChl *a* are in close contact within the baseplate structure [J. Dostál et al., J. Phys. Chem. Lett. 5, 1743 (2014)]. This finding was tested via HB spectroscopy (5 K) [6]. Our results demonstrated that the four excitonic states identified by 2DES likely correspond to contamination of the baseplate with the FMO antenna and possibly the RC. In contrast, HB revealed a different excitonic structure of the baseplate chromophores; where excitation is transferred to a localized trap state near 818 nm via exciton hopping, which leads to emission near 826 nm [6].

Science objectives for 2015-2016:

The key goals are: 1) provide additional insight into photoconversion, EET pathways, and conformational complexity in the B800-B840 and B800-B820 complexes from *Alc. vinosum*; 2) continue exploring (theoretically) the Frenkel exciton parameters for optical spectra of bRCs and their mutants while tracing the high-energy excitonic component of the special pair; 3) explain the origin of femtosecond processes observed via resonant HB spectroscopy in the mutated bRCs; 4) provide an uniform description of HB data and femtosecond pump-probe data obtained recently for various WSCP; 5) continue studies of electron exchanges in various photosynthetic proteins; 6) complete the manuscripts on: *i)* the modeling of resonant HB spectra in weakly coupled dimers in the presence of uncorrelated EET; *ii)* the effects of different shapes of spectral densities on the dynamics observed in PCs; and *iii)* the excitonic structure and dynamics observed in various FMO complexes.

References to work supported by this project in 2015:

1. A. Chauvet; Jankowiak, R.; Kell, A.; Picorel, R.; Savikhin, S. *"Does the Singlet Minus Triplet Spectrum with Major Photobleaching Band Near 680-682 nm Represent an Intact Reaction Center of Photosystem II?*" J. Phys. Chem. B 119, 448 (**2015**).
2. M. Reppert; Kell, A; Pruitt, T.; Jankowiak, R. *"Comments on the Optical Lineshape Function Formulas Involving Multi-Phonon Transitions"* J. Chem. Phys. 142, 094111 (**2015**).
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5. J. Chen.; Kell, A.; Acharya, K.; Kupitz, C.; Fromme, P.; Jankowiak, R. *"Critical Assessment of the Emission Spectra of Various Photosystem II Core Complexes"* Photosynth. Res. 124, 253 (**2015**).
6. A. Kell; Chen, J.; Jassas, M.; Tang, J.; Jankowiak, R. *"Alternative Excitonic Structure in the Baseplate (BChl a–CsmA Complex) of the Chlorosome from Chlorobaculum tepidum"* J. Phys. Chem. Lett. 6, 2702 (**2015**).
7. A. Kell; Bednarczyk, D.; Acharya, K.; Chen, J.; Noy, D.; Jankowiak, R. *"New Insight into Water-Soluble Chlorophyll-Binding Proteins from Lepidium virginicum*" submitted to J. Phys. Chem. B **(2015)**.

Photobiohybrid Solar Fuels

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David W. Mulder, Staff Scientist, NREL
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Overall research goals:

A long-term objective of this project is to understand the mechanisms of how photochemical energy generated by light-harvesting molecules is converted to chemical bonds by redox enzymes. Semiconducting nanoparticles saturate at high photon intensities, can be controlled to absorb over a wide spectral region, and can be directly coupled to enzymes *via* formation of molecular complexes to drive catalytic transformations. Photochemically driven energy transduction in nanomaterial-enzyme complexes can be used to help establish the principles controlling electron transfer across interfaces. A reaction of interest for this project has been the photochemical production of H_2 by hydrogenases. In photosynthetic microbes hydrogenases couple to low potential reductant pools to help maintain electron flow under anaerobic-aerobic transitions. The ubiquitous role of H_2 as an energy carrier in microbial systems is underscored by significant structural-functional diversity among the different hydrogenase enzyme classes. We are investigating active site coordination environment, substrate transfer pathways and cofactor compositions of hydrogenases towards understanding enzyme catalytic function, and integration with different charge-transfer reactions and molecules.

Significant achievements (2013-2015):

Biophysical analysis of [FeFe]-hydrogenase and the catalytic mechanism. The [FeFe]-hydrogenase from the green alga *Chlamydomonas reinhardtii* (CrHydA1), consisting of the catalytic H cluster, has been analyzed using electron paramagnetic resonance (EPR) and Fourier transform infrared (FTIR) spectroscopy of samples under various reducing and oxidizing conditions towards developing a model of catalytic H_2 activation. In an effort to isolate hydride intermediates, we studied a Cys-to-Ser (C169S) variant of CrHydA1 that decouples proton and electron transfer at the H cluster. Compared to native CrHydA1, the FTIR and EPR of reduced C169S showed differential enrichment of transient reaction intermediates. This included a higher population of $[4Fe\text{-}4S]^{1+}$ subcluster that correlated with the presence of a 2Fe subcluster with high frequency νCO modes (Figure 1). DFT optimized catalytic site models suggest assignment to a $[4Fe\text{-}4S]^{1+}$-Fe(II)Fe(II)-[H] state. This agrees with H→D exchange combined with IR that showed a shift in the 2Fe subcluster μ-CO IR band, which is consistent with a *trans-effect* on μ-CO from a terminally bound hydride (designated as "H" in $[4Fe\text{-}4S]^{1+}$-Fe(II)Fe(II)-[H] state) on Fe_D.

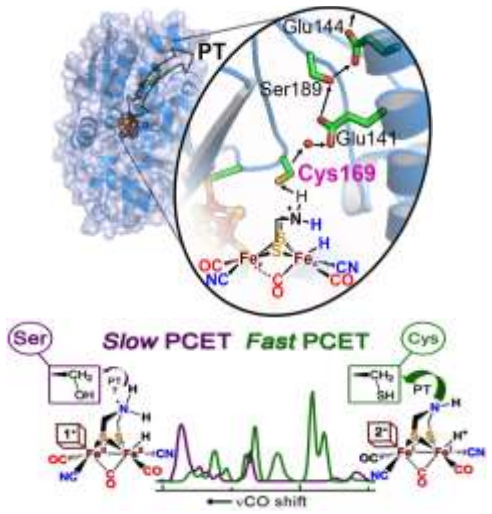

Figure 1. Magnified view of the CrHydA1 [FeFe]-hydrogenase proton-transfer relay and catalytic site (Top). Comparison of FTIR spectra of C169S (magenta) and native CrHydA1 (green) (Bottom).

Computational modeling of proton-transfer in [FeFe]-hydrogenase. The free energies along proton-transfer (PT) pathways in [FeFe]-hydrogenase were investigated using QM/MM and umbrella sampling techniques. Key residues, including an H cluster proximal Cys were identified, along with pK_a estimations from a thermodynamics integration method, and were used to model the PT profiles to the H cluster showing preference for the Cys/Water/Glu/Ser/Glu pathway identified in Figure 1.

Solar energy conversion and catalysis in photobiohybrid complexes. We have shown that the [FeFe]-hydrogenase CaI from *Clostridium acetobutylicum* self-assembles with mercaptopropionic acid capped CdTe and CdS nanoparticles (NP) to form photocatalytic complexes. Under illumination, NP light absorption and charge-separation leads to interfacial electron transfer into CaI at a surface localized [4Fe-4S]-cluster. Ultrafast measurements showed photoexcited electron-transfer from NP's occurs at rates of 10^7 s^{-1}. We investigated how altering NP diameter, which changes both the charge recombination kinetics and the free energy (ΔG_{ET}) of electron-transfer (Figure 2), affects electron-transfer rates and H_2 production in NP-CaI complexes. Surprisingly, the rate of the NP→CaI electron-transfer step was minimally affected by lowering ΔG_{ET}, suggesting a gated, or chemically coupled, interfacial electron-transfer process. As a result, H_2 production was controlled by competition between electron transfer and recombination, where slower recombination in larger NPs allowed for higher photon-to-H_2 conversion and yields.

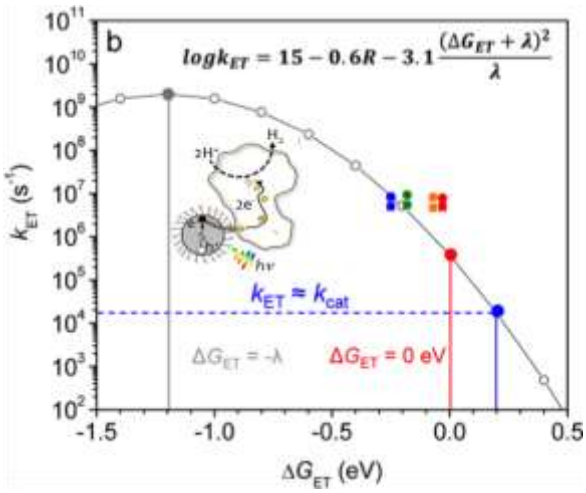

Figure 3. Interfacial electron transfer rates (k_{ET}) versus ΔG_{ET} for CaI-CdTe (blue, 2.0; green, 2.2, orange, 2.8; red 3.2 nm).

Science objectives for 2015-2016:

- Apply both steady-state and time-resolved cryo-FTIR of [FeFe]-hydrogenase, and [FeFe]-hydrogenase/NP complexes to resolve the electronic properties of H cluster reduced states.
- Determine the mechanism of electron injection from NP→CaI, and the function of His-ligation to the distal [4Fe-4S] cluster in interfacial electron-transfer to CaI.
- Mössbauer spectroscopy (with Dr. Y. Guo at Carnegie-Mellon University) of the reduced CrHydA1 C169S variant showed a significant population of a $[4Fe\text{-}4S]^{1+}$-Fe(II)Fe(II) H cluster, with hyperfine sublevel correlation EPR ^1H and ^2D spectra (with Dr. S. Dikanov at the University of Illinois) indicating the presence of exchangeable protons. Further spectroscopic analysis of reduced CrHydA1 will be completed to verify assignment(s) of H cluster hydride states.

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Controlling Electron Transfer Pathways in Photosynthetic Proteins

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Overall research goals: Photosynthetic reaction centers (RCs) are protein-cofactor complexes that convert light energy into chemical energy in a series of extremely efficient transmembrane electron transfer (ET) reactions. The X-ray structures of RCs reveal two pseudo-symmetrical branches of cofactors (Fig. 1) that are functionally asymmetric; bacterial RCs use the A pathway exclusively. The goal of the project is to identify a mutant RC that utilizes the B pathway for quinone (Q_B) reduction with the same high yield as that of the A pathway in the native complex, thereby gaining fundamental insight into the factors necessary for *de novo* design of efficient multistep ET. Previous attempts at rational design have provided neither the means nor understanding necessary to engineer efficient B-branch ET.

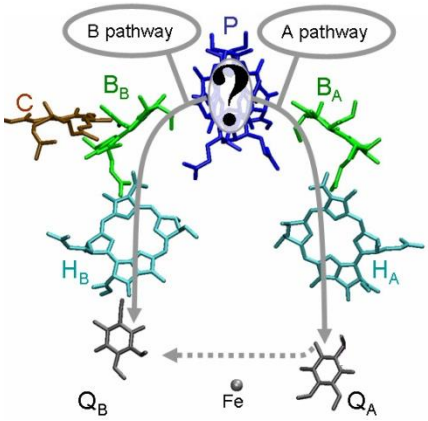

Fig. 1. *Arrangement of cofactors in the bacterial RC.*

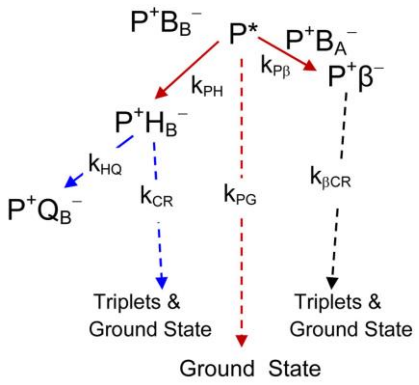

Fig. 2. *States, target processes, and key $P^+H_B^-$ branching point.*

Towards this end, we have developed both semi-directed molecular evolution approaches that streamline mutagenesis and RC isolation and high-throughput time-resolved spectroscopic screening assays. By design, a large number of RC variants can be examined for the yield of $P^+Q_B^-$ arising from exclusive use of the B-side cofactors. The experimental vehicles are the RCs from *Rhodobacter capsulatus* and *Rhodobacter sphaeroides*. Our goal is to find mutant(s) that unlock efficient, transmembrane, B-side charge separation and limit competing charge-recombination reactions (Fig. 2). Polarizable residues near B_B were identified in an initial screening of mutants as one means to enhance B-side ET. A set of these mutants was the kernel of our first publication.[1]

Significant achievements 9/2013 – 9/2015: Improvements to our mutagenesis and screening methods have enabled the preparation of ~625 mutants with ~550 RCs screened. Fig. 3 shows the results of the millisecond assay for the yield of $P^+Q_B^-$ relative to wild type (~100%, via Q_A) for a subset of nearly 400 mutants. The colors of the bars reflect different RC templates used and different residues targeted for saturation mutagenesis. Most mutants utilize a scaffold (YFHV; Fig. 3, left inset) that displays substantial B-pathway activity in the assay (Fig. 3; main, yellow bar). The new mutant sets recently developed and screened add to the list of constructs that show significantly improved utilization of the B-side cofactors compared to the initial YFHV template. The sets have targeted additional residues in the vicinity of the B-side cofactor B_B to further enable B-side activity and near B_A to handicap the A path. Expansion of mutagenesis targets into other regions of the RC revealed that a single residue near H_B significantly increases the efficiency of secondary ET from H_B to Q_B in some cases.[2] Forward ET to Q_B (~7.5 ns) becomes the favorable decay pathway for $P^+H_B^-$ in a mutant RC where M131

is changed from Val to Glu, largely due to slowing the competing charge-recombination process from ~4.5 ns to ~10 ns. On the A side in wild-type RCs, the time constant for the analogous $P^+H_A^-$ charge recombination process is ~10 ns and, most interestingly, the residue near H_A that is related by C_2 symmetry to M131 near H_B is a Glu (at L104). Combining this favorable result on secondary electron transfer (Val to Glu at M131 near H_B) with mutations near B_B that enhance primary electron transfer to the B side (such as Phe to Asn at L181) has not yet led to improvements in both steps simultaneously.

