Towards Biohybrid

Artificial Photosynthesis

by

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A Dissertation Presented in Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

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Graduate Supervisory Committee

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ABSTRACT

A vast amount of energy emanates from the sun, and at the distance of Earth, approximately 172,500 TW reaches the atmosphere. Of that, 80,600 TW reaches the surface with 15,600 TW falling on land. Photosynthesis converts 156 TW in the form of biomass, which represents all food/fuel for the biosphere with about 20 TW of the total product used by humans. Additionally, our society uses approximately 20 more TW of energy from ancient photosynthetic products i.e. fossil fuels. In order to mitigate climate problems, the carbon dioxide must be removed from the human energy usage by replacement or recycling as an energy carrier. Proposals have been made to process biomass into biofuels; this work demonstrates that current efficiencies of natural photosynthesis are inadequate for this purpose, the effects of fossil fuel replacement with biofuels is ecologically irresponsible, and new technologies are required to operate at sufficient efficiencies to utilize artificial solar-to-fuels systems. Herein a hybrid bioderived self-assembling hydrogen-evolving nanoparticle consisting of photosystem I (PSI) and platinum nanoclusters is demonstrated to operate with an overall efficiency of 6%, which exceeds that of land plants by more than an order of magnitude. The system was limited by the rate of electron donation to photooxidized PSI. Further work investigated the interactions of natural donor acceptor pairs of cytochrome c₆ and PSI for the thermophilic cyanobacteria *Thermosynechococcus elongatus BP1* and the red alga *Galderia sulphuraria*. The cyanobacterial system is typified by collisional control while the algal system demonstrates a population of prebound PSI-cytochrome c₆ complexes with faster electron transfer rates. Combining the stability of cyanobacterial PSI and kinetics of the algal PSI:cytochrome would result in more efficient solar-to-fuel conversion. A second priority is the replacement of platinum with chemically abundant catalysts. In this work, protein scaffolds are employed using host-guest strategies to increase the stability of proton reduction catalysts and enhance the turnover number.
without the oxygen sensitivity of hydrogenases. Finally, design of unnatural electron
transfer proteins are explored and may introduce a bioorthogonal method of introducing
alternative electron transfer pathways in vitro or in vivo in the case of engineered
photosynthetic organisms.
I dedicate this work to my family and friends whose encouragement and support enabled me to achieve it.
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**Abstract:**

Introduction:

Materials And Methods:

- Growth of *G. sulphuraria* and PSI Isolation
- Purification of *G. Sulphuraria* Cyt c₆
- Purification of *T. elongatus* Cyt c₆
- Cloning Expression and Purification of Recombinant *G. sulphuraria* Cyt c₆
- PSI Intact Protein Mass Spectrometry
- EPR Spectroscopy
- Cyt c₆ Reduced Minus Oxidized Difference Spectra
- Cyt c₆ Midpoint Potential Measurements
- Transient Absorption Spectroscopy
- Homology Modeling of *G. sulphuraria* PsaA, PsaB, PsaF of PSI and PetJ (cyt c₆)
- Rigid Body Docking

Results:

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EVOLUTION OF REACTION CENTER MIMICS TO SYSTEMS CAPABLE OF GENERATING SOLAR FUEL

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My contributions included design, writing, figure preparation and editing of the manuscript. See Appendix I for permission details)

Abstract:

Capturing and converting solar energy via artificial photosynthesis offers an ideal way to limit society’s dependence on fossil fuel and its myriad consequences. The development and study of molecular artificial photosynthetic reactions centers and antenna complexes and the combination of these constructs with catalysts to drive the photochemical production of a fuel helps to build the understanding needed for development of future scalable technologies. This review focuses on the study of
molecular complexes, design of which is inspired by the components of natural photosynthesis, and covers research from early triad reaction centers developed by the group of Gust, Moore, and Moore to recent photoelectrochemical systems capable of using light to convert water to oxygen and hydrogen.

Introduction:

Human activity and especially our reliance on burning fossil fuels has affected planet wide systems, resulting in a precarious future for the global ecosystem (Rockström et al. 2009). The magnitude of the energy reaching the Earth from the sun makes solar energy conversion a likely part of any alternative energy future that does not rely on the use of fossil fuel yet still satisfies society’s energy demand (Armaroli and Balzani 2007). The immense scale of solar irradiance, however, means little without an efficient means of converting it to useful forms such as electricity or an energy-dense and transportable fuel. Developing a system for converting an abundant and readily available precursor into a fuel using solar radiation as the sole energy input is the primary goal of artificial photosynthesis (Bard and Fox 1995; Gust et al. 2009).

The motivation for and possible impact of the wide scale use of artificial photosynthesis to meet human societal energy needs can be illustrated with reference to Fig. 1. Taking into account reflection and absorption of light by the atmosphere, around 65,000 TW reaches the hydrosphere and 15,600 TW reaches land (Sorensen 2010). Photosynthesis converts a portion of this irradiance into energy stored in reduced carbon, with the net amount of biomass produced in a year [net primary production (NPP)] totaling ~112 Pg of fixed carbon (Ito 2011). Converting this amount of reduced biomass produced per year to a rate of energy conversion using the conversion factor of 41.3 kJ g⁻¹ of fixed carbon (Archer and Barber 2004) yields an average rate of ~156 TW,
evenly distributed between terrestrial and marine production (Cramer and Field 1999; Field 1998; Ito 2011). This biomass in turn supports nearly all life on the planet. Humans consume an increasing portion, currently ~25 % (Haberl et al. 2007). An important issue we face is that human appropriation of net primary production (HANPP) comes at the expense of the remainder of biology, and continued population growth will increase the human demand on NPP for food and material. The conversion of land to support HANPP is thought to be driving several Earth systems over boundaries established by natural

Figure 1 – Solar radiation and photosynthetic conversion. Rates of solar energy reaching various portions of the Earth system (Sorenson 2010). While ~80,000 TW reach the surface of the Earth, only 15,600 TW fall on land. About half is within the wavelength range of the photosynthetic pigments; a small fraction of this is stored as chemical energy for the biosphere, i.e., NPP, at an average rate of ~156 TW.
cycles operating on the geological time scale (Barnosky et al. 2012; Rockström et al. 2009).

Therefore, any solar-to-fuel system must be vetted against its impact on HANPP; an increase will accentuate an already tenuous ecological situation (Barnosky et al. 2012; Rockström et al. 2009). Figure 2 outlines energy accounts comparing terrestrial NPP, HANPP, the remainder of NPP after human harvest, and the current non-food energy consumption of modern society. Part (a) illustrates the current state: the human requisition of NPP sums to 20 TW (Haberl et al. 2007). Driving the global gross domestic product currently consumes ~17 TW, mainly supplied by fossil fuels (IPCC 2011). Fully displacing fossil fuels with crop-derived biofuel, as shown in (b), must come directly out of NPP. Though carbon neutral, such a scenario is not sustainable as HANPP would increase to nearly 50 % NPP. Such a substantial consumption of the total available terrestrial biomass would likely upend the balance of life on the planet as it has been known for the entirety of human existence. Alternatively, as shown in (c), an artificial photosynthetic system (possibly including a synthetic biological organism or photosynthetic microbe, the culture of which does not displace native organisms or crops) could supply human energy needs while not depleting the biomass needed to support the global ecosystem. We term this converted solar energy APP or artificial photosynthetic production. This situation would have to satisfy several caveats. For instance any such system should occupy surface area already altered by human activity and minimize the amount of additional land converted for human use. This basic argument seeks not only to focus just on the surface footprint needed to convert solar irradiance to forms of energy usable by humans but also to consider that any system should not compete for sunlight or land currently used for food production or accessible
to nature. To do so will only increase HANPP and further endanger the stability of the global ecosystem.

In order to exceed current productivity without increasing HANPP, the field of artificial photosynthesis seeks the development of solar-to-fuel systems capable of converting light energy to chemical energy stored in a dense, transportable fuel with efficiencies much greater than those of natural photosynthesis. Such a system, as opposed to that of solar-to-electricity (photovoltaic cells), allows for the storage of solar energy and separates its points of generation and utilization in both time and space. As such, artificial photosynthesis can provide a direct substitute for fossil fuels, which are ideal energy carriers with respect to their high energy densities and easy transport.

Although designing and constructing such a system comes with enormous challenges, the natural process of oxygenic photosynthesis offers us a guide.

Oxygenic photosynthesis uses the energy of visible light to carry out the oxidation of water and reduction of carbon dioxide to form oxygen and reduced carbon fuel. Absorption of actinic photons by antennas and reaction center pigments of the...
photosynthetic machinery initiates charge separation and migration in the reaction center, generating spatially separated oxidizing and reducing equivalents. This conversion of solar energy into electrochemical energy is followed by the production of oxygen from water at the oxygen evolving complex (OEC), the generation of reduced nicotinamide adenine dinucleotide phosphate (NADPH), and creation of a proton gradient across the thylakoid membrane. Dissipation of this proton motive force across the membrane produces adenosine triphosphate (ATP) via ATP synthase, and ATP and NADPH then power the dark reactions of the Calvin–Benson cycle leading to the assimilation of CO$_2$.

Artificial photosynthesis does not seek to reproduce the natural process, but rather to adapt its basic science to meet the needs of humans. Akin to the process of photosynthesis, an artificial reaction center for solar fuel production needs to absorb light in the visible and near-infrared, generate a charge-separated state upon photoexcitation, spatially separate and stabilize the charge-separated state, and then transport the photo-generated oxidizing and reducing equivalents to catalysts to carry out the chemical reactions necessary for the production of a fuel (e.g., oxidation of H$_2$O and production of H$_2$). In addition, efficient solar-to-fuel systems incorporating abundant materials are paramount. The study of the underlying photophysical and photochemical processes of model constructs and photoelectrochemical cells will inform the design and aid in the development of such systems.

Much of the research of the Gust, Moore, and Moore group has centered on the development and study of artificial reaction centers with the ultimate objective of constructing solar-to-fuel systems. Here, we provide an overview of this work from the first triad reaction centers to the solar water splitting photochemical systems currently under study. The focus of this review is intended as a concise summary of how the group
has approached the major challenge of developing sustainable solar energy to fuel technologies, a task that needs input from and is being addressed by many research groups.

**Molecular reaction centers**

An artificial reaction center seeks to perform the same photochemical processes as those observed in the natural system. With the ultimate goal of using light energy to form a fuel, the key functions include: absorption of light across the visible and near-infrared spectra, rapid and efficient transfer of excitation energy from antenna pigments to the reaction center, fast photoinduced charge separation with quantum yields near unity, and prevention of photodamage. Artificial constructs capable of emulating many aspects of the photosynthetic process have been studied, but developing complete systems capable of efficient conversion of light energy into a fuel remains a challenge. The work outlined below shows a research trajectory aimed at this ultimate goal.

**Triad reaction centers**

Covalently linking two molecular analogs of the cofactors involved in the photosynthetic reaction center (e.g., chlorophylls, carotenoids, pheophytins, quinones) produces some of the simplest artificial reaction centers. Study of porphyrin–quinone (P–Q) constructs in particular guided early work in the field carried out by many different researchers. While a P–Q dyad could form the P⁺–Q⁻ charge-separated state with proper illumination, the extremely fast decay of this state limits its usefulness (Gust and Moore 1989). In the natural system, sequential electron transfer steps following photoinduced charge separation spatially separate positive and negative charges across a substantial distance, which greatly slows recombination. Following this design principle,
incorporating a third component, one thermodynamically competent for carrying out a second electron transfer after photoinduced charge transfer, proved essential for prolonging the lifetime of the charge-separated state. Synthesis and subsequent study of a carotenoid–P–Q triad (C–P–Q) (Moore et al. 1984) marked a major improvement in stabilizing charge separation in an artificial reaction center and established a platform for engineering improved artificial reaction centers as well as studying other processes characteristic of photosynthesis.

This first triad reaction center, compound 1, consisted of a ditolylporphyrin covalently bonded via amide linkages to a benzoquinone electron acceptor and a carotenoid secondary electron donor. Molecule 1 and subsequent carotenoporphyrin-acceptor triads characteristically adopt a linear conformation in solution without folding of the appended groups back over the plane of the porphyrin (Gust et al. 1987; Moore et al. 1984). Transient spectroscopic studies of C–P–Q show that illumination with visible light (600 nm) generates the porphyrin first excited singlet state, C–1P–Q, with a portion of this species then decaying to the first charge-separated state, C–P*–Q*. Competing with recombination, a second electron transfer from the carotenoid to the porphyrin radical cation produces the final charge-separated state C*–P–Q*. Monitoring the transient absorbance of the oxidized carotenoid moiety (in the 970 nm region) shows that the final charge-separated state has a lifetime of 170 ns in dichloromethane, increasing to 2.5 µs in electrolyte-saturated solvent.

Table 1 contains a summary of the results for 1 and several other reaction centers described herein. The final state preserves 1.1 eV of the 1.9 eV of the porphyrin first excited singlet state (Gust et al. 1993a). The improved stability of the final charge-separated state of this complex relative to those of the preceding dyads resides in the greater spatial separation, and therefore electronic decoupling, of the separated charges
and the endergonic steps hindering the overall energetically favorable charge recombination.

The C–P–Q triad in turn provided a foundation for further elaboration and improvement in reaction center design and function. Several iterations of the triad reaction center are shown in Fig. 3. Alterations of the electronic coupling, as in changing the orientation of the amide linker in 4 and 5 (Kuciauskas et al. 2000), redox properties (Kodis et al. 2004), and type of acceptor moiety (Gust et al. 1991; Liddell et al. 1997) in the reaction center led to triad complexes with improved lifetimes and yields of the final charge-separated state. The extension of the triad design led to the creation of tetrad and pentad complexes which demonstrated the feasibility of carrying out multiple electron transfer steps across larger complexes resulting in greater spatial separation of the charges and long lifetimes for the charge-separated state (Gust et al. 1988a; Gust et al. 1990; Gust et al. 1993b; Gust et al. 1988b).

### Table 1 - Final charge-separated state lifetimes, quantum yield for the formation of the final charge-separated state, and experimental conditions for selected artificial reaction centers

<table>
<thead>
<tr>
<th>Compound</th>
<th>Solvent</th>
<th>( \tau^a )</th>
<th>( \Phi^b )</th>
<th>( \lambda_{ex.} ) (nm)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Dichloromethane</td>
<td>170 ns</td>
<td>0.04</td>
<td>600</td>
<td>(Moore et al. 1984)</td>
</tr>
<tr>
<td></td>
<td>Dichloromethane</td>
<td>2.5 ( \mu s )</td>
<td>0.25</td>
<td>600</td>
<td>(Moore et al. 1984)</td>
</tr>
<tr>
<td>2</td>
<td>2-Methyltetrahydrofuran</td>
<td>60 ns</td>
<td>0.96</td>
<td>590</td>
<td>(Bahr et al. 2000)</td>
</tr>
<tr>
<td>3</td>
<td>2-Methyltetrahydrofuran</td>
<td>57 ns</td>
<td>0.95</td>
<td>600</td>
<td>(Kodis et al. 2004)</td>
</tr>
<tr>
<td>4</td>
<td>2-Methyltetrahydrofuran</td>
<td>170 ns</td>
<td>0.14</td>
<td>590</td>
<td>(Liddell et al. 1997)</td>
</tr>
<tr>
<td></td>
<td>2-Methyltetrahydrofuran</td>
<td>1.5 ( \mu s )</td>
<td>0.10</td>
<td>590</td>
<td>(Liddell et al. 1997)</td>
</tr>
<tr>
<td>5</td>
<td>2-Methyltetrahydrofuran</td>
<td>340 ns</td>
<td>0.88</td>
<td>590</td>
<td>(Kuciauskas et al. 2000)</td>
</tr>
<tr>
<td>6</td>
<td>2-Methyltetrahydrofuran</td>
<td>1.3 ns</td>
<td>0.69</td>
<td>560</td>
<td>(Kuciauskas et al. 1999)</td>
</tr>
<tr>
<td>7</td>
<td>2-Methyltetrahydrofuran</td>
<td>240 ns</td>
<td>0.86</td>
<td>560</td>
<td>(Kodis et al. 2006)</td>
</tr>
<tr>
<td>8</td>
<td>2-Methyltetrahydrofuran</td>
<td>8.9 ns</td>
<td>0.80</td>
<td>480</td>
<td>(Kodis et al. 2006)</td>
</tr>
<tr>
<td>9</td>
<td>2-Methyltetrahydrofuran</td>
<td>15.3 ns</td>
<td>0.96</td>
<td>480</td>
<td>(Kodis et al. 2002)</td>
</tr>
<tr>
<td>10</td>
<td>1,2-Difluorobenzene</td>
<td>230 ps</td>
<td>~1</td>
<td>480</td>
<td>(Terazono et al. 2009)</td>
</tr>
<tr>
<td>11</td>
<td>Benzonitrile</td>
<td>3.8 ( \mu s )</td>
<td>0.52</td>
<td>740</td>
<td>(Megiatto et al. 2012)</td>
</tr>
</tbody>
</table>

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* Lifetime and quantum yield given for the final charge-separated state of the respective reaction center
* Saturated with tetra-n-butylammonium tetrafluoroborate
* 77 K (all other values in table at room temperature)
* \( M = 2H \) (freebase form, see Figure 5)
* \( M = Zn \) (Zn inserted form, see Figure 5)
The introduction of a fullerene electron-accepting unit in place of a quinone advanced the performance of the artificial reaction centers (Liddell et al. 1997). Although, not observed in any known biological system, the use of a fullerene acceptor moiety offers definite advantages in imitating the photochemical processes observed in natural photosynthesis. With small solvent and internal reorganizational energies upon reduction and relative insensitivity of the radical anion to the solvent dielectric constant.
compared to quinones, fullerenes perform remarkably well in reaction center constructs (Imahori et al. 1996; Liddell et al. 1997; Liddell et al. 1994). In comparison with triad complexes employing quinones, those with fullerene acceptor moieties in general show more rapid photoinduced charge separation and slower charge recombination, can perform charge separation in a variety of solvents and even at low temperature in glasses (Kuciauskas et al. 2000; Liddell et al. 1997), and can recombine to triplet excited states rather than the ground state (Kodis et al. 2004; Liddell et al. 1997). Such behavior is characteristic of the natural system, making fullerenes an ideal example where compounds alien to biology can substantially aid in the development and performance of overall biomimetic artificial systems (Kodis et al. 2002).
The first iteration of the C–P–C₆₀ based triad featuring a β-alkyl substituted porphyrin, 4, formed the final C•+–P–C₆₀• state with a yield of 0.14 in 2-methyltetrahydrofuran (Liddell et al. 1997). Spectroscopic studies showed that the decay of this state, with a lifetime of 170 ns, led to the formation of the carotenoid triplet state, 3C–P–C₆₀. Progressive molecular engineering of the complex by introduction of a lower potential carotenoid (compare 4 and 5) (Kuciauskas et al. 2000), introduction of a ditolylophyrin in 2 (Bahr et al. 2000), and then dimesitylophyrin in 3 (Kodis et al. 2004) steadily improved the performance of the reaction center, producing complexes capable of obtaining quantum yields for the final charge-separated state near unity. The trajectory of triad reaction centers from the first C–P–Q complexes to the later fullerene-containing analogs demonstrated the ability of synthetic molecular constructs to transform efficiently light energy to chemical potential with charge-separated state lifetimes long enough for conversion of the conserved excitation energy to a useful form, for example by formation of chemical bonds or generation of a pH gradient across a membrane (Steinberg-Yfrach et al. 1997; Steinberg-Yfrach et al. 1998).

Antenna-reaction centers

The single chromophore in a triad reaction center limits the total usable region of the spectrum; nature, by contrast, employs several different pigment molecules such as chlorophylls, carotenoids, cyanobilins, and erythrobilins for wide coverage of the solar spectrum. In the photosynthetic process, antenna pigment-protein complexes absorb most of the actinic light and channel the resultant excitation energy to the reaction center leading to charge separation (Blankenship 2002).

In an artificial context, multiporphyrin antenna-reaction center complexes provided the basis for later multichromophore arrays and also established a means of studying
energy transfer to a reaction center. An antenna moiety consisting of four Zn(II)tetrarlyporphyrins—a central Zn(II)porphyrin connected to the meso positions of three peripheral porphyrins—was attached to a free base porphyrin fullerene reaction center [(PZP)3–PZC–P–C60, 6]. This system established the feasibility of specifically exciting the antenna to induce charge separation in the attached reaction center (Fig. 4) (Kuciauskas et al. 1999). Optimizing the design of the (PZP)3–PZC–P–C60 reaction center by replacing the diarylporphyrin of 6 with tetraarylporphyrin in 7, and thereby increasing the potential of the electron donating porphyrin, provided more thermodynamic driving force for electron transfer from the antenna porphyrins to the radical cation of the freebase porphyrin and led to a hole delocalization in the antenna and longer lifetime of the final charge-separated state (Kodis et al. 2002). The structural

Figure 5 – Antenna-reaction center complex 8.
differences between 6 and 7 also led to different HOMO orbital types in the freebase porphyrin moieties: the b-alkyl substituents of the freebase porphyrin in 6 induce an a1u-type HOMO with nodes at the meso positions, whereas the freebase porphyrin of 7 without β substituents and mesityl groups at the 10,20-meso positions exhibits an a2u-type HOMO with lobes at the meso positions. These differences in the HOMO orbital structure combined with the steric influence of the β-alkyl groups of 6, which results in an increased average dihedral angle and less π–π overlap between the meso aryl group and the porphyrin macrocycle, also create a substantial increase in the rate of singlet–singlet energy transfer rates, better antenna function, and increased quantum yield of final charge separation for 7 as compared to 6 (Kodis et al. 2002). Extending the concept of this design, an antenna-reaction center complex comprised of five bis(phenylethynyl)anthracene (BPEA) antenna molecules linked to a hexaphenylbenzene core and covalently linked to a porphyrin–fullerene complex 8 (Fig. 5), carried out efficient charge separation with better coverage of the spectrum from 430 to 480 nm (Kodis et al. 2006).

Further elaboration of the antenna-reaction center came with the development of complex 9 (Fig. 6), consisting of several different chromophore moieties. Centered on a hexaphenylbenzene core, two BPEA, two borondipyrromethene, and two Zn(II)tetraarylporphyrin dyes comprised an antenna complex with coverage across the visible spectrum (Terazono et al. 2009). Coordination of a dipyridyl functionalized fullerene to the two adjacent Zn(II)porphyrins of the antenna complex completed the formation of a multiantenna-reaction center complex 9. Excitation of any of the antenna pigments in the complex leads to rapid light-induced electron transfer to the fullerene. Developing molecular design strategies for broad spectral coverage with funneling of
excitation energy to charge transfer centers, such as those outlined above, plays a central role in the development of efficient solar-to-fuel systems.

Photoregulation

During photosynthesis, the process of non-photochemical quenching enables the

Figure 6 – Antenna-reaction center complex 9.
organism to avoid the buildup of energetic, and damaging, intermediates that would otherwise form under high light intensities (Horton et al. 2012; Müller et al. 2001). Avoiding destructive intermediates formed under such conditions could likely increase the longevity of artificial systems in a similar manner. A pentad reaction center, consisting of a porphyrin primary donor, fullerene acceptor, two antenna pigments, and a dihydroindolizine (DHI) photochrome control moiety, demonstrated the ability of an artificial reaction center to reversibly change the quantum yield of charge separation based on the intensity of incident light (Straight et al. 2008). Under low white light levels, in which the DHI exists primarily in the closed form, the reaction center carries out light-induced charge separation with a quantum yield of 0.82. Under high white light intensities, the quantum yield of a solution of the pentad decreases to 0.27 as photoisomerization converts some of the photochrome to its open, betaine form (BT), a conformation competent for rapidly quenching the porphyrin excited singlet state by energy transfer. Upon forming BT at high white light intensities, the quenching of the porphyrin excited state prevents charge separation in the reaction centers of the isomerized molecules, markedly reducing the overall quantum yield for this process.

*Charge separation across a membrane*

Along with light capture and formation of a charge-separated state, an artificial solar-to-fuel system must convert incident light energy to some other useful form. In photosynthesis, photoinduced charge transfer across the thylakoid membrane is coupled to proton shuttling across the bilayer, thereby establishing a proton gradient. Dissipation of the proton imbalance back across the membrane then drives the production of ATP via a transmembrane ATP-synthase enzyme. Producing constructs capable of mimicking
this process can offer insight into the design and development of artificial systems for solar energy conversion.

A study to this end demonstrated that illumination of a phospholipid bilayer impregnated with C–P–Q type reaction centers and separating a solution containing a sacrificial electron donor from that with an acceptor species results in the passage of photocurrent in a circuit bridging the bilayer membrane (Seta et al. 1985). Building off this work, imbedding a similar C–P–Q type reaction center in the bilayer of a liposome set the basis of a system capable of pumping protons across the lipid layer (Steinberg-Yfrach et al. 1997). The overall amphiphilic nature of the C–P–Q complex used directs the asymmetric insertion of the reaction center into the liposome with the carotenoid moiety toward the interior and the more polar quinone to the exterior. Excitation of the complex generates an oxidizing potential inside and a reducing potential around the periphery of the liposome. A freely diffusing quinone electron/proton carrier within the membrane of the liposome with a midpoint potential between that of the oxidized carotenoid and reduced quinone moieties of the reaction center shuttles protons across the membrane, resulting in the acidification of the interior of the liposome. This results in the creation of a light-induced potential gradient across the bilayer.

In order to harness the proton motive force generated in this system, a CF₅₆F₇-ATP synthase was incorporated into the liposome along with the reaction center and redox mediator components (Steinberg-Yfrach et al. 1998). With proton translocation driven by the photocycle described above, dissipation of the pH gradient coupled to the catalytic conversion of ADP and P₇ to ATP as carried out by the CF₅₆F₇-ATP synthase resulted in the net conversion of incident light energy into that of a high-energy chemical species. Quantitative analysis of the system reveals that in low light the absorption of 14 photons
results in the production of 1 ATP molecule and, with illumination by 633 nm light, roughly 4% of the absorbed energy is conserved in the form of a chemical bond.