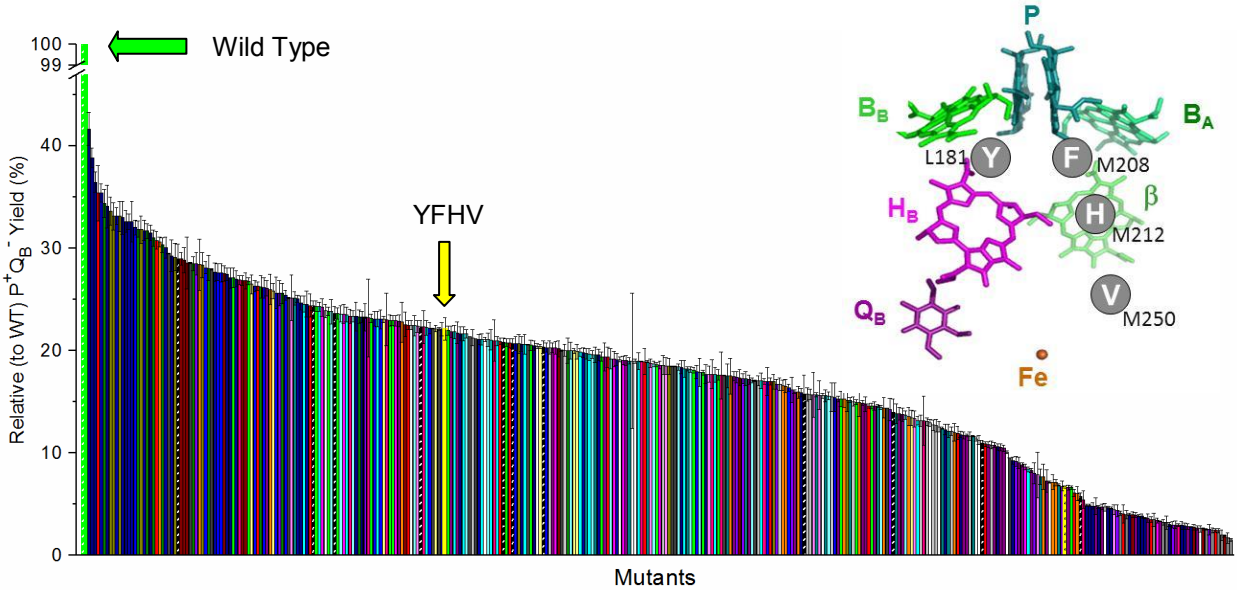

Fig. 3. *Yield of $P^+Q_B^-$ relative to wild type in 375 mutants of two species as assayed by a millisecond screening assay using 7-ns excitation flashes (main panel); the YFHV template RC (top right).*

The efficient (measurement and analysis time, sample requirements) millisecond screen was further validated by ultrafast time-resolved studies on a set of eight mutants to obtain the rates and yields of the primary and secondary events. Such work anchors the continued use of the high-throughput screen for identifying promising mutants. Papers are currently being drafted on these studies and additional sets of mutants, including analogous sets that demonstrate marked differences in functional consequences of substitutions depending on the species studied. Our overall approach is clearly bearing fruit, providing unanticipated insights into how to control the directionality of charge separation and the efficiencies of the electron-transfer processes.

Science objectives for 2015-2016 and beyond:

- Iterate combinations of mutations, pairing favorable substitutions with those in new regions.
- Mine datasets to correlate properties of substituted residues with observed photochemistry.
- Refine photochemical screens to detail intricacies of events in a larger sampling of mutants.
- Select photocompetent, phenotypic revertants that grow via B-side cofactors exclusively.

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The Energy Budget of Steady-State Photosynthesis

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Overall research goals:

The project goals focus on understanding how photosynthesis balances its energy budget, matching the output of energy in NADPH and ATP to precisely meet biochemical demands. Our specific aims concern the mechanisms of 1) cyclic electron flow (CEF) which acts to generate ATP to balance the chloroplast energy budget; 2) the transport and sharing of ATP among the chloroplast, cytoplasm and mitochondria; 3) the partitioning of proton motive force into ΔpH and $\Delta\psi$.

Significant achievements 2013-2015:

1) There are two distinct types of CEF, one through the NDH complex, which is analogous to Complex I of respiration, that the other through ferredoxin:quinones reductase, FQR, which in higher plants FQR is thought to involve the PGR5 and PGRL1 proteins. We show that these two pathways are distinct in their energetics and regulation and likely serve distinct metabolic and regulatory functions (1, 2).

2) The mechanisms of CEF regulation are not well understood. We provide evidence that that H_2O_2 is a missing link between environmental stress, metabolism, and redox regulation of NDH-linked CEF in higher plants (3).

3) We showed that, in contrast to the NDH pathway, the FQR pathway is rapidly regulated by the redox status of regulatory thiols (4), and this mode of regulation probably operates in Chlamydomonas (5).

4) We demonstrated that the chloroplast NADPH dehydrogenase complex (NDH), a homolog to respiratory Complex I, pumps approximately two protons from the chloroplast stroma to the lumen per electron transferred from ferredoxin to plastoquinone, effectively increasing the efficiency of ATP production via CEF by two-fold compared to the FQR- pathway. This proton pumping activity also allows a (new) non-canonical mode of photosynthetic electron transfer, allowing electron transfer from plastoquinol to NADPH to be driven by the thylakoid proton motive force possibly helping to sense or remediate mismatches in the photosynthetic budget.

5) CEF needs to be tightly regulated to prevent accumulation of excess pmf. Work on this grant also contributed significantly to our recent observations that large extents of the $\Delta\psi$ component of pmf accelerates the production of singlet O_2 by PSII leading to photodamage, possibly explaining a large fraction of the sensitivity of photosynthesis to fluctuating illumination.

6) We reinvestigated a widely-used fluorescence signal previously used to assess CEF, and show that it has been misinterpreted (6). Our reassessment led us to reassess a large fraction of past literature, especially those on the PGR5 pathway, suggesting that a major effect of *pgr5* is on regulation of the chloroplast ATP synthase rather than on CEF.

7) Isolation of novel mutants that activate CEF *in vivo*. In order to understand the mechanism and control of CEF, we isolated, mapped and/or characterized a series of mutants that display high CEF, i.e the *hcef* mutants. Recently, we have mapped several more mutations with high CEF. One of these, *hcef5*, appears to have high FQR-linked

CEF. Another, *hcef6*, is a nonsense mutation Q334 (889C>T) in a protein of unknown function that is localized to the chloroplast. The *hcef4* mutation is in an enzyme of the Calvin-Benson cycle, suggesting that futile cycling of chloroplast metabolism can lead to increased ATP demands, as we previously proposed.

8) Finally, we presented a new model for how cytochrome bc complexes tame reactive intermediates to conduct the Q-cycle (7).

Science objectives for 2013-2014:

- Determine the mechanisms *hcef* mutants
- Determine if the chloroplast nucleotide transporter acts to balance ATP/NADPH budget during fluctuations in metabolic demands
- Test the hypothesis that PSI damage is regulated by the pmf, and indirectly by CEF and the ATP synthase.

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Cyanobacterial Photoreceptor Systems for Regulation and Optimization of Energy Harvesting

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All photosynthetic organisms rely on antenna complexes to support sufficient light capture for phototrophic growth and net carbon fixation, but biosynthesis of these antennae requires a large investment of energy and fixed carbon. Such investments are particularly problematic for photosynthetic microbes, which lack large reserves of stored chemical energy. This collaborative research project seeks to expand the knowledge base of cyanobacterial photoreceptor proteins that enhance the efficiency of solar light energy capture and carbon fixation. We focus on the GAF-domain biliprotein photoreceptors, phytochromes and cyanobacteriochromes (CBCRs). Such bilin-based light sensors regulate a broad range of dynamic adaptive responses, including shade avoidance in land plants, complementary chromatic acclimation (CCA) and far-red-light-induced photoacclimation (FaRLiP) responses in cyanobacteria, and induction of light-harvesting complexes in nonoxygenic photosynthetic bacteria. Phytochromes and CBCRs comprise the largest family of cyanobacterial photoreceptors and span a broad spectral range from the near ultraviolet to the near infrared.

Overall research goals: Our ongoing studies examine how representative members of this family function and elucidate the biological processes that they regulate. This project combines approaches ranging from ultrafast characterization of photochemical processes through biochemical analysis of signal transduction and genetic examination. A longer-term goal of these studies is to use these light-sensing proteins as synthetic tools for regulating the expression of arbitrary target genes in response to the color or intensity of ambient light. Such tools can be used to tailor cyanobacteria and other photosynthetic species for more efficient, sustainable, and carbon-neutral biological capture of sunlight and conversion and storage of that light as chemical energy.

Significant achievements 2013-2015: By combining transient and solution NMR spectroscopy with biochemical analysis of wild-type and mutant recombinant sensors, our studies have yielded new insights into how these sensors see specific colors of light, how chromophore-binding GAF domains transmit photophysical cues (light absorption) to biological outputs elsewhere in the same molecule, and how these molecules interface with the rest of the cell to trigger photobiological responses. We have ***discovered and elucidated four CBCR tuning mechanisms*** that explain much of their photosensory diversity, ***provided the first explicit biochemical model for CBCR signaling***, ***characterized the intrinsic heterogeneity of chromophore-protein photodynamics of CBCRs*** that underlie their diverse quantum efficiencies, and ***genetically identified the CBCR photoreceptor that regulates phototaxis*** of *Nostoc punctiforme* hormogonia.

Science objectives for 2015-2016: Ongoing studies seek to understand the molecular basis of newly discovered bilin-based sensor families with distinct photocycles that extend into the near infrared (***Lagarias***). We will exploit multi-dimensional NMR spectroscopic techniques to provide atomic level structure and inform mechanistic models for light signal transfer within the protein scaffold of representative CBCRs (***Ames***). These studies parallel transient kinetic analyses from the ultrafast (sub-ns) to secondary (ns-ms) timescales and cryotrapping of intermediates to probe structural changes in the photoexcited chromophore to changes in the protein structure (***Larsen***). Development of portable CBCR-based systems for optogenetic applications in photosynthetic and non-photosynthetic organisms is an ongoing, long-term objective of our studies. These studies exploit the ever-expanding natural diversity of bilin-based sensors and re-engineered sensors with known molecular outputs that will interface with ongoing efforts of other BES-funded investigators.

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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 2013-15. (A) The binding and activation of substrate water molecules in the oxygen-evolving complex of photosystem II. The light-driven four-electron water oxidation reaction occurs at the tetranuclear manganese-calcium-oxo (Mn_4Ca-oxo) catalytic cluster in the oxygen-evolving complex (OEC) of PSII. 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. (a) In previous studies, we had unambiguously resolved the individual spectroscopic signatures of the substrate water molecules that are directly ligated to the Mn_4Ca-oxo cluster in the S_2 state of the OEC of PSII.[2] These experiments provided a direct handle to monitor the catalysis of the substrate water molecules in the solar water oxidation reaction. We have recently used the structure of the bound substrate water molecules in the OEC to investigate the role of the Ca^{2+} ion in the solar water oxidation reaction. The Mn_4Ca-oxo cluster of PSII consists of three manganese ions and a calcium ion that form a distorted cubane and fourth manganese ion that is a dangler. The structural and functional differences that are induced by metal ion substitution can provide valuable insight on the role of the Ca^{2+} ion in water oxidation. By exploiting the power of 2D ^1H hyperfine sublevel correlation (HYSCORE) spectroscopy, for the first time, we elucidate the electronic structure of the substrate waters in the Mn_4Sr-oxo cluster in the S_2 state of Sr^{2+}-substituted PSII.[3] (b) The super-oxidized Mn(III)Mn(IV) state of manganese catalase from *L. plantarum* is an excellent proteinaceous model of the Mn_4Ca-oxo cluster in the OEC. Recently, we have utilized 2D HYSCORE spectroscopy to determine the high-resolution electronic structure of the dinuclear manganese center of superoxidized manganese catalase.[4] Based on the magnetic parameters of synthetic dimanganese models, manganese catalase, and those of the S_2 state of the OEC of PSII determined in our laboratory, we provide valuable insight on the impact of the protein environment on the coordination geometry of catalytic multi-nuclear manganese clusters. **(B) The tuning and control of quinone cofactors in photosynthetic reaction centers**. Quinones are widely used as electron transport cofactors in photosynthetic reaction centers (RC). It is thought that the structure and substituent groups of the quinone, the location of the quinone cofactor, the geometry of its binding site, redox potential and 'smart' matrix effects from the surrounding protein environment greatly influence the functional properties of quinones in photosynthesis. (a) We have examined an extensive library of naphthoquinone models by cyclic voltammetry, *cw* and pulsed EPR spectroscopy to understand the factors that influence the functional tuning of these cofactors.[5] (b) PSII contains identical plastoquinones in the primary and secondary quinone acceptor sites, Q_A and Q_B, that exhibit very different functional properties. The

primary quinone, Q_A, is a single electron acceptor, while the secondary quinone, Q_B, undergoes PCET reactions. We have utilized 2D ^{1}H HYSCORE spectroscopy to directly probe the structure of light-induced PCET intermediates of quinone models to understand the mechanism of coupled charge transfer at the Q_B cofactor.[6] **(C) The functional specificity of the tyrosine residues of PSII: Mechanism of proton-coupled electron transfer at Y_Z and Y_D.** The D1 and D2 polypeptides that form the core of PSII each contain a redox-active tyrosine residue, Y_Z and Y_D, respectively. Recent models invoke Y_Z in the O_2 evolution reaction as an abstractor of protons and/or hydrogen atoms from the substrate water molecules in the OEC. In contrast, Y_D does not participate in rapid electron transfer in the O_2 evolution reaction. We have developed pulsed 2D HYSCORE and high-frequency electron nuclear double resonance (HF ENDOR) spectroscopy methods to structurally characterize the $Y_Z^\bullet$ and $Y_D^\bullet$ PCET intermediates of PSII. These studies provides direct 'snapshots' of functional PCET intermediates and, for the first time, makes it possible to detail the mechanism of PCET in biological solar energy transduction.[7,8] Furthermore, we have used pure quantum mechanical calculations to elucidate the smart matrix effects from the surrounding protein environment that determine the distinct functional tuning of the Y_Z and Y_D residues of PSII.[9]

Science Objectives for 2015-16. (i) We have determined the electronic structure and substrate activation in the S_2 state of the OEC and we plan to extend these studies to include the higher S-state intermediates in the solar water oxidation reaction. (ii) We are investigating the mechanism of PCET at the secondary quinone acceptor of PSII and (iii) we plan to develop experimental models to further elucidate the mechanism of light-induced PCET at the redox-active tyrosine residues of PSII.

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Molecular Genetic Dissection of Chloroplast Fe Homeostasis

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Overall Research Goals: Iron is essential for virtually all life forms because of its broad function as a catalyst, particularly of redox reactions and reactions involving O_2 chemistry. Despite its abundance on earth, iron has limited bioavailability because of its relative insolubility in the Fe(III) oxidation state, which is the prevalent form in most aerobic environments. Organisms have therefore evolved complex pathways for iron mobilization and storage, typically involving chelation and redox chemistry (often multiple sequential steps) followed by biosynthesis of heme, inorganic Fe/S or other clusters, and incorporation into protein. Despite the occurrence of sophisticated acquisition mechanisms, in the natural world, photosynthetic organisms can be chronically under-nourished for iron, which limits primary productivity. For instance, about 30% of the oceans are iron-limited, and a similar fraction of the world's croplands have poor iron availability. Therefore, primary photosynthetic metabolism often occurs in a situation of iron-deficiency stress in nature, requiring the operation of iron economy mechanisms – sparing and recycling. The mechanisms underlying iron recycling are not known, but it is clear that iron storage, at least transiently, must be involved. Ferritin, an iron storage molecule, has been proposed to serve in this capacity, and the occurrence and expression of genetic information for ferritin has been suggested to contribute to the success of pennate diatoms in iron-poor ocean environments. The Merchant group is interested in dissecting the pathways of iron metabolism in algae by combining classical genetic and genomic approaches in a "reference (model) organism" with state of the art chemical methodologies for monitoring and visualizing iron with high spatial and temporal resolution. The objective is to understand the mechanisms underlying iron economy in algae, especially 1) during the transition from photoautotrophy to photoheterotrophy, and 2) the role of ferritin and of a newly described organelle for metal homeostasis, the acidocalcisome.