In a subsequent study, the same C–P–Q reaction center was used in conjunction with a quinone-like molecule, ability to bind Ca$^{2+}$ of which is modulated by the redox status (Bennett et al. 2002). This system was capable of pumping Ca$^{2+}$ ions against a concentration gradient by the asymmetric arrangement of the C–P–Q reaction center in liposomes. Although the quantum yield was only 1%, a significant electrical potential was measured across the membrane extending Mitchell’s mechanism of accumulating membrane potential using a redox loop to divalent cations in addition to protons.

![Figure 7 – BiP containing triad reaction centers 10 and 11.](image)
Mimicking proton control during electron transfer

The Tyrosine$_Z$–Histidine$_{190}$ (Tyr$_Z$–His$_{190}$) pair of photosystem II (PSII) is thought to undergo proton-coupled electron transfer (PCET) as it serves as an electron transfer mediator between P$_{680}$ and the OEC (Faller et al. 2003; Rappaport and Diner 2008). The interaction between Tyr$_Z$ and His$_{190}$ likely serves to tune the potential of the tyrosine residue so that it lies between that needed for the reduction of the photogenerated P$_{680}^+$ and the potential for oxidizing the OEC. Serving as an intermediary between P$_{680}$ and the OEC, Tyr$_Z$–His$_{190}$ prevents charge recombination that would otherwise compromise the catalytic activity of the Mn$_4$O$_5$Ca cluster of the OEC. This is especially important given that four oxidizing equivalents must accrue on the OEC to carry out the oxidation of one molecule of water. To improve artificial systems, utilizing a similar design feature could also aid in preventing back electron transfer after photoinduced charge separation.

With the natural system as a template, we designed a porphyrin construct 10 (Fig. 7) bearing a benzimidazolephenol (BiP) moiety with the phenolic hydrogen capable of forming a hydrogen bond with the lone pair electrons of the imidazole nitrogen (Moore et al. 2008; Moore et al. 2010). Chemically functionalizing the porphyrin to adsorb to the surface of a colloidal TiO$_2$ nanoparticle enables the assembly of 10 as shown in Fig. 7. This molecule–nanoparticle complex is reminiscent of the triad reaction centers discussed earlier, consisting of a porphyrin light absorber (PF$_{10}$), TiO$_2$ primary electron acceptor, and BiP electron donor (Moore et al. 2008). Electron paramagnetic resonance studies of this complex reveal phenoxy radical formation in the complex upon excitation of the porphyrin as a result of light-induced charge separation. Given that the potential for the oxidation of the BiP moiety lies at 1.00 V versus SCE, the photo-formed BiP$^{++}$–PF$_{10}$–TiO$_2^{-}$ state is thermodynamically competent for water oxidation.
A continuation of this study led to the development of 11 (Fig. 7), a fully organic triad complex with a tetracyanoporphyrin (TCNP) serving as the primary acceptor in place of a TiO$_2$ nanoparticle (Megiatto et al. 2012). This complex bears considerable similarity to the Tyr$_Z$–His$_{190}$–P$_{680}$–Pheo$_{D1}$ portion of the electron transport chain in PSII. Time resolved spectroscopic investigation of this reaction center in benzonitrile reveals that with excitation of TCNP the complex undergoes an initial electron transfer between the PF$_{10}$ and TCNP resulting in Bi–PhOH–PF$_{10}$•–TCNP•. Competing with charge recombination, a second, rapid electron transfer then occurs between the benzimidazolephenol and oxidized PF$_{10}$, presumably resulting in BiH$^+$–PhO–PF$_{10}$–TCNP•. This state forms with a quantum yield of 0.52 and persists with a 4 µs lifetime, which is longer than those of many of the carotenoporphyrin–acceptor reaction centers of prior studies. Presumably, the final charge-separated state reflects transfer of the phenolic proton to the nitrogen of the benzimidazole. Based on reduction potentials for model compounds (Moore et al. 2010), the PF$_{10}$• would not generate sufficient driving force for the formation of the Bi–PhOH•–PF$_{10}$–TCNP•, implying a PCET leading to the formation of the BiH$^+$–PhO–PF$_{10}$–TCNP• state. The long-lived charge-separated state and high potential of the oxidized BiH$^+$–PhO$^*$ (1.04 V vs. SCE), make this reaction center type an ideal candidate for incorporation into the photoanode of a photoelectrochemical device for light driven water splitting.

**Employing reaction centers for water splitting**

Thus far, we have illustrated artificial photosynthetic systems for light absorption and charge separation via photoinduced electron transfer. Fuel production requires “wiring” such reaction centers to catalysts. One approach to doing so is to combine...
electrode architectures reminiscent of those used in dye sensitized solar cells with artificial reaction centers to produce photoelectrochemical water splitting cells.

In collaboration with the Mallouk lab, we developed a photoanode composed of a transparent fluorine-doped tin oxide (FTO) conductive glass support bearing a nanoparticulate TiO$_2$ semiconducting layer to which is adsorbed a trisbipyridylruthenium(II) [Ru(bpy)$_3^{2+}$]-hydrated iridium oxide (IrO$_x$•H$_2$O) complex (Fig. 8) (Youngblood et al. 2009). A photoelectrochemical cell (PEC) consisting of this electrode in combination with a platinum cathode in aqueous solution demonstrated overall photolytic water splitting to oxygen and hydrogen, with the application of a small bias.

This system resembles a triad-type reaction center with a Ru(bpy)$_3^{2+}$ light absorber and primary electron donor, a TiO$_2$ primary acceptor, and an IrO$_x$•nH$_2$O secondary electron donor. Ideally, four sequential photocycle turnovers consisting of excitation of the Ru(bpy)$_3^{2+}$, electron transfer to the TiO$_2$ resulting in current flow to the platinum counter electrode, and hole transport to the IrO$_x$•nH$_2$O, results in the conversion of water to oxygen at the IrO$_x$•nH$_2$O surface and production of hydrogen at the platinum cathode. While overall water splitting was observed, spectroscopic study of the system revealed that the charge recombination from the TiO$_2$ to the oxidized dye occurred nearly an order of magnitude faster than the forward electron transfer from the IrO$_x$•nH$_2$O to regenerate the ground state dye. This charge recombination imposes a major limitation on the performance of the system and represents a critical area for improvement.
In PSII, Tyr\textsubscript{Z} serves the function of charge transfer mediator between P\textsubscript{680} and the OEC and limits the occurrence of charge recombination. In order to address the analogous recombination in our system, we turned to the use of the benzimidazolephenol of the BiP–PF\textsubscript{10}–TiO\textsubscript{2} triad (Fig. 7) as a redox mediator in the photoelectrochemical system. Chemically modifying the BiP with a dicarboxylate functionality enabled the production of colloidal IrO\textsubscript{x}•nH\textsubscript{2}O particles decorated with both the BiP mediator and 2-dicarboxyethylphosphonic acid (CEPA) (Zhao et al. 2012). Study of a PEC similar to that of the earlier work but using an FTO–TiO\textsubscript{2} photoanode bearing co-immobilized Ru(bpy)\textsubscript{3}\textsuperscript{2+} dye and BiP/CEPA–IrO\textsubscript{x}•nH\textsubscript{2}O shows that the BiP mediator improves the performance of the system. As compared to a cell without the mediator (using only CEPA capped IrO\textsubscript{x}•nH\textsubscript{2}O), higher photocurrents and greater overall efficiency (about a factor of three) are observed. Transient bleaching recovery traces showed that the improved performance results from faster regeneration of the ground state.

Figure 8 – Schematic representation of the photoanode compartment of a PEC. Electrons and protons from water oxidation migrate to the cathode for fuel production.
state dye from the first oxidized state with the presence of BiP in the system (Zhao et al. 2012). Thus, BiP serves a similar role to that of TyrZ in PSII by preventing recombination losses after light-induced charge separation.

Conclusion:

This account shows our research group’s trajectory from building simple molecular reaction center constructs to developing complete systems for the conversion of solar energy to a fuel. Efficient and economical systems which can generate a sustainable fuel from sunlight and a widely available precursor such as water are requisite to meeting future human energy demand in a way that does not endanger the diversity of life on the planet or the health and wellbeing of its inhabitants. Developing and improving the technologies for such systems represents a principal challenge of the modern world. The complications of fulfilling energy demand requires a host of sustainable technologies for varying locales. Certainly, solar energy will play a substantial role, and chemical fuels provide the greatest possible flexibility of usage.

We have pursued this challenge through the study of artificial reaction centers designed to mimic aspects of photosynthesis. As the only process capable of converting solar energy to a chemical fuel on a planet wide scale, photosynthesis provides a model for doing so by artificial means. The development and study of molecular reaction centers has culminated in the development of a first generation of dye-sensitized PECs for solar-to-fuel conversion. Substantial work remains in advancing the viability of this system. For instance, we are exploring the synthesis and use of new dye-catalyst constructs (Sherman et al. 2011) and catalytic materials based on more abundant elements (Wee et al. 2011). Ultimately, the grand challenge of supplying sustainable energy requires contributions from many researchers and all fields of science; our best
hope lies in developing a range of renewable energy technologies and contributing to a well informed and well equipped global society.
SELF-ORGANIZED PHOTOSYNTHETIC NANOPARTICLE FOR CELL-FREE HYDROGEN PRODUCTION

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Abstract:

There is considerable interest in making use of solar energy through photosynthesis to create alternative forms of fuel. Here, we show that photosystem I from a thermophilic bacterium and cytochrome-c₆ can, in combination with a platinum catalyst, generate a stable supply of hydrogen in vitro upon illumination. The self-organized platinization of the photosystem I nanoparticles allows electron transport from sodium ascorbate to photosystem I via cytochrome-c₆ and finally to the platinum catalyst, where hydrogen gas is formed. Our system produces hydrogen at temperatures up to 55 °C and is temporally stable for > 85 days with no decrease in hydrogen yield when tested intermittently. The maximum yield is ~5.5 mmol H₂ h⁻¹ mg⁻¹ chlorophyll and is estimated to be ~25-fold greater than current biomass-to-fuel strategies. Future work will further improve this yield by increasing the kinetics of electron transfer, extending the spectral response and replacing the platinum catalyst with a renewable hydrogenase.

Introduction:

The world petroleum production rate is predicted to reach its peak before the middle of the century (Tsokounoglou et al. 2008) and the higher cost of recovering recalcitrant deposits is expected to drive up the price of petroleum-derived fuels. Although fossil fuels rely on photosynthesis-driven biomass accumulation from millions of years ago, there is hope that directly harnessing photosynthesis can shorten the cycle time for creating fuels from solar energy (Moore 2005). Biomass-derived fuels are potentially a
clean, renewable and sustainable source of fuel, but several challenges exist. Current biomass-to-fuels schemes yield relatively low fuel value per unit land area; indeed, ethanol produced from switchgrass has a projected gross fuel value yield of 12 liters of gasoline per hectare per day equivalent (Pimentel and Patzek 2005). All biomass-to-ethanol technologies produce a dilute mixture of water and ethanol, which needs to be distilled. This accounts for ~20% of the production cost of fuel-grade ethanol (Pimentel and Patzek 2005). Furthermore, billions of tons of biomass must be harvested, transported, processed and converted to replace the petroleum used for gasoline in western Europe alone.

One way to improve production yield is to use solar energy directly, as plants do in photosynthesis. Recently, there has been considerable interest in green algae because they can, in principle, use photosynthesis to produce hydrogen from sunlight and water (Ghirardi 2000; Ghirardi et al. 2007). This hydrogen production is facilitated by

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2H^+ + H_2 \rightarrow H_2
\]

**Figure 9** – Schematic of the electron flow in the photosystem I catalytic nanoparticle. The monomeric form of the *T. elongatus* PSI is shown with chlorophyll cofactors (magnesium ligated in macrocycle shown in green van der Waals radius) and the protein colored by secondary structure (helical regions in purple, beta sheets in yellow and unstructured regions in brown). A partially docked form of the cyt c₆ is shown, providing the re- reduction of P₇₀₀, deriving electrons from the oxidation of ascorbate (Asc⁺₁→ Asc⁺ + e⁻). The platinum clusters on the stromal surface of PSI are shown as grey stars catalysing the reduction of protons to hydrogen with electrons of lower potential via the energy of the absorbed photon, and PSI.
specialized pigment–protein complexes known as reaction centers, which span the membranes of plants, algae, cyanobacteria and bacteria (Deisenhofer and Michel 1991; Golbeck 1993). In oxygenic photosynthesis, two reaction centers, photosystem II (Iwata and Barber 2004; Kálmán et al. 2008) and photosystem I (PSI) (Grotjohann and Fromme 2005; Jensen et al. 2007; Nelson and Ben-Shem 2005), function together in the Z-scheme to transfer electrons derived from water (via photosystem II) to reduce NAD(P)⁺ (via PSI) producing both oxygen and ATP (Barber 2003). Coupling either platinum nanoclusters (Evans et al. 2004; Millsaps et al. 2001) or covalently linked hydrogenase (Ihara et al. 2006a; Ihara et al. 2006b) to the acceptor end of PSI complexes can harvest the photochemically produced electrons to reduce protons to hydrogen in vitro. Similarly, it has been shown that reaction centers integrated into solid-state devices can be used to produce a photovoltaic current (Das et al. 2004).

Here, we show that a stable supply of hydrogen can be generated using a platinum catalyst and a system made of PSI isolated from the thermophilic cyanobacterium T. elongatus and a recombinant form of cytochrome-c₆ (cyt c₆) protein as illustrated in Fig. 9. The thermostable PSI, which is shown to be more stable than PSI from mesophilic cyanobacteria, shows enhanced hydrogen evolution rates up to 55 °C. Our system can evolve hydrogen for three months without special treatment or chemical preservatives. Under optimized conditions, we estimate that the gross fuel production rate per unit area for these complexes exceeds the best biomass-to-fuel schemes by a factor of 25. We believe that these properties and the renewable nature of PSI and cyt c₆ may lead to a sustainable and efficient way to produce alternative fuel.
Isolation of PSI and Recombinant Cyt C₆ from *T. elongatus*:

To enable the direct in vitro production of hydrogen, we isolated highly purified and highly dispersed PSI particles from both the mesophilic cyanobacterium *Synechocystis PCC 6803* and the thermophilic cyanobacterium *T. elongatus*. In both PSI preparations, the lowest chlorophyll-containing band of the sucrose gradient typically contained nearly
pure trimeric PSI (Fig. 10a). However, the upper band contained significant impurities of the more fluorescent PSII and phycobilisome (PBS) as observed by low-temperature fluorescence (Fig. 10d). Because Tris-tricine sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE; Fig. 10b) revealed the presence of a small amount of PBS proteins, anion exchange chromatography was subsequently used to ensure consistent purity and to concentrate the final preparation of PSI trimers. Transmission electron microscopy (TEM) of the complexes confirmed the uniformity and purity of the trimeric PSI nanoparticles (Fig. 10e). Furthermore, the size distribution of these nanoparticles was investigated by sedimentation velocity (SV) analysis and revealed a single large peak at ~21S (Fig. 10c, blue line). In contrast, the chlorophyll-containing band from a PSI sucrose gradient isolation revealed a much smaller monomeric PSI (with a sedimentation coefficient of 12S) and some PSII as indicated by low-temperature fluorescence (Fig. 10d). For reference, we have included a scaled outline of the PSI trimer structure as determined by Jordan and colleagues (Jordan et al. 2001) (Fig. 10e), with the solvent-accessible surfaces of each of the monomers shown in red, blue and purple and their three respective stromal exposed surfaces shown in green. Outlines of these dimensions were drawn in red and placed over several of the nanoparticles observed in the TEM, further confirming the uniformity of our PSI preparation.

To facilitate rapid re-reduction of P700 (the special pair of PSI) in vitro, it is necessary to have an abundant source of the physiological electron donor cyt c6 (Evans and Krogmann 1983; Ki Ho et al. 1979). The complete T. elongatus genome (Nakamura 2002) revealed only one cyt c6 gene containing a signal peptide. Using gene-specific primers, the full-length sequence for cyt c6 was cloned into the pET21b vector and the recombinant protein isolated as described in the Supporting Information. The correct folding and heme insertion of this recombinant protein were confirmed by the reduced-
minus-oxidized difference spectrum (Fig. 15) which indicates a β-peak at 552.1 nm, identical to published values for native cyt c₆ isolated from a variety of related cyanobacteria (Cho et al. 1999).

Thermal Stability of Purified PSI and Cyt C₆:

Circular dichroism (CD) spectroscopy was used to measure the thermostability of both PSI and cyt c₆ by monitoring the non-covalent pigment organization of PSI and the protein secondary structure of cyt c₆. The visible CD spectra of both the mesophilic cyanobacterium *Synechocystis PCC 6803* and the thermophilic cyanobacterium *T. elongatus* (Fig. 16a,b) were similar, as expected for the homologous complexes. The absorbance between 450 and 550 nm is from the bulk antenna chlorophyll associated with PSI, whereas the split exciton signal near 700 nm is derived from the P₇₀₀ special pair (Anta et al. 2006). Figure 11 illustrates the effects of temperature on the arrangement of these pigments. Although both complexes are stable to ~40 °C, approximately half of the observed absorbance of the bulk chlorophyll is lost by ~55 °C.

Figure 11 – Thermal stability of photosystem I monitored by circular dichroism. The normalized maximal absorbance for each of the datasets shown in Fig. 16 are shown as a function of temperature; green symbols indicate data for *Synechocystis PCC 6803* and the red symbols indicate *T. elongatus*. In both cases, circles indicate antenna chlorophyll (positive peak at 515 nm) and the triangles indicate the split exciton signal originating from P₇₀₀ (minima at 700 nm).
for PCC 6803 and ~90 °C for T. elongatus. Interestingly, the chlorophyll associated with P_{700} maintains its native structure until much higher temperatures for both organisms, maintaining 80% of the maximal absorbance until 70 °C and 90 °C for Synechocystis and T. elongatus, respectively. Furthermore, CD experiments indicated cyt c_{6} has a T_{m} of 81 °C (Fig. 16c).*

Rate-Limiting Step in PSI-Mediated Hydrogen Evolution:

Although PSI-mediated photo-production of hydrogen has been demonstrated with PSI isolated from spinach leaves (Evans et al. 2004; Millsaps et al. 2001) and cyanobacteria (Ihara et al. 2006b), this work investigates a similar reaction using thermophilic cyanobacterial PSI. Initial experiments examined the parameters for platinizing T. elongatus PSI to support photo-dependent hydrogen evolution. Subsequently, the stability of the system was demonstrated and the effect of each reagent on the rate of hydrogen production was studied.

The role of each component was determined by a sequence-of-addition experiment. The initial reaction containing PSI and [PtCl_{6}]^{2-} was allowed to proceed for two light/dark (L/D) cycles, after which the sacrificial electron donor sodium ascorbate was added (marked by a black arrow in Fig. 12a) in the dark, and the rate of hydrogen evolution was continuously monitored at 25 °C (Fig. 12a). Very low hydrogen evolution was observed during light cycles before or after ascorbate addition, indicating that ascorbate is inefficient for P_{700}^{+} re-reduction. However, addition of cyt c_{6} (black arrow with asterisk in Fig. 12a) after six L/D cycles supported a rapid, short-lived burst of H_{2} evolution that decreased sharply by the next light cycle. This behaviour indicates rapid reduction of the pool of reduced cyt c_{6} as the platinum catalyst deposits on PSI, causing

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*a This note is added after the original printing to indicate that interactions with binding partners is less stable than the pigment arrangement indicates, see figure 22B.
the rate of H$_2$ evolution to drop. The system is in equilibrium when the rate of cyt c$_6$ reduction by ascorbate matches the level of P$_{700}$ photo-oxidation, causing H$_2$ evolution to become constant with each light cycle.

The platinum catalyst was shown to be tightly associated with PSI nanoparticles using energy-dispersive X-ray spectroscopy of previously platinized PSI nanoparticles that had been re-isolated and dialyzed overnight. As shown in Fig. 12b, this sample clearly contains platinum, as indicated by the energy levels of the emitted X-rays with the rate of H$_2$ evolution to drop. The system is in equilibrium when the rate of cyt c$_6$ reduction by ascorbate matches the level of P$_{700}$ photo-oxidation, causing H$_2$ evolution to become constant with each light cycle.

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characteristic energies (2.05, 11.07/11.25 and 9.36/9.44 keV). Finally, the catalytic activity of the previously platinized PSI was confirmed to not require additional platinum (Fig. 12c). Limited hydrogen evolution was observed before the addition of ascorbate and cyt c₆. The lack of hydrogen evolution detected on addition of ascorbate indicates that the re-isolation b of PSI following platinization effectively removed cyt c₆. Moreover, addition of fresh cyt c₆ increased the rate of hydrogen evolution by a factor of ~80. These experiments indicate that the initial platinization results in platinum integration into PSI that is both stably associated and catalytically active. Although the exact molecular-level structure of this platinization is not known, electron microscopy analysis indicates that the platinum forms electron-dense complexes associated with PSI (Fig. 17) Following this burst in hydrogen evolution, we observe a slow decay in hydrogen evolution that can be fit to a single exponential decrease with a half-life of 3.8 h (shown in Fig. 12c).

The light saturation experiment was performed with platinized T. elongatus PSI nanoparticles similar to those described above. The hydrogen production rate increased with increasing light intensity (Fig. 12d) and began to plateau as the light intensity approached 800 µE m⁻² s⁻¹; however, this curve is sigmoidal with a distinct lag phase. Although these data cannot be well fit to a simple single-substrate kinetic model (dashed line), it can be fit to a cooperative model (solid line) with a Hill coefficient of ~2.5. The shape of this curve is different from the light saturation response for monomeric PSI isolated from spinach (Millsaps et al. 2001), which was linear in the low light region. Because the T. elongatus PSI (Fig. 10e) is trimeric, the platinum catalyst can potentially accept electrons from more than one of the PSI P₇₀₀ in the trimeric complex. Such cooperativity between adjacent PSI monomers explains how a single electron from a single P₇₀₀ could facilitate molecular hydrogen production, which requires two electrons.

b In order to clarify the original article, re-isolation of PSI refers to isolating the PSI from the platinization reaction mixture.
to reduce two protons. This sigmoidal response indicates that at higher light levels the probability of simultaneously photo-exciting two PSI reaction centers increases within a trimer, thus yielding a higher and ‘cooperative’ mechanism of hydrogen production.

Another difference between the spinach and \textit{T. elongatus} light responses was the light level at which the hydrogen yield was observed to plateau. In spinach, the plateau was at \textasciitilde 200 mE m$^{-2}$ s$^{-1}$, whereas in \textit{T. elongatus} the plateau was at \textasciitilde 800 mE m$^{-2}$ s$^{-1}$. This may be the result of the considerable difference in the chlorophyll antennae-to-$P_{700}$ ratio in the organisms. The plant PSI has much larger antennae, approaching \textasciitilde 300 chlorophyll/$P_{700}$ whereas the \textit{T. elongatus} PSI is \textasciitilde 95 chlorophyll/$P_{700}$. This approximately threefold difference in antenna size would account for the difference in light dependence.

**Evaluation of Hydrogen Production:**

To evaluate the temporal stability of this cell-free hydrogen evolution system, we tested one preparation of platinized PSI repeatedly over three months. The temporal details of this experiment are described in the Supporting Information. These data show that our PSI preparations maintain operational stability for extended time periods. Representative data from three L/D cycles throughout this 85-day experiment are shown in Fig. 13a. By directly comparing the hydrogen yield observed initially with that observed after 85 days, it is clear that the yields are identical, as shown in Fig. 13b. This indicates that without special precautions or additives, the platinized \textit{T. elongatus} PSI complex is capable of constant hydrogen evolution for more than 85 days of intermittent testing and storage.

To optimize hydrogen evolution from this thermophilic PSI system, we examined the effect of temperature on hydrogen evolution rates in vitro. We have already
determined that cyt c₆ and PSI have Tₘ values of 81 °C and ~90 °C, respectively (Fig. 11 and Fig. 16c) and observed an 80% increase in hydrogen yield when the temperature was

Figure 13 – Thermal and temporal stability of hydrogen evolution. a.) A single sample was repeatedly measured for ~86 days. Selected data are shown: three light/dark (L/D) cycles following 60 h platinization; exhaustive dialysis to remove platinate (S1); followed by 170 h at 25 °C (three L/D cycles shown and remaining as S2); three L/D cycles at 60 °C; stored for 64 days at 4 °C (S3); sample was re-tested for three L/D cycles at 25 °C; stored for 5 days (S4); finally tested for three L/D cycles. b.) Integrated hydrogen yield of three L/D cycles shown at the beginning before S2 (solid line) and at the end after S4 (dashed line), both at room temperature, 25 °C. c.) Temperature–activity measurements of T. elongatus PSI. d.) Ascorbate consumption. Addition of 1 mM ascorbate at t = 68 h (arrow) increased yield, suggesting the initial 2 mM ascorbate has become limiting after the first 17 L/D cycles. The decrease in activity is fit by a single exponential in panels c and d. e.) Comparison of hydrogen yield of T. elongatus (open circles) and Synechocystis (open triangles) PSI with increasing temperature. Integrated hydrogen yield over 2 h light cycles are normalized by dividing by their respective 25 °C yields.
raised to 60 °C (Fig. 13a). We therefore repeated this temperature dependence measurement using a fresh platinized PSI preparation (Fig. 13c). The yield of integrated hydrogen evolution increased between 25 °C and 55 °C, as shown in Fig. 13c. Further increase in the temperature above 55 °C (the physiological growth temperature) resulted in a slow decrease in the rate of hydrogen evolution. A slight decrease in the hydrogen evolution rate as a function of temperature was observed (Fig. 13c) above 55 °C (Durán et al. 2005). However, a similar decrease in the rate of hydrogen evolution with subsequent L/D cycles where the temperature was fixed at 25 °C was observed (Fig. 12c). Fitting both of these observed decreases in hydrogen evolution (Figs 12c and 13c) yielded a single exponential decay with a similar half-life of 3.76 h. This similarity in kinetics suggests some system component is slowly losing its activity during multiple L/D cycles.

Being the only consumable component, it is possible that ascorbate is degraded or consumed to sub-saturating levels during longer experiments. To test the limiting role of ascorbate, we ran a 70-h experiment as shown in Fig. 13d. We observed a decrease in yield best fit by the sum of two exponentials (half-lives 19.8 h and 4.64 h). The faster decay is similar to that observed in Figs 12c and 13c. At ~70 h, fresh ascorbate was injected (black arrow, Fig. 13d) yielding an immediate ~80% increase in H₂ evolution, with no decay evident for the next 30 h. This experiment shows that part of the decrease in yield is due to ascorbate consumption, which is reflected in this slow phase. The initial loss of activity is possibly a result of catalyst poisoning. Platinum and other platinum group metal catalysts are sensitive to poisoning by a wide range of compounds including sulfur-containing compounds (Du et al. 2005), heterocyclic nitrogen-containing compounds (Hegedus and Mathe 2002) and other surface adsorbing molecules. Chlorophyll, a five-membered heterocycle, is a known poison for some metal-based catalysts (Irandoust and Edvardsson 1993). It is possible that either free chlorophyll or
some other degradation product is responsible for this decrease in activity. A second explanation is that chlorophyll could be lost from PSI over time, leading to a direct decrease in the antenna size and rate of photon capture. During extended periods (> 24 h) there is a measurable loss of chlorophyll from the PSI complex as determined by absorption spectrometry (Fig. 18). This chlorophyll release could account for the initial change in hydrogen evolution rate over time, either by its poisoning of the platinum catalyst or by simply reducing the amount of chlorophyll in PSI or some combination of these processes. However, in long-term experiments (Fig. 13a) the rate becomes constant, indicating that the system is stable for months of operation after an early initial decrease in activity.

To compare the temperature dependence of hydrogen production for PSI from *T. elongatus* and *Synechocystis PCC 6803*, the integrated yield of hydrogen for a 2-h light cycle was measured and plotted against temperature. It is clear that for *T. elongatus* the hydrogen yield increased up to 55 °C before it began to decrease (Fig. 13c,e); however,
the hydrogen yield for *Synechocystis PCC 6803* decreased steadily and was barely detectable at 60 °C. The experiments above illustrate the thermal (> 55 °C) and temporal (> 2,000 h) stability achievable with these hydrogen-evolving nanoparticles. The hydrogen evolution rates shown in Fig. 12c (~80 nmol H\textsubscript{2} h\textsuperscript{-1} (mg chlorophyll)\textsuperscript{-1}) equal or exceed those previously reported using platinized PSI from plants (Evans et al. 2004; Millsaps et al. 2001).

In the final experiment, reaction conditions were altered to allow comparison to a recent publication (Grimme et al. 2008) and are described in the Methods. Under these conditions, a maximum rate of > 5.5 µmol H\textsubscript{2} h\textsuperscript{-1} (mg chlorophyll)\textsuperscript{-1} was obtained (Fig. 14). Our maximum rate is of the same magnitude as that recently reported for a tethered PSI nanoparticle complex used by Grimme and colleagues (Grimme et al. 2008). In that study, pre-formed platinum/gold nanoparticles coupled directly to cyanobacterial PSI were used to evolve hydrogen. They reported a rate of ~45 µmol H\textsubscript{2} h\textsuperscript{-1} (mg chlorophyll)\textsuperscript{-1}. Unlike the self-organizing *in situ* platinization procedure used in the current study, this group rebuilt the native PSI to incorporate a recombinant form of the PsaC subunit containing a free cysteine that allowed the covalent attachment of preformed platinum–gold nanoparticles. The peak rate for our system is also an order of magnitude higher than the hydrogen evolution rates of ~580 nmol H\textsubscript{2} h\textsuperscript{-1} (mg chlorophyll)\textsuperscript{-1} that have been reported using a [NiFe]-hydrogenase from *Ralstonia eutropha* fused directly to PsaE of PSI from *Synechocystis PCC 6803* (Ihara et al. 2006b). An additional advantage of the *in situ* approach is that it requires at least a 50-fold lower level of platinum salts (70 µM versus 3.4 mM H\textsubscript{2}PtCl\textsubscript{6}) than the rebuilt tethered system. This is an important difference in light of the potential sustainability of such a hydrogen source. The platinized system can maintain a high level of hydrogen production over long periods (> 2,000 h), whereas the hydrogenase system described
above becomes inactive in less than 200 min and the stability of the system of Grimme and colleagues using a rebuilt PSI complex has not been tested beyond 12–16 h.

Discussion:

In summary, we have demonstrated a simple ‘rewiring’ of the electron transport pathway of PSI that permits a productive interface with a self-organized platinum catalyst (Fig. 9). This light-driven catalytic production of hydrogen is both temporally and thermally stable. Simple optimization of this self-organized system leads to rates close to those recently reported by researchers working with synthetically attached catalysts. Additionally, we showed that increasing the temperature to 55 °C increases our rates by a factor of ~15 (Fig. 13c), which considerably exceed the rates of other available methods while retaining the benefit of a self-organizing system. If scaled linearly, a solar collector 1 acre in size with a solution depth of 10 cm operating at 55 °C would be capable of producing hydrogen with an energy yield equivalent to that of 300 liters of gasoline per hectare per day (gross yield, ignoring production separation and distribution energy costs; see Supporting data). This potential yield is more than an order of magnitude higher than the gross yield in terms of gasoline equivalents of agricultural biomass systems such as corn-based ethanol (5.43 liters per day per hectare), soy based biodiesel (1.42 liters per day per hectare) or projected yields of switchgrass-produced ethanol (12.1 liters per day per hectare). Comparing this fuel production rate to the average available solar radiation at latitudes in the middle of the US, this system is capable of converting ~6% of solar radiation into usable fuel. This system provides a more direct route to fuel production with no need for the harvesting, converting, fermenting and distilling processes involved in conversion of biomass to ethanol. Moreover, other processing and transportation costs would be much lower because the bio-platinum hybrid catalyst is
reused through many cycles, unlike in single-use methods such as biomass accumulation. Finally, the fact that our PSI operates with high thermal tolerance suggests that this approach may be viable in non-arable regions with high solar irradiances. This is in contrast to the cultivation of biofuels that may compete directly with agricultural production.

Methods:

*Growth of T. elongatus*

The thermophilic cyanobacterium *T. elongatus BP-1* was grown in 2-l airlift fermenters (Bethesda Research Labs) in nitrilotriacetic acid (NTA) media. The temperature was held at 55 °C with continuous illumination by fluorescent lights. The light level was increased as the cultures approached higher densities to a maximum of 50 μE m⁻² s⁻¹. Cells were collected during late log phase by centrifugation for 10 min at 7,000 g, and washed once in wash buffer (20 mM 2-(N-morpholino)ethanesulphonic acid (MES) pH 6.5, 5 mM MgCl₂ and 5 mM CaCl₂) before storage at -20 °C until use for PSI preparation.

*Isolation of PSI*

Frozen cells were resuspended in wash buffer and 500 mM sorbitol. The resuspended cells were adjusted to a chlorophyll a content of 1 mg ml⁻¹ and homogenized using a Dounce homogenizer. Lysozyme was added to 0.2% (w/v) and the mixture was incubated for 2 h at 37 °C with shaking. The resulting mixture was centrifuged for 10 min at 7,000g and the light blue supernatant was discarded. The pellet was resuspended in the wash buffer. The volume was adjusted again so that the chlorophyll a concentration was 1 mg ml⁻¹; the mixture was then passed twice through French Press (Amino) at a cell
pressure of 20,000 psi. The highly fluorescent lysate was centrifuged at 50,000 \( g \) for 20 min, and the supernatant was discarded. The crude membrane fragments collected in the pellet were washed in wash buffer supplemented with 3 M NaBr and then twice in the initial wash buffer. The final washed membrane fragments were adjusted to a chlorophyll a concentration of 1 mg ml\(^{-1}\) and dodecyl–maltoside (\( \beta \)-DDM) was added to a final concentration of 0.6% w/v and the mixture incubated for 20 min at 20 °C in darkness with gentle stirring. The insoluble material was removed from the solubilized membrane mixture by centrifugation at 50,000 \( g \) for 30 min. The supernatant was separated immediately from the pellet and then loaded onto 10–30% sucrose gradients with 60% cushion; all solutions in the gradient also contained 20 mM MES pH 6.5 and 0.03% \( \beta \)-DDM. Density gradient centrifugation was performed at 80,000g at 10 °C for 16 h. The lowest green band contained the trimeric PSI complex; these bands were collected and pooled using a large syringe. Pooled PSI samples were slowly diluted fivefold by addition of 20 mM MES pH 6.5 with 0.03% \( \beta \)-DDM (w/v), and then loaded onto a POROS 20HQ (Applied Biosystems) anion exchange column and eluted with a linear 0 to 400 mM MgSO\(_4\) gradient. The MgSO\(_4\) was removed by dialyzing against 20 mM MES pH 6.5, 500 \( \mu \)M CaCl\(_2\), 500 \( \mu \)M MgCl\(_2\) and 0.03% \( \beta \)-DDM, and aliquots were stored at -80 °C for future use.

**Secondary structure deconvolution and thermal stability of PSI and cyt c6**

CD of purified PSI was measured in the visible region from 350 to 750 nm using an Aviv 202 (AVIV Biomedical). Spectra were collected across a temperature range of 15–95 °C in increments of 3 °C; at each temperature the sample was equilibrated for 3 min before collecting the data with a step size of 1 nm and integration of 1 s. Similarly, the cyt c\(_6\) was scanned between 15 and 95 °C in 5 °C increments.

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Real-time hydrogen detection and calibration

An in-house developed continuous flow system was used for hydrogen detection. A humidified nitrogen stream flowing at 50 ml min⁻¹ carried the hydrogen produced in the reaction chamber to an in-line Figaro tin oxide hydrogen sensor (model TGS821, Figaro Engineering). The signal from the hydrogen sensor was transmitted to a Keithley model 2000 auto-ranging digital multimeter via a bridge amplifier as described previously (Millsaps et al. 2001). The sample was illuminated by means of a dual Fiber-Lite A3200 fiber-optic illuminator with an OSRAM 150 W quartz halogen lamp (Dolan-Jenner Industries). Light was filtered through a 590-nm long-pass filter and measured with a Licor quantum flux meter (Model Li-189). For the light saturation and temperature dependence experiments, a Fisher Scientific fiber-optic illuminator (Model 12-562-36) fitted with dual fiber-optic cables was used, and an USHIO 150 W halogen projector lamp (USHIO INC) calibrated using the LI-1800 portable spectroradiometer (LI-COR). At each power setting on the fiber-optic illuminator, the micro-processor-controlled LI-1800 spectroradiometer was used for rapid acquisition of spectroradiometric and photometric data.

PSI-mediated hydrogen evolution. Platinization reactions were carried out by incubation of cyt c₆, PSI (T. elongatus or Synechocystis) and 0.5 mM Na₂[PtCl₆] in a thermostatically controlled photo-bioreactor as described previously (Millsaps et al. 2001), except that 20 mM MES at pH 6.4 was used to buffer the reaction. The molar ratio of cyt c₆ to T. elongatus PSI was maintained at 10:1, unless otherwise stated. Typical reactions contained PSI (80 mg chlorophyll ml⁻¹), 282 µmol cyt c₆, 0.5 mM Na₂[PtCl₆], 20 mM MES buffer pH 6.4 and 20 mM (1 mM in some cases) NaAsc, at 25 °C. All reaction components, except NaAsc, were combined and injected into the

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⁹ Image of experimental setup included in (Millsaps et al. 2001)
photobioreactor. After anaerobiosis was achieved (~1 h) the NaAsc was added to the reaction. Samples were illuminated as described above. A Chrontrol Model XT microprocessor-based timer was used to cycle the light on and off at intervals of 2 h. The photo-bioreactor was shielded from external light. After 24 h of the light-catalyzed platinization and hydrogen evolution, the reaction mixture was dialyzed in 20 mM MES buffer pH 6.4, to remove excess hexachloroplatinate. In some cases, sucrose density gradient centrifugation was used to remove small analytes but had the additional effect of separation of the PSI complexes and cyt c₆.

Temperature–activity measurements were carried out as described above using the dialyzed platinized PSI preparation. The reactions were carried out in 20 mM MES pH 6.4 with 20 mM NaAsc as the sacrificial electron donor. No additional cyt c₆ or Na₂[PtCl₆] were added to these reactions. An external water bath was used to incrementally increase the temperature during successive 2 h L/D cycles, allowing the reaction to equilibrate during the dark cycles. A thermocouple was inserted into the reaction mixture to directly measure the temperature of the reaction. For long-term stability, hydrogen evolution was measured as above, and the data were integrated as a function of time with a smoothing window of 13 data points (Fig. 12b), demonstrating the compatible yield upon long-term storage.

For the peak yield experiment in Fig. 13, the PSI complexes were platinized as described above, except that the chlorophyll and hexachloroplatinate concentrations were reduced to 5 µg ml⁻¹ and 70 µM, respectively. After ten light cycles, the PSI complexes were recovered and purified by density gradient centrifugation. The purified PSI complexes were resuspended in reaction media at a cyt c₆ to P700 molar ratio of 75, with an illumination level of ~1,200 µE m⁻² s⁻¹, a NaAsc concentration of 100 mM and a temperature of 25 °C.
Supporting Information:

Optimized *E. coli* expression, secretion and assembly of *T. elongatus* recombinant cyt c6

The SDS-PAGE analysis of typical purification is shown in Supplemental Fig. 15A. This purification first involves the use of IMAC followed by anion exchange chromatography. The combination of these two steps can be used to produce purified protein as is shown in Supplemental Fig. 15a, lane 2. The native environment of heme is confirmed for this recombinant protein by the reduced minus oxidized difference spectra shown in Supplemental Fig. 15b. This spectra indicates a β-peak at 552.1 nm which is identical to published values for native cyt c6 isolated from a variety of related cyanobacteria (Cho et al. 1999). Finally, the purity and heme coordination of this protein is also evident from the ratio of the A552/A274 of the reduced form of cyt c6. We observed a ratio of 2.1 for the purified protein, considerably higher than the best reported value (Diaz et al. 1994) indicating both high purity and uniform heme insertion.

Thermal stability of *T. elongatus* cyt c6

The structural stability of the recombinant cyt c6 was determined by monitoring the secondary structure using circular dichroism Aviv 202 (AVIV Biomedical, Lakewood, NJ). The temperature was ramped from 20°C to 94°C at 1° per min. At each temperature, the sample was equilibrated for 5 min and the spectrum was recorded and baseline corrected, the corrected spectra was deconvoluted using the CDPRO software with the IBASE3 reference set of 37 soluble proteins (http://lamar.colostate.edu/~sreeram/CDPro). The results are shown in Supplemental Fig. 16c. Since the cytochrome is primarily alpha helical and since this is the most
accurately predicted secondary structure, we used the helical content to determine the $T_m$ for denaturation.

**Evaluation of temporal sustainability of hydrogen evolution**

The initial platinization of PSI was conducted using a 10:1 ratio of cyt c$_6$ to P$_{700}$ in the presence of NaAsc and hexachloroplatinate for 60 h at 25°C; three representative L/D cycles are shown on the left of Fig. 12A. Upon termination of the reaction, the PSI preparation was dialyzed overnight (indicated as S1, for Segment 1) to remove small reactant molecules and by-products (Fig. 12A). There was no visible precipitation of platinum in the reaction. The dialyzed PSI preparation was re-introduced to the reaction vessel, fresh NaAsc was added; H$_2$ production was observed without the addition of hexachloroplatinate further demonstrating that the Pt catalyst was stably associated with PSI. After 170 h of hydrogen evolution at 25°C, shown schematically as S2, the temperature was increased to 60°C for 20 h to determine if the system was thermally stable, as expected for thermophilic cyanobacterial proteins. The H$_2$ evolution rate at 60°C was not only stable but actually increased by 80%. Following this temperature increase the platinized Pt-PSI biomimetic catalyst was stored at 4 °C for an extended period of 64 days (S3) and the rate of hydrogen evolution was re-measured at 25°C for 29 L/D cycles and observed to be comparable or possibly a bit higher than the rates observed earlier at 25 °C, however this may be due to fresh NaAsc being added at the beginning. Finally, the sample was stored another 5 days (S4) and then retested, showing only a slight decrease from the initial values.
Methods

Purification of cyt c6 by IMAC & HPLC. IMAC was performed using Ni-Sepharose (Pharmacia, GE Healthcare) chromatography column. The column was rinsed with 3 column volumes ddH$_2$O and then equilibrated with 3 column volumes of 50 mM Tris (pH 8.0). The total periplasmic fraction was passed over the column at a maximum rate of 3 ml/min. The column was washed with 3 column volumes of 50 mM Tris and then eluted with 50 mM Tris (pH 8.0) and 300 mM imidazole. The IMAC purified cyt c$_6$ was then purified using anion exchange chromatography using POROS HS resin and a BioCAD 202 Chromatography workstation.

SDS/PAGE and chemiluminescent heme stain. Purified cyt c$_6$ was subjected to Tris-Tricine-SDS as described by Schagger & Von Jagow (Schagger and Vonjagow 1987) and stained with Coomassie Brilliant Blue R250. In order to further confirm the presence of the heme-containing holoprotein, cyt c$_6$ was additionally subjected to Tris-Tricine-SDS followed by electroblotting and chemiluminescent heme staining as described previously.

Energy Dispersive Spectroscopy. Energy levels of the emitted X-rays at 2.05, 11.07/11.25, and 9.36/9.44 KeV indicate the presence of platinum. The presence of carbon (0.28 KeV), nitrogen (0.39 KeV), oxygen (0.52 KeV), magnesium (1.25/1.30 KeV), sulphur (2.31 KeV), chlorine (2.62 KeV) and iron (6.4 KeV) were also indicated by the energy levels of the emitted X-rays.
Spectral analysis of chlorophyll in PSI and supernatant. Hydrogen evolution was carried out for 14 L/D cycles with platinized PSI complexes (90 \( \mu g \) Chl/ml, 10 mM NaAsc, 10-molar excess cyt c\(_6\) to PSI ratio, 20 mM MES, pH 6.4) at 30 °C and illumination at 300 \( \mu E \) m\(^{-2}\) s\(^{-1}\). Mock samples were treated similarly yet they were kept placed in a second reactor that was kept in the dark. After 56 h (equivalent to 14 L/D cycles) 1.5 ml samples were removed from both the mock treatment and the hydrogen evolving sample and centrifuged at 50,000 \( g \). The supernatant was collected and the remaining green pellet was resuspended in 1 ml of 90% methanol, shaken using a MP Biomedical FastPrep-24 and spun at 21,000 \( g \) for 5 min. This methanol extraction was further diluted 10 fold and the spectra was collected on a Cary 50 Bio UV/Visible spectrometer using a 1 mm pathlength quartz cuvette. The 50,000 \( g \) supernatant was also measured yet this sample was not extracted in 90% methanol nor was it diluted (shown in Supplemental Figure 18).

![Image](image1.png)

**Figure 15 – Characterization of T. elongatus cyt c6.** Cytochrome c\(_6\) was cloned into pET21d (Novagen) and used for expression in order to reduce the \( P_{700}^+ \) in this work. **a.** Tris-Tricine SDS-PAGE of purified cyt c\(_6\) with no significant impurities. After much optimization, large amounts of cyt c\(_6\) were obtained at this purity. **b.** In order to test the reversible redox activity and estimate concentration, reduced-minus-oxidized difference spectroscopy was used in which the cyt c\(_6\) demonstrates the expected maximum at 552.1 nm and the extinction coefficient of Synechocystis PCC 6803 was used.
Figure 16 – Thermal Stability of PSI and cyt c6. a. & b.) Visible CD absorbance data for PSI derived from Synechocystis and Thermosynechococcus measured between 15 and 95 °C, respectively. Lowest temperature scans are shown in violet and proceed though blue, green yellow and finally red for the highest temperature. The visible CD absorbance of PSI is a function of pigment arrangement/environment and is a sensitive indicator of the functional state of PSI; the fine structure of these spectra is lost as the temperature is increased. c.) The thermal stability of cyt c6 was also investigated using UV-Vis CD spectroscopy. Using the CDPro software package, the data was deconvoluted as indicated in the main text and the resulting values are plotted as a function of temperature. Helical content is plotted with red circles, unordered with green diamonds, turn with inverted purple triangles and beta strand with blue squares. Using the helical content and fitting with a sigmoidal Boltzmann distribution, we estimate the Tm value to be 80.6 ± 1.0 °C.

Figure 17 – TEM of individual PSI particles showing electron dense particles following platinitization and staining with 0.5% potassium phosphotungstic acid. Images were collected on a Hitachi H800 TEM at 50,000 magnification. Seven individual particles are shown to reveal the somewhat irregular electron dense labeling that may represent bound metallic platinum particles. Sample preparation was the same as described in figure 10.
Figure 18 – Spectral analysis of chlorophyll loss. a.) This represents the spectrum of the mock PSI treatment (green line) and the hydrogen evolving sample (red line) after 56 h (14 L/D) after extraction with 90% methanol. b.) This is the spectrum of the 50,000 × g supernatant following 14 L/D cycles of hydrogen evolution. The inset represents an enlargement of the chlorophyll absorbance at 675 nm. The arrows indicate the α- and β-peaks of the cyt c6 that also remains in the supernatant.
IN VITRO KINETICS OF P700+ REDUCTION OF THERMOSYNECHOCOCCUS ELONGATUS PHOTOSYSTEM I PARTICLES BY RECOMBINANT CYTOCHROME C6 USING A JOLIET-TYPE LED SPECTROMETER

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Abstract:

The reduction rate of photooxidized Photosystem I (PSI) with various natural and artificial electron donors has been well studied by laser-flash absorbance spectroscopy. The electron transfer rate from various donors to P700+ has been measured for a wide range of photosynthetic organisms encompassing cyanobacteria, algae, and plants. PSI
can be a limiting component due to tedious extraction and purification methods required for this membrane protein. In this report, we analyze the reduction kinetics of isolated photooxidized PSI from *Thermosynechococcus elongatus* (*T.e.*) by recombinantly expressed (*T.e.*) cyt c6 via a Biologic Joliot Type Spectrometer (JTS-10) LED pump-probe spectrometer. We use a standard sample mix containing only 3\( \mu \)g/ml chl a, and vary cyt c6 concentration, temperature, pH, and ionic strength, all of which show similar trends to the reported literature utilizing higher PSI concentrations with laser-based spectrometers. Our results do however indicate kinetic differences between actinic light sources (laser vs. LED), and we have attempted to resolve these effects by varying our LED light intensity and duration. We can conclude that our findings from the LED based JTS-10 system reveal that a molecular crowding effect exists at high protein concentration and that additional kinetic phases are evident during multiple turnover events of P\(_{700}^+\) reduction by cyt c6.

**Introduction:**

In oxygenic and anoxygenic photosynthetic organisms, photosynthetic electron transport requires a soluble electron carrier protein to couple the process of electrogenic proton pumping via the b\(_{6f}/bc\)_1 membrane complex with the light activated charge separation associated with the reaction center of Photosystem I (Blankenship 2002). In oxygenic cyanobacteria and algae, two separate metalloproteins have evolved to perform this physiological role, a heme-containing protein, cytochrome c\(_{553}\) (also called cyt c\(_6\)), and a type 1 copper protein, plastocyanin (PC) (Wood 1978). Despite no sequence or structural similarities, both proteins have reversible 1-electron metal redox centers and are capable of binding to the luminal side of PSI in the thylakoid membrane and donating an electron to P\(_{700}^+\) (Bohner et al. 1980). Although functionally
interchangeable, cyt c₆ is considered to be the original, ancestral protein and is still found in primitive, thermophilic cyanobacteria (Beissinger et al. 1998; Nakamura 2002). However, as iron became limited in aquatic environments following the great oxygenic event (Navarro et al. 2011), evolution yielded an alternative, copper containing electron donor, PC, which reduced the dependence of these photosynthetic organisms on scarcely available iron. In some algae both cyt c₆ and PC genes still exist with PC being primarily expressed under iron-limiting conditions (Ho and Krogmann 1984). In vascular plants only PC has been shown to reduce PSI, although there is a cyt c₆-like protein expressed in Arabidopsis (Gupta et al. 2002), this protein is unable to donate electrons to P₇₀₀⁺ (Molina-Heredia et al. 2003).

Direct reduction of the oxidized special pair (P₇₀₀⁺) by these soluble donors occurs on the luminal surface of PSI. This electron transfer step has been previously studied with a variety of eukaryotic and prokaryotic systems, both in vivo and in vitro with isolated/purified proteins, along with artificial donors to P₇₀₀⁺ (Baker et al. 2014; Gourovskaya et al. 1997; la Rosa et al. 2002). Previous kinetic models have suggested that there are three mechanisms for P₇₀₀⁺ reduction by either PC or cyt c₆: oriented collision, complex formation, and complex formation with interface rearrangement (Hervás et al. 1995). These models have been proposed as evolutionary intermediates in the optimization of surface interactions between donor and acceptor proteins, with less specificity in cyanobacterial systems to more high affinity interaction in algal and higher plant systems (Hervás et al. 2005; Hervás et al. 2003; Hippler et al. 1999; Olesen et al. 1999).

The rate of electron transfer/P₇₀₀⁺ reduction has been characterized mainly by laser-based transient absorption spectroscopy at various wavelengths, characteristic of either donor or acceptor proteins (Hervás et al. 1992; Mamedov et al. 1996; Olesen et al. 1999).
Reported rate constants vary from micro- to milli-second lifetimes with varying interpretations accounted for quantitatively by single, biphasic (Díaz-Quintana et al. 2003), and three component (Jin et al. 2001) exponential models typically under pseudo-first order kinetics. Moreover, this variation still holds even when comparing the kinetics of different cyanobacterial systems, where multi-phasic rate differences are dependent on species, measurement apparatus, protein concentration, temperature, ionic strength, and mutations of protein surface charges (Balme et al. 2001; HATANAKA et al. 1993; Hippler et al. 1998).