Significant achievements: (2013-2015): 1. Acetate-grown Chlamydomonas cells sacrifice the photosynthetic apparatus in Fe-limited growth conditions but CO_2-grown cells do not. We are using comparative transcriptomics to discover components of the acetate-signalling pathway. Physiological characterization (cyanide-sensitive respiration capacity, O_2-evolution capacity, Kautsky curve, Fv/Fm, total organic C and N (for biomass) and elemental profile) of the transition from photoautotrophy to photoheterotrophy indicates that a program to degrade the photosynthetic apparatus is initiated 4 to 6 h post-acetate supplementation. Therefore we have isolated and sequenced high quality RNA in a time course after acetate addition to distinguish changes in abundances of RNAs encoded in all 3 genomes. The transcriptome time course may identify gene expression changes that occur during acetate-induced degradation of the photosynthetic apparatus as well as during proliferation of mitochondrial mass for increased oxidative phosphosphorylation capacity.

2. We had noted that the total iron content of cells varied as a function of iron supply in the medium with 4.5x over-accumulation of iron in the iron-excess situation. This raised the question of where the excess iron is located, since ferritin abundance actually decreases in the overload situation. We found that ferritin-associated iron is constant in Chlamydomonas: iron-limited cells with high ferritin abundance have very little iron associated with the protein while iron-overload cells contain very little ferritin but the protein is nearly saturated with iron. This finding emphasizes the importance of monitoring the metalation state of ferritin for a more accurate picture of iron distribution.

Therefore, we focused on iron-localization using NanoSIMS in thin sections of fixed Chlamydomonas cells in collaboration with Dr. Jennifer Pett-Ridge (at LLNL) and confocal fluorescence microscopy using a synthetic iron probe, IP1, for live cell imaging in collaboration with Dr. Chris Chang (UC-Berkeley). We localized the non-ferritin associated iron to an intracellular acidic membrane-bound compartment that also contains phosphorus and calcium. This compartment is analogous to the vacuole (in yeast) or lysosome (in animal cells) and has been called the acidocalcisome. The acidocalcisome has high polyphosphate content and stores various types and amounts of metal ions, including Cu(I), Fe(II) and Zn(II). Chlamydomonas *vtc* mutants (from Dr. Arthur Grossman) are blocked in the biogenesis of this compartment. We found that the *vtc* mutants cannot hyper-accumulate iron, which underscores the importance of this compartment for iron storage. Preliminary results indicate that *vtc* mutants are sensitive to iron-limitation under phototrophic growth, suggesting that this compartment is a source of iron for maintenance of the photosynthetic apparatus in iron-limited photo-autotrophic cells.

To distinguish the role of ferritin vs. the compartment in handling iron in an overload situation, we monitored the movement of iron from the medium into iron-limited cells using iron isotope pulse-chase methodology. Iron-limited cells have very high iron assimilation capacity. Iron supplied (as Fe57) in the medium is rapidly taken up: it appears first in ferritin and then in the acidocalcisome compartment at the expense of ferritin. We suggest that chloroplast ferritin is an intermediate during iron trafficking from the medium to the acidocalcisome. Mutants lacking the acidocalcisome cannot hyper-accumulate iron, emphasizing the importance of the compartment for iron homeostasis. Pulse-chase analysis shows that assimilated Fe57 stays ferritin-associated, consistent with a role for ferritin as an intermediate in iron traffic from the medium to the acidocalcisome.

The bio-available pool of iron may be critical for survival of photoautotrophic organisms in an iron-deficient environment. Interestingly, the selectivity of IP1 for Fe(II) implies that iron is in the +2 oxidation state in the compartment (compatible with the acidic environment) whereas ferritin iron is in the +3 oxidation state.

Science objectives for 2015-2016:

1. We will complete the analysis of the transcriptome of cells transitioning from phototrophy to heterotrophy with the objective of identifying components involved in iron recycling from the plastid to the mitochondria. We are also interested in assessing whether there is coordination between the nucleus and plastid during acetate-induced degradation of the photosynthetic apparatus and whether lncRNAs might be involved in iron-responsive signal transduction pathways.
2. We will address the role of ferritin in loading the acidocalcisome by a reverse genetic approach.

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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 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, nonphotochemical quenching (NPQ) mechanisms that regulate photosynthetic light harvesting are induced. In algae and plants, the qE type of NPQ is turned on and off rapidly by changes in thylakoid lumen pH, whereas the slower qI type downregulates PSII during long-term light stress. Our specific aims are (1) to investigate the regulation of the novel, stromal violaxanthin de-epoxidase (VDE) enzyme found in *Chlamydomonas*, (2) to analyze the relationship between structure and function in PsbS and LHCSR, (3) to dissect the mechanism of SOQ1-related antenna qI, (4) to model energy transfer and NPQ in grana thylakoid membranes, and (5) to develop methods for performing single-shot and snapshot transient absorption spectroscopy.

Significant achievements 2013-2015: By chloroplast fractionation, we showed that the VDE enzyme in both *Chlamydomonas* and *Arabidopsis* is associated with the stromal side of the thylakoid membrane. We established an assay for this enzyme *in vitro* with isolated thylakoids. We used site-directed mutagenesis and transient expression in *Nicotiana benthamiana* to identify mutants of LHCSR and PsbS that are constitutively activated for quenching, and we generated loss-of-function LHCSR and VDE mutants of *Nannochloropsis oceanica* that affect qE. We engineered an *Arabidopsis* line with a lutein epoxide cycle as its only xanthophyll cycle. Through a genetic screen for suppressors of *soq1 npq4* in *Arabidopsis*, we found that a chloroplast lipocalin in the thylakoid lumen is necessary for SOQ1-associated qI. The fluorescence lifetime snapshot method developed in the previous period was devised to enable measurements on whole leaves. Using *Arabidopsis* wild-type, *npq4*, and *npq1* mutants, we showed the PsbS acts only as a catalyst in speeding up the onset of qE. In contrast zeaxanthin appears to play an explicit role in the quenching process. We also showed that zeaxanthin plays an important role in regulating light harvesting in Photosystem I. We have developed the structure-based model for PSII supercomplexes into a model for large (300 nm x 300 nm) patches of grana membrane, that enables fluorescence yields and decay curves to be calculated directly from structure and appropriate quantum information.

Science objectives for 2015-2016:

- Determine the cofactors and regulation of the *Chlamydomonas* VDE enzyme.
- Characterize the lutein epoxide cycle and its role in NPQ.
- Use constitutively active PsbS to identify its interaction partners.
- Establish a system for site-directed mutagenesis of LHCSR in *Nannochloropsis*.

- Investigate interactions between SOQ1 and the chloroplast lipocalin using biochemical and genetic approaches.
- Using the membrane model we will explore models for NPQ benchmarking with experimental fluorescence snapshot data.
- Develop a transient absorption "snapshot" method to follow the yield of carotenoid cations during adaptation to high light.

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Dynamics of Photosynthetic Membrane Formation, Structure, and Regulation

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Overall research goals: In plants and algae, solar energy conversion into chemical energy occurs within a complex architecture of photosynthetic membranes inside chloroplasts. By integrating programs in plant and algal photosynthesis, genetics, biophysics, advanced imaging, ultrafast spectroscopy, and theoretical modeling, our long-term goal is to obtain a detailed molecular description of the organization of the photosynthetic membrane, the control of its composition and structure, and the membrane dynamics occurring during thylakoid biogenesis and regulation of photosynthesis. Our specific aims are (1) to investigate membrane structure and dynamics during thylakoid formation, (2) to use mutants, synthetic membranes, and modeling to analyze the relationship between structure and function in thylakoids, and (3) to image and model the dynamics of thylakoid membranes during regulation. We follow protein rearrangements in photosynthetic membranes using a range of cutting-edge imaging techniques, which are ideally suited to reveal complex structural changes in the membrane at different levels of spatio-temporal resolution. The imaging approaches are integrated with parallel modeling and theoretical efforts, which will provide new insights into the fundamental biophysical mechanisms governing membrane formation, protein interactions, and the partitioning of protein complexes in the photosynthetic membrane.

Significant achievements 2013-2015: Using atomic force microscopy (AFM), we completed experiments analyzing the dynamic changes in biophysical properties of the thylakoid membrane during a state transition and the structural attributes of grana thylakoids from *Arabidopsis thaliana*. We made progress in developing a novel near-field optical scanning microscopy (cathodoluminescence-activated imaging via resonant energy transfer, CLAIRE) that combines the high spatial and temporal resolution of a scanning electron microscope (SEM) with the non-invasiveness of light microscopy at length scales well below the diffraction limit. Briefly, our strategy is to use a tightly focused electron beam to generate nanoscale regions of optical excitation in a cathodoluminescent (CL) film that supports a thylakoid membrane encapsulated in an aqueous environment. The role of the CL film is to convert electrons to optical excitations that can probe aqueous biological samples at a spatial resolution approaching that of the electron beam. These optical excitations in the CL film will undergo Förster resonance energy transfer (FRET) with nearby protein-bound acceptor fluorophores in the thylakoid membrane. We successfully obtained an appropriate material out of which to fabricate the CL film in the imaging device and characterized the film properties, including its ability to FRET with nearby dye molecules, and we measured that the optical spot size that we can generate within the film upon electron beam excitation is of diameter <20 nm. To enable simulation of membrane systems that are as large as grana. we developed a dissipative particle dynamics algorithm for graphical processing units that is 25 times faster. We developed a grand canonical Monte Carlo algorithm that allows for flow of lipid or protein material from the edges of a simulation box; this reservoir technique can simulate lipid and protein flows between the grana and stroma lamellae. We also developed a coarse-grained molecular model of the grana thylakoid membrane including the PSII-LHCII complex and lipids to

study the self-organization of these proteins into various supramolecular complexes. We isolated mutants of the unicellular green alga *Chlamydomonas reinhardtii* that affect thylakoid formation and/or structure based on altered pigmentation in the dark or confocal fluorescence imaging and obtained super-resolution fluorescence images of thylakoid architecture in live *Chlamydomonas* cells using structured illumination microscopy (SIM).

Science objectives for 2015-2016:

- Use high-speed AFM to record images of PSII supercomplex dynamics in thylakoid membranes.
- Use live-cell SIM and focused ion beam scanning electron microscopy (FIB/SEM) to follow assembly of chlorophyll-containing protein complexes and the formation of thylakoid membranes in *y5* and other chloroplast biogenesis mutants of *Chlamydomonas*.
- Extend our work on membrane reconstitution to include LHCII and associated proteins, using either electroformation or a new microfluidic jetting technique to control transmembrane protein incorporation.
- Refine our protocols for fabricating free-standing scintillator films and develop a liquid cell for CLAIRE in order to perform proof-of-concept imaging in aqueous environments.
- Implement fluorescence lifetime imaging (FLIM) to study mechanisms affecting light harvesting.
- Explore the role of lateral diffusion and structural change using our model for grana membrane excitation dynamics and try to extend this model to multiple grana layers.

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Two-Dimensional Electronic Spectroscopies for Probing Coherence and Charge Separation in Photosystem II

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Overall research goals:

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

1) What is the electronic structure of the PSII RC?
2) What are the charge separation pathways in the PSII RC?
3) Does coherence facilitate charge separation in the PSII RC?

Significant achievements (2014-2015):

We have developed a novel implementation of 2DES with improved sensitivity[1]. We also recently wrote a review paper on experimental implementations of 2DES to highlight the strengths and weaknesses of different approaches that have been developed thus far[2].

We have applied our improved 2DES setup to studies of the PSII RC and we have observed coherent dynamics[3]. There is currently considerable debate in the 2DES spectroscopy community about the physical origin of coherent dynamics and their potential importance for photosynthetic function. Supported by simulation work and extensive characterization of the coherences we believe that they have mixed electronic and vibrational character and that they are functionally important for charge separation. To assign the origin of the experimentally observed coherences and determine their influence on charge separation, we performed theoretical simulations of the dimeric special pair of the PSII RC including explicit coupling to discrete vibrations, obtaining good agreement with the experimental data. Further simulations using an exact hierarchical equations of motion (HEOM) method demonstrated that resonant vibrations speed up the initial step of charge separation in the PSII RC. This speed-up occurs when the vibrational frequency is close to excitonic resonance. *This may represent an important design principle for enabling the high quantum efficiency of charge separation in oxygenic photosynthesis.*

We are currently studying the coherent dynamics of the monomer pigments and comparing how they differ from the PSII RC. We have developed a high sensitivity spectroscopy for measuring coherent dynamics that is faster and provides higher signal-to-noise data than 2DES[4]. This method has enabled us to observe coherence in the weak anion bands of the PSII RC. This is an ongoing study but thus far these observations support the importance of coherence for charge separation.

This year we also obtained our first data from a new experiment that combines Stark spectroscopy and 2DES. This experiment was designed to help distinguish between energy transfer and charge separation, a challenging task due to the lack of clear spectral signatures of charge transfer states. Linear Stark spectroscopy has proven to be a valuable method for uncovering charge transfer states in photosynthetic reaction centers. With Stark 2DES, we have extended this approach to enable time-resolved measurements of how the charge transfer states

participate in the charge separation process. The new experimental approach and representative data on the PSII RC are shown in Figure 1.

Finally, we have also performed polarization-dependent 2DES studies of the PSII "core" preparation, consisting of the intact PSII RC complex (with quinones) and the neighboring antenna complexes CP43 and CP47. With this data we will combine excitonic models of the CP43 and CP47 complexes with our D1D2-RC model to simulate 2DES spectra from the PSII core. This will enable us to address questions about possible differences in excitonic coupling in the reaction center between D1D2-RC and PSII core samples, as well as gain insight into the energy landscape that guides energy transfer from the antennae to the RC.