In this work we have characterized the in vitro kinetics of $P_{700}^+$ reduction using purified PSI and recombinantly expressed cyt c$_6$ from the thermophilic cyanobacteria *Thermosynechococcus elongatus*. This characterization has been performed using a Biologic JTS-10, a Joliet-type LED pump-probe spectrometer (Biologic SAS, Claix, France). Although this instrument does not have the kinetic resolution of laser-based systems with shorter actinic flashes, it is capable of resolving the cytochrome c$_6$ reduction kinetics of *T. elongatus* $P_{700}^+$ as a recovery from the bleaching of the ground state at 705 nm or as the appearance of the absorbance of $P_{700}^+$ at 810 nm. The compact size, ease, affordability, and high sensitivity of this instrument make it ideal for measuring many of the parameters that affect the kinetics of electron transport in vitro and possibly in the field. The common design and solid-state nature of the JTS-10 will permit many labs to compare results using a common instrument. We have determined the effects of donor concentration, temperature, ionic strength, pH, and total protein on this electron transfer process. The reduction kinetics fit well to a two-phase recovery with half-lives of the fast and slow phase of 6.7 and 62.3 ms respectively (Fig. 21A, 10:1 trace). We introduce a model for extended actinic periods relative to the single turnover experiments to explain the observed two-phase kinetics. In addition, through site-
directed mutagenesis we are making selective amino acid changes that may increase the 
affinity and stability of interactions between the PsaF (Lumenal PSI subunit) and cyt c₆. 
Since we are working in vitro we do not need to be constrained by the physiological need 
to cycle between the b₆/f complex and PSI, thus we can maximize the interaction 
between PSI and cyt c₆ and thereby enable higher yield of either photosynthetic 
hydrogen production or photocurrents in vitro.

Materials and Methods:

*PSI isolation and recombinant cytochrome c₆ expression/purification*

PSI particles from *T. elongatus* were isolated and purified using sucrose density 
gradient centrifugation and anion exchange chromatography as described in 
(Iwuchukwu et al. 2010). Recombinant *T. elongatus* cytochrome c₆ was co-expressed 
with the maturation pathway for c-type cytochromes in *E. coli* and purified from the 
periplasm as described in the previous work (Kranz et al. 1998).

*JTS-10 Configuration And Data Collection*

Basic configurations of JTS utilize an “Orange Ring” array of LEDs (630 nm peak 
emission) for the actinic source at 3000 μE/m²/s intensity (unless otherwise noted), with 
the probe LEDs emitting at either 705 nm or 810 nm using the appropriate interference 
filters for each LED wavelength. Data collection begins with 15 discrete points (500 ms 
 apart) to establish a pre-actinic flash baseline, followed by 200 points of post-actinic 
flash, exponentially increasing from 60 μs to 10 s. The first 15 points (pre-actinic flash) 
and the last 10 points are used for a linear regression of each sample trace. Data shown 
are an average of three individual traces with a subtraction of a non-actinic trace,
accounting for any actinic activity given off by the probe LEDs. Data was analyzed and fitted using the GraphPad Prism® software (Fig. 19).

Sample Setup

Samples are comprised of purified *T. elongatus* PSI at 3 µg chlorophyll/ml or 34 nM (assuming 100 chl a/PSI), with a 10-fold molar excess of recombinantly expressed *T. elongatus* cytochrome c₆ as described previously (Iwuchukwu et al. 2010). The mix also includes 2 mM sodium ascorbate, 0.1 mM methyl viologen, 5 mM MgCl₂, 5 mM MgSO₄, brought up to 1 mL of 20 mM MES buffer (pH 6.4) with 0.03% n-Dodecyl-β-D-maltoside (Glycon Biochemicals, Germany).
**Laser Flash Photolysis**

Kinetic experiments at 830 nm were performed on an in-house constructed system. For the detection beam, a Newport 150 mW diode was used. For single turnover excitation, a Spectra Physics Q-switched Nd:YAG laser was frequency doubled to 532 nm to provide a saturating laser flash with pulse width of 5-8 ns. The power of each laser pulse reaching the sample and the detector was limited using neutral density filters. Data sets were averaged 8-32 times with 5 s between each laser flash. Signals were detected with an OSI Optoelectronics PIN-10D photodiode and were amplified using a Femto DHPCA-100 High Speed Variable Gain Amplifier. Signals were digitized and recorded using a Tektronix 3012B oscilloscope and a LabView® program written in-house. Data sets were fitted with 1st, 2nd, and 3rd order exponential models in Prism®.

**Results:**

**Illumination/Photo-bleaching Parameters**

Both PSI and cyt c₆ were purified using the methods described previously (Iwuchukwu et al. 2010). The purity of both proteins was determined by SDS-PAGE (Fig. 20A) and BN-PAGE (Fig. 20B). The BN-PAGE indicates that yield and homogeneity of PSI was primarily in the trimeric form with an apparent molecular weight of 800 kDa with very little PSI monomer (apparent molecular weight of 400 kDa) and a very small amount of PSII monomers or dimers. Although these molecular weights appear low, this has been observed for well-characterized PSI preparations and may be due to an altered ratio of coomassie to protein in the blue-native gel system relative to the standards (Li et al. 2014). The lack of PSII was also confirmed from the low temperature fluorescence at 77K which had a single prominent peak at 728 nm (Fig. 20C) and lacked the
characteristic fluorescent peak at 695 nm. This far red emission peak is also
case of the trimeric form of PSI since the monomer form of PSI has an emission
peak blue shifted several nm to 725 nm as demonstrated in our previous work (Li et al.
2014).

The JTS-10 is an LED based spectrometer and the ability to completely photo-
oxidize P_{700} using this actinic light source needed to be confirmed. The amplitude of
absorbance change at 705 nm was monitored with varying the length of the LED
illumination from 10 μs to 7.5 ms. Fully photooxidized samples containing 34 nM PSI (3
μg chlorophyll/ml) displayed a plateau in the intensity of the photobleaching after 2.5 ms
at 3000 μE/m²/s (Fig. 21A). The actinic LED intensity was also varied from 12 to 3000
μE/m²/s with three different exposure times (5, 15, and 45 ms). The sigmoidal shape of
this light intensity effect on photobleaching reached the same saturating value yet took
considerable higher light levels when the illumination period was reduced from 45 ms to
15 and 5 ms. The complete conversion of P_{700} to P_{700}^{+} required a flash duration longer
than 5 ms at intensities below 3000 μE/m²/s as indicated by maximum ΔAbs_{705nm} (Fig.
21B). Considering the required actinic flash duration to reach complete P_{700}^{+} formation
for this LED based system is 2–3 ms and previously reported rates of charge separation (approximately 1 μs) to form the (P$_{700}^+$, F$_B^-$) pair (Breiter and Leibl 2001), suggests that multiple turnover events of P$_{700}^+$ reduction by cyt c$_6$ may occur when actinic flash durations in the millisecond range are used. We also investigated the effect of cyt c$_6$:PSI ratio on the signal intensity. Measurements were performed in triplicate at four concentrations of cyt c$_6$, ranging between 10 and 60-fold molar excess to PSI, each of which gave similar absorbance amplitudes at each flash duration.

The solid state design of the JTS-10 allowed P$_{700}$ activity to be measured with very dilute samples. We tested the signal-to-noise of the ΔAbs$_{705\text{ nm}}$ measurement as a function of P$_{700}$ concentration. We observed that we could clearly see P$_{700}$ photobleaching and get a high quality fit of the reduction kinetics all the way down to 0.1 μg chlorophyll/ml. This is significantly lower concentration of PSI than was used in similar reports in literature range from 10 μg chlorophyll/ml (HATANAKA et al. 1993) (100 x more sensitive) to 0.75 mg chlorophyll/ml (Hervás et al. 1995) (7500 x more sensitive), which indicates the sensitivity of the optical design and photodiodes used in the JTS-10.

Figure 21 – Total P$_{700}$ photo-oxidation displayed as a function of flash duration and flash intensity. a.) 34nM of PSI was used with 10, 20, 40, and 60-fold molar excess of cytochrome and a using the “orange ring” lamp at 3000 μE/m²/s. b.) 34nM of PSI was used with a 10-fold molar excess of cytochrome and flash intensity ranged from 12 to 3000 μE/m²/s.
**Cyt c₆/P₇₀₀ ratio**

In most cyanobacteria P₇₀₀⁺ is reduced via cyt c₆ in vivo. This process is similar to the activity of plastocyanin in plants and algae. We have over-expressed and purified the mature, holo form of cyt c₆ with the addition of a C-terminal His-tag. We have then used this purified form of cyt c₆ to reduce P₇₀₀⁺. The oxidized cytochrome is then reduced slowly via sodium ascorbate (data not shown). In the absence of cyt c₆, reduction of P₇₀₀⁺ did not occur within our timeframe of our experimental regime (Fig. 22A, bottom trace). The reduction rates of P₇₀₀⁺ were analyzed with both single and double exponential models and the residuals were determined. As can be seen from the amplitude of the residuals, the single exponential fit of the data was unable to accommodate the data adequately especially in the first 100 µs (Fig. 22A), suggesting an additional component relative to previously reported rates in the reduction rate of P₇₀₀⁺ by cyt c₆ of *T. elongatus*. We find that the amplitude or contribution of this fast phase does not change much as we increase the cyt c₆/PSI ratio and stays around 10% of the total amplitude. The small contribution of the fast phase is consistent with our two-phase model, such

**Figure 22** – Total P₇₀₀ re-reduction as a function of cyt c₆ concentration (molar excess). **a.** 34nM of PSI was used with 0, 1, and 10-fold molar excess of cytochrome. Residuals of P₇₀₀ re-reduction shows better fit to two phases as compared to one. **b.** 34 nM of PSI was used with excess molar ratios of cyt c₆ ranging from 0 to 100. Rates of two phases (k_fast & k_slow) compared to single-phase rate (k_obs). k_fast fits to a single exponential while k_slow and k_obs follows a linear fit. The % k_fast shows all ratios have about 10% fast phase.
that the slow phase ($k_{\text{slow}}$) closely matches the single exponential rate ($k_{\text{obs}}$), where they both increasing linearly with increasing cytochrome concentration and at low cyt c₆ concentration, accounts for the majority of the reduction process, while the rate of the fast phase ($k_{\text{fast}}$) increases with increased cyt c₆ concentration in a single exponential manner (Fig. 22B). We report a value of $1.27 \times 10^6$ (M$^{-1}$s$^{-1}$) for the observed rate constant with cytochrome concentration ranging from 34 nM to 3.4 µM (Fig. 22B).

**Effects of Temperature**

Measurement temperature was also analyzed, ranging from 5 – 60 °C, showing a similar trend in a linear increase in $k_{\text{slow}}$ and a single exponential increase in $k_{\text{fast}}$ with increasing temperature (Fig. 23A). The temperature dependence of the $k_{\text{slow}}$ is shown at three different protein ratios, all of which display similar rate increases. Samples measured at 5°C and 60 °C showed similar absorbance amplitudes, indicating both proteins were still active and stable, while higher temperatures of 65 °C and 70 °C gave

![Figure 23](image)

**Figure 23** – Temperature dependence of the observed rate constant at three different ratios of Cyt c₆ to PSI. **a.)** As temperature goes from 5 to 60°C, there is a linear increase in $k_{\text{slow}}$ and single exponential increase in $k_{\text{fast}}$, with %$k_{\text{fast}}$ ~10% (not shown). **b.)** Increased P₇₀₀ re-reduction rate ($k_{\text{slow}}$) as the measurement temperature is raised from 5 to 60°C for 2.5, 10, and 40-fold molar excess of cytochrome to PSI. **c.)** Arrhenius plot displays an inflection point between 40 and 45°C.
much lower amplitudes that continued to decrease with time (not shown), indicating possible protein degradation or irreversible unfolding. The temperature effect was also plotted onto an Arrhenius plot which we then calculated the activation energy ($E_a$) to be ($28.3, 23.1, \text{and} 21.7$ kJ/mol) for 2.5, 10, and 40 molar excess of cyt $c_6$: PSI respectively (Fig. 23B). An inflection point can be seen between 40 and 45 °C. This is approaching the physiological temperature that we grew the organism at (50°C) but is much higher than the temperature that we used to culture the $E. \text{coli}$ for over-expression of the cyt $c_6$.

It is possible that there is some thermally induced conformation change in one or both of these proteins that caused the $E_a$ to become some what less above 40°C. This observation is similar to what has been observed for both plastocyanin and cyt $c_6$ when tested from the thermophile $\text{Phormidium}$, where they also observed that the rate plateaued above 40°C (Balme et al. 2001).

**Effects of pH and Ionic Strength**

The $P_{700}^+$ reduction rate increased with decreasing pH (8.2 to 5.5) (Fig. 24A), which agrees with previous reports utilizing similar donor proteins with pI’s around that of $T.e.$ cyt $c_6$ (5.5) (Hervás et al. 1992; Medina et al. 1993; Takabe et al. 1983). In addition to varying pH, changing the buffer composition between MES and TES also appears to have

![Figure 24](image-url)
an added effect as seen in Fig. 24A, where two separate fits are used for the two buffers between pH’s of 6.6 and 6.9. Effects of ionic strength also agreed with previous reports (Balme et al. 2001) with a rise in $P_{700}^{+}$ reduction rate with increasing ionic strength by increasing MgCl$_2$ concentration which reaches a plateau of about 30 mM for our sample mix (Fig. 24B). This has been interpreted as the weakening of any electrostatic forces between cyt c$_6$ and psaF subunit of PSI that might be repulsive (Balme et al. 2001).

**JTS-10 LED vs. Laser Flash Photolysis**

Laser flash photolysis was performed on the same sample measured in the JTS-10 with the LED actinic source. Although the probe beam of the YAG laser-based system was 830 nm, we demonstrate that 705 nm and 810 nm detection on the JTS yield virtually identical $P_{700}^{+}$ reduction kinetics (Fig. 25). The measured change in absorbance at 705 nm and 810 nm correlated well with difference spectra of *T. elongatus* $P_{700}$ from previous reports, showing the absorption change to be about 8 times more intense at 705 nm and in the negative direction (Nakamura et al. 2011). The bleaching at 705 nm is the disappearance of $P_{700}$ while the concomitant appearance of the broad 800-840 nm peak.

![Figure 25](image)

**Figure 25** – Comparison of $P_{700}$ re-reduction rate as a function of measurement wavelength. **a.** 705 nm shows about 8 times the change in amplitude of absorbance in the negative direction as 810 nm. **b.** Superimposition of normalized recovery curves.
is the direct observation of \( P_{700}^+ \). Results showed similar total \( P_{700}^+ \) recovery times (80 ms for JTS and 90 ms for laser) for both actinic light sources, with the main difference being in the curve fit of the laser data showing a single phase in reduction as compared with the JTS-10’s two phases (Fig. 26).

In order to detect a measurable signal with our laser system, the chlorophyll content had to be increased 10-fold to 340 nM / 30 μg chlorophyll ml\(^{-1}\) and with that caveat, the same sample was then analyzed using the LED based JTS-10 yielding an unexpected increase in the percent \( k_{\text{fast}} \) of our bi-phasic results. This led to the analysis of the contribution of the fast phase of the \( P_{700}^+ \) reduction rate as with increased total PSI concentration from 20 nM to 540 nM, keeping cyt \( c_6 \):PSI ratio constant, resulting in the rise of \( \%k_{\text{fast}} \) from less than 10% to nearly 90%.

**Discussion:**

In our investigation of the electron transfer between recombinantly expressed *T. elongatus* cytochrome \( c_6 \) and purified PSI, we utilized a pulsed LED spectrometer to monitor the reduction kinetics of \( P_{700}^+ \). As seen from the results, we can conclude that with adequate light intensity and duration, the system reaches a maximal \( \Delta \text{Abs}_{705\text{nm}} \) or
saturation level of P$_{700}$ oxidation. The rate of P$_{700}^+$ reduction was monitored as a function of electron donor (cyt c$_6$) concentration, resulting in a recovery curve exhibiting two kinetic components. This suggested either two competing processes or a multiple step process of P$_{700}^+$ reduction. At a 34 nM PSI concentration, we see that the slower component closely resembles that of the overall or combined rate constant, k observed ($k_{obs}$), increasing linearly with higher amounts of cyt c$_6$, suggesting a predominantly diffusion driven system. The faster component was negligible accounting for ~10% of the total decay amplitude. Reported rate constants for P$_{700}^+$ reduction by cyt c$_6$ of *T. elongatus* range from 1.7 $\times$ 10$^6$ (M$^{-1}$ s$^{-1}$) (Hatanaka et al. 1993) to 5.9 $\times$ 10$^6$ (M$^{-1}$ s$^{-1}$) (Proux-Delrouyre et al. 2013), in which the variability can be attributed to variations in sample set-up and measurement conditions. The linear increase in the $k_{slow}$ and $k_{obs}$ indicates that this system is either at dilute sub-saturating concentrations of cyt c$_6$ or the interaction is collisional and saturation is not expected.

With previous reports suggesting that cyanobacterial PSI lacks specificity for its electron donor (Hervás et al. 2005), we are surprised to see the bi-phasic nature of the re-reduction kinetics since the interaction appears to be collisional. This suggested an instrument/light source specific origin where the longer actinic duration in the millisecond time-frame is the main factor. This is developed further in the discussion.

This is also supported by the same trend in rate increase as the measurement temperature was raised from 5 to 65 °C. The activation energy ($E_a$) can be further extrapolated by plotting the rate increase on an Arrhenius plot, which yielded a higher $E_a$ for lower cyt c$_6$ concentrations. This suggested more energy is required for this reaction when lower electron donor is present.

The dependence of P$_{700}^+$ reduction kinetics was then investigated as a function of pH, which also influenced the kinetics of the reduction. We created a pH profile
indicating the highest observed rate at pH 5.5 which steadily decreasing rates as the pH is increased to 8.2, however, this may not represent the optimal rate as it was the lowest pH investigated. As the acidic residues’ pKa’s are around 4.5, additionally lowering pH is unlikely to further increase the rate, but rather it may begin to lower. This correlated with previously described results (Hervás et al. 1992; Medina et al. 1993; Takabe et al. 1983), all of which seem to agree the lower the pH gives the donor protein a favorable charge with their pI’s all fairly lower than 7. Depending on the source organism, this will vary with regards to any electrostatic surface charges of cyt c₆ and the respective psaF subunit on the lumen of PSI. Along with a pH effect, we also see that the rates can be affected by varying the buffer, between MES and TES. This has been observed in other studies dealing with similar proteins to generate a difference in end-product yield (H₂) (Ellis and Minton 2006; Lubner et al. 2011), although this may have been attributed to reducing the required potential for proton reduction, here we see that it also improves the interaction with the electron donor.

Molecular crowding can impact kinetic rates via two contributions. One, large macromolecules will occupy more volume, creating an increased effective solute concentration and subsequent decrease in water activity, thereby increasing reaction rates by decreasing diffusion lengths. The other opposing effect is that the higher viscosity results in decreased protein diffusion rates (Ellis and Minton 2006; Kirchhoff 2008). Many kinetic models have been described for the donor/PSI interaction, with variability being attributed to the different source organisms (Hervás et al. 2005; Hervás et al. 1995; Hervás et al. 1994). In eukaryotes, the electron donor to PSI is predominantly acidic, interacting with a well conserved positively charged docking site on the N-terminus of the psaF subunit of PSI via electrostatic interactions (Ben-Shem et al. 2003; Hervás et al. 2003). This has been suggested by previous studies to result in a
biphasic kinetic rate where the electron transfer of the preformed donor/PSI complex gives rise to the initial fast phase and the following slow phase is attributed to donor/PSI complex formation (Fromme et al. 2003; Hervás et al. 2003; Hervás et al. 1995). This is not the case for cyanobacterial systems however, where a simpler diffusion based collisional mechanism is thought to exist for electron transfer from donor to PSI, suggesting the kinetic rates would be better fitted to monophasic model in single-turnover experiments that utilizes an actinic laser (Hervás and Navarro 2011).

In further analyzing the longer flash duration used in the JTS-10, we see that the light history and the total protein concentration play significant roles in determining the kinetic behavior of the system. As the PSI concentration is increased we observe the effects of molecular crowding with increasing cyt c₆ concentration. To compare our results with multiple turnovers, we performed flash photolysis with a Nd:YAG laser system with pulse width of 10 ns, allowing only a single turnover of P₇₀₀. In this analysis, even with a similar rate of P₇₀₀ recovery, we are able to see only one phase, which would represent a simple collision rate of cyt c₆ to P₇₀₀ as dominated by the second order rate constant of complex formation. We attribute this kinetic difference to the relatively long actinic light source duration where the system will undergo multiple P₇₀₀ turnovers of oxidation/reduction.
During prolonged illumination, P$_{700}$ is photooxidized very rapidly, and the majority of the P$_{700}$ population accumulates in oxidized state regardless of cyt c$_6$ concentration. In this regime, the oxidized P$_{700}^+$ may have a different affinity for cyt$_2^{2+}$ and in any case as its concentration is increased and the equilibrium is shifted toward complex formation. The electrostatic potential surface has previously been suggested by computational studies to depend heavily on the oxidation state of the heme (Autenrieth et al. 2004); similar effects for P$_{700}$ have not been investigated but are certainly possible and would explain the kinetic behaviors we observe. During long actinic periods, the cycle of (cyt$_2^{2+}$ + P$_{700}^+$ → cyt$_3^{3+}$ + P$_{700}$) can have populations contributing various rates depending on the relative rate constants (Fig. 27). If $k_2 < k_3 < k_1$, the complex will arrive at a nonzero
concentration dependent steady state and \( k_3 \) will become directly observable following the actinic period. As the total protein concentration increases, the complex formation between Cyt\(^{2+} \) and \( P_{700}^+ \) is also favored, further increasing the pool of [cyt:P\(_{700}\)], leading to a higher percentage of the fast \( k_3 \) phase being observed versus the slower complex formation of \( k_2 \). Within one 5 ms \( P_{700} \) oxidation/reduction cycle, it is suspected that the conversion of Cyt\(^{2+} \) to Cyt\(^{3+} \) is much less than 10% of the total cyt \( c_6 \) population, where ascorbate is in excess, leading to Cyt\(^{2+} \) depletion to be insignificant. While these observations differ from single-turnover laser-based observations, the sensitivity is much greater requiring less sample, and more importantly, the direct observation of the first-order decay of the electron transfer is possible. This situation is precluded by the single-turnover experiment, which requires a sequential formation of each population, of which the first is a relatively low probability event, and eliminates the observation of the second faster step.

While we find that variation exists between our LED-based JTS-10 configuration and a laser-based system, the ability to conduct experiments at lower protein concentrations is a significant advantage of the simpler system. However we should add that for experiments with sub-microsecond characteristic times or preformed, dark complexes, the abilities of laser-based systems coupled with ultrafast detection will remain most appropriate.
CHARACTERIZATION OF PHOTOSYSTEM I FROM GALDIERIA SULPHURARIA, AN ANCIENT RED ALGAE, AND THE EVOLUTION OF ITS NATIVE ELECTRON DONOR, CYTOCHROME C₆

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Abstract:

Photosystem I (PSI) receives electrons from a soluble redox protein, either the copper-containing plastocyanin (Pc) or iron-containing cytochrome c₆ (cyt c₆). Cyt c₆ is regarded as the original redox protein for this role with Pc evolving later due to iron limitation. The key interaction leading to the association with Pc occurs via the PsaF subunit of PSI, which has inserted a complementary basic domain. Here we present comparative transient absorption spectroscopy data for the interaction between the PSI-cyt c₆ for two thermophilic microorganisms: the cyanobacteria *Thermosynechococcus elongatus* and the red alga *Galdieria sulphuraria*. In addition being extremophiles, they also both use only cyt c₆ as an electron donor for PSI. Although midpoint potentials of both cytochromes are similar, the reduction of PSI occurs faster in *G. sulphuraria*. Also unlike *T. elongatus*, the kinetics of *G. sulphuraria* PSI reduction displays biphasic kinetics similar to the PSI-Pc interaction observed in plants. Homology modeling of PSI and cyt c₆, reveal complementary charged surfaces that may be involved in interaction. These kinetic results support a long-range electrostatic mechanism of PSI-cyt c₆ interaction, which may produce a PSI-cyt c₆ complex. As a primitive alga, it is evolutionarily interesting that *G. sulphuraria* displays a similar biphasic electron transfer mechanism as observed in plants. The fact that the PsaF gene is retained in the
chloroplast genome suggests that this mode of electron transfer may have existed and preceded its transfer to the host nucleus during endosymbiosis. This suggests that the progenitor cyanobacteria may also have evolved an electrostatic interaction between PsaF and the earliest electron donor, cyt c6. Further work will be needed to temporally place this process relative to the evolution of plastocyanin.

Introduction:

Oxygenic photosynthesis is one of the central biogeochemical processes influencing every aspect of chemistry occurring at the surface of the planet (Falkowski and Woodhead 1992). Namely, maintaining a source of molecular oxygen in the atmosphere, providing reduced compounds for biomass i.e. the oxidant and the fuels for respiration, on which all aerobic life is dependent (Falkowski and Godfrey 2008). Sometime after the many ionic chemical species (e.g. sulfur and ferrous iron) and molecular compounds (e.g. methane) and eventually the atmosphere of the ancient earth were oxidized and molecular oxygen accumulated during great oxidizing event between 2.22 and 2.06 billion years ago (Holland 2006; Karhu and Holland 1996). This was a fundamental change that affected the entire biosphere, with the exception of niche environments (e.g. freshwater sediments/swamps, rice paddy fields, peat bogs, etc.) where ancient metabolisms that do not utilize molecular oxygen continue to thrive. An additional consequence in aquatic environments was the insolubility of ferric iron, which has eventually become a major limitation to primary production in large areas of oligotrophic oceans.