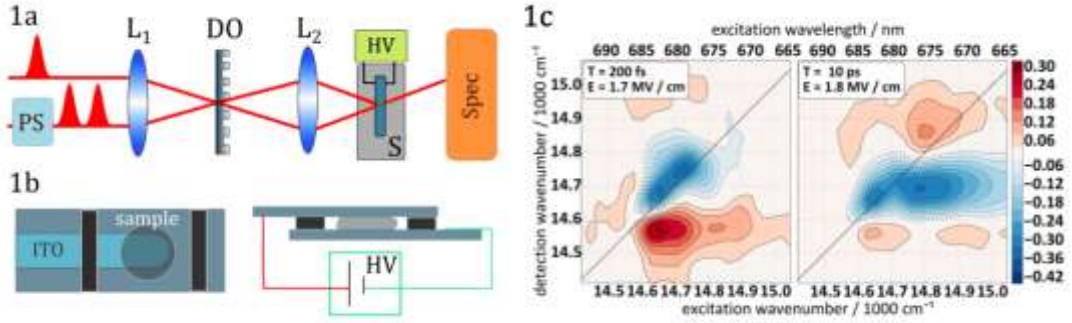

Figure 1a: Experimental setup for Stark 2DES (DO: diffractive optic, HV: high voltage, PS: pulse-shaper). **1b**: Stark sample cell geometry: 1.7 MV/cm is applied across indium tin oxide electrodes in an immersion cryostat. **1c:** Stark 2DES data from the PSII RC at 77K at different waiting times throughout the charge separation process. The data shown are difference spectra that show the changes in the 2DES data due to the Stark field, revealing the states and kinetic processes of charge separation.

Science objectives for 2015-2016:

- Perform additional Stark 2DES measurements of the PSII RC and PSII cores and use these to test and refine excitonic and charge separation models of the PSII RC.
- Compare the PSII RC and Chl a coherent dynamics to better understand the signatures of vibronic coupling in 2DES and their functional importance in the PSII RC. Demonstrate that our modeling can capture the relevant coherent dynamics, 2D peakshapes and relaxation processes.
- Perform further anion band experiments and combine experimental and theoretical analysis of the observed coherent dynamics and their role in charge separation.
- Combined experimental-theoretical analysis of the energy transfer processes in the PSII core complex.

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Spatial Regulation of Chloroplast Division and Physiological Significance

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Overall research goals: Chloroplast division increases chloroplast numbers and compartment size during leaf growth and development, and is therefore essential for achieving maximum photosynthetic productivity. A striking feature of the division process is that it is restricted to the middle of the chloroplast, producing a large population of small organelles of similar size and shape. The aims of the funded research are to probe the mechanisms by which spatial regulation of chloroplast division-site positioning is achieved, investigate the relationship between chloroplast morphology, chloroplast movement and photosynthesis, and identify genetic loci potentially involved in the control of chloroplast size, number and cell coverage.

Significant achievements 2013-2015:

Spatial regulation of division-site placement: The cytoskeletal proteins FtsZ1 and FtsZ2 coassemble to form the cytokinetic FtsZ ring ("Z ring") inside the stroma. We showed that the Arabidopsis chloroplast division protein ARC3 mediates division-site placement by directly inhibiting Z-ring assembly at non-division sites (Zhang et al., 2013). However, ARC3 also localizes to the mid-plastid, where it interacts with the inner envelope membrane (IEM) protein PARC6, also a negative regulator of Z-ring assembly. To further probe PARC6 function, we performed topological analysis, which showed it has a single transmembrane domain and a topology equivalent to that of the IEM division protein ARC6 (Fig. 1), from which PARC6 evolved by gene duplication. We found that the stromal region of PARC6 interacts not only with ARC3, but, like ARC6, also with FtsZ2. Overexpression of PARC6 inhibited FtsZ assembly in Arabidopsis, but not in a heterologous yeast system, suggesting the negative affect of PARC6 on FtsZ assembly is a consequence of its interaction with ARC3. The intermembrane space (IMS) region of PARC6 interacted with that of the outer envelope membrane (OEM) protein PDV1, which recruits the dynamin-related protein DRP5B to the OEM, where DRP5B forms an external contractile ring. These and other findings supported primarily by our DOE-funded research suggest a model in which PARC6, together with ARC6, coordinates the FtsZ and DRP5B rings across the envelope membranes, and that PARC6, via its interaction with ARC3, promotes Z-ring remodeling during division (Fig. 1) (Zhang, Chen et al., submitted).

Relationship between chloroplast morphology, chloroplast movement and photosynthesis: We collaborated with David Kramer and coworkers to develop an imaging platform for simultaneous measurements of chloroplast movements and chlorophyll fluorescence on intact plants in white light. Dual imaging, involving reflectance and fluorescence measurements of the same pulsed red light, also allowed us to develop an approach to correct calculations of non-photochemical quenching for interference from chloroplast movements. Measurements in three Arabidopsis chloroplast division mutants having only 1-2 giant chloroplasts in their mesophyll cells showed that their photosynthetic phenotypes in response to high-light stress are due predominantly to their altered chloroplast size and shape rather than to their diminished chloroplast movement capacity (Dutta, Cruz et al., in press).

QTL mapping for genes controlling chloroplast size/number. We scored Cvi/Ler near-isogenic lines and identified a major QTL affecting chloroplast size and number in a small region bearing two genes, one of which is a strong candidate as the causal gene. Experiments to confirm that the Cvi allele is responsible for the increased size and decreased number of chloroplasts in this accession are in progress.

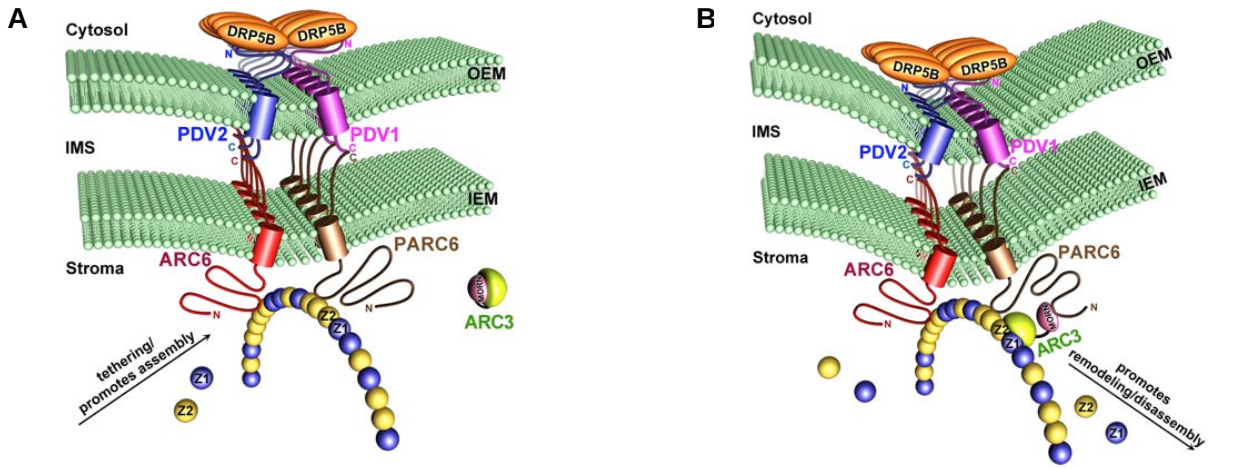

Figure 1. Working model of the chloroplast division complex emphasizing the roles of PARC6 and ARC6. **(A)** The topology of PARC6 in the IEM is equivalent to that of ARC6. Z-ring assembly is restricted to the mid-plastid in part by ARC3 (Zhang et al., 2013). The Z ring is tethered to the IEM by ARC6 through direct interaction with FtsZ2 in the stroma. ARC6 positions PDV2 to the division site in the OEM through direct interaction of their IMS regions. PARC6 functions similarly downstream of ARC6, interacting with FtsZ2 in the stroma and positioning PDV1 at the division site through direct interaction in the IMS. PDV1 and PDV2 independently recruit DRP5B from the cytosol to the OEM, but both are required for full DRP5B contractile activity. Thus PARC6 and ARC6 coordinate the FtsZ and DRPB5B rings across the envelope membranes, enabling them to function together to constrict the chloroplast. **(B)** PARC6 recruits ARC3 to the division site, possibly during constriction. PARC6 binds to a region of ARC3 called the MORN domain, allowing ARC3 to interact with the Z ring. This interaction may facilitate Z-ring remodeling and disassembly during constriction. Other details omitted for simplicity are reviewed in Osteryoung and Pyke, 2014. N, N-terminus; C, C-terminus; Z1, FtsZ1; Z2, FtsZ2. Note that proteins shown are not meant to represent stoichiometric ratios, as these have not been established. From Zhang, Chen et al., submitted.

Science objectives for 2015-2016:

- Determine the functional roles of selected regions of ARC3, including the MORN domain (Fig. 1), in regulating Z-ring and division-site positioning in chloroplasts.
- Confirm that the Cvi-1 *FtsZ2-2* allele is responsible for the Cvi-1-like chloroplast morphology in the affected NILs. If so these results would suggest that allelic variation in *FtsZ2-2* contributes to natural variation in chloroplast size and number in Arabidopsis.

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Two Redoxin Proteins Are Involved in the Maintenance of Photosystem Stoichiometry in the Cyanobacterium *Synechocystis* sp. PCC 6803

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Overall research goals: The research objectives of this project are to discover redox-related protein factors and study the role of these factors in maintaining and/or protecting the photosynthetic apparatus in cyanobacteria.

Significant achievements 2015: We found two proteins containing thioredoxin folds in *Synechocystis* 6803 that are involved in the maintenance of photosystem I and II (PSI and PSII) stoichiometry. Inactivation of one of these, Slr1796, specifically reduced PSI content and activity (Fig. 1A-C), but phycocyanin (PC) and PSII contents were unaffected. Slr1796 is involved in the regulation of PSI complex biogenesis at a post-transcriptional level, and it appears that this regulation occurs at a late step during PSI assembly. The deletion of another redox-related gene, *sll0685*, resulted in significant loss of both PC and chlorophyll (Fig. 1D). Blue native PAGE analysis showed PSI content also largely reduced (Fig. 1E). PSII-mediated oxygen evolution rate and room temperature chlorophyll fluorescence from the Δ*sll0685* mutant were similar to those of WT cells. These findings describe new redox-related protein factors that determine the stoichiometry of photosystems in the photosynthetic apparatus.

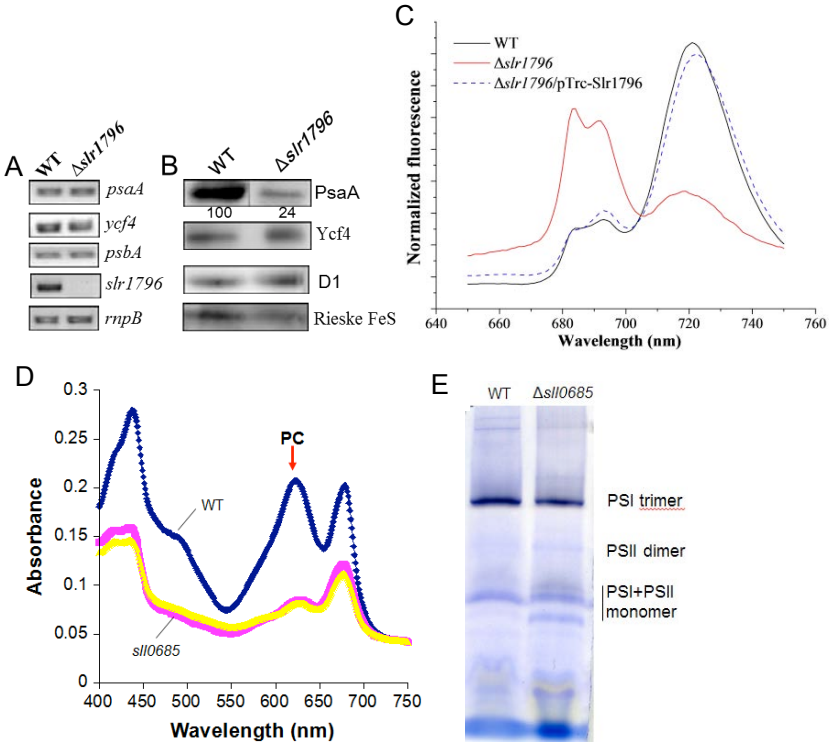

Figure 1. (A) Transcript and (B) protein levels for representative PSI and PSII proteins in WT and Δ*slr1796* strains. (C) 77K Chlorophyll *a* fluorescence measurements in WT, Δ*slr1796*, and the *slr1796*-complemented strains. (D) Whole cell absorption spectra showed that on a cellular basis Δ*sll0685* mutant has both lower phycocyanin (625 nm peak) and chlorophyll (676 nm peak) compared to WT. (E) Blue native PAGE analysis of thylakoid proteins in WT and Δ*sll0685* mutant.

Science objectives for 2015-2016:

- Our findings have described the importance of thioredoxin fold-containing proteins in regulating the composition of the photosynthetic apparatus and we are characterizing this family of proteins.
- During the next six months, we will complete our analysis of both of these proteins, which will result in the preparation of two manuscripts.

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Proteins in Control of Optimal Pathways for Proton-Coupled ET

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Overall research goals: Our natural photosynthetic research focuses on resolving fundamental mechanisms of photochemical energy conversion in photosynthetic proteins and using this information in the design of bio-inspired materials for solar fuels production. The efficient charge separation that occurs within reaction center (RC) proteins is the most important step of photosynthetic solar energy conversion. The experimental approach includes the application of a suite of advanced, multi-frequency, time-resolved magnetic resonance techniques together with capabilities to prepare specialized RC samples.

Significant achievements 2013-2015:

Directionality of Electron Transfer in Type I Reaction Center Proteins. Both Type I and Type II RCs contain two branches of potential electron transfer chains, labeled A and B (see Figure 1). In Type II RCs, light-driven primary electron transfer (ET) reactions take place exclusively through the A-branch of redox-active components (unidirectional ET). Unlike Type II RC, the directionality of ET in the Type I RC has been a long debated question. Recently, using advanced high-frequency time-resolved EPR methods, we were the first to definitively prove that under strongly reducing conditions ET in PS I proceeds down both nearly symmetrical cofactor branches. To prove that the reduction condition of the PSI preparation does not influence our conclusion on directionality of ET in PSI and to clarify the degree of the ET asymmetry, we have spectroscopically characterized biochemically modified PSI RCs wherein the terminal acceptor iron-sulfur centers, F_A/F_B, and F_X, have been sequentially removed to prevent secondary ET from phylloquinones (A_1) to F_X (Figure 1). For these modified RCs, we find that ET occurs along both A- and B- branches and the ratio of ET through the A- and B- branches is close to 1.

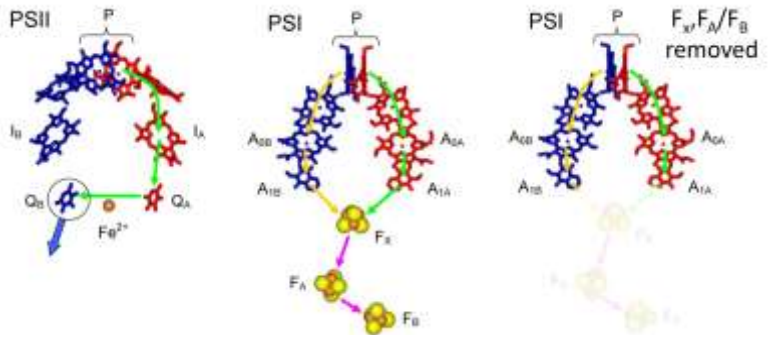

Figure 1 Schematic structure and ET pathways in Type II (left), Type I (center), Type I with iron sulfur complexes removed (right) RCs. Red – A-branch, Blue – B-branch.