Oxygenic photosynthesis begins with the process of oxygen evolution. Electrons are extracted from water, and are then moved though three macromolecular complexes (PSII, b6/f complex, and PSI) in the thylakoid membrane that are responsible for
generation of a proton gradient and production of reductants for the reduction of carbon
dioxide (Blankenship 2002). This process begins at oxygen evolving complex of the first
pigment-protein complex, photosystem II (PSII), and the photo-oxidation of its pigment
cluster P680 (Durrant et al. 1995; Zouni et al. 2001). Four successive oxidations at PSII
result in the accumulation of 4 oxidation equivalents and the oxidation of two water
molecules producing molecular oxygen (Kok et al. 1970), 4 protons in the luminal side of
the thylakoid, and liberating 4 electrons for the electron transfer (ET) reactions. These
ET reactions are often coupled to proton transfers and as such, are capable of storing
energy by perturbing the concentration of these ions across the thylakoid. After two
internal electron transfer reactions, two electrons exit PSII via the first soluble carrier,
plastoquinol, which is lipid-soluble and shuttles the electrons in pairs to the cytochrome
b6/f complex. Although no photochemistry occurs in the b6/f complex, the plastoquinol
is oxidized liberating protons originating from the stroma into the lumen, and a series of
proton-coupled ETs, known as the Q-cycle, occur further increasing the amount of
energy stored across the membrane. Another soluble carrier, either cytochrome c₆ (cyt
c₆) or plastocyanin (PC) is then employed to shuttle the electrons through the aqueous
compartment of the lumen to the photo-oxidized pigment pair P₇₀₀⁺ of photosystem I
(PSI). Through the absorption of an additional photon in PSI, the electron is lowered in
potential and transferred from the lumen via chlorophyll, and three iron sulfur clusters
into the stroma where it is accepted by the iron-sulfur protein ferredoxin. The reduction
potential of the electron is then lowered sufficiently so that it is utilized by ferredoxin-
NADP reductase to produce NADPH, which is used for many reduction reactions in the
cell such as reduction of CO₂ or H⁺. Furthermore, the protons which have been pumped
from the stroma into the lumen flow back into the stroma turning the ATPase, and the
energy temporarily stored as a proton gradient from the previous proton/electron
transfers are conserved in chemical bonds in ATP which is widely used to drive unfavorable reactions in vivo (Blankenship 2002).

This is a brief description of oxygenic photosynthesis under linear electron transport, which is responsible for the far-reaching effects of photosynthesis as a portion of the biogeochemical processes for oxygen and carbon. The molecular mechanisms and evolutionary history of photosynthesis are necessarily complex, however, understanding the molecular mechanistic details is of central scientific interest. Photosynthesis incorporates 112 petagrams (Pg) of atmospheric carbon (Ito 2011), of which ~25% is currently used by human society (Haberl et al. 2007). Although intense effort is still needed as the overall process of photosynthesis is not very efficient in high irradiance (Blankenship et al. 2011), and future energy solutions are suggested to rely, at least in part, on photosynthetic products, creating dangerous ecological conditions. While some aspects of photosynthesis operate near perfect efficiency (e.g. light-harvesting under low irradiance, charge separation without recombination), other processes require much improvement (e.g. CO₂ concentration and fixation efficiency, multiple pigments for dual-threshold PS machinery). Bio-inspired versions of the efficient processes may guide technological advances, while improvements to the natural system may also drastically increase its efficiency. The societal impacts include agriculture/food production, global population/ geopolitical stability, energy, climate and global ecological stability.

Researchers have been exploring every aspect of photosynthesis for many years, however many questions remain unanswered, especially with regards to the evolutionary progression of the photosynthetic complexes and processes. Organisms that have continuously occupied these niche environments without undergoing such dramatic changes over geological time are capable of providing insight into the early stages of evolution of photosynthesis. A good example of one type of environment occupied by
these ancient organisms is hot springs, which are dominated in their chemistry by abiotic geochemical processes. Acid-thermal environments are typically dominated by red algae of the order Cyanidiales, whose members are primitive eukaryotes with a single chloroplast and a single mitochondrion (Rothschild and Mancinelli 2001). The genera present in this order are *Cyanidium*, *Cyanidioschyzon*, and *Galdieria* reclassified from previous literature (Albertano et al. 2000). Most algae have plant-type monomeric PSI and chlorophyll-a binding membrane embedded light harvesting complexes (LHCR); in addition, *Galdieria* also has phycobilisomes for additional light harvesting. Also in contrast with most other plants and algae, in *G. sulphuraria*, the genomic copies of PsaF (the subunit of PSI responsible for binding of soluble donor) and cyt c₆ remain in the chloroplast, and there is no copy of plastocyanin in either the chloroplast or nuclear genome (Vanselow et al. 2009).

In this study we have characterized the PSI of *Galdieria sulphuraria* and its interaction with cyt c₆, its soluble donor. Although the organization of genes involved closely resembles that of the progenitor cyanobacteria, the interactions between PSI and cyt c₆ are not adequately described by a purely collisional model. While most cyanobacterial PSI:cyt c₆ partners fall in this category, *G. sulphuraria* PSI:cyt c₆ interaction appears as an early introduction of an electrostatically stabilized binding site. It likely represents a starting point for the evolution of tight PSI:donor interactions and the accompanying fast ET rates observed in those systems that predates the evolution of PC.
Materials And Methods:

Growth of *G. sulphuraria* and PSI Isolation.

*G. sulphuraria* was grown in 11 L flasks at 42°C as in previous studies (Thangaraj et al. 2010). Cultures were harvested by centrifugation once they reached sufficiently high density (OD_{730} >2). Cells were resuspended in MM buffer (20 mM MES at pH 6.0, 10 mM CaCl₂, 10 mM MgCl₂, and 500 mM mannitol) and PMSF was added at a final concentration of 1 mM. The cell suspension was then lysed using a bead beater (Biospec Products, Bartlesville, OK). The thylakoids were collected by centrifugation at 12,000 x g for 30 min at 4 °C. β-Dodecyl-maltopyranoside (β-DDM) was added at 0.6% (w/v) and stirred for 30 min at room temp with a final chlorophyll (chl) concentration of 1 mg/ml. Detergent solubilized material was clarified by centrifugation at 58,000 rpm in a Beckman Type 70 Ti rotor for 45 min. The PSI containing material was collected by gently mixing the loose portion of the pellet. The resuspended detergent solubilized material was then loaded onto an anion exchange column (Fast Flow Q-Sepharose, GE Healthcare Life Sciences) and eluted by 0 to 200 mM MgSO₄ gradient. The PSI containing fractions were then concentrated and loaded onto a gel filtration column as a final step of purification resulting in homogeneous PSI samples (see figure 22B).

Purification of *G. Sulphuraria* cyt c₆.

The soluble portion of of the *G. sulphuraria* lysate was used for purification of cyt c₆. Before breaking the cells, they were homogenized in 20 mM phosphate buffer, 5 mM sodium ascorbate and mixture of protease inhibitors (1 mM PMSF, 1 mM benzamidine and 1 mM ε-aminocaproic acid). Cells were disrupted by using glass beads of 0.5 mm dia in a bead beater (Biospec products, Inc., OK, USA), with a spin time of 1 min, followed by
a break time of 30 sec for cooling and this process is repeated for 20 times. The glass beads were washed with 20 mM phosphate buffer and the suspension was centrifuged at 4500 rpm for 2 minutes. Following centrifugation, the pellet was discarded and the supernatant was sonicated (Model 300V/T, Ultrasonic homogenizer, Biologics, Inc., VI, USA) for 1 min at 60% power after adding solid NaCl, to reach a final concentration of 50 mM. The sonicated supernatant was centrifuged at 9000 rpm for 20 min and precipitated material was discarded. Solid ammonium sulfate was slowly added to the supernatant to reach a final concentration of 40% under vigorous stirring and pH was continuously monitored. Small amounts of NaOH were added to keep the pH values close to 7. This solution was stirred for 20 min at 4°C and centrifuged at 8850 rpm for 20 min. Ammonium sulfate was added again to the resulting supernatant to reach a concentration of 100%, and pH was adjusted as mentioned above, and stirred for 60 min. After centrifugation at 8850 rpm for 20 min, the pellet was resuspended in 40 to 50 ml of 10 mM phosphate buffer (pH 7.0) and dialyzed (3500 MWCO, 54 mm width, 34 mm diameter, Spectrum laboratories Inc., CA, USA) against 4 l of the 10 mM phosphate buffer (pH 7.0) for 43 h, with at least one buffer replacement. The dialyzed solution was loaded on to the Q-Sepharose column (GE Healthcare Life Sciences) previously equilibrated with 20 mM phosphate buffer (pH 7.0). A linear gradient from 20 mM to 100 mM was applied to wash and elute the cyt c₆, which was eluted just after the gradient began. The cytochrome c₆ containing fractions were pooled and concentrated by using 5K cut-off ultrafiltration spin filters (Amicon Ultra-15, Millipore Corporation, MA, USA) and centrifuged at 4000 rpm at 4°C (Sorvall SH-3000). The concentrated protein solution were purified further by gel filtration column containing Sepharose-CL6B (Amersham Biosciences, USA) on Akta FPLC system GE Healthcare Life Sciences) with a
flow rate of 1 ml/min. Purified cyt c₆ were again concentrated and stored in 20 mM phosphate buffer at -80 °C.

**Purification of T. elongatus cyt c₆**

*T. elongatus* cyt c₆ was purified as previously described (Beissinger et al. 1998).

**Cloning Expression and purification of recombinant G. sulphuraria cyt c₆**

The coding sequence of cyt c₆ of *G. sulphuraria* was expression optimized and purchased with flanking NdeI and XhoI sites. The resulting fragment was subcloned into pET30 bearing a C-terminal extension of LEHHHHHH for immobilized metal-affinity chromatography purification. The resulting plasmid was used along with pRGK333 bearing the system I heme maturation pathway for increased capacity of maturation for c-type cytochromes (Feissner et al. 2006) to transform BL-21(DE3) *E. coli* as previously described for *T. elongatus* cyt c₆ (Iwuchukwu et al. 2010). Expression cultures were supplemented with 5-aminolevulinic acid (0.4 mM) for increased heme production and maintained under selection for each plasmid using both kanamycin (30 mg/L) and ampicillin (100 mg/L).

**PSI intact protein mass spectrometry**

Performed as in previous work on PSII (Thangaraj et al. 2010). The initial separation was achieved with LC MS and concomitant fraction collection (LC-MS+). Fractions of interest were further investigated using nano-electrospray FT ion-cyclotron resonance MS experiments (FT-MS). Both collisionally activated and electron capture dissociation were used to confirm the subunits of PSI.
**EPR spectroscopy**

EPR spectra were collected on a Bruker (Elexsys E580) spectrometer equipped with an Oxford liquid helium cryostat and temperature controller. The spectrometer conditions were as follows: temperature 12K; power 50mW; microwave frequency 9.41 GHz; Modulation amplitude 20 Gpp. Spectra were obtained by averaging 4 scans. The PSI samples were concentrated in 2 mM chl concentration. For the reduction of the [4Fe-4S] clusters the samples were treated in two ways: a) Samples were chemically reduced with 40mM sodium Hydrosulfite in 100 mM glycine at pH 10.0 and incubated in the dark for 30 min, the samples were frozen in liquid nitrogen in the dark, so called dark reduced samples, b) samples were chemically treated with 40 mM sodium dithionite in 100 mM Tricine at pH 8, incubated in the dark for 30 minutes and the samples were frozen in the dark. To promote an electron transfer from P700 to the [4Fe-4S] clusters the samples were illuminated at 200K for 10 min for the photo-induced charge separated state.

**Cyt c₆ Reduced minus Oxidized Difference spectra**

Absorbance spectra of cyt c₆ were collected in the oxidized and reduced states using a split beam UV-Vis spectrophotometer (Shimadzu UV-2550) with 1 cm pathlength quartz micro cuvettes with a total volume of 100 µL. Oxidized spectra were collected using potassium ferricyanide (2.5 mM) as an oxidant which was included in both the sample and reference cuvettes. Identical amounts of cyt c₆ were added for the reduced sample in the presence of 2.5 mM sodium ascorbate. Multiple spectra were collected even at higher oxidant/reductant levels to ensure complete oxidation or reduction, respectively.
**Cyt c₆ midpoint potential measurements**

Cyt c₆ samples were prepared at 0.5 mg/ml in 100 mM sodium phosphate buffer at pH 7.00. Measurements were performed using a CH Instruments 650 electrochemical workstation in the standard 3-electrode setup using a glassy carbon working electrode, platinum mesh counter electrode, and a saturated calomel electrode as a reference. Buffers were saturated with argon during the measurements, which were performed at room temperature. Charging current was subtracted using SOAS (Fourmond et al. 2009) as well as identification of peak positions for midpoint potential calculations. The midpoint is reported as the average of the anodic and cathodic peak positions of the background subtracted scans.

**Transient absorption spectroscopy**

Nanosecond transient absorption measurements were performed with excitation at 630 nm from an optical parametric oscillator driven by the third harmonic of a Nd:YAG laser (Ekspla NT342B). The pulse width was ~4-5 ns, and the repetition rate was 5 Hz. The detection portion of the spectrometer (Proteus) was manufactured by Ultrafast Systems (the instrument response function was ca. 4.8 ns). The probe light from a 150 W xenon lamp was filtered using a long band pass filter RG 780. Transient absorption kinetics of P₇₀₀⁺ at 810 nm were recorded. Samples contained 30 µg/ml chl a and a 10-fold molar excess of cyt c₆ as well as 2 mM sodium ascorbate, 0.1 mM methyl viologen, 5 mM magnesium chloride, 5 mM magnesium sulfate, 20 mM MES (pH 6.4), and 0.3% (w/v) β-DDM.
Homology Modeling of G. sulphuraria PsaA, PsaB, PsaF of PSI and PetJ (cyt c₆)

The PSI subunits, PsaA, PsaB and PsaF were modeled based on alignments generated between the G. sulphuraria sequences and the Pisum sativum PSI structure (pdb id 1QZV). Likewise, the template used for the cyt c₆ was pdb id 1C6S of Thermosynechococcus elongatus. Alignments were generated using the SALIGN algorithm of MODELLER (Sali and Blundell 1993) with the default parameters, the percent similarity between all templates and targets were above 70% using the Gonnet62 substitution matrix and are therefore likely to be accurate for homology modeling (Marti-Renom et al. 2000).

Rigid Body Docking

Rigid body docking was performed using HEX (Ritchie and Kemp 2000). 20,000 separate docking results were collected and ranked by lowest energy using steric and electrostatic considerations.

Results:

Characterization of the G. sulphuraria PSI complex

Galdieria sulphuraria was cultured to high density, collected by centrifugation and lysed using a bead mill. The resulting membranes were then solubilized using β-Dodecylmaltopyranoside, and separated initially by anion exchange chromatography as shown in Figure 28A. The first major peak containing material that does not interact strongly with the column contained mainly phycobiliproteins (PBS), followed by the peak centered around 30 min which contains monomeric PSI, and the final peak centered around 45 min was primarily composed of PSII. The PSI fraction contained
PBS following the anion exchange chromatography, and was further purified to homogeneity using gel filtration, see Figure 28B. Following the preparative gel filtration column, the sample was concentrated and passed through the column again; no PBS were observed. The purified PSI was separated on a denaturing Tris/Tricine SDS-PAGE alongside well-characterized PSI preparations shown in Figure 28C. The large subunits of PSI run aberrantly on SDS-PAGE as they are extremely hydrophobic and are not fully...
denatured by the solubilization solution, for comparison a trimeric complex from *T. elongatus* was included as well as the monomeric complex from the green algae *C. reinhardtii*. Since *G. sulphuraria* has genes for PBS, light harvesting complex 1 (LHCR1), and PsaL, there are components typically considered cyanobacterial-like (e.g. PBS and PsaL) as well as algal/plant-like (e.g. LHC1); however, the PSI complex clearly resembles the algal architecture and has LHC1 antennae complexes. The LHC type antenna is consistent with TEM observations that suggest the antenna is larger than the typical algal/plant PSI (Thangaraj et al. 2011).

The creation of the reduced [4Fe-4S] cluster EPR signal in a previously uncharacterized PSI complex such as *G. sulphuraria* is indicative of the environment of the [4Fe-4S] clusters and is therefore important for the characterization of the reaction centers. Although the EPR signals of the [4Fe-4S] clusters $F_A$ and $F_B$ are not fully understood in relation with the structure and the orientation of the clusters, their g-values are as follows: $F_A^-$ has rhombic features with g-values of 2.05, 1.95 and 1.85; $F_B^-$ has rhombic features with g-values of 2.07, 1.93 and 1.88. The well-studied signals from *T. elongatus* (Vassiliev et al. 2001) (Figure 28D trace C) were used as a control for sample treatment, focusing on reaction center and electron transfer activities during illumination. Freezing a PSI complex during continuous illumination promotes two or more electrons through $P_{700}$ to both $F_A$ and $F_B$, producing a so-called ‘interaction spectrum’ with g-values of 2.05, 1.94, 1.92 and 1.88 (Figure 28D trace B). The interaction spectrum results from the close distance between the two spin systems, $F_A^-$ and $F_B^-$, and reflects magnetic coupling between the two paramagnetic centers. The $F_A^-/F_B^-$ interaction spectrum is best observed at temperatures below 20 K. In order to reduce both $F_A$ and $F_B$, the PSI complexes were incubated with 50 mM sodium dithionite at pH 10 in darkness. The resulting spectrum measured at 10 K (Figure 28D trace A) is similar to the photo-
induced interaction spectrum (Figure 28D trace B). The EPR signals from $F_A/F_B^-$ interactions in *Galdieria* show a clear peak at $g=1.85$ possibly due to slightly different orientation of the [4Fe-4S] clusters compared to *T. elongatus*. The detailed study of the [4Fe-4S] cluster properties is beyond the scope of this paper and it is still under investigation. The photo-accumulated signal from *Galdieria* $P_{700}^+$ (Figure 28D trace b) appears to be similar to that of *T. elongatus* at $g = 2.0023$.

Identifications of the PSI subunits was accomplished made by top-down mass spectrometry (Thangaraj et al. 2010). Purified PSI from *G. sulphuraria* was loaded onto a reverse phase column, and the eluent was continuously flowing into an ESI-MS/MS instrument. The resulting chromatogram and masses of identified PSI subunits are shown in Figure 29. All subunits of PSI that were found in the genome were identified, with nuclear encoded subunits displaying post-translational modifications typical of general import into chloroplasts (i.e. transit peptides are removed). Other possible post-translational modifications are indicated on Table 1. Interestingly, all five isoforms of LHC1 were observed indicating that the accessory antennae size of the *Galdieria* PSI may be larger than other plant/algal PSI and is consistent with the previously reported high
chlorophyll:P$_{700}$ ratio and increased antenna size observed by TEM (Thanagaraj et al. 2011). The presence of the different LHCRs (except LHCR4) was confirmed by tandem mass spectrometry of peptides obtained by trypsin digestion of proteins in fractions collected concomitant with the MS (LC-MS+).

Table 2 - Summary of the intact protein mass spectrometry data for G. sulphuraria PSI. Proposed post-translational modifications are indicated in the final column.

<table>
<thead>
<tr>
<th>PSI Subunit</th>
<th>TMH$^a$</th>
<th>Elution Time (min)</th>
<th>Calc. Avg. Mass (Da)$^b$</th>
<th>Exp. Avg. Mass (Da)$^c$</th>
<th>Proposed PTMs$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PsaA</td>
<td>11</td>
<td>83.8</td>
<td>83,963</td>
<td>83,980</td>
<td>1-18 removed</td>
</tr>
<tr>
<td>PsaB</td>
<td>11</td>
<td>87.2</td>
<td>82,519</td>
<td>82,475</td>
<td>none</td>
</tr>
<tr>
<td>PsaC</td>
<td>0</td>
<td>37.7</td>
<td>8,710</td>
<td>8,710</td>
<td>none</td>
</tr>
<tr>
<td>PsaD</td>
<td>0</td>
<td>43.6</td>
<td>16,010</td>
<td>16,013</td>
<td>none</td>
</tr>
<tr>
<td>PsaE</td>
<td>0</td>
<td>32.9</td>
<td>8,455</td>
<td>8,461</td>
<td>1-54 removed</td>
</tr>
<tr>
<td>PsaF</td>
<td>1</td>
<td>68.6</td>
<td>20,441</td>
<td>20,439</td>
<td>1-8 removed</td>
</tr>
<tr>
<td>PsaI</td>
<td>1</td>
<td>98.6</td>
<td>4,050</td>
<td>4,078</td>
<td>Formyl-Met</td>
</tr>
<tr>
<td>PsaJ</td>
<td>1</td>
<td>85.2</td>
<td>4,752</td>
<td>4,780</td>
<td>Formyl-Met</td>
</tr>
<tr>
<td>PsaK/G</td>
<td>2</td>
<td>78.5</td>
<td>e</td>
<td>9,025</td>
<td>none</td>
</tr>
<tr>
<td>PsaL</td>
<td>2</td>
<td>90.7</td>
<td>15,297</td>
<td>15,299</td>
<td>none</td>
</tr>
<tr>
<td>PsaM</td>
<td>1</td>
<td>71.9</td>
<td>3,153</td>
<td>3,181</td>
<td>Formyl-Met</td>
</tr>
<tr>
<td>Lhcr1</td>
<td>3</td>
<td>69.4</td>
<td>28,940</td>
<td>28,940</td>
<td>1-43 removed</td>
</tr>
<tr>
<td>Lhcr2</td>
<td>3</td>
<td>74.0</td>
<td>19,687</td>
<td>19,687</td>
<td>1-45 removed</td>
</tr>
<tr>
<td>Lhcr3</td>
<td>3</td>
<td>77.5</td>
<td>20,109</td>
<td>20,069</td>
<td>1-43 removed</td>
</tr>
<tr>
<td>Lhcr4</td>
<td>3</td>
<td>67.6</td>
<td>20,461</td>
<td>20,462</td>
<td>1-45 removed</td>
</tr>
<tr>
<td>Lhcr5</td>
<td>3</td>
<td>65.8</td>
<td>20,530</td>
<td>20,529</td>
<td>1-36 removed</td>
</tr>
</tbody>
</table>

$^a$ TMH - Transmembrane Helices  
$^b$ Calc. Avg. Mass - calculated based upon translation of the gene sequence  
$^c$ Exp. Avg. Mass - experimentally determined average mass  
$^d$ PTMs - Post-Translational Modifications  
$^e$ The related sequences of PsaK and PsaG exist in duplicate in G. sulphuraria and were not predicted here.

**Comparative donor properties of cyt c$_6$ of T. elongatus and G. sulphuraria**

The nature of the interaction between PSI and its natural donor cyt c$_6$ was also investigated to determine if it dominated by collisional frequency as in cyanobacteria or long-range electrostatic attractions as in more evolved organisms. In this study, experiments were carried out using recombinant cyt c$_6$ (unless otherwise indicated) which was quantified as described previously, (Cho et al. 1999; Metzger et al. 1997) although the wild-type protein was also purified from the original organisms, T.
elongatus (Beissinger et al. 1998) and G. sulphuraria (this work). The spectral properties of the recombinant cyt c₆ samples are shown in Figure 30A and 30B. The oxidized and reduced spectra are seen in panel A in solid and dashed lines, respectively. These spectra are typical of c-type cytochromes, and the maxima of the α bands are 552.2 and 552.8 nm for the recombinant cyt c₆ of G. sulphuraria and T. elongatus, respectively. Since the extracted WT cyt c₆ from T. elongatus and G. sulphuraria cultures only yielded a small amount, they were used as a control to ensure that the redox properties of the recombinant proteins were not significantly perturbed (Table 2).

Midpoint potentials of cyt c₆ samples were measured with a three-electrode setup under potentiostatic control. Data of the recombinant T. elongatus cyt c₆ (Tecyt c₆) is shown in Figure 31 and is as a representative for all cyt c₆ cyclic voltammetry. Data has been processed using the SOAS (Fourmond et al. 2009) software package to remove noise, charging current, and to determine peak positions. The midpoint potentials of the Tecyt c₆’s are 342 and 323 mV vs NHE for the wild-type and recombinant cyt c₆’s respectively. The recombinant and wild-type Tecyt c₆ samples have a slight discrepancy of ~20 mV, however, this is a mild increase in driving force (i.e. slightly more reducing
potential) for $P_{700}^+$ reduction and if there is any change in the kinetics this would cause an overestimation of the kinetics of $P_{700}^+$ reduction. The midpoint potential between wt and recombinant $Gscyt$ c$_6$’s were nearly identical at 291 and 293 mV vs NHE, respectively, indicating that the thermodynamic driving force has not been altered.