Imaging Proton-Coupled Electron Transfer Pathways. Owing to transient features of the TR-ENDOR technique, the spin density distribution can be followed in time and provides important information about the protein environment's response to photoinduced ET. Spectral analysis of the ^{2}H-ENDOR of deuterated Q_A substituted into protonated purple bacterial RCs demonstrates that positions of the ENDOR lines, recorded shortly after ET, do not coincide with the positions of the same peaks in the thermalized spectrum. We provide direct spectroscopic evidence that ET induces a small-scale reorganization at the level of the global H-bonding network. Orientational dependence of the matrix proton TR-ENDOR spectra of deuterated purple bacteria RCs in deuterated buffer allows us to identify a number of exchangeable protons involved in the ET process. These protons are located close to the primary acceptor pheophytin and belong to Trp100L and Glu104L amino acid residues. This finding supports our previous

hypothesis that matrix relaxation responsible for the ET regulation in the bacterial RCs occurs around the pheophytin cofactor.

Mimic of PCET at the TyrD Residue of Photosystem II. Our previous studies of the proton-coupled ET (PCET) in PSII at cryogenic temperatures where both proton and protein motions are limited, demonstrate that electron transfer, proton movement and associated conformational changes of the Tyr*D*-binding pocket are individual steps of the PCET reaction. In collaboration with researchers from ASU we designed and spectroscopically characterized bioinspired organic/inorganic hybrid systems able to mimic intricate protein/radical interactions that ultimately allow the generation of highly energetic but long-lived radicals through PCET reactions, thereby mimicking a key step in in water oxidizing catalysis in PSII (Figure 1). This is a challenging step in the development of highly efficient artificial photosynthetic devices.

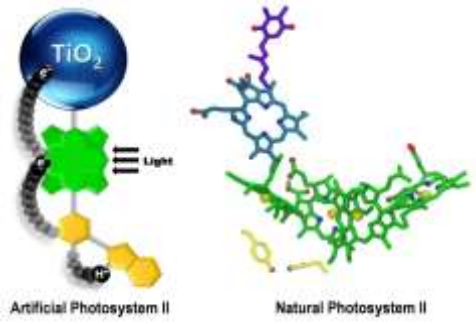

Figure 2. Schematic presentation of bioinspired hybrid system (left) that mimics PCET water oxidizing reactions in PSII (right).

Science objectives for 2015-2016:

Based on our previous work we will be using forefront time-resolved multi-frequency EPR capabilities to characterize the structure (docking place) of the novel protein-catalyst biohybrids for H_2-productions as well as PCET reactions in these systems. For structure characterization the double electro-electron resonance in combination with technique of magnetic triangulation will be used. PCET processes will be monitored by following the light-induced transformation of the redox states of the RC's cofactors and molecular catalyst.

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The Homodimeric Type I Reaction Center of *Heliobacterium modesticaldum*

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Overall research goals: Our research objectives are to understand the structure, function and organization of the homodimeric Type I reaction center (RC) found in *Heliobacterium modesticaldum* (HbRC). Our goals are to answer the following questions: (i) What are the important functional characteristics of the inter-polypeptide F_X [4Fe-4S] cluster that serves as the terminal electron acceptor? (ii) Which proteins can serve as electron acceptors from F_X? (iii) What is the role of the loosely bound menaquinone in electron transfer within the RC and can it serve as a terminal electron acceptor? (iv) What are the functional properties of the HbRC in which the major bacteriochlorophyll (BChl) *g* pigment has been converted to chlorophyll *a*? (v) What are the 3-dimensional structures of the HbRC core, and the PshB1 and PshB2 F_A/F_B-containing polypeptides? The overall idea is to 'jump start' the study of a simple Type I reaction center, so that with the acquisition of high-resolution X-ray crystal structures, mutagenesis techniques, biochemical protocols, and biophysical tools, structure/function studies can be carried out on a simplified reaction center that works at the reducing end of the biological redox scale.

Significant achievements 2013-2015:

(1) Characterization of the F_X [4Fe-4S] cluster. We had previously shown that the reduced F_X cluster has an unusual EPR signal that was interpreted as an $S = 3/2$ ground state. This $g \sim 5$ signal has been confirmed in both HbRC cores reduced in the dark in strongly reducing conditions and in membranes illuminated under weakly reducing conditions. We have measured the reduction potential of the F_X cluster by EPR and optical spectroscopic techniques and determined that it is –500 mV (vs. NHE), which is much less reducing than the F_X cluster of Photosystem I (PSI).

(2) Work on heliobacterial ferredoxin-like proteins. We have purified recombinant versions of four candidate ferredoxin-like proteins predicted from the genome of *H. modesticaldum*. Two of these, PshB1 and PshB2, are highly expressed and probably serve as the major electron acceptors of the HbRC. They have reduction potentials near -450 to -500 mv (vs. NHE). We also studied two polypeptides, HM_2505 and FdxB, that are expressed at a lower level. FdxB is in the nitrogenase gene cluster and likely plays a role in N_2 fixation. The HbRC appears to be capable of using all four ferredoxins as electron acceptors *in vitro*.

(3) Demonstration that the HbRC can use menaquinone as a terminal electron acceptor. Illumination of heliobacterial membranes in the presence of ascorbate results in reduction of menaquinone (MQ) to menaquinol (MQH_2). There seems to be a competition between the light-driven reduction of the MQ pool and the oxidation of MQH_2 by the cyt b_6c complex, which determines the steady-state level of quinone reduction. This indicates that the HbRC is a functional hybrid between PSI and the type II RCs.

(4) Conversion of a homodimeric HbRC into a functional heterodimeric HbRC. In the presence of light and O_2, BChl *g* and BChl *g'* are converted at the same rate into 8^1-OH-Chl a_F and 8^1-OH-Chl a'_F (where F is farnesyl). The loss of P_{800}^+ photooxidiation and flavodoxin reduction are not linear with the loss of BChl *g* but instead obey a model in which both the BChl *g*′/BChl *g*′ and the BChl *g*′/Chl a'_F special pairs are functional, but the Chl a'_F/Chl a'_F special pair is not. At 70% conversion of BChl *g*, the functional RCs consist of a 4.2:1 ratio of Chl *a*′/BChl *g*′ special pairs relative to Chl

a′/Chl *a′* special pairs. It is therefore statistically possible to obtain a majority population of HbRCs with heterodimeric special pairs by selective pigment oxidation.

(5) Crystallization of the Heliobacterial RC. We have extended our purification protocol for the HbRC, adding a second detergent solubilization step to obtain protein that crystallizes in a well-ordered fashion. Recent X-ray diffraction data has been obtained with a resolution in the range of 2.8 to 3.0 Å. Efforts to obtain phase information are underway.

(6) Creation of a genetic transformation system for *H. modesticaldum*. We have identified in the *H. modesticaldum* genome the major DNA methyltransferases linked to restriction endonucleases and expressed them in *E. coli*. This allows protection of plasmids before conjugation into the heliobacterium. Using this system we have successfully transformed this species with plasmids that replicate in other Firmicutes.

Science objectives for 2015-2016:

1. **Finish work on recombinant candidate electron acceptors:** measure reduction potentials and test the ability of the HbRC to reduce them.
2. **Create an *in vitro* system to monitor quinone reduction by the HbRC:** reconstitute the purified HbRC into liposomes that have been pre-loaded with MQ and attach recombinant cyt c_{553} to NTA-labeled lipids via a hexahistidine tag; use this system to study the quinone reduction mechanism by biochemical and biophysical means.
3. **Study the effect on primary charge separation when BChl *g* is converted to a Chl *a*-like molecule:** carry out high-field pulsed EPR and ENDOR studies on solutions and single crystals of the HbRC to determine the electronic properties of the unique Chl *a′*/BChl *g′* special pair.
4. **Obtain structure of the HbRC core by X-ray crystallography:** obtain phasing data by anomalous X-ray scattering from Fe or other methods; use this to produce a model of the HbRC
5. **Produce stable deletion mutants in *H. modesticaldum:*** use the CAS9/CRISPR system to create gene deletions of several target genes (*pshA, pshB1, pshB2, mqnA, etc.*).

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Toward time-resolved circular dichroism spectroscopy of photosynthetic proteins: accessing excitonic states

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

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

Few femtosecond and nanosecond TRCD spectrometer designs have been proposed recently, but their application to physical research has been typically limited to few test samples that possess strong CD properties. We will build on the current knowledge and advance the TRCD technique to measure *weak* CD signals and apply this technique to solve long standing scientific problems in photosynthesis.

Significant achievements 2014-2015:

1) The study of triplet excitonic states of closely spaced pigments has been conducted both experimentally and theoretically. Several artificial dimers and multimers of chlorophyll and chlorophyll-like molecules have been constructed and their triplet excitonic energies were characterized via weak phosphorescence, measured by ultra-sensitive time-gated phosphorescence spectrometer developed in our laboratory in the previous DOE grant period. The results were compared with excitonic model, where triplet-triplet (T-T) couplings for each system were calculated from known structures via overlap of molecular orbitals. The results revealed, that (i) the T-T coupling between two Chl-like molecules depends dramatically on the mutual orientation of the molecular planes of the two interacting molecules facing each other. In an extreme case the interaction fluctuates around zero as one molecular plane is slid across the plane of the other molecule, while in the other case the interaction monotonically increases with the molecular overlap area. For example, T-T coupling between the RC special pair in PSII is about 50% larger, than that in PS I, in spite of the fact that special pair molecules in PSI are closer to each other, than in PS II (4.7 Å for PSI and 7.4 Å for PSII). Both experiment and theory also suggest that environmental shift of triplet state energy is often larger or on par with the excitonic energy shifts even for very closely spaced molecules. A comprehensive manuscript on these results is in preparation.

2) In line with the time plan for the 2014-2015 year, a nanosecond TRCD spectrometer was built and tested, producing first transient CD kinetics associated with triplet-triplet energy transfer within

FMO complexes (Figure 1). Several changes have been introduced to the originally proposed design to achieve that goal, and few more will be introduced to enhance its performance.

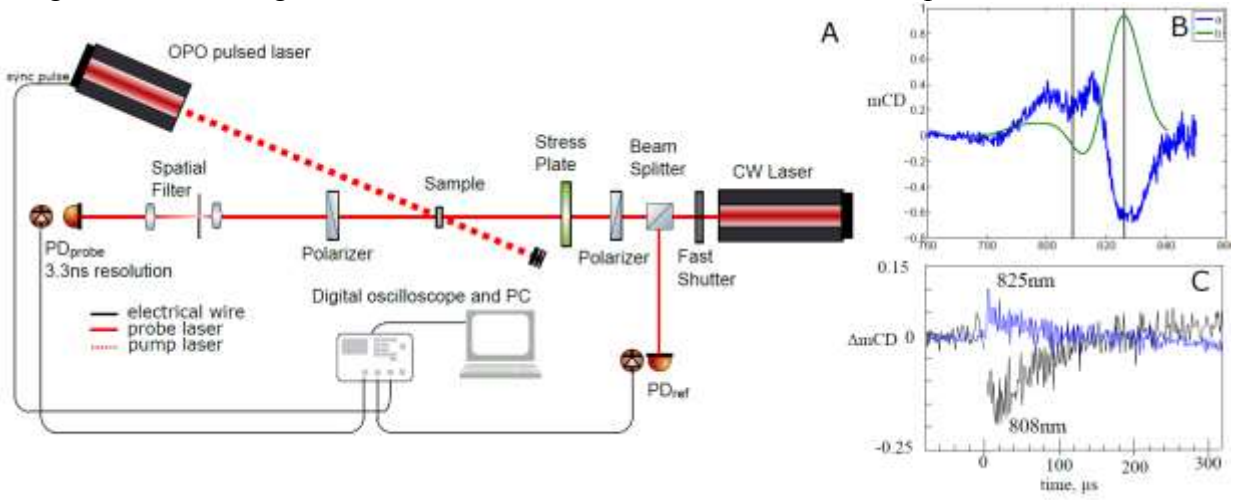

Figure 1. (A) The simplified schematic of the nanosecond TRCD spectrometer. (B) The measured steady state CD spectrum of the FMO in ground state (a) and the expected difference CD spectrum (b) calculated in the model where pigment #3 in FMO is in triplet state. (C) The measured TRCD signals upon exciting FMO at 600 nm probed at 808 and 825 nm have opposite signs and reveal two "rise" kinetics (1μs and 10 μs) followed by 50μs decay.

3) The program for computer simulation of expected TRCD signals for triplet excites states has been compiled for future analysis of the data (see, for example, smooth trace b in Figure 1B).
4) A dedicated Ti:S laser system was built and the work began on construction of the femtosecond TRCD with dual beam modulation that will be able to detected CD signal changes on the order of 10^{-6}.

Science objectives for 2015-2016:

- The nanosecond TRCD spectrometer will be further improved by introducing electronic CD compensator instead of the current mechanical one, which will significantly improve S/N and the reproducibility of magnitude of the TRCD traces measured at different wavelengths;
- The FMO TRCD will be measured across the exciton spectrum and compared with the proposed excitonic structures of FMO;
- The femtosecond TRCD spectrometer will be built and applied to a test system - FMO complex;
- The work on direct detection of primary charge separation in PS I RC will be initiated.

References to work supported by this project 2014-2015:

1. Jankowiak R, Savikhin S. Mechanism of Primary Charge Separation in Photosynthetic Reaction Centers. In: The Biophysics of Photosynthesis, eds. John Golbeck and Art van der Est, Springer New York, (2014)
2. Chauvet A, Jankowiak R, Kell A, Picorel R, Savikhin S. Does the Singlet Minus Triplet Spectrum with Major Photobleaching Band Near 680–682 nm Represent an Intact Reaction Center of Photosystem II? J. Phys. Chem. B, pp 448–455, (2015)
3. S Kihara, DA Hartzler, GS Orf, RE Blankenship, S Savikhin. The Fate of the Triplet Excitations in the Fenna–Matthews–Olson Complex. The Journal of Physical Chemistry B 119 (18), 5765-5772 (2015)

The Glucose 6-Phosphate Shunt Around the Calvin-Benson Cycle

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Overall research goals: The research objective is to study regulation of carbon metabolism to identify mechanisms used by plants to optimize the storage of energy as reduced carbon. The current focus is on the glucose-6-phosphate shunt in which carbon that is expected to go through the non-oxidative branch of the pentose phosphate cycle instead goes through the oxidative branch. The sources of carbon for the G6P shunt are stromal phosphoglucoisomerase (PGI) and the glucose 6-phosphate transporter (GPT2). It is hypothesized that the oxidative branch of the pentose phosphate pathway, the G6P shunt, normally occurs at about 15% of the rate of the non-oxidative branch. The shunt may help stabilize photosynthesis but at the cost of three ATP per carbon going through the shunt. This increases the ATP deficit and could lead to cyclic electron flow (CEF).