**Kinetics of $P_{700}^+$ reduction by cyt c$_6$**

The oxidation of $P_{700}$ was initiated by a 5 ns laser pulse at 630 nm resulting in a single turnover of PSI which results in the transfer of one electron from $P_{700}$ to methyl viologen, which is capable of reducing oxygen, the terminal electron acceptor in the experimental condition. As a direct comparison between a well-characterized cyanobacterial PSI cyt c$_6$ interaction and that of *Galdieria*, $P_{700}$ concentrations were normalized for each sample and photooxidized in the presence of 10-fold molar excess of the appropriate cyt c$_6$. Excitation energy transfer was very fast (much faster than time resolution of transient absorption spectrometer employed) converting $P_{700}$ into a long-lived charge separated state $P_{700}^+\cdot[4Fe4S]^-$. In the case of the *T. elongatus* PSI/cyt c$_6$ pair, the interaction is as described previously and similar to the *Synechocystis PCC6803*
PSI/cyt c₆ pair, where the ET kinetics are explained by a single slow phase. The *T. elongatus* displays the single exponential decay with a lifetime of 9.89 ms. In the case of the *G. sulphuraria* PSI/cyt c₆ interaction, a single kinetic component is unable to explain the observed data; although the new phase is only one order of magnitude faster than the collisionally controlled system, *G. sulphuraria* shows a biphasic decay of P₇₀₀⁺ with lifetimes of 1.86 ms (83.6%) and 22.3 ms (16.4%). Differences in the observed kinetics between the two PSI/cyt c₆ pairs can be explained by different cyt c₆ binding interactions involved since the midpoint potentials for each of the cytochromes and the P₇₀₀⁺'s (Nakamura et al. 2011) are very similar, almost identical.
In order to elucidate individual residues that may be responsible for this interaction, homology models were constructed for the *G. sulphuraria* PSI and cyt c₆ using templates from spinach (Ben-Shem et al. 2003) (PDB ID: 1QZV) and *T. elongatus* (Jordan et al. 2001) (PDB ID: 1C6S), respectively. The interaction interface required the modeling of PsaA, PsaB, and PsaF of PSI. The percent similarity between the spinach and *G. sulphuraria* sequences were all greater than 70%. Likewise, the similarity of the cyt c₆ proteins of *G. sulphuraria* and *T. elongatus* was above 60%. All of these template-target sequences are sufficiently related to produce reasonable models using the automodel routines of MODELLER. The cyt c₆ model revealed that, like Pc, one face of the protein was significantly more acidic than the other. Initially, 20 models were generated for both
the PSI and cyt c₆ surfaces; the lowest energy models were chosen for docking calculations. The docking calculations were performed using shape complementarity and electrostatic considerations. The lowest energy docked structure is seen in Figure 33, and the acidic face of the cyt c₆ is facing the luminal loops of PsaF, which contain predominately basic residues are likely involved in electrostatic interactions with Cyt c₆ are shown in ball and stick representation with orange carbons and blue nitrogen with key interaction indicated within cyan.

In this orientation, the edge-to-edge distance between the heme of the cyt c₆ and the special pair of chlorophyll a in PSI is ~15Å. This distance would likely be shorter in the

![Figure 34 – PetJ vs psaF Charge Analysis.](image)

a.) petJ alignment from cyanobacteria(1-7), algae(8-23), and plants (24-25). Heme coordinating cysteins are highlighted in yellow. Potential psaF interacting interface region are boxed (A & B) with their charges shown on the left. b.) psaF alignment from cyanobacteria(1-7), algae(8-23), and plants (24-25). Charged residues are highlighted red (acidic) and blue (basic). Regions A and B are potential cytochrome interaction sites as indicated by (Vanselow et al. 2009) and their net charges are shown on the left. c.) Plot of cytochrome charge (region A) vs psaF charge (region B). Cyanobacteria are represented as circles, algae as diamonds and squares, and plants as triangles. Plastocyanin are shown in open square and triangles.
actual complex as the docking calculations were performed with the structures as rigid bodies. The main acidic residues of Gscyt c₆ responsible for interactions with PsaF are likely Glu 46, Asp 68, and Glu 72. Further mutational studies would be required to confirm the involvement of these positions. From left to right, the acidic residues of Cyt c₆ are Glu 46, Glu 72 and Asp 68. Likewise the Cyt c₆ model is shown in yellow ribbons with acidic residues in ball and stick with yellow carbons and red spheres for oxygen.

Alignment of PetJ from cyanobacteria (1-7), algae (8-23), and plants (24-25) are shown in Figure 34A. Potential PsaF interacting regions (A & B) are also shown with their respective net charges. Algal PetJ sequences appear to have more negative A and B regions than the cyanobacteria. The two plants are shown to have more neutral A and B regions, as these are predicted to be the Cyt c₆A version of cytochromes. The same is shown for PsaF luminal domain in Figure 34B. The potential cytochrome interacting regions (A & B) appear to contain more basic charges in plants and algae than in cyanobacteria as previously identified (Vanselow et al. 2009). A summary plot of PsaF vs PetJ charge is shown in Figure 34C. Cyanobacterial proteins seem to fit into a less charged lower left region (A), while algae (C) and plants (B) contain more charges on both interacting protein interfaces, suggesting potential electrostatic interactions. The acquisition of plastocyanin in plants are indicated by the lack of charged residues in their PetJs (B), yet with much more basic charges in the PsaF luminal domain and negative charges in the PsaF interacting regions (D) of plastocyanin (Pc). The shift in donor charges from region (B) to (D) indicates the transition from a basic collisional interaction between eukaryotic psaF and cyt to an electrostatic interaction with psaF and pc, with algal cyt c₆ fitting in between with region (C). It is also of note that Cyanidioschyzon (#22 in the alignments of Figure 34), a close ancestor of G. sulphuraria, has the same negatively charged cytochrome, yet a rather neutral lumen region of psaF. This is
indicative of a potential introduction of an electrostatic binding interaction by *Galdieria* in the evolution of a positively charged rsaF lumen domain.

Discussion:

Previous work using TEM and single particle analysis has indicated that the PSI of *G. sulphuraria* is monomeric as in other algae and plants, however, there are likely differences in the accessory antennae organization (Thangara et al. 2011). While the authors suggest that the core of PSI remained constant, there were abundant particles in the population that were of an appropriate size and shape to contain up to eight LHCR accessory antennae, twice as much as the spinach PSI structure (Ben-Shem et al. 2003). This is consistent with the comparison between PSI of *G. sulphuraria* and *C. reinhardtii* seen in Figure 28C, similar amounts of chlorophyll were loaded on the gel, however the LHC of *G. sulphuraria* are much more prominent. Although other particle types were observed in significant abundance as well, these results together with the observation of all five isoforms of the LHCR proteins in this study suggest that the antennae of *G. sulphuraria* is structured and/or possibly regulated differently than in plants and other algae, possibly in response to quality or quantity of light in its original environmental niche or interaction with PBS.

As an emerging tool in elucidation of atomic level detail of large membrane protein complexes, we have shown that top-down mass spectrometry may be used on these extremely hydrophobic complexes such as PSI, as has already been demonstrated for PSII (Thangaraj et al. 2010). Relative abundance of the antennae proteins may therefore be investigated as a function of various environmental conditions such as light intensity or wavelength. In this study, all subunits of PSI were observed including PsaK, which was overlooked in the genomic sequence data, but present in the mass spectrometry
data. The resolution was adequate enough to suggest post-translational modifications in some cases and readily identified cleavage sites of transit peptides, which may serve to improve predictive algorithms such as TargetP and ChloroP (Emanuelsson et al. 2000; Emanuelsson et al. 1999).

Comparative functional assays were carried out to make clear the differences of several intrinsic properties of *G. sulphuraria* with relative to *T. elongatus* PSI, which also utilizes only cyt c₆ as a donor. The EPR spectra (Figure 28D) indicate that there are differences in the environment of F₅₅/F₅₈ for *G. sulphuraria* relative to other PSI samples in the literature (Vassiliev et al. 2001). Both recombinant cyt c₆ samples used in this study had difference spectra that are typical of c-type cytochromes and displayed reversible electrochemistry. The recombinant *T* cyt c₆ has a slightly lower potential, but this decrease in midpoint potential would likely increase the kinetics if the rate were primarily controlled by thermodynamic properties (i.e. more overpotential is available). Instead we propose that the kinetics of this interaction are dominated by geometric/spatial constraints imposed by the type of binding interaction, which is collisional for *T. elongatus* and may consist of specific electrostatic interactions for *G. sulphuraria*. Major indications of binding specificity for the *G. sulphuraria* PSI:Gscyt c₆ complex include biphasic reduction kinetics when using Gscyt c₆ as a donor for GsPSI. None of the previous points are true for *T. elongatus* PSI:Tcyt c₆ interaction. Structural features that lead to this interaction in the GsPSI:Gscyt c₆ proteins are the acidic face of cyt c₆ and the insertion of a basic patch into PsaF. The insertion of the basic residues in PsaF is well studied in organisms using Pc as a donor to P₇₀₀, however this is the first observation of electrostatic binding and biphasic ET kinetics in an organism using only cyt c₆ as a donor to PSI.
Our modeling data suggests that \textit{Gscyt} c\textsubscript{6} interacts with PsaF in an analogous manner to Pc:PSI of plants which is in agreement with previous studies focusing on PsaF (Vanselow et al. 2009); although these models are not accurate in atomic detail, but they should allow identification of key residues with reasonable accuracy, namely, Glu46, Asp68, Glu72. The amino acids identified are spatially and chemically similar to the key residues identified in similar studies of \textit{C. reinhardtii}. The similarity between the Pc and \textit{Gscyt} c\textsubscript{6} interactions is remarkable, and indicates that transfer of PsaF to the nucleus did not necessarily precede the insertion of the basic residues of into PsaF, as this has not occurred in \textit{G. sulphuraria}. The extremely acidic environmental conditions occupied by \textit{G. sulphuraria} may account for increased iron solubility, and therefore, \textit{Galdieria} may not have been subject to this evolutionary pressure that led other organisms to depend on the copper-containing Pc. This particular evolutionary history may not be unique to \textit{G. sulphuraria}, and the so-called Pc binding site may have originally accommodated cyt c\textsubscript{6} with bound specificity instead of a mere collisional interaction.
CATALYTIC TURNOVER OF [FEFE]-HYDROGENASE BASED ON SINGLE-MOLECULE IMAGING

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My contributions included image analysis, structural analysis and independent confirmations of CaHydA concentrations and identity as well as design, writing, figure preparation and editing of the manuscript.
Abstract:

Hydrogenases catalyze the interconversion of protons and hydrogen according to the reversible reaction: $2\text{H}^+ + 2e^- \rightleftharpoons \text{H}_2$ while using only the earth-abundant metals nickel and/or iron for catalysis. Due to their high activity for proton reduction and the technological significance of the $\text{H}^+ / \text{H}_2$ half reaction, it is important to characterize the catalytic activity of [FeFe]-hydrogenases using both biochemical and electrochemical techniques. Following a detailed electrochemical and photoelectrochemical study of an [FeFe]-hydrogenase from Clostridium acetobutylicum (CaHydA), we now report electrochemical and single-molecule imaging studies carried out on a catalytically active hydrogenase preparation. The enzyme CaHydA, a homologue (70% identity) of the [FeFe]-hydrogenase from Clostridium pasteurianum, CpI, was adsorbed to a negatively charged, self-assembled monolayer (SAM) for investigation by electrochemical scanning tunneling microscopy (EC-STM) techniques and macroscopic electrochemical measurements. The EC-STM imaging revealed uniform surface coverage with sufficient stability to undergo repeated scanning with a STM tip as well as other electrochemical investigations. Cyclic voltammetry yielded a characteristic cathodic hydrogen production signal when the potential was scanned sufficiently negative. The direct observation of the single enzyme distribution on the Au-SAM surface coupled with macroscopic electrochemical measurements obtained from the same electrode allowed the evaluation of a turnover frequency (TOF) as a function of potential for single [FeFe]-hydrogenase molecules.

Introduction:

Hydrogenases are of growing interest due to their utilization of common metals at their active sites and ability to catalyze the $2\text{H}^+ + 2e^- / \text{H}_2$ redox system under nearly
activationless conditions, with concomitant energy storage or release (Armstrong 2004; Evans and Pickett 2003; Frey 2002; Vincent et al. 2007; Volbeda et al. 2005). They serve as models for catalysis of this most fundamental of redox reactions. Interest in the catalytic abilities of hydrogenases for hydrogen generation has led to various electrochemical studies (Butt et al. 1997; Goldet et al. 2009; Parkin et al. 2006; Vincent et al. 2005) as well as photoelectrochemical hydrogen production studies (Hambourger et al. 2008).

A typical [FeFe]-hydrogenase is CaHydA, a 65.4 kDa protein with a highly conserved [6Fe-6S] catalytic H-cluster as well as three [4Fe-4S] and one [2Fe-2S] accessory clusters. The proximity of the distal [FeS] cluster to the exterior of the protein allows electrons to be transferred directly from an external redox partner, presumably through a chain of [FeS] clusters, to the H-cluster, where catalysis occurs and hydrogen is produced or oxidized.

Direct electrochemical measurement of catalytic currents on macroscopic electrodes as a function of applied potential has yielded valuable information (Vincent et al. 2007). The CaHydA [FeFe]-hydrogenase, as well as other [FeFe]-hydrogenases, reportedly show a preference for proton reduction over hydrogen oxidation (Frey 2002), leading them to be investigated as a means of producing hydrogen. On the other hand, hydrogenases biased toward hydrogen oxidation could serve as catalysts in fuel cells (Jones et al. 2002). The mechanism of bias in either hydrogen oxidation or proton reduction is not clear: electrochemical studies have shown that these catalysts appear to operate very near the prevailing thermodynamic potential of the H⁺/H₂ couple and therefore cannot differentially favor the reduction or oxidation.

Numerous electrochemical studies with both [NiFe]- and [FeFe]-hydrogenases have been performed at carbon electrodes (Alonso-Lomillo et al. 2007; Butt et al. 1997;
while a handful have been performed on gold and modified gold electrodes (Hoeben et al. 2008; Krassen et al. 2009). Although most studies utilize hydrogenase adsorbed on an electrode as an ensemble average of orientations (Leger and Bertrand 2008), attempts have been made to specifically orient the protein on the electrode surface (Rudiger et al. 2005; Ruediger et al. 2010). A suitably oriented molecule allows for better defined interfacial electron transfer (Leger et al. 2002a) and the possibility of observing the largest current densities attainable for a given system. Maximum current densities are necessary to observe maximum turnover, and the number of molecules participating in the reaction must be known in order to calculate the TOF. Previously, surface coverage of hydrogenases on electrodes was either estimated on the basis of the electroactive surface area of an electrode, or by inhibiting the enzyme to reveal nonturnover signals from redox cofactors inside the protein (Armstrong 2002; Heering et al. 1998). While the latter technique can be useful and has provided the first measurements of electroactive surface coverage, it has only been demonstrated in a limited number of cases with specific hydrogenases, most notably a [NiFe]-hydrogenase from Allochromatium vinosum (Pershad et al. 1999). Calculation of the electroactive surface coverage of enzyme enabled Armstrong et al., to calculate accurate TOFs for various hydrogenases (Jones et al. 2002; Parkin et al. 2006). One study has also shown that a [NiFe]-hydrogenase from Thiocapsa roseopersicina can be imaged with a scanning tunneling microscope in a Langmuir Blodgett film; I–V curves of the protein film were reported (Nakamura et al. 1998). The ability to measure redox processes of simple proteins through SAMs has also been investigated previously where one-electron oxidations and reductions were taking place (Song et al. 1993). More complicated, multielectron or catalytic processes have also been measured through
SAMs. However those studies did not involve the use of single-molecule topographic techniques such as STM to determine surface concentrations (Haas et al. 2001; Krassen et al. 2009). Knowing the actual surface coverage on an atomically flat surface is crucial to accurately calculate the catalytic TOF, measured as a function of applied potential, per single adsorbed enzyme molecule.

The measurement of the maximum TOF of hydrogenase molecules is the most direct indication of catalytic prowess. Such a measurement requires accurate knowledge of the concentration of active protein and of substrates and products. However, performing this measurement in solution remains a formidable challenge because saturating concentrations for both substrates cannot be realized. Varying the substrate (H⁺) concentration as necessary to allow for a typical biochemical assay to determine the turnover of the hydrogenase is not possible because the protein is rendered inactive outside a narrow pH range (Hambourger et al. 2008; Leger et al. 2004; Leger et al. 2002b).

In this study, we investigate the enzymatic turnover of an [FeFe]-hydrogenase, CaHydA from Clostridium acetobutylicum, which is homologous (70% identity) (King et al. 2006) with the [FeFe]-hydrogenase from C. pasteurianum, CpI, for which structural data exists (Pandey et al. 2008; Peters et al. 1998). The CaHydA was chosen for this study due to its high catalytic activity for hydrogen production and the relative ease of recombinant expression (King et al. 2006). The enzyme was adsorbed onto a negatively charged SAM on a flat gold surface for investigation by electrochemical scanning tunneling microscopy (EC-STM) techniques and macroscopic electrochemical measurements. The EC-STM imaging allowed quantitative determination of the number of bound enzyme molecules. The bound enzymes were catalytically active, and cyclic voltammetry (CV) yielded a characteristic cathodic hydrogen production signal when the
potential was scanned sufficiently negative. The quantitative determination of the enzyme distribution on the Au-SAM surface coupled with macroscopic electrochemical measurements obtained from the same electrode allowed the evaluation of a TOF as a function of potential for single [FeFe]-hydrogenase molecules.

Experimental Section:

Electrochemical scanning tunneling microscopy (EC-STM) studies were performed with a Pico-SPM (Agilent, AZ) using a Nanoscope E controller and a bipotentiostat (Agilent, AZ). Imaging experiments were performed in a homemade Teflon cell using as a working electrode an atomically flat Au (111) substrate prepared by thermally evaporating ~130 nm of gold (Alfa Aesar 99.999%) onto freshly cleaved mica surfaces under ultra high vacuum ($2 \times 10^{-8}$ Torr). The STM tips were prepared by mechanically shearing Pt/Ir wire (80/20, 0.25 mm diameter) and coating the tips with Apiezon wax. All tips had <1 pA leakage current, the tunneling current set point was 200–400 pA with a 10 nA/V current amplifier, and a 100 mV sample bias was typically applied. All Au (111) substrates were annealed with a hydrogen flame prior to use and subsequently immersed in ethanolic solutions of mercapto-carboxylic acids to form self-assembled monolayers (SAMs). The resulting SAMs were imaged without protein to ensure formation of the SAM and cleanliness of the surface. The CaHydA was subsequently adsorbed *in situ* to the monolayer with 0.1 M phosphate buffer, pH 7.0, as the supporting electrolyte and imaged in EC-STM mode to control substrate potential. All EC-STM images were recorded with a substrate potential of -400 mV vs Ag/AgCl. Titration of protein to control surface coverage results in the adsorption of protein to the modified gold surface over the course of several minutes to a few hours to reach equilibrium.
The [FeFe]-hydrogenase CaHydA from \textit{C. acetobutylicum} was purified and expressed in \textit{Escherichia coli} and assayed for H\textsubscript{2} evolution activity according to previously reported procedures (Adams and Mortenson 1984). Due to the extreme oxygen sensitivity of [FeFe]-hydrogenases (Adams 1990; Baffert et al. 2008; Goldet et al. 2009), all work was performed in an anaerobic chamber (Coy Laboratory Products) under strictly anaerobic conditions (2–3\% H\textsubscript{2}, bulk N\textsubscript{2}, <1 ppm O\textsubscript{2}). Under anaerobic conditions and at ambient temperature (~25°C), CaHydA is stable for several hours in the electrochemical STM setup with little or no observed loss in electrocatalytic activity. The specific activity units (U) are defined as 1 \(\mu\text{mol} \) H\textsubscript{2} produced min\(^{-1}\) mg\(^{-1}\) of enzyme from sodium dithionite reduced methyl viologen (7.5 mM) as an electron donor. This study used two separate CaHydA preparations with nominal solution-based specific activities for H\textsubscript{2} production of 177 and 280 U mg\(^{-1}\), which correspond to TOFs of 192 s\(^{-1}\) and 303 s\(^{-1}\) respectively. All data were normalized to both the surface coverage of protein and the specific activity.

Cyclic voltammetry was performed with a CH Instruments 650C electrochemical workstation using a platinum wire counter electrode and either a silver wire quasi reference or a silver/silver chloride reference electrode. The Au (111) substrate modified with a carboxylate-terminated SAM was used as a working electrode. All CV was performed in the same EC-STM cell, with a geometric surface area of 0.283 cm\(^2\). The same sample was used for imaging by EC-STM inside an anaerobic chamber as well as anaerobic CV in a typical three-electrode configuration. All CVs were recorded in 0.1 M phosphate at pH 7.0 with a scan rate of 50 mV/s.
STM images were scrutinized by eye to estimate the number of proteins on the surface. Several 100 nm by 100 nm images from different areas of the electrode were analyzed, particles were counted, and an average coverage was obtained, which was then used to estimate the number of proteins on the geometric surface. Variation in surface coverage among images was <10% for a given sample. To verify the methodology of counting individual particles in order to quantify enzymes on the electrode, two of the STM image-quantified samples were tested for iron content via a ferrozine iron-binding assay adapted from previously reported procedures (Carter 1971; Stookey 1970). The quantity of iron determined by this procedure differed from that determined by counting the images by 20–50% underestimation, indicating that qualitatively the method of
counting the images is valid. Due to the minute amounts of protein in the experiments, ($\sim10^{-12}$ mol of protein/cm$^2$), the difficulty of recovering protein from surfaces, and detection limitations by UV-vis absorption measurements, data from the STM images alone were used to calculate a TOF in this report. Recovered samples were also analyzed by SDS-PAGE to confirm that the molecular weight of the adsorbed protein was consistent with CaHydA (see Supporting Information).

Results:

**Assembly on SAMs**

SAMs were prepared on atomically flat gold electrodes with carboxylic-acid terminated alkanethiols. The acid moieties, which are negatively charged at the pH of these experiments, are designed to interact with the large regions of positive charge that are present on the protein surface. Ideally, such interactions are both strong and serve to orient the enzyme molecules all with the same face toward the gold. We employed SAMs having 3–11 carbon atoms in order to alter the distance between the protein and the electrode surface. Figure 35a–c depicts the proposed interaction between the positively charged protein surface and the negatively charged monolayer surface based on electrostatic considerations (Kubiak-Ossowska and Mulheran 2010). Although, the relative energies of this orientation and others were not rigorously calculated; the area of exposed positive charge of CaHydA interacting with the carboxylated surface of the SAM is maximized in these structures. Recently, Brown et al. have shown that CdTe nanocrystals capped with 3-mercaptopropionic acid interact with a region of positive charge on the surface of CaHydA to form a stable complex for the photoproduction of hydrogen (Brown et al. 2010). Our system presumably utilizes the same region of positive charge, which surrounds the proposed binding site for the negatively charged
ferredoxin during in vivo electron transfer (King et al. 2006). This is the point where electrons are thought to enter the CaHydA electron transfer chain. Cyclic voltammetric studies were performed on these Au-SAM electrodes with bound CaHydA and large catalytic electrochemical signals for hydrogen production were observed (Figure 35d), suggesting that the orientation of the protein on the electrode is favorable for electron transfer (ET).

**EC-STM**

EC-STM was chosen for imaging in this work for its ability to provide high-resolution imaging in a liquid medium as well as potentiostatic control of both tip and substrate. With CaHydA stably adsorbed onto carboxylate-terminated SAMs, the
resulting surface was imaged, revealing random, relatively uniform coverage (Figure 36a–d). The electrochemical signal (Figure 35d) on the Au-SAM electrodes resembles that on PGE electrodes (Hambourger et al. 2008), both in observed current density for short carbon chains, and waveform. It has been previously demonstrated that, under conditions like these, hydrogenase molecules free in solution do not contribute significantly to the catalytic current (Bianco and Haladjian 1992; Leger et al. 2002b). This suggests that the enzyme-surface interaction is stable and that the catalytic current can be ascribed to the enzyme molecules detected in the STM images.

The apparent height of the protein on the surface reflects the magnitude of the current flowing through the enzyme between the tip and gold substrate. This current changes with substrate potential (EC-STM) due to the properties of the redox active cofactors incorporated in the protein, specifically the distal [FeS] cluster which serves as an initial acceptor for intramolecular ET. As the substrate potential is shifted negative, the Fermi levels of both working electrodes (i.e., the tip and substrate) will bracket the redox level of the protein, at some point meeting resonant tunneling conditions and giving rise to an increase in the tunneling current through the protein. A higher current is observed at roughly -0.6 V vs Ag/AgCl, the potential at which proton reduction occurs, which is closely matched to the redox properties of the [FeS] clusters. Under these conditions, topographical STM images show larger apparent heights for the redox active hydrogenase (see Figure 36b–d). When the Fermi levels of the electrodes are positive or negative of the midpoint of the redox center (i.e., nonresonant tunneling conditions), the height appears smaller due to the decreased protein conductivity. Theoretically this observation should give a Gaussian distribution of heights versus substrate potential as the potential is swept past the midpoint of the redox center. This phenomenon has been recorded previously for redox proteins such as azurin (Chi et al. 2005) as well as other
redox-active small molecules (Tao 1996). In our case, only one side of the curve can be observed due to limits imposed by solvent reduction at the tip at increasingly negative potentials.

**Cyclic Voltammetry**

The overall electrochemical properties of such electrodes may be studied using standard cyclic voltammetric techniques. A catalytic hydrogen production current due to enzymatic turnover is observed when sufficient driving force is applied to the electrode as seen in Figure 37a. For accurately measuring these currents, samples with high-density surface coverage (0.5–2 pmol/cm²) were prepared as seen in the inset of Figure 37a. The observed catalytic current is directly related to the amount of hydrogen produced from the enzyme. Taken together, the catalytic current and surface coverage (Figure 37a inset) were used for the calculation of a TOF per adsorbed CaHydA. The distance between the electrode and enzyme was altered by varying the SAM alkyl chain length from three to eleven carbons. As a result, the current densities at a given voltage declined exponentially with increasing SAM length. Figure 37b depicts typical CVs of
CaHydA on Au-SAM electrodes of lengths from three to eleven carbons, showing a decreasing catalytic current (i.e., turnover) for increasing chain length.

Discussion:

From the catalytic hydrogen production current recorded through SAMs of various lengths and the number of enzyme molecules per electrode area counted from the STM images of each electrode, a TOF per molecule of CaHydA can be calculated at any given applied potential. The TOF per protein at -0.7 V was plotted as a function of SAM length and fitted to an exponential decay function (Figure 37c) according to eq 1, where I is the tunnel current at a distance d from the electrode, I₀ is the limiting current in the absence of the SAM layer, and β is the electronic decay constant.

\[ I = I_0 e^{-\beta d} \]

An incremental distance per carbon in the alkyl chain of 1.25 Å was calculated on the basis of 109.5° carbon–carbon bond angles and a carbon–carbon bond length of 1.54 Å. From this, we calculated the length of the SAM alkyl chain and estimated the distance (d) through which electron transfer between the enzyme surface and the electrode must occur. The experimental electronic decay constant was determined to be 0.82 (0.16 Å⁻¹). This agrees well with values reported in the literature for self-assembled monolayers of alkanethiols and is consistent with values obtained from drastically different techniques, including single-molecule junction measurements (Cui et al. 2002). The exponential decay behavior is a consequence of control of the catalytic rate by ET through the SAM to the protein. ET through the protein itself to the catalytic site is the same for all SAM-protein constructs and, we conclude, does not control the catalytic rate at the potentials investigated.
Extrapolation of this plot to a distance of zero, approximating a CaHydA molecule in direct contact with the bare gold electrode surface where eq 1 simplifies to \( I = I_0 \), gave a TOF of \( \sim 21,000 \) (12,000 s\(^{-1}\)) at pH 7.0, which is higher than previous estimates in the literature for hydrogenases (Haas et al. 2001; Jones et al. 2002; Leger et al. 2004). Methods using nonturnover signals for estimates of protein coverage have given a TOF upward of 10,000 s\(^{-1}\) (Vincent et al. 2007). Catalytic current normalized by STM-derived surface density thus provides an average of the TOF for individual enzyme molecules for any given potential within the window of the electrochemical signal for CaHydA. This number does not account for inactive protein at the electrode interface or enzymes in an orientation unfavorable for interfacial ET and is thus a lower limit for turnover.

The electrochemical signal observed in cyclic voltammetry and the potential at which proton reduction occurs agree well with those reported in the literature for various electrode surfaces (Hambourger et al. 2008; Krassen et al. 2009; Rudiger et al. 2005), confirming that the kinetics of ET through the protein are not altered in this system. Theoretically, a limiting cathodic current should be observed when the rate of enzymatic proton reduction is slow compared to the rate of interfacial ET from the electrode to the protein. When these conditions are satisfied, the limiting current is directly related to the maximum turnover of the enzyme for the reduction of protons at a given pH. As the CVs indicate, even at the relatively low \([H^+]\) used in our experiments, a limiting current was not observed. This may show that at these currents, a maximum turnover was not reached. There are other explanations for the observed lack of limiting currents. In one interpretation, a dispersion of nonspecific electrostatic protein-surface interactions (see Figure 35), such as could be the case with our system, preclude the direct observation of a limiting current (Leger et al. 2002a).
As a complement to the STM images, additional measures were taken to ensure the identity and quantity of CaHydA. To confirm the identity of the protein adsorbed to the surface, SDS-PAGE was run with samples recovered from the Au (111) substrates used for STM imaging experiments against purified CaHydA. Matching bands were observed at 65 kD (see Supporting Information). The detection of this small amount of protein was also consistent with the STM images in that three samples were combined for the SDS-PAGE, which was just sufficiently above the limit of detection for visualization by silver staining (Schagger 2006; Schagger and Vonjagow 1987; Winkler et al. 2007). As an independent estimate of amount the CaHydA present on the STM substrate, samples were recovered under denaturing acidic conditions, and the Fe was quantified using a modified ferrozine assay (Carter 1971; Stookey 1970). Although this assay was near its limit of detection, the amount of Fe observed was also qualitatively consistent with the STM imaging results (see Supporting Information).

Conclusion:

We have designed a method for immobilizing an [FeFe]-hydrogenase, CaHydA, on an atomically flat gold electrode for topographic and electrochemical experiments on catalytically active samples. Direct observation of immobilized protein via EC-STM techniques shows the actual surface coverage which, when coupled with macroscopic electrochemical analysis, allows calculation of TOF as a function of potential at the single molecule level. From our analysis, we observe a TOF of \(~1000\ \text{s}^{-1}\) for CaHydA on a three-carbon SAM, and when extrapolated to bare gold, the TOF is estimated to be \(~21,000\ \text{s}^{-1}\) at \(-0.7\ \text{V vs Ag/AgCl}\). Using a bipotentiostat, resonant tunneling at potentials consistent with the redox midpoints of cofactors within the protein was observed. The \(\beta\)-value of 0.82 \(\text{Å}^{-1}\) for electron transfer through alkanethiol SAMs measured via determination of
catalytic TOF is consistent with values obtained from radically different techniques, such as STM break junction. Taken together, the number of molecules calculated from the images, the exponential fit to the number of carbons in the SAMs, the value of $\beta$, and the indication of the redox activity for FeS centers form a consistent picture of electrocatalytic activity of CaHydA on SAMs. However, these results do not fully address the question of heterogeneity in the enzyme’s catalytic activity or interaction with the electrode. The stability of immobilized CaHydA on SAMs under these conditions augurs well for future experiments to explore catalytic detail at the single molecule level.

Supporting Information:

Reagents and Protein Activity

Aqueous solutions were prepared with reagent grade water (Barnstead Easypure UV/UF compact reagent grade water system, 18.3 MΩ cm). All chemicals purchased were reagent grade or higher purity and used without further purification. [FeFe]-hydrogenase CaHydA from Clostridium acetobutylicum was expressed and purified as reported previously (King et al. 2006). Enzyme isolation, electrochemical studies and STM experiments were all performed in an anaerobic chamber under strictly anaerobic conditions (2–3 % H$_2$, bulk N$_2$, < 1ppm O$_2$) with a palladium catalyst to remove trace O$_2$ (Coy Laboratory Products, Inc.). Purified hydrogenase could be stored for months under anaerobic conditions at 4 °C in a buffer consisting of 50 mM Tris, 5 mM NaCl, 5 mM sodium dithionite, 5 % (v/v) glycerol. Protein concentrations were determined by the Lowry-Peterson assay and were found to be \( \sim 0.2 \) mg ml$^{-1}$ (Peterson 1983). Enzymatic activity for hydrogen production was determined in the presence of 25 mM methyl viologen reduced by excess sodium dithionite (50 mM) in 0.05 M Tris buffer pH 8.0 at
23 °C or 37° C. Assays performed in 0.1 M phosphate buffer pH 7.0 gave similar results. Activity assays for hydrogen production were determined by gas chromatography (5 Å molecular sieve column, thermal conductivity detector, Ar carrier gas). 500 µL of headspace gas were transferred with a locking airtight syringe (Hamilton). A gas standard (1% H₂, bulk N₂) was used to calibrate the gas chromatograph to ± 10% of the measured value. Activity assays were adapted from a literature procedure (Adams and Mortenson 1984). Activity units (U) reported for CaHydA are defined as 1 µmol H₂ produced per minute. This work utilized two separate protein preparations with specific activities of 177 and 280 U mg⁻¹.

**Homology Modeling and Electrostatics**

The homology model of the CaHydA [FeFe]-hydrogenase was constructed from alignment with 1FEH in the PDB database using modeler and was constructed using the salign command within modeler (Sali and Blundell 1993). Electrostatic surface potentials were generated using the PBEQ web server using default settings (Im et al. 1998; Jo et al. 2008) and visualized in VMD (Humphrey et al. 1996) with +1 kcal/(mol e⁻) isosurface in blue the -1 kcal/(mol e⁻) isosurface in red.
Electrochemical and STM Methods

Electrochemical scanning tunneling microscopy studies were performed with a Pico-SPM head (Agilent, AZ) utilizing a Nanoscope E controller and bipotentiostat (Agilent, AZ). All STM experiments were performed in an anaerobic chamber (Coy Laboratory Products, Inc.) with a laboratory constructed stabilization mechanism to isolate vibration. Imaging experiments were performed in a specially fabricated Teflon cell of 6 mm diameter utilizing an atomically flat Au (111) substrate as a working electrode prepared by thermally evaporating ~130 nm of gold (Alfa Aesar 99.999 %) onto freshly cleaved mica surfaces in a UHV chamber (~2 x 10^-8 torr). The Teflon cell, which incorporates a Pt wire counter electrode, was cleaned in boiling piranha (98 % H₂SO₄, 30 % H₂O₂ = 3:1 v/v) for ~30 min and rinsed three times with water (18.3 MΩ cm, Nanopure). Either a freshly polished Ag wire quasi reference or a freshly made Ag/AgCl reference electrode was utilized in the EC-STM sample cell. STM tips were prepared by mechanically shearing Pt/Ir wire (80/20, 0.25 mm diameter) and coating with them.

Figure 38 – a.) Blank Au surface modified with 3-mercaptopropionic acid/ethanethiol (1:1) SAM. b.) Same SAM modified surface with adsorbed, catalytically active CaHydA.
Apiezon wax. All tips had $<1$ pA leakage current, the tunneling current set point was 100-400 pA with a 10 nA/V amplifier and a 100-200 mV bias. All Au (111) substrates were annealed with a H$_2$ flame prior to use and subsequently immersed in either a 1mM solution of 6-mercaptohexanoic acid in ethanol (200 proof), an ethanolic solution of 3-mercaptobutrylic acid/ethanethiol (1:1, 1mM total thiol concentration), an ethanolic solution of 4-mercaptobutyric acid or an ethanolic solution of 11-mercaptoundecanoic acid (1mM) overnight (22-24 h). The resulting self-assembled monolayers were imaged without protein to ensure formation of the SAM and cleanliness of the surface. CaHydA was subsequently adsorbed to the monolayer and imaged in EC-STM mode with 0.1 M phosphate buffer, pH 7.0 as the supporting electrolyte.

Figure 39 – Representative cyclic voltammograms of blank Au-SAM surfaces and samples with adsorbed hydrogenase. All scans were recorded with a scan rate of 50 mV/s and are not corrected for surface coverage or activity.
Cyclic voltammetry was performed with a CH Instruments 650C electrochemical workstation using a platinum wire counter electrode and either a silver wire quasi reference or a silver/silver chloride reference electrode. The Au (111) substrate modified with a carboxylate terminated SAM was used as a working electrode. All cyclic voltammetry was performed in a home-built electrochemical STM cell housed inside the anaerobic chamber and set-up in a typical three-electrode configuration. All values in this work are reported against the Ag/AgCl (saturated KCl) scale. Reported potentials can be converted to the standard hydrogen electrode (SHE) scale by adding 0.199 V to the potential reported against Ag/AgCl. All cyclic voltammograms were recorded in 0.1 M phosphate, pH 7.0 with a scan rate of 50 mV/s.

Au (111) working electrodes were utilized for EC-STM studies with cyclic voltammetry subsequently performed on the same, catalytically active samples. Self-assembled monolayer formation followed annealing with a H₂ flame. The Au (111) electrodes were placed at the bottom a specially fabricated Teflon STM cell and immersed in ~150 µL of anaerobic 0.1 M phosphate buffer, pH 7.0. The area of the working electrode inside the cell was 0.283 cm². A Pt wire counter electrode and either a
Ag/AgCl or a Ag wire reference electrode was used in the electrochemical cell. The bare surfaces were imaged in EC-STM mode and 5-10 µL of CaHydA was subsequently injected into the working solution depending on protein concentration. Protein adsorption was followed by imaging and cyclic voltammetry on the same, catalytically active samples.

For comparison, cyclic voltammetry was performed on pyrolytic graphite edge (PGE) electrodes, which were constructed by sealing 3/16” PGE rods (GE Advanced Ceramics) into a Teflon tube with a steel rod electrical contact. PGE electrodes were polished prior to use with 0.05 µm alumina slurry (Bioanalytical Systems) and sonicated briefly in deionized water. CaHydA films were prepared on the electrode by drying protein solution with a flow of argon gas over the electrode surface. Protein films were subsequently immersed into anaerobic 0.1 M phosphate buffer for cyclic voltammetric studies. A high surface area platinum mesh (Alfa Aesar) counter electrode was used and separated behind a porous glass frit (Vycor®, Bioanalytical Systems). Observed current densities were greater than those observed on modified gold electrodes, although
comparable to the shortest SAM modified Au electrode. The onset potential for hydrogen production was not altered between the two types of working electrodes. Values reported in this work are against the Ag/AgCl (saturated KCl) scale.

**Protein Recovery and Quantification**

Protein samples were recovered from STM substrates with a sample solubilization buffer consisting of Tris (150 mM), glycerol (30% v/v), 2-mercaptoethanol (6% v/v), sodium dodecyl sulfate (12% w/v) and coomassie brilliant blue G-250 (0.05% w/v) or by subjecting the samples to a high concentration of salt (~0.5 M) to disrupt the electrostatic interaction between SAM and protein. The resulting solution was recovered from the substrate and run with SDS-PAGE against pure, soluble protein in figure 43. This resulted in matching bands at 65 kD, thus confirming the identity of the protein on the surface. Protein recovered from STM samples in a similar manner was also subjected to a ferrozine iron binding assay to quantify the amount of protein on the surface.
CaHydA incorporates 20 iron atoms into cofactors in its structure, allowing for protein quantification via iron quantification. A standard curve from ferrous chloride was used to calibrate the quantity of iron from the recovered protein samples resulting in the data seen in figure 42.

![Silver stained SDS-PAGE confirming identity of protein recovered from STM surfaces. Left lane: standards. Middle lane: recovered CaHydA from STM substrates. Right lane: purified CaHydA](image)

Figure 43 - Silver stained SDS-PAGE confirming identity of protein recovered from STM surfaces. Left lane: standards. Middle lane: recovered CaHydA from STM substrates. Right lane: purified CaHydA
PROTEIN SECONDARY-SHELL INTERACTIONS ENHANCE THE PHOTOINDUCED HYDROGEN PRODUCTION OF COBALT PROTOPORPHYRIN IX

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Abstract:

Hydrogen is an attractive fuel with potential for production scalability; if inexpensive, efficient molecular catalysts utilizing base metals can be developed for hydrogen production. Here we show for the first time that cobalt myoglobin (CoMyo) catalyzes hydrogen production in mild aerobic conditions with turnover number of 520 over 8 hours. Engineered variants in which specific histidine residues in proximity of the active site were mutated to alanine result in modulation of the catalytic activity, with the H64A/H97A mutant displaying activity 2.5-fold higher than wild type. Compared to free Co-Protoporphyrin IX, incorporation into the myoglobin scaffold results in a 4-fold increase in photoinduced hydrogen production activity. Our results demonstrate that
protein scaffolds can augment and modulate the intrinsic catalytic activity of molecular hydrogen production catalysts.

Introduction:

In the quest for alternative, sustainable fuels to address increasing societal needs, molecular hydrogen has arisen as a forerunner due to being potentially carbon independent, energy-rich, and transportable (Bockris 1972; Lewis and Nocera 2006). At the moment, however, hydrogen production relies on steam reforming of hydrocarbons at high temperatures or on the use of precious metal catalysts, such as platinum, and can’t be scaled up in a sustainable manner. Nature, in contrast, utilizes a class of enzymes called hydrogenases, which contain an unusual bimetallic active site ([FeFe] or [NiFe]) and reversibly catalyze the reduction of protons to molecular hydrogen (Faiella et al. 2013; Lubitz et al. 2014; Peters et al. 1998; Vignais et al. 2001). Remarkably, hydrogenases function in weakly acidic conditions, at low overpotentials, with non-noble metals, and catalyze hydrogen production with high turnover frequencies (5000-21000 s⁻¹) (Armstrong and Hirst 2011; Madden et al. 2012). However, the application of these enzymes to scalable hydrogen production is hampered by their difficulty of overexpression and high oxygen sensitivity (Armstrong and Fontecilla-Camps 2008; Fontecilla-Camps et al. 2007) (Armstrong and Fontecilla-Camps 2008; Fontecilla-Camps et al. 2007). For these reasons, chemists have sought to develop robust organometallic catalysts as alternatives to the natural enzymes (Camara and Rauchfuss 2012; Daresbourg et al. 2003; Helm et al. 2011; Tard and Pickett 2009). Among those, cobalt macrocycle catalysts are an attractive option, as they often exhibit structural heterogeneity, which allows for tuning of catalytic properties, low overpotentials, and high oxygen tolerance; however they function mostly in organic solvents with strong

Recently, a number of cobalt porphyrin catalysts have been reported for their ability to both electrochemically and photochemically produce hydrogen at high turnover numbers, implicating them for use in dye-sensitized fuel cells (Lee et al. 2011; Natali et al. 2014). Porphyrin and related macrocycles serve as cofactors in proteins, raising the possibility that such natural proteins could be used as scaffolds to accommodate hydrogen production catalysts (Kleingardner et al. 2014; Robertson et al. 2013). Initial work utilizing cobalt-functionalized microperoxidase 11 (CoMP11), however, showed that the catalytic activity decays after about 15 minutes due to porphyrin degradation, likely from porphyrin surface exposure to the environment (Kleingardner et al. 2014; Robertson et al. 2013).
Here, we present an alternative approach by which a cobalt-derivatized porphyrin is buried into a protein scaffold, myoglobin. This well-folded, stable protein scaffold binds heme (Fe-Protoporphyrin IX) leaving the metal in a pentacoordinate state; the sixth position is available for coordination to dioxygen and other substrates. Myoglobin withstands mutagenesis at positions close to the vacant axial site, facilitating the engineering of catalytic sites into the scaffold (Lu et al. 2009; Yeung et al. 2009). In addition, myoglobin readily accommodates unnatural cofactors (Brucker et al. 1996; Li et al. 2000; Ueno et al. 2005; Ueno et al. 2007). In the case of Co-Protoporphyrin IX, incorporation into myoglobin provides additional second sphere and long-range interactions, while protecting the cofactor from degradation. Our results show that CoMyo (Figure 44) catalyzes photoinduced production of hydrogen with high efficiency, and that activity is modulated by engineered mutations.

Results and Discussion:

Preparation of CoMyo was accomplished with cobaltous protoporphyrin IX (CoPP(IX)) using established methods (Brucker et al. 1996). The electronic spectra of the air-oxidized protein displays absorbance maxima at 425, 534, and 567 nm, typical of Co(III) porphyrins (Figure 48). These maxima are red shifted relative to other peptide-based catalytic systems, which exhibit maxima at 415, 530, and 560 nm, indicating shielding CoPP(IX) from the aqueous environment (Kleingardner et al. 2014; Mathura et al. 2013). Reduction of the holo protein at pH 7.5 with a 500 molar excess of sodium dithionite resulted in shift of the Soret and q-bands to 394 and 557 nm, respectively, corresponding to a Co(II) species (Figure 48).

We then assayed the ability of CoMyo and CoPP(IX) to catalyze the reduction of protons to hydrogen by cyclic voltammetry (CV) (Figure 45). CoPP(IX), which has low
Figure 45 – Cyclic voltammagrams of 1.5 µM CoMyo in the absence (black) and presence (red) of oxygen in a 200 mM Tris-HCl, 100 mM NaCl, pH 7.5 solution at 100 mV/s with a 0.28 cm² glassy carbon working electrode.

solubility in water, was studied in acetonitrile (MeCN) with 0.1 M (n-Bu₄N)(PF₆) as the supporting electrolyte. (Figure 49) A quasi-reversible wave appears at -1.17 V vs SHE after referencing to ferrocene monocarboxylic acid, assigned to the reduction of Co(I) to Co(0); addition of para-toluenesulfonic acid to the anhydrous acetonitrile results in the onset of a catalytic wave at -1 V, thus implicating this redox couple as the catalytically active species (Figure 50) (Lee et al. 2011). In the case of CoMyo, the onset of a strong catalytic wave is observed at -0.95 V against SHE in the CV scans of CoMyo in 200 mM Tris-HCl buffered solution, concealing the Co(I)/Co(0) peak (Figure 45). This is 200 mV more positive relative to the catalytic wave of a bare glassy carbon electrode (Figure 51). These values are in agreement with previous work on aqueous cobalt macrocycles, however, the catalytic onset potential is approximately 100 mV closer to the thermodynamic value compared to other water-soluble cobalt porphyrins assayed (Natali et al. 2014).
The peak catalytic current for scans down to -1.26 V is linearly dependent on CoMyo concentration (Figure 52). The catalytic current was found to be strongly dependent on the pH of the solution, as expected for a molecular hydrogen generating system (Figure 53). CV scans were also performed in unsealed electrochemical cells open to the ambient atmosphere; negligible catalytic current loss was observed after introduction of oxygen into the electrochemical setup (Figure 45). However, loss of activity of the CoMyo system is seen below pH 6, due to protonation of the ligating His93 in the myoglobin core and loss of the porphyrin from the hydrophobic core of myoglobin.

To confirm the loss of CoPP(IX) from the active site, rinse tests were performed (Figure 54). Consecutive scans on a 1.5 µM sample of CoMyo at a pH of 7.5 showed negligible loss of catalytic current. Removing the electrode from the CoMyo solution, rinsing, and placing into fresh buffer without CoMyo yielded no catalytic current, indicating no degradation of the catalytic system at the electrode surface to form an electroactive film. Upon lowering of the pH to 4.5, consecutive scans on CoMyo in solution yielded a catalytic wave, as expected. When the electrode was rinsed and placed into buffer containing no CoMyo, the catalytic wave observed persisted, indicating deposition of CoPP(IX) onto the electrode surface. UV-Vis spectra of CoMyo at pH 4 revealed a red shift of the Soret band to 427 nm, consistent with the loss of coordination by the axial His93 (Figure 55) (Wang et al. 2003). Because of this loss of coordination, we chose to characterize the CoMyo system at pH levels above 6.5, avoiding characterization of a mixture of myoglobin-bound and unbound CoPP(IX).

We investigated whether incorporation of catalysts into a hydrophobic environment could modulate the intrinsic activity of CoPP(IX) by assessing hydrogen production in the presence of a photosensitizer and sacrificial electron donor. In a typical experiment, samples were irradiated with 1100 W/m² of visible light (>400) in the presence of 1 mM
Ru(Bpy)$_3^{2+}$ and 100 mM sodium ascorbate, a sacrificial electron donor, over the course of 12 hours. Hydrogen evolution was quantified by analyzing aliquots of headspace volume of the anaerobic cuvette via gas chromatography as a function of time. We found that the CoMyo system efficiently produces hydrogen under these conditions, achieving turnover numbers (TON, i.e. moles product / moles catalyst) ranging from 230–520 with a maximal average turnover frequency of 1.08 min$^{-1}$ for the H64/97A at pH 6.5. Direct comparison of photoinduced catalysis of the CoMyo system to free-CoPP(IX) showed that incorporation of the porphyrin into the protein scaffold increased the TON by a factor of 3 on average (Table 1). TONs of porphyrin-based catalysts have strong inverse relationship with concentration, as activity is lost as dimers form (Natali et al. 2014); in contrast, CoMyo proved to be resilient to changes in catalyst concentration, yielding the same TON for conditions tested (1 µM – 5 µM). In pH dependence studies maximal photoinduced hydrogen production was observed at pH 7, and was lower as pH either increased or decreased. As pH rose, lower concentrations of protons resulted in the lower
TON. However, at lower pH values, catalysis was expected to increase with increasing proton concentration. We observed that as the two distal histidines in the active site (His64 and His97) become protonated, with pKₐ value estimates of ~5 and 5.6, respectively, the catalytic activity of CoMyo is decreased (Bashford et al. 1993).

To verify this hypothesis, we generated three mutants of myoglobin, in which histidines were exchanged for non-ionizable alanine: H64A, H97A, and H64/97A (Figure 44). We found that the H64A and H97/64A point mutants increased catalytic activity of CoMyo at pH 6.5 to 331 and 512 TON, respectively, compared to the 231 TON observed for WT (Figure 46). However, the H97A mutant reduced the TON to 120, only slightly higher than the porphyrin alone (TON = 100). Electrochemical analysis of the mutants at pH 7 revealed an increase of current at -1.26 V vs. SHE for both the H64A and double mutant and a reduce of catalytic current of H97A, but no change of the onset of catalytic hydrogen reduction (Figure 47).

Table 3 - pH dependence of photoinduced hydrogen production of WT CoMyo

<table>
<thead>
<tr>
<th>pH</th>
<th>TON</th>
<th>TOF (min⁻¹)</th>
<th>Enhancement</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.5</td>
<td>234</td>
<td>0.47</td>
<td>2.13</td>
</tr>
<tr>
<td>7</td>
<td>518</td>
<td>1.47</td>
<td>4.32</td>
</tr>
<tr>
<td>7.5</td>
<td>454</td>
<td>0.85</td>
<td>2.84</td>
</tr>
</tbody>
</table>

[a] Conditions: 1 M potassium phosphate; *Enhancement over CoPP(IX) assayed under the same conditions.

Two factors could play a role in the change in the catalytic peak height upon removal of the two active site His. First, removing His64 from the proximity of the CoPP(IX) catalysts removes a slight positive charge at physiological pH levels, as well as the potential for His64 to compete for proton binding. The loss of this positive charge will shift the redox potential of the catalyst more positive, allowing for easier reduction of protons at the catalytic site. Characterization of the electrochemical pH dependence of the H64A mutant shows a stronger dependence of catalytic activity on pH, a direct result of these two changes. (Figure 56).
Secondly, closer inspection of the active site of CoMyo reveals a hydrogen bond forming between the His97 residue and a CoPP(IX) propionic acid group. Removing this residue destabilizes the binding of the porphyrin, altering steric constraints on the porphyrin and increasing the degrees of freedom between the scaffold and the catalyst, lowering its catalytic ability by diminishing enhancing contacts with the scaffold. It has been characterized with heme in the myoglobin active site, and the H97D mutation increases lability of the cofactor by a factor of 38 (Hargrove and Olson 1996). In the system presented, this is realized through loss of scaffold-mediated enhancement of catalytic activity, as the H97A mutant is nearly indistinguishable from the free porphyrin. During photoinduced hydrogen production, removal of this residue also increases the solvent-accessibility of the active site, and may allow for increased interaction between the photosensitizer and the catalyst, increasing the TON of the H97/64A mutant relative to the H64A mutant.