Significant achievements 2014-2015: Tools for studying the two sources of carbon for the G6P shunt have been built. The stromal PGI is under investigation by measuring the Km for F6P in one direction and G6P in the other. Measuring in the direction of F6P to G6P was found to be problematic because the assay generates 6-phosphogluconate, a suspected inhibitor of PGI. Adjusting the assay conditions has allowed us to now make reliable measurements with crude extracts. To separate possible post-translational effects we have had the Arabidopsis stromal PGI gene synthesized and will express it in *E. coli*.

The other source of G6P for the stroma is the GPT2 transporter. GPT2 gene expression is highly variable, being induced after a change to high light or high CO2. We have confirmed that putting plants into high light causes a significant increase in message for GPT2. However, if the CO_2 concentration is dropped to force plants into negative carbon balance because of photorespiration when plants are put into high light, the message level for GPT2 declines. This was unexpected, we had hypothesized that message level would increase helping increase carbon flux into the stroma.

We are engineering plants to control the expression of GPT2 (Table 1). We have begun using experiments with the constitutively on and off plants. We

Promoter	Gene	Regulation	Status
CaMv 35S	GPT2	Constitutive on	5 lines in T3
Alc-Ind	GPT2	Inducible on	
CaMv 35S	GPT2 RNAi	Constitutive off	
Alc-Ind	GPT2 RNAi	Inducible off	

Table 1. *Arabidopsis* lines under construction for studying the role of GPT2 in regulating and stabilizing photosynthesis. The Alc-Ind promoter system consists of a CaMV promoter-AlcR that codes for an ethanol receptor protein and AlcA, a promotor that responds to AlcR bound to ethanol (Weise et al., 2012).

Fig. 1. Wild-type Col-0 and three lines transformed with GPT2 expressed by a 35S promoter two days after being switched from 30 to 80 μmol m^{-2} s^{-1}.

found that putting constitutively on plants in a light intensity significantly above that at which they were grown causes severe stunting and anthocyanin accumulation (Fig. 1). In addition, plants constitutively expressing GPT2 accumulate starch throughout their life (Fig. 2).

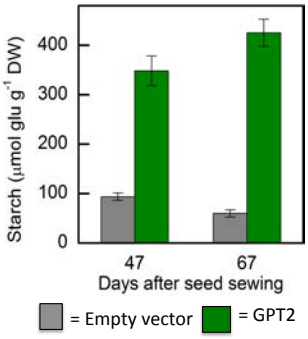

Fig. 2. Starch measured in the afternoon in empty vector controls and plants expressing GPT2 on a 35S promoter.

Finally, we tested whether GPT2 overexpression increased the rate of cyclic electron flow (CEF) in order to supply additional ATP used in the futile cycle. Two methods for assessing CEF agreed that CEF is greater in GPT2 overexpressing plants than empty vector plants. In collaborative work with Drs. Deserah Strand and David Kramer we have shown that the *hcef*1 mutant that has high cyclic electron flow and lacks stromal fructose bisphosphatase (FBPase) has elevated expression of GPT2. We hypothesize that the loss of stromal FBPase is compensated by export of TP to the cytosol, conversion to G6P, and import of G6P through the GPT2. While this allows some non-oxidative branch pentose phosphate pathway (net CO_2 fixation) it also stimulates the oxidative branch leading to loss of ATP, which is compensated by cyclic electron flow.

Science objectives for 2015-2016:

- Metabolomics - Measure G6P levels in the stroma by non-aqueous fractionation
- Determine the properties of PGI and glucose-6-phosphate dehydrogenase (G6PDH) – There are preliminary indications that these enzymes have unusual properties that regulate the G6P shunt. Activities will be determined in crude extracts and in proteins made from plant genes expressed in *E. coli*.
- Engineer plants with unregulated PGI – This will test whether PGI is kinetically limiting during photosynthesis and what are the consequences when too much F6P is converted to G6P.
- Metabolic flux analysis – This will be aimed at finding the slow labeling pool of carbon, we will specifically test the hypothesis that maltodextrin made during starch synthesis is the slow-to-label carbon pool. We will make a double mutant Starch phosphorylase KO plus isoprene emission so that isoprene can be used as a readout of the degree of labeling of Calvin-Benson cycle intermediates.

Sharkey TD (2015a) Understanding carbon partitioning and its role in determining plant growth Plant Cell Environ **in press**

Sharkey TD (2015b) What gas exchange data can tell us about photosynthesis. Plant Cell Environ **in press**

Weise SE, Aung K, Jarou ZJ, Hardy AC, Carr DJ, Sharkey TD (2012) Engineering starch accumulation by manipulation of phosphate metabolism of starch. Plant Biotechnology Journal **10:** 545-554

Weise SE, Carr DJ, Bourke AM, Hanson DT, Sharkey TD (2015) The *arc* mutants of *Arabidopsis* with fewer large chloroplasts have a lower mesophyll conductance. Photosynth Res **124:** 117-126

RNA Quality Control in the Chloroplast

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Overall research goals: The research objectives are to understand the functions and regulation of novel RNAs expressed from the chloroplast chromosome by: (1) characterizing the ribonucleases that shape the chloroplast RNA population; and (2) functionally characterizing antisense and noncoding RNAs (asRNA, ncRNA). The chloroplast chromosome is fully transcribed on both its strands and RNases are required to eliminate deleterious or nonfunctional RNAs; failure to do so compromises photosynthesis and can cause embryo lethality. High-throughput techniques have identified a large population of RNAs that were previously unstudied. These may form double-stranded structures with regulatory properties, or encode novel proteins that act in photosynthetic pathways.

Significant achievements to date: This phase of our project began at a prior PI meeting, and led to the identification of more than 100 ncRNAs. Subsequent approaches validated functions for two of them, both related to maturation of chloroplast ribosomal RNAs, one being a positive regulator and the other a negative regulator. Because many of the ncRNAs were transcribed on the opposite strand to known genes, they have the potential to form sense-antisense RNA duplexes. The impact of such base pairing was demonstrated dramatically when the chloroplast ribonuclease RNase J was repressed: double-stranded RNA accumulated to very high levels and as a result, translation of sense RNAs was impeded. Thus, the chloroplast must use quality control to ensure that only desired asRNAs accumulate. Further investigation of ncRNAs, coupled with an independent genome-scanning approach, suggests that some "non-coding" RNAs, as well as previously unstudied regions of the chloroplast genome, may encode novel proteins. Confirmation of these results is a future objective. Finally, all the above analysis depended on the ability to analyze large transcriptome datasets effectively. For this purpose, we have created a user-friendly bioinformatic pipeline optimized for the concurrent analysis of both chloroplast transcripts, including splicing, editing and 5'/3' end maturation.

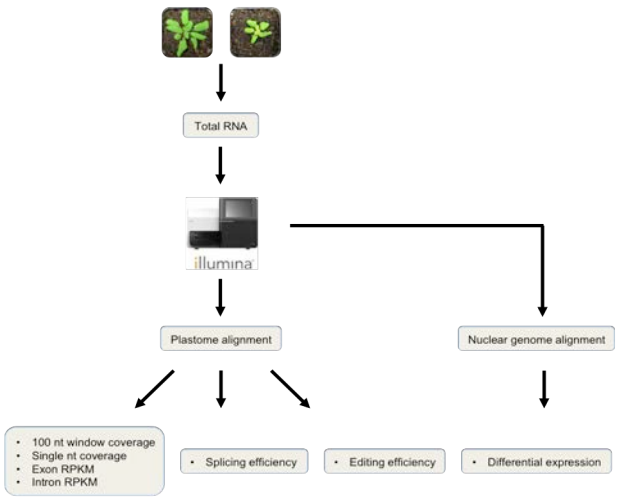

Fig. 1. RNA-Seq pipeline. The flow chart illustrates RNA isolation, sequencing, alignment of chloroplast and nuclear reads, and assessment of expression characters using customized scripts.

Science objectives for 2015-2016:

- Analysis of numerous transcriptomes has revealed that the chloroplast RNA population, including ncRNAs, is particularly responsive to heat stress, which also has a dramatic effect on photosynthesis. We will complete the characterization of this response, which will allow a future functional focus on the most highly regulated transcripts.
- Preliminary findings that the chloroplast may encode more proteins that previously thought must be supported by data proving that these proteins are in fact translated and accumulate. Ribosome footprinting and proteomic techniques will be applied to validate at least some of these candidates.

References to work supported by this project 2011-2015:

1. Hotto A, Schmitz RJ, Fei Z, Ecker J, Stern DB (2011) Unexpected diversity of chloroplast non-coding RNAs as revealed by deep sequencing of the Arabidopsis transcriptome. *Genes Genomes Genet.* 1:559-570.
2. Germain A, Kim D, Gutierrez R, Stern DB (2012) RNase II preserves chloroplast RNA homeostasis by increasing mRNA decay rates, and cooperates with polynucleotide phosphorylase in 3' end maturation. *Plant J.* 72:960-971.
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5. Castandet B, Hotto AM, Fei Z, Stern DB (2013) Strand-specific RNA sequencing uncovers chloroplast ribonuclease functions. *FEBS Lett.* 587:3096-3101.
6. Hotto AM, Castandet B, Gilet L, Higdon A, Condon C, Stern DB (2015) *Arabidopsis* chloroplast Mini-Ribonuclease III participates in rRNA maturation and intron recycling. *Plant Cell* 27:724-740.

Mechanism of Protein Transport on the Twin Arginine Translocation Pathway

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Overall research goals: The research objectives are to distinguish between two competing models of the mechanism through which proteins are transported across the thylakoid membrane on the Tat pathway by: (1) testing the proteinaceous pore model by measuring the amount of Tha4 required to transport proteins of different sizes, and exploring the consequences of transport under Th4-limiting conditions; (2) Testing the membrane defect model by examining the dependence of the transport reaction on the negative hydrophobic mismatch found in trans-membrane helices in Tha4, Hcf106 and cpTatC, and by examining treatments known to cause thinning of the thylakoid membrane for their ability to stimulate protein transport on the Tat pathway and *to cause a decrease in the pmf energy required for such transport to occur; (3)* sep arating the energy required for assembly of the translocon and from that required for protein translocation. Resolution of the mechanism of Tat protein transport, which takes as substrates proteins critical for photosynthetic light reactions, wi ll direct future research into this pathway toward a fuller understanding of the role of membrane biophysical properties in affecting chloroplast biogenesis and homeostasis.

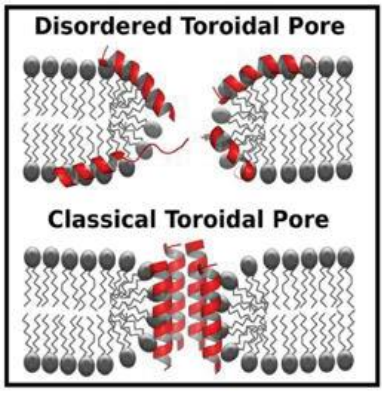

Figure 1. Structures of toroidal pores formed under the influence of CPPs (red).

Significant achievements 2013 – 2015: Our initial investigations into the mechanism of action of the Tat translocon focused on the prediction that protein transport on this unique pathway went via toroidal pores (Fig. 1), and so should be stimulated by arginine-rich, cell-penetrating peptides. This is indeed the case (Fig. 2), and such stimulation is shown to be accomplished by a decrease in the energy required to affect translocation (Fig. 3). We additionally also showed that stimulation of the Tat pathway could be affected by addition of the amphipathic region of one of the translocon subunits

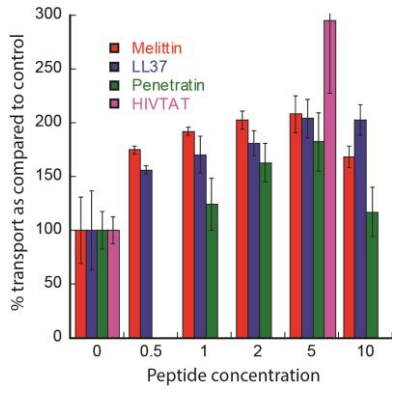

Figure 2. Stimulation of transport of iOE17 across the thylakoid membrane on the Tat pathway by cell penetrating peptides.

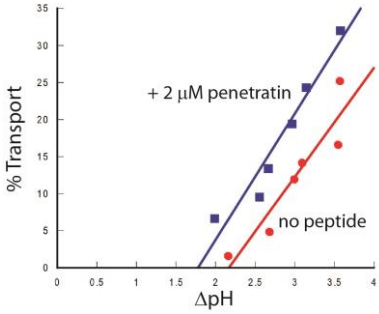

Figure 2. Penetratin lowers the energy threshold for thylakoid transport of iOE17. ΔpH was measured with NED for 6 min, and then the sample was analyzed for protein transport.

(Fig. 3). These experiments are consistent with the Tat pathway operating via non-proteinaceous, toroidal pores.

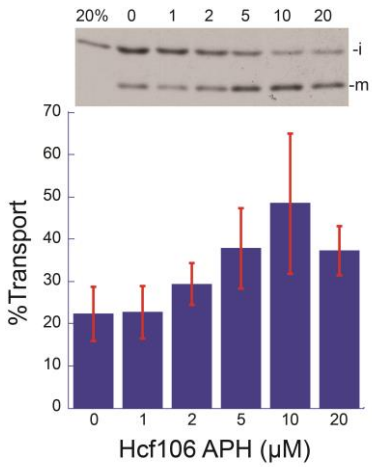

Figure 3. Stimulation of iOE17 transport by the APH of Hcf106 at the concentrations indicated.

Science objectives for 2015 – 2017:

- We will to test a central tenet of the proteinaceous pore model of Tat transport that requires proteins of different sizes to be translocated by a pore made of different numbers of Tha4 subunits. Should this prediction not be borne out by experiment, the proteinaceous pore mode will necessarily be retired.
- We will test a key part of the toroidal pore model of Tat transport that states that membrane thinning, a prelude to toroidal pore formation, is set up by hydrophobic mismatch in the short transmembrane anchors of the translocon subunits Tha4 and Hcf106. We will also continue our exploration of other mechanisms of membrane thinning and their effect on Tat transport.
- We will perform detailed measurements of the energetic requirements for the assembly of the Tat translocon and subsequent translocation. The methodology used will be based on those developed earlier in the lab to calculate the Gibbs free energy requirement for Tat protein transport.