Figure 47 – Cyclic voltammograms of 1.5 µM myoglobin mutants in 200 mM Tris-HCl, 100 mM NaCl, at pH 7 at a scan rate of 100 mV/s with a 0.28 cm² glassy carbon working electrode. Traces correspond to WT (Black), H97A (Green), H64A (Blue), H64/97A (Red).
The variation of photoinduced catalysis of hydrogen production by these mutants confirms that the protein environment plays a major role in the overall increase of catalysis of the CoPP(IX), affecting not only the stability of the catalyst but also the competing processes and redox activity in the active site. These effects are reminiscent of the role of the protein matrix in [FeFe] hydrogenases, in which it augments and modulates the intrinsic activity of the diiron center through secondary shell and long range interactions (Knorzer et al. 2012; Lubitz et al. 2014). This concept was exploited by burying CoPPIX within the hydrophobic pocket of myoglobin and resulted higher turnover number in photocatalytic experiments. Compared to CoMP11, which is a helical peptide covalently linked to a porphyrin, the myoglobin scaffold presents several advantages. First, the scaffold efficiently protects the porphyrin cofactor from degradation, resulting in sustained photoinduced catalytic activity over 8 hours. In contrast, the electrocatalytic activity of CoMP11 ceased after 15 minutes. Second, the increased complexity of the protein scaffold surrounding the porphyrin allowed us to introduce specific mutations in the second coordination sphere, thus modulating the activity of the protein. Further optimization of the system via directed mutagenesis may be possible. Third, the scaffold is robust and can be functionalized on the surface for attachment to a number of photosensitizers or solid-support materials, allowing for development of a heterogeneous catalytic system. Finally, the non-covalent nature of the interaction between the porphyrin and myoglobin allows for the identity of the catalyst studied to be altered from the native protoporphyrin IX.

In conclusion, we have demonstrated that the proton reducing activity of CoMyo can be modulated by mutation of the amino acids that are near the metal center and allows studying the effect of secondary shell interactions on catalysis, utilizing non-covalent linkages to facilitate catalysis in aqueous environments that would otherwise be limited
to organic solvents. The characterized CoMyo system shows significant enhancement of photoinduced hydrogen production for a simple macrocyclic complex, CoPP(IX), due to stabilization of the catalyst by incorporation into a hydrophobic, protein scaffold. Along with the ability to stabilize the catalyst, point mutants of myoglobin affected the catalysis of the CoPP(IX), suggesting that \textit{in vitro} selection techniques could be used to enhance the catalytic activity of CoMyo. Future studies will aim to optimize both the catalyst, as well as the protein environment solubilizing the catalyst, to achieve efficient photoinduced hydrogen production.

Experimental Section:

Point mutants were prepared by the method of Gibson et al. and were confirmed by direct sequencing of the PUC19 vector harboring the insert (Gibson et al. 2010). CoMyoglobin was prepared following previously reported procedures (Hargrove and Olson 1996; Springer and Sligar 1987). Briefly, BL21 E. coli harboring the pMB413a vector, purchased from Addgene, were induced with 100 µM IPTG at an OD$_{600}$ of 0.6 and grown for 5 hours. Harvested cells were lysed by continuous sonication on ice. Clarified lysate was brought to 60% saturation with Ammonium Sulfate and the precipitated proteins discarded. The solution was then brought to 95% saturation, in which myoglobin precipitated. Resuspended proteins were ran over a 1 m, Sephadex G50 Size Exclusion Column, followed by batch bound to DEAE cellulose. Unbound myoglobin was concentrated and heme was removed using the acid:acetone method (Hargrove and Olson 1996). CoPP(IX), 10 molar excess, was dissolved in 90% water, 10% pyridine, and added slowly to a solution of apomyoglobin. After 1 hour of incubation, excess porphyrin was removed through desalting on a GE healthcare PD10 column. Protein concentration was calculated using the extinction coefficient of 170,000 M$^{-1}$ cm$^{-1}$ at the Soret.
**Electrochemical Methods**

Electrochemical experiments were carried out using a CH-instruments 1242B potentiostat. For all electrochemical measurements, a three-electrode system was used. The electrodes used were a 3-mm diameter glassy carbon working electrode with a surface area of 0.28 cm\(^2\), platinum mesh counter electrode, and a saturate calomel reference electrode. All potentials were normalized to SHE by the addition of 240 mV to the SCE values. All electrolyte solutions were degassed by incubation in a Coy anaerobic chamber for 2 days prior to use. Working electrodes were polished with 1 µm alumina for 5 minutes, followed by 10 minutes of sonication, prior to use.

**Photoinduced \( \text{H}_2 \) Production**

Irradiation was performed using a 450W xenon lamp with a 400 nm cutoff filter, irradiating at a constant 1100 W/m\(^2\) throughout the experiment. For each experiment, 1 mM Ru(Bpy)\(_3\)\(^{3+}\), 100 mM sodium ascorbate, and the desired catalyst were added to a 1 M potassium phosphate buffer at the appropriate pH. The 400 µL total reaction volume was added to a custom made airtight 1 mm cuvette and degassed extensively with argon prior to illumination. During irradiation time course 100 µL samples of the headspace were removed with a gas-tight syringe and injected directly for analysis by GC. Calibration was achieved by injection of various volumes of a 1% \( \text{H}_2 \), 99% \( \text{N}_2 \) gas mixture onto the GC.
Supporting Information:

Materials

All chemicals were purchased from commercial suppliers and used without further purification. UV-Vis spectra were acquired on a Varian Cary 50 Bio Spectrophotometer. Gas chromatography was carried out on a SRI instruments, Model no. 310C GC using a 5Å molecular sieve column with a thermal conductivity detector and argon carrier gas.

Photophysical Parameters

Quantum efficiency was calculated starting from the irradiance spectra of the 450W Xenon Arc lamp. Individual light wavelengths were converted to photon energy through the use of SI equation 1.

\[ E = \frac{hc}{\lambda} \]

Equation 1

Each wavelength was then converted to photons irradiated for each experiment following SI equation 2:

\[ \text{photons irradiated} = \frac{\text{Incident radaiton in} \ \frac{W}{m^2} \times \text{time} \times \text{Irradiated area}}{\text{Sum of photon energy}} \]

Equation 2

Integration under the curve from 400-550 nm (Ru(Bpy)_3^{2+} absorbance bands) resulted in total photons available for photoexcitation during the experiment time course. Quantum efficiency was then calculated by SI Equation 3:
Quantum Efficiency = \[
\frac{2 \times \text{mol } H_2 \text{ evolved}}{\text{mol photons}} \times 100 \]

Equation 3

Utilizing the above equations, quantum efficiency for 2.5 µM the H64/97A mutant was calculated to be 0.0035 %.

Figure 48 – UV-Vis traces of 2.6 µM CoMyo in 100 mM KPi pH 7.5 in the oxidized (black) and dithionite reduced (blue) states.
Figure 49 – Cyclic Voltammogram of a 1 mM CoPP(IX) solution in MeCN with 0.1 M \((n\text{-Bu}_4\text{N})\text{PF}_6\) (solid line), compared to a blank scan (dotted line), at 100 mV/s with a glassy carbon working electrode.

Figure 50 – Cyclic voltammograms of 250 µM CoPP(IX) in MeCN with 0.1 M \((n\text{-Bu}_4\text{N})\text{PF}_6\) with increasing concentrations of tosic acid. (Black: 0 mM, Red: 1.2 mM, Green: 3.6 mM, Blue: 4.8 mM, Orange: 7 mM) Scan rate was 100 mV/s with a glassy carbon electrode.
Figure 51 – Cyclic Voltammograms of a blank glassy carbon electrode (red) compared to 1.5 µM H64A mutant (black) at pH 7.0 in 100 mM KPi, 200 mM KCl at a scan rate of 100 mV/s.

Figure 52 – Cyclic voltammograms of WT CoMyo at varied concentrations in 100 mM Tris-HCl, 200 mM NaCl, pH 7.5 at 100 mV/s with a glassy carbon electrode. Inset: Linear fit of current at -1.26 V vs SHE for various concentrations of CoMyo.
Figure 53 – Cyclic voltammograms of 1.6 µM WT CoMyo at varied pH levels of 100 mM Tris-HCl, 200 mM NaCl at 100 mV/s with a glassy carbon electrode. Inset: Current at -1.26 V vs SHE for varied pH levels.

Figure 54 – Cyclic voltammogram in pH 4.5, 100 mM Citrate, 100 mM NaCl of the blank scan (grey dashed), after addition of 2.6 µM WT CoMyo (black), and after placing rinsed electrode back into fresh buffer (red).
Figure 55 – Cyclic voltammogram in pH 7.5, 100 mM Tris-HCl, 100 mM NaCl of the blank scan (grey dashed), after addition of 5.5 µM WT CoMyo (black), and after placing rinsed electrode back into fresh buffer (red).

Figure 56 – UV-Vis spectra of 2.6 µM WT CoMyo in 100 mM Tris, 100 mM NaCl, pH 7.5 (black trace) and 2.6 µM WT CoMyo in 100 mM Citrate, 100 mM NaCl, pH 4.5.
Figure 57 – Catalytic current at -1.26 V for WT (black) and H64A (red) CoMyo at various pH levels in 100 mM KP, 200 mM KCl with a glassy carbon working electrode at 100 mV/s. Data were normalized to current at pH of 6.8 to account for variation in catalytic activity of the two systems.

Figure 58 – Typical GC standardization curve obtained for photoinduced hydrogen production assays. Data were fit to the line $y = 42.90x + 0.003$
Figure 59 – Photoinduced hydrogen production of 2.5 µM WT CoMyo under varied light conditions. White area corresponds to irradiation with 1100 W/m², grey corresponds to no irradiation of the sample.
IMPROVED STABILITY OF A BIOTINYLATED DIIRONHEXACARBONYL PROTON REDUCTION CATALYST BY SUPRAMOLECULAR CONFINEMENT UTILIZING STREPTAVIDIN IN A HOST-GUEST STRATEGY

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Abstract:

Supramolecular confinement has been demonstrated to prolong the stability and in some cases enhance the catalytic prowess of hydrogenase active site mimetic organometallic complexes. Here we demonstrate the introduction of a biotinylated diironhexacarbonyl into streptavidin with stoichiometric binding. The FTIR spectrum of the resulting host-guest hybrid demonstrates less broadening and peak shifts indicative of interactions between the catalyst and protein. The photoinduced catalysis of proton reduction to yield molecular hydrogen is enhanced by a factor of 3-4 in the bound state. Future work includes mutagenesis of relevant positions in order to further enhance the stability and provide proton relays to also increase substrate availability and therefore the reaction rate as well.
Introduction:

Nature manages to efficiently catalyze chemical reactions that are difficult or even impossible to facilitate using synthetic methods by the intricate structural features of proteins. One such example is the [Fe-Fe] hydrogenases that reversibly catalyze proton/hydrogen interconversion in microorganisms. The active site of the enzyme consists of a cuboidal [4Fe-4S] cluster connected to a diiron catalytic site ligated by a non-proteinogenic 1,3-azadithiolate unit through a single cysteine side chain (Figure 60)(Fontecilla-Camps et al. 2007). The remaining ligands of the diiron catalytic site are also nonproteinogenic, and are exotic organometallic ligands such as –CO and –CN (Peters et al. 1998). The –CN remains hydrogen bonded with the protein to restrain the active site in ‘rotated structure’ for effective catalysis; this enthalpic structure of the
diiron site exposes a vacant site for substrate binding in both oxidized (Fe[II]-Fe[II]) and reduced form (Fe[II]-Fe[II]) (Darensbourg et al. 2003).

Structural and functional mimetic complexes of naturally occurring hydrogenase active sites have been the center of efforts to elucidate the mechanistic details of the hydrogen evolution reaction as well as the development of commercial catalysts for the abundant production of clean sustainable fuels. One of the primary goals of the biomimetic approaches is to realize organometallic active sites within proteins or supramolecular cavities that impose similar stereochemical confinement as observed in the natural enzyme, and is otherwise unavailable in organometallic compounds. Few supramolecular assemblies of biomimetic [Fe-Fe] hydrogenase models have been reported so far, although a myriad of organometallic complexes are abundant in the literature. Pickett et al. reported that incorporation of a well studied diiron complex containing 1,3 propanedithiolate as bridging ligand, in an Fmoc-Leu based hydrogel dramatically increases its stability towards UV-irradiation (Frederix et al. 2012). In an elegant study, Darensbourg and colleagues incorporated a simple organometallic complex based on 1,3 propanedithiolatodiironhexacarbonyl into a cyclodextrin (Singleton et al. 2010). This supramolecular assembly is more efficient in photocatalytic hydrogen production by comparison with the catalyst alone, indicating the advantage of supramolecular confinement of these diiron catalysts (Li et al. 2012). Further studies have demonstrated that incorporation of such parent diironhexacarbonyl complexes in supramolecular arrangements dramatically increases water solubility and robustness towards photocatalytic hydrogen production (Jian et al. 2013; Wang et al. 2013; Wang et al. 2011a). Taken together, these results point to supramolecular confinement as a promising avenue to ameliorate the catalytic properties of simple organometallic mimics of diiron hydrogenases.
Despite these promises, however, existing approaches do not allow facile optimization of the host system. To overcome this limitation, a well-characterized protein-based host system was chosen that could easily be modified by altering its amino acid sequence. This platform utilizes the streptavidin-biotin interaction, which has long been exploited in biotechnological and biomaterial applications because of unique strength of this noncovalent binding. Also, streptavidin is extremely stable: it is resistant to denaturation within a wide range of pH, temperature, and organic solvents, including high concentrations of chemical denaturants such as urea and guanidinium hydrochloride (Green 1965). Finally, the valeric chain of biotin can be modified as needed without affecting the binding constant significantly (Heinisch and Ward 2010; Lin et al. 1999; Lo et al. 2004; Lo and Hui 2005; Lo and Lee 2004). Based on these reports, a biomimetic proton reduction catalyst could likely be chemically conjugated to a biotin molecule and sequestered within streptavidin to generate a stable supramolecular species (Figure 61). As a proof of concept, we biotinylated a simple diironhexacarbonyl complex containing a propanedithiolate bridging ligand; the adduct was incubated with wild-type streptavidin, and the complex characterized for its ability to generate hydrogen.
Experimental Section:

All chemicals were purchased from commercial sources of the highest quality available unless otherwise specified. All reactions were performed under an argon environment. Solvents were dried when necessary following conventional literature protocols. D-Biotin was purchased from Alfa-Aesar, and Streptavidin was purchased from prozyme. All other chemicals were purchased from Sigma-Aldrich. Compound 1A (figure 62) was synthesized using reported protocol without any modification (Hicks et al. 2008).

Synthesis of 1B:

950mg (4.11mmol) of compound 1A and 1.5 gm of NaSH, xH$_2$O (26.78 mmol, dry weight) were dissolved in 21 ml of ice-cold methanol: the solution was stirred in an ice bath for 2 hours and left stirring for overnight at room temperature. Finally, excess NaSH was quenched with equivalent amount of TFA and the resulting mixture was evaporated to dryness under reduced pressure to yield a 1B (figure 62) as a yellow liquid. The Yellow liquid was dissolved in dichloromethane and washed with 50 ml water in...
triplicate and dried over anhydrous Na$_2$SO$_4$ and evaporated to dryness under reduced pressure. Resulting yellowish liquid was used for the next step without any purification.

*Synthesis of 1C*

To the crude dithiolate 1B obtained from previous step in 20 ml toluene, 3.14 g (6.24 mmol) of Fe$_3$(CO)$_{12}$ was added under an inert atmosphere and the resulting solution was refluxed for overnight. A change in color from green to red was observed. The solvent was evaporated to dryness under reduced pressure. Resulting deep brown solution was treated with excess ethyl acetate to yield a deep red solution and a brown precipitate. The solution was transferred to a centrifuge tube and centrifuged at 10,000 × g for 30 minutes to separate the insoluble iron oxide particles. The deep red solution was carefully decanted off and evaporated under reduced pressure. Resulting red compound (1C figure 62) was purified on a silica gel column using (2:3) ethyl acetate:hexane as an eluent. Isolated yield = 245 mg (14% over two steps). $^1$H NMR (DMSO, δ [ppm]): 1.23 (m, 1H), 1.52 (dd, 2H), 2.73(dd, 2H), 3.18 (t, 2H), 4.74 (t, 1H); $^{13}$C NMR (DMSO, δ [ppm]): 25.00, 46.27, 64.53, 208.09

*Synthesis of 1D*

Compound 1D was synthesized by coupling D-Biotin and 1C in DMF using HATU as coupling agent. 180.56 mg (0.74 mmol) of Biotin and 308 mg (0.74 mmol) of compound 1C (figure 62) and 281.37 mg (0.74 mmol) of HATU was dissolved in 6 ml of anhydrous DMF and 185.22 mg of dry DIPEA was added to the mixture. Resulting mixture was stirred under an inert atmosphere overnight. Next day, DMF was evaporated under reduced pressure and resulting yellowish red crude oil was purified on a silica gel column using 10% MeOH in DCM. $^1$H NMR (DMSO, δ [ppm]): 1.19-1.67 (m, 9H), 2.28-2.34 (m,
Incorporation of 1D in Streptavidin and purification:

Due to low solubility in water, Biotin-HM was dissolved in DMSO as a stock and used for subsequent reactions and experiments. For the incorporation of Biotin-HM in streptavidin, a stock solution of 2 mg/ml of streptavidin in 100 mM Tris, pH 7.5 was incubated with 2- to 3-fold excess of Biotin-HM from a stock DMSO solution. After 15 minutes, resulting solution was passed through a PD10-desalting column (GE healthcare) previously equilibrated with the same buffer. Yellow fractions from the column were collected in 1 ml fractions and analyzed by UV-Vis spectroscopy.

Determination of binding properties of Biotin-HM with Streptavidin

Binding stoichiometry for Biotin-HM with streptavidin was analyzed using well-established 4'-hydroxyazobenzene-2-carboxylic acid (HABA) displacement assay (Skander et al. 2004) HABA binds to streptavidin with low micromolar dissociation constant and HABA-SA complex gives rise to absorbance near 500 nm. In a typical HABA displacement assay, 10 µM of Streptavidin in 20 mM phosphate, 150 mM NaCl, pH 7.5 was saturated with 500 uM HABA. After incubation at room temperature for 30 minutes, resulting solution was used for spectrophotometric titration. From a stock DMSO solution of Biotin-HM, incremental amount of ligand were added to the HABA saturated Streptavidin, and decrease of absorbance at 500 nm was monitored.

Results and Discussion:
Synthesis and Incorporation of Biotin-HM in Streptavidin: As previously mentioned above, a simple well-characterized model of the [Fe-Fe] hydrogenase mimic was chosen for this study. 3-bromo-2-(bromomethyl)propan-1-ol (1A, Figure 62) was used, which was obtained from 3-bromo-2-(bromomethyl)propanoic acid by diborane reduction following reported protocols with high yield (Hicks et al. 2008). The dibromo unit was replaced with a dithiol unit using NaSH. Refluxing the crude dithiol compound (1B) with Fe\(_3\)(CO)\(_{12}\) in Toluene resulted formation of alcohol functionalized diironhexacarbonyl
compound 1C. Initial coupling trials with 1C and Biotin under normal coupling conditions, for example EDC coupling at both room and elevated temperature remained unsuccessful. In presence of a stronger coupling agent, HATU, 1D was synthesized in high yield and with homogeneity. The biotinylated complex was characterized using 1H/13C NMR, FTIR and also ESI-MS. Biotin-HM is sparingly soluble in water, whereas solubility dramatically increases upon addition of streptavidin (SA) indicating binding of the organometallic moiety within protein environment. The binding stoichiometry of Biotin-HM was determined in figure 63 by the displacement of 2-(4-Hydroxyphenylazo)benzoic acid (HABA) (Skander et al. 2004). HABA binds to streptavidin in a (1:1) stoichiometric ratio as D-Biotin but with much lower affinity and thus it can be stoichiometrically replaced by biotin in a titration experiment. HABA-streptavidin complex absorbs in the visible range, with maximum at 500 nm; and displacement with biotin causes loss of absorbance. In this assay, streptavidin is saturated with HABA using 50 molar excess of HABA and incubated for 30 minutes at room temperature. Biotin-HM was titrated in and absorbance at 500 nm was plotted as a function of biotin-HM concentration. We found that the stoichiometry of binding for

![Figure 63](image)

**Figure 63 – a.)** HABA displacement assay of streptavidin by Biotin-HM. Absorbance at 500 nm of a HABA saturated was monitored as a function of Biotin-HM concentration; **b.)** Chemical structure of HABA.
biotin-HM to streptavidin is approximately 1:0.7, which is consistent with the activity of the commercial streptavidin. Thus, introduction of the diiron-hexacarbonyl moiety at the valeric chain of biotin does not affect binding stoichiometry.

**UV-Vis and FTIR Spectroscopy**

Because of the extremely high affinity of streptavidin for biotin, streptavidin-incorporated Biotin-HM can be purified away from unbound Biotin-HM using a desalting P10 column. The structural integrity of the diiron complex was verified by UV-vis and by FTIR spectroscopy. Purified Streptavidin: Biotin-HM assembly shows two distinct bands in its UV-Vis absorption spectrum, centered around 329 nm and 474 nm which is characteristic of Fe$_2$S$_2$ butterfly complexes and consistent with reported literature (Jones et al. 2007; Roy et al. 2012) (Figure 64). This also proves capture of Biotin-HM in streptavidin matrix. The electronic environment of the diiron cluster can be probed by FTIR by analyzing the coupled -CO stretching frequencies. Due to low solubility of Biotin-HM in water, a direct comparison of the complex in presence and
absence of streptavidin in identical experimental conditions turned out to be quite difficult. Instead, we compared thin films of Biotin-HM from a solution of 20% methanol in water on a FTIR CaF$_2$ window in presence and absence of streptavidin. FTIR spectra of Biotin-HM show 3 major peaks at 1992, 2030, 2073.5 cm$^{-1}$, which are assigned to the coupled stretching frequency of –CO, consistent with related organometallic analogues (Jones et al. 2007; Roy et al. 2012; Sano et al. 2011). In the presence of twofold molar excess of SA, all three peaks are shifted to 1990, 2034, 2074 cm$^{-1}$ implying a change in environment of the –CO which is attributed to the binding of Biotin-HM to SA. Taken together, the spectroscopic information collected support the presence of a structurally intact biotinylated diiron hexacarbonyl center, which is incorporated within the desired streptavidin scaffold. Incorporation results in confinement within the protein scaffold, and more importantly, results in interaction of the cluster with the surrounding protein matrix.
We then investigated the hydrogen production efficiency of the catalyst as a function of supramolecular confinement by the streptavidin host. Diironhexacarbonyl catalysts of are known to generate hydrogen when reduced by excited photosensitizers and when the reducing equivalents are provided electrochemically. First we adopted a well established protocol, in which Ru(Bpy)$_3$ is photo-excited by visible light: this excited species then generates the active redox state, Fe(I)Fe(0), from the resting Fe(I)Fe(I) either by oxidative or reductive quenching. Fe(I)Fe(0) center then reduces proton through formation of an intermediate Fe(I)Fe(II)H entity (Kluwer et al. 2009; Li et al. 2008; Na et al. 2008; Song et al. 2009; Streich et al. 2010; Wang et al. 2011a; Wang et al. 2010; Zhang et al. 2010). In this assay (Figure 65), Biotin-HM gives rise to 504 nmol of hydrogen resulting in a TON of 98.13; the conditions used were 5.14 µM Biotin-HM in 100 mM citrate buffer, pH 4.5 in presence of 100 mM ascorbate as a sacrificial electron donor and 150 µM of Ru(Bpy)$_3$ as photosensitizer. When Biotin-HM is bound within

Figure 65 – Photocatalytic hydrogen production using Ru(bpy) as a photosensitizer. Black trace is the Biotin-HM alone and the Blue trace is the confined biotin-HM bound within streptavidin.

Photocatalytic and Electrochemical Hydrogen Production
streptavidin, TON increases to 293 or 1510 nmol H\textsubscript{2} along with the lifetime of the catalyst. This result supports the use of protein-based hybrid materials to augment the intrinsic catalytic activity of organometallic complexes. In general, simple [Fe-Fe] hydrogenase mimics containing diironhexacarbonyl are inefficient as hydrogen production catalysts because of their instability towards long irradiation times during which they are degraded to intermediates that are not catalytically viable (Wang et al. 2011b). We hypothesize that streptavidin insulates the catalyst from unwanted side reactions and degradation, resulting in increased overall stability and lifetime.

We also investigated electrochemical proton reduction property of Biotin-HM in presence and absence of streptavidin. In 100 mM Citrate, 100 mM NaCl, pH 4.5, a catalytic current on the reductive scan was observed corresponding to proton reduction as shown in Figure 66. Although a miniscule amount proton reduction was observed on the bare electrode, the amount of hydrogen generated through this process is insignificant when compared to the catalyzed reaction. When streptavidin was added to the solution, magnitude of the catalytic current was diminished in a significant fashion. This phenomenon can be explained considering the insulation of the catalyst from active

Figure 66 – a.) Cyclic Voltammogram of Biotin-HM in absence (black line) and presence of Streptavidin (blue line) in solution using 100 mM citrate, 100 mM Sodium Chloride, pH 4.5. b.) Cyclic Voltammogram of Streptavidin–Biotin-HM complex thin film on the surface of a pyrolytic graphite electrode (black trace, right panel), CV scan for bare electrode under the same condition is shown in blue trace. CV conditions pyrolytic graphite working electrode, saturated calomel reference electrode, and platinum mesh counter electrode.
electrode surface upon binding to the protein. Streptavidin has a pI of 5.0, so we hypothesized that at pH 4.5, positively charged streptavidin can be electrostatically adsorbed on the negatively charged glassy carbon-working electrode. Indeed, when a thin film of the streptavidin-Biotin-HM complex is dried on a glassy carbon electrode surface, it gives rise to a significant catalytic wave corresponding to proton reduction (Figure 6). Considering the ease and robustness of existing methods for covalent attachment of streptavidin to solid surfaces, the catalytic enhancement observed opens up the possibility of designing streptavidin-functionalized electrode surfaces for electrocatalytic hydrogen production.

Conclusions