<u>References to work supported by DOE funds 2013 – 2015</u>:

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4. Wang, X, Wei, Y., Shi, L-X., Ma, X and Theg, S.M. (2015) New Isoforms and Assembly of Glutamine Synthetase in the Leaf of Wheat (*Triticum aestivum L.*). J. Exp. Bot. *in press*, published on line August 24, doi:10.1093/jxb/erv38

Site-Selective Characterization of Plastocyanin with Infrared Spectroscopy

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Overall research goals: Unlike electron transfer between small molecules, biological electron transfer occurs between redox centers imbedded within proteins with environments that are structurally and electrostatically heterogeneous and undergo fluctuations on a broad range of timescales, challenging its rigorous characterization with currently available techniques as they are incapable of resolving dynamics on a sufficiently fast timescale and/or of specifically probing different parts of the electron transfer complexes. Toward addressing this problem, we draw upon the inherent high temporal resolution of linear and multidimensional IR spectroscopy with the spatial resolution afforded by the site-selective incorporation of frequency-resolved vibrational reporter groups, including carbon deuterium (C-D) and cyano (CN) moieties, to characterize the local environments, conformations, and dynamics at specific parts of proteins to elucidate their contribute to the binding and subsequent electron transfer between redox proteins.

Significant achievements 2013-2015: We have characterized the Cu site of plastocyanin (Pc) by unique incorporation of C-D bonds at the Met97 ligand and showed the vibrations to be highly sensitive to changes at the Cu center. We then used IR spectroscopy to characterize Pc bound to its physiological electron transfer partner, cytochrome (cyt) *f*, and observed spectral changes that suggest formation of a stronger Cu-Met97 interaction. To extend our studies toward understanding the role of dynamics in the activity of Pc with cyt *f*, we have site-selectively incorporated CN probes at four sites in Pc and are applying 2D IR spectroscopy to measure the local dynamics and generate a position-dependent model of their involvement in binding to cyt *f*. Concurrently we are working toward developing new, better IR probes for multidimensional IR experiments of proteins, and have shown that isotopic labelling increases vibrational lifetime of the CN probes, extending the experiments' dynamic range.

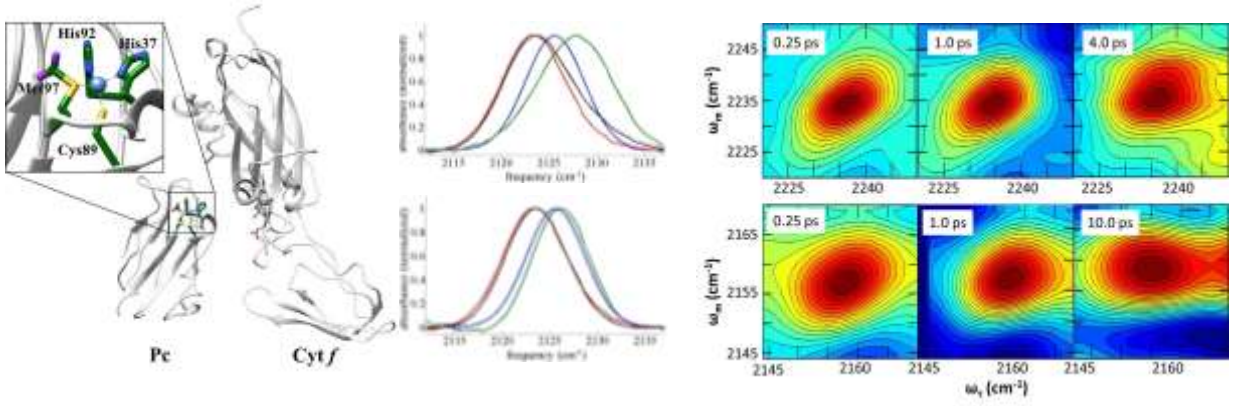

Figure 1. Left Panel: structural model of Pc and cyt *f* complex. Inset: Cu site of Pc showing in purple location of C-D bonds incorporated at Met97. PDB 1TU2. Middle Panel: Top: FT IR spectra of d_3Met97 in Cu(II) unbound (blue), Cu(I) unbound (red), Cu(II) bound (green), and Cu(I) bound (black) Pc. Bottom: FT IR spectra of d_3Met97 in Cu(II) unbound (blue), Cu(I) unbound (red), Zn substituted Pc (green) and Cu(I) reduced at pH 4 (black). Right Panel: time-dependent 2D IR spectra of CNPhe36 (top) and $^{13}C^{15}N$Phe36 (bottom) Pc.

Science objectives for 2015-2016:

- Complete 2D IR studies of selectively CN-labeled Pc variants and determine the changes in dynamics along interface upon binding to cyt f.
- Initiate non-equilibrium, visible-pump, 2D IR studies of Pc to probe response of protein dynamics to electronic perturbation.

References to work supported by this project 2007-2009:

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Photosynthetic Biomimetic Approaches for Investigating Fundamental Mechanisms in Photosynthesis

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Overall research goals: This program investigates fundamental design principles and mechanisms for solar energy conversion in photosynthesis using a biomimetic approach. Biomimetic architectures offer opportunities to explore a greater range of structures and chemical functionalities than can be investigated with native photosynthetic proteins. A comparison of natural and biomimetic photosynthesis allows fundamental mechanisms and cross-cutting principles to be resolved. Key research targets include the investigation of photosynthetic mechanisms for coupling of solar photons-to-fuels, including the harvesting single electron excited states to drive sequential electron transfer, charge accumulation, and multi-electron water-splitting and solar fuels catalysis.

Significant achievements 2013-2015: We have developed a photosynthetic biomimetic approach that exploits a family of multi-heme c-cytochrome "molecular wires" found in the *Geobacter* genome and produced in native and engineered forms using an *E. coli* expression system. The multi-heme cytochromes are used as frameworks for testing photosynthetic design principles for coupling single electron excited states to charge accumulation and multi-electron catalysis. A recent highlight has been the biomimetic duplication of primary photosynthetic electron transfer. A universal feature of primary events in photosynthesis is the ultrafast, activationless light-induced electron transfer, which proceeds along either one or both of two symmetry-related pathways in different types of reaction centers. Using ruthenium tris-bipyridyl derivatives as photosensitizers that are covalently linked to cysteine residues placed at a variety of locations on the tri-heme cytochrome PpcA through site-directed mutagenesis, we demonstrate constructs that mimic photosynthetic ultrafast, activationless, light-induced electron transfer. In particular, we have found constructs that allow either a single electron transfer pathway, or bifurcated, dual symmetry-related pathways which function without loss of activity

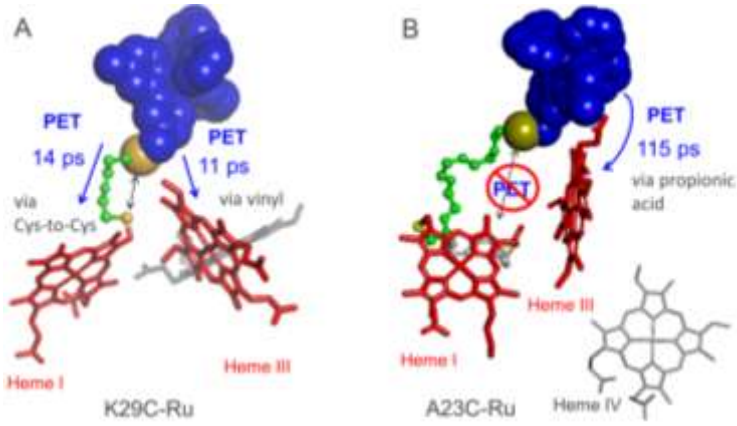

Figure 1. Representative structures from MD simulations for two PpcA constructs bearing covalently-linked ruthenium *tris*-bipyridyl, $Ru(bpy)_3$, attached at engineered cysteine residues. The figures show only the $Ru(bpy)_3$ (blue) and heme (hemes I and III red, heme IV, grey) cofactors. **Part A** shows construct K29C-Ru and **Part B** shows A23C-Ru which support bidirectional and monodirectional primary electron transfer, PET, respectively.

down to 2.8 K. This duplication of core features of primary photosynthesis offers opportunities to investigate fundamental mechanisms for the first electron transfers in photosynthesis, and to expand on these designs to create artificial biomimetic reaction centers that support charge accumulation and multi-electron catalysis.

Science objectives for 2015-2016:

- Investigate activationless, ultrafast, variable pathway electron transfer in photosynthetic biomimics using a range of inorganic, organic, and chlorin (chlorophyll-based) photosensitizers. These studies will examine the roles that the molecular structures and energetics of the photosensitizers play in determining pathways for primary light-driven electron transfer.
- Investigate activationless, ultrafast, variable pathway electron transfer in photosynthetic biomimics, using native and engineered multi-heme cytochrome frameworks with altered redox properties of the cofactors. These studies will examine the roles that the molecular structures and energetics of the electron-accepting/donating cofactors play in determining directionality of primary electron transfer in photosynthetic mimics.
- Extend on the biomimetic designs to create artificial reaction centers that support charge accumulation and multi-electron catalysis. Initial research will target multiple, sequential photo-oxidation of transition metals for oxygen evolving catalyst, OEC, assembly. Research will include the engineering of amino acid sequences in the PpcA frameworks for metal-binding and function as electron donors to linked photosensitizers.

References to work supported by this project 2013-2015:

1. O. Kokhan, N.S. Ponomarenko, P.R. Pokkuluri, M. Schiffer, K.L. Mulfort, D.M.Tiede. Bidirectional photoinduced electron transfer in Ruthenium(II)-*tris*-Bipyridyl modified PpcA, a multi-heme c-type cytochrome from *Geobacter sulfurreducens*, *J. Phys. Chem. B*, 119, 7612-7624 (2015).
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3. Nature-driven Photochemistry for Solar Hydrogen Production. L. M. Utschig, S.R. Soltau, D. M. Tiede, (2015) *Invited Article: Current Opinion in Chemical Biology, 25:1-8.*
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sulfurreducens reveals the structural origin of their functional difference. *Biochim. Biophys. Acta Bioenergetics*, 1837, 920-928 (2014).

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Photosynthetic-Inspired Systems for Solar Hydrogen Production

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Overall research goals: Natural photosynthetic energy research is aimed at resolving fundamental mechanisms of photochemical energy conversion in photosynthetic proteins. These basic studies provide us with insight into how to use Nature's optimized photochemistry to drive non-native chemical reactions. Currently, we are designing new bio-inspired systems that capture and convert the sun's energy and store it in the energy-rich bond of hydrogen, a clean, carbon-neutral and renewable energy source. Specifically, we are (1) developing new energy conversion strategies that couple the photon energy, which is efficiently captured as a stabilized charge separation across the native photosynthetic reaction center (RC), to the direct synthesis of hydrogen using synthetic first-row transition metal catalysts and (2) creating a new class of small protein-based photocatalytic complexes that replicate essential design features of RCs and enable the spectroscopic discernment of the structure and processes crucial to solar-driven proton reduction. Our combined effort at the forefront of biochemical and spectroscopic experimental approaches provides an opportunity for breakthroughs in the resolution of fundamental mechanisms for coupling photons to fuels in photosynthetic-inspired hybrid systems, a necessary step forward in the development of optimized systems for solar fuels.

Significant achievements 2013-2015:

Protein Delivery of a Ni Catalyst to PSI for Solar driven H_2 Production. We have prepared the first solar fuel hybrid that couples PSI light-driven chemistry to H_2 production using a synthetic nickel diphosphine molecular catalyst $[Ni(P_2^{Ph}N_2^{Ph})_2](BF_4)_2$. The protein environment enables photocatalysis at pH 6.3 in completely aqueous conditions using inexpensive, earth abundant elements. Additionally, we have developed a strategy for incorporating the Ni molecular catalyst with the native acceptor protein of PSI, flavodoxin. The binding pocket of flavodoxin stabilizes the Ni molecular catalyst which, in the absence of the protein environment, has low solubility and rapidly degrades in water. Photocatalysis experiments with this modified flavodoxin demonstrate a new mechanism for biohybrid creation that involves protein-directed delivery of a molecular catalyst to the reducing side of PSI for light-driven catalysis. This approach provides the potential for self-repair of the biohybrid system with a mechanism for introducing fresh catalyst to the acceptor end of PSI and creates new opportunities for solar fuel production that merges synthetic inorganic and biochemical capabilities.

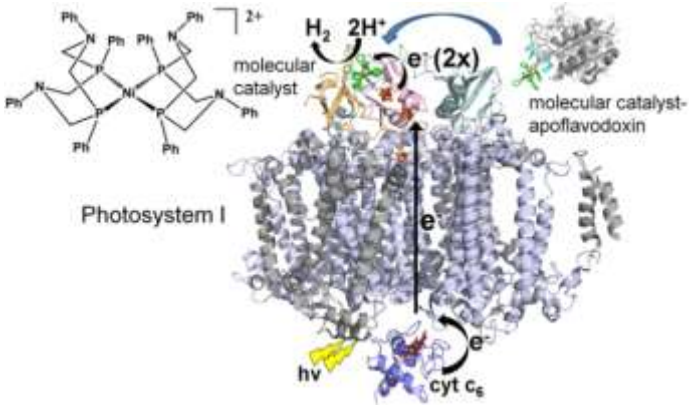

Figure 1. Photocatalytic scheme of H_2 production from a PSI-Ni molecular catalyst hybrid complex and Ni-ApoFlavodoxin.

Examination of Light-driven Electron Transfer in Processes involved in Photocatalytic H_2 Production. We have targeted the development of a mini reaction center/catalyst hybrid that enables the spectroscopic characterization and monitoring of dynamic light-induced electron transfer reactions involved in hydrogen production. Our design strategy involves a small soluble electron transfer protein, ferredoxin (Fd), as a scaffold for the controlled covalent binding of a Ru photosensitizer (Ru) and a cobaloxime catalyst (Co). The resultant complex, termed Ru-Fd-Co, is functional for hydrogen production. Electron paramagnetic resonance (EPR) and transient optical spectroscopic experiments provide direct evidence of a long-lived (>1.5 ms) Ru(III)-Fd-Co(I) charged separated state formed via an electron relay through the Fd [2Fe-2S] cluster, initiating the catalytic cycle for $2H^+ + 2e^- \rightarrow H_2$.

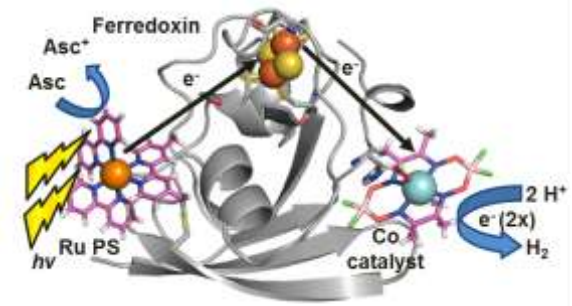

Figure 2. Photocatalytic H_2 production scheme in a Ru-Fd-Co biohybrid with electron transfer from PS to catalyst via the [2Fe-2S] cluster of Fd.

Science objectives for 2015-2016:

- PSI has proven to be an excellent photochemical module, and we will continue our studies of PSI-molecular catalyst constructs and electron carrier protein-based strategies to connect PSI photochemistry to molecular catalyst hydrogen production: (1) comparing delivery of catalysts to the acceptor docking site of PSI via covalent (Fd) vs. non-covalent (flavodoxin) interactions, (2) exploring opportunities for self-repair of the photocatalytic system, and (3) spectroscopically characterizing dynamic interprotein-catalyst ET pathways.
- A new direction involves examining the self-assembly of catalysts to PSI within thylakoid membranes. A membrane environment provides opportunities for coupling reductive chemistry to intact electron transfer pathways, thereby removing the necessity of sacrificial donors.
- We will expand our new class of small protein-based photocatalytic complexes by creating complimentary flavodoxin adducts. This will allow us to spectroscopically determine the role of the [2Fe-2S] cluster in Fd, explore protein-catalyst structures and interactions, and monitor light-induced electron transfer events as related to the mechanism of H_2 production.

References to work supported by this project 2013-2015:

1. S. C. Silver, J. Niklas, P. Du, O. G. Poluektov, D. M. Tiede, L. M. Utschig, "Protein Delivery of a Ni Catalyst to Photosystem I for Light-Driven Hydrogen Production." *J. Amer. Chem. Soc.*, **2013**, *135*, 13246-13249.
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Structure and Function of Rubisco Activase from Higher Plants

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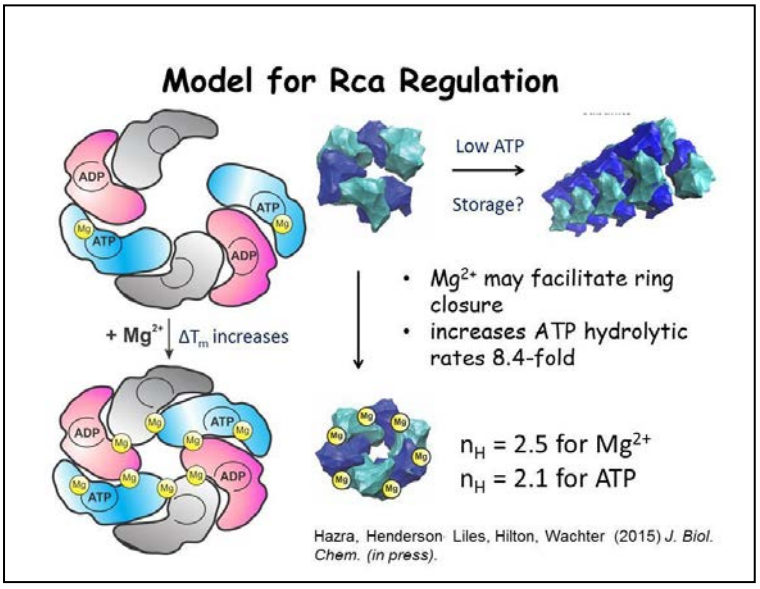
Overall research goals:

This project addresses the regulation of higher plant carbon assimilation by Rubisco activase (Rca), a chemo-mechanical motor protein essential in maintaining Rubisco activity. Rca is a ring-forming ATPase that plays a critical role in regulating the conversion of energy derived from photosynthetic electron transport into chemical storage forms. The proposed work aims to employ biophysical, structural and mechanistic approaches to answer fundamental questions in Rubisco regulation. We seek to elucidate Rca conformational states sampled during the catalytic cycle, to interrogate the physical interaction between Rca and Rubisco, and to unravel the mechanistic enzymology of ATPase and Rubisco reactivation activities. Long-term, we are interested in gaining a comprehensive understanding of the Rca-mediated mechanism of Rubisco remodeling.



Higher plant Rubisco requires the synergistic action of several enzymes to cope with self-inhibition intrinsic to turnover in an oxygenic atmosphere. With decreasing CO_2 and increasing O_2, increasing amounts of xylulose bisphosphate (XuBP) and pentodiulose bisphosphate (PDBP) are produced, compounds that resemble the substrate and prevent Rubisco carboxylation activity by obstructing its active sites. The release of these inhibitors is substantially accelerated by the chemo-mechanical action of Rca. Equally important, Rca plays a key regulatory role in the dark-to-light transition of the stroma by facilitating the release of ribulose bisphosphate (RuBP) from decarbamylated Rubisco sites. In addition, the dissociation of the nocturnal inhibitor carboxyarabinitol phosphate (CA1P) is also mediated by Rca.

This project aims to provide a significant milestone towards a comprehensive understanding of all co-regulatory mechanisms that enhance or diminish the rate of carbon fixation in higher plants. Arguably, the ultimate goal is to develop the predictive power required to re-engineer photosynthetic pathways for sustained biomass accumulation under changing climate conditions.

Significant achievements in 2014-2015:

Mechanistic enzymology: The ATPase activity of 0.005 mM tobacco Rca was monitored under steady-state conditions, and global curve fitting was utilized to extract kinetic constants. The k_{cat} was best fit by 22.3 ± 4.9 min^{-1}, the K_m for ATP by 0.104 ± 0.024 mM, and the K_i for ADP by 0.037 ± 0.007 mM. Without ADP, the Hill coefficient for ATP hydrolysis was extracted to be 1.0 ± 0.1, indicating non-cooperative behavior of homo-oligomeric Rca assemblies. However, the addition of ADP was shown to introduce positive cooperativity between two or more subunits (Hill coefficient 1.9 ± 0.2), allowing for regulation via the prevailing ATP/ADP ratio. ADP-mediated activation was not observed,

whereas larger amounts led to competitive product inhibition of hydrolytic activity. The catalytic efficiency increased 8.4-fold upon cooperative binding of a second magnesium ion (Hill coefficient 2.5 ± 0.5), suggesting at least three conformational states (ATP-bound, ADP-bound, empty) within assemblies containing an average of about six subunits. The addition of excess Rubisco (24:1, L8S8:Rca6) and crowding agents did not modify catalytic rates. However, high magnesium provided for thermal Rca stabilization. We propose that magnesium mediates the formation of closed hexameric toroids capable of high turnover rates and amenable to allosteric regulation. We suggest that in vivo, the Rca hydrolytic activity is tuned by fluctuating [Mg^{2+}] in response to changes in available light.

Rca assembly mechanism: We report a fluorescence correlation spectroscopy (FCS) study of the assembly pathway of the AAA+ protein Rubisco activase (Rca), a ring-forming ATPase responsible for activation of inhibited Rubisco complexes for biological carbon fixation. A thermodynamic characterization of simultaneously populated oligomeric states appears critical in understanding Rca structure and function. Using cotton beta-Rca, we demonstrate that apparent diffusion coefficients vary as a function of concentration, nucleotide and cation. Using manual fitting procedures, we provide estimates for the equilibrium constants for the step-wise assembly, and find that in the presence of ATPγS, the K_d for hexamerization is ten-fold lower than with ADP (~0.1 vs. ~1 micromolar). Hexamer fractions peak at 30 micromolar and dominate at 8-70 micromolar Rca, where they comprise 60-80% of subunits with ATPγS, compared to just 30-40% with ADP. Dimer fractions peak at 1-4 micromolar Rca, where they comprise 15-18% with ATPγS and 26-28% with ADP. At 30 micromolar Rca, large aggregates begin to form that comprise ~10% of total protein with ATPγS and ~25% with ADP. FCS data collected on the catalytically impaired WalkerB-D173N variant in the presence of ATP provided strong support for these results. Titration with free magnesium ions lead to the disaggregation of larger complexes in favor of hexameric forms, suggesting that a second magnesium binding site with a K_d value of 1-3 mM promotes critical subunit contacts. We propose that closed-ring toroidal hexameric forms are stabilized by binding of Mg·ATP plus Mg^{2+}, whereas Mg·ADP promotes continuous assembly to supra-molecular aggregates such as spirals.

Science objectives for 2015-2017:

First, we aim to elucidate the structural features of higher plant Rca oligomers in different nucleotide-bound states. By utilizing a variety of methods commonly employed in structural biology, we hope to characterize the conformational changes required to accommodate different classes of sites within ring-like assemblies. Second, we plan to compare the assembly mechanisms of Rca proteins from different plant species in relation to their respective activities, and to monitor the physical interaction of Rca oligomers with the Rubisco client protein. Third, we aim to use methods in mechanistic enzymology to characterize subunit cooperativity and rate-limiting events in the Rca ATPase and reactivation cycles.

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The Mn_4Ca Cluster in Photosystem II and Inorganic Complexes Studied Using *In Situ* and Steady State X-ray Absorption and Emission Spectroscopy

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Overall research goals: The objective of this proposal is to understand the mechanism by which H_2O is oxidized to O_2 in Photosystem II (PS II). We propose to develop and utilize new methodologies of X-ray spectroscopy and crystallography using synchrotron and X-ray free electron laser (XFEL) sources. The focus of our studies are: 1) What are the geometric and electronic structural changes of the catalytic Mn_4C_a cluster of PS II? 2) How does the protein environment and the catalytic center interact with each other to carry out the sequential events during the four-electron water oxidation reaction, under ambient conditions? 3) Development of new X-ay spectroscopic techniques to study the natural and artificial photosynthetic systems.

Significant achievements 2013-2015:

1) We have shown using X-ray spectroscopy that the magnetic isomorphism seen in the S_2 state, and characterized by the EPR signals at g=2 (low spin, LS) and g=4.1 (high spin, HS), are well correlated with structural changes in the Mn_4CaO_5 cluster. The changes between the HS and LS states in the S_2 state are different from that seen on advancing from the S_2 to the S_3 state. These results have important implications for the mechanism of water exchange and water oxidation.
2) Polarized XAS studies have shown that the removal of Ca has a minimal effect on the structure of the Mn_4O_5 core cluster (Fig. 1). Although, Ca is required for the S_2 to S_3 transition and for O_2 evolution, it is surprising that removal of Ca does not significantly alter the structure of the Mn cluster.
3) Mn inorganic complexes relevant to the water oxidation catalyst have been investigated using *in situ* X-ray absorption and emission spectroscopy and electrochemistry. The valence to core emission spectra of a series of oxo-bridged complexes were used to show the sensitivity of this method to the protonation state of the bridged ligand atoms. A series of homologous Mn complexes in oxidation states II though IV was used to study the charge density distribution, which showed that in the higher oxidation states the charge was widely delocalized on to the ligands.

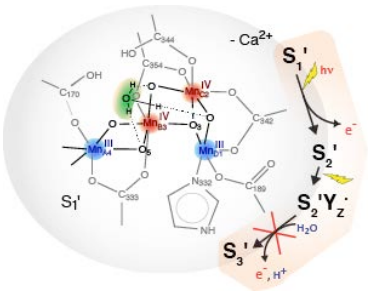

Fig.1. The proposed model for the Ca-depleted Mn_4O_5 cluster, with the kinetics of S-state transitions.

Science objectives for 2015-2016:

- We will pursue studying inorganic systems of relevance to PS II with X-ray methods, especially, *in situ* X-ray emission and absorption spectroscopy of electro/photo-chemical water-oxidation catalysts.
- We will study the solution and single-crystal Mn and Ca EXAFS studies of the S_2, S_3 and S_0 states, along with the use of newer methods for site-specific and range-extended EXAFS methods, for studying the geometric structure. The distance resolution that can be obtained from these new methods, will be combined with the XFEL data, to determine the structural changes and the mechanism of the O-O bond formation.
- High energy-resolution fluorescence detected (HERFD) XANES and high-resolution RIXS methods will be applied to PS II and inorganic models to study the electronic structure and the changes. We will also use X-ray emission spectroscopy, especially the valence to core $K\beta_{2,5}$

transitions, to study the electronic structure changes in the ligands of the catalyst during the enzymatic cycle in natural and inorganic systems.

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Taking Snapshots of Photosynthetic Water Oxidation: Femtosecond X-ray Diffraction and Spectroscopy Using X-ray Free Electron Lasers

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Overall research goals: The objective of this proposal is to understand the mechanism by which H_2O is oxidized to O_2 in Photosystem II (PS II). We propose to develop and utilize new methodologies of X-ray spectroscopy and crystallography using synchrotron and X-ray free electron laser (XFEL) sources. The focus of our studies are: 1) What are the geometric and electronic structural changes of the catalytic Mn_4C_a cluster of PS II? 2) How does the protein environment and the catalytic center interact with each other to carry out the sequential events during the four-electron water oxidation reaction, under ambient conditions? 3) Development of new X-ay spectroscopic techniques to study the natural and artificial photosynthetic systems.

Significant achievements 2013-2015:

1) We developed the method of simultaneous X-ray diffraction (XRD)/X-ray emission spectroscopy (XES) to collect time-resolved data at room temperature using XFELs. Using this method, we collected data from PS II from all the S-states. We demonstrated that we can proceed through the entire S-state cycle, including time-points between the S_3 and S_0 states. The S-state intermediates of PS II were generated by multiple visible laser excitations, with 0.5 s intervals between flashes to achieve S-state turnover. We used a similar system to measure O_2 evolution yields using $H_2{}^{18}O$ labelled water and optimized the flow rate, laser power and time interval required for S-state turnover. The Kβ emission spectra show that the Mn cluster was undamaged and advanced through the S-states. The XES data from the 250 µs time point after the S_3 state showed that the Mn cluster was more similar to the S_3 state than the S_0 state providing evidence that the Mn cluster was not yet reduced by 250 µs after the third flash indicating that the O-O bond formation is subsequent to that time (Fig. 1). The anomalous diffraction signal from Mn could be isolated from all the S-states and the transient 250 µs time point, that identifies electron density from the Mn atoms of the Mn_4CaO_5 cluster.

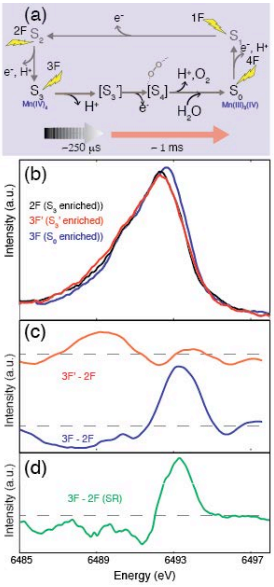

Fig. 1. The Mn Kβ emission spectra from XFEL that shows that the S_3 and S_0 spectra are similar to that from the cryogenic data collected using synchrotron radiation. The 250 µs time-point shows the first room temperature data from PS II using XFELs.

2) We collected the undamaged Mn L-edge spectrum, using the XFEL, from the S_1 state of PS II. We used a specially designed 100-element zone-plate spectrometer, that discriminates the Mn Lα signal from the overwhelming signal from the O Kα from the aqueous solution. Theoretical simulations for understanding the spectrum and the electronic structure of the Mn cluster is underway. We have made headway in collecting the spectrum from a high-spin Mn^V complex, *in situ*, using a specially designed low-temperature sample injector with an in-built reaction chamber, that will be important for answering the question of whether a high valent Mn is involved in the mechanism of O-O bond formation.

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

XFEL data requires fewer diffraction images than previously expected. We have developed a new drop on demand acoustic transducer-based sample delivery method, that shows promise to study the time-points between the S_3 and S_0 states, because of the precise control we can achieve in the time-intervals and the visible illumination protocols. This procedure also allows us to study larger crystals, that usually show diffraction to higher resolution. Finally, using this method we have shown that we can collect the XES data from one time-point in less than 30 mins making time-resolved spectroscopy a reality.

Science objectives for 2015-2016:

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

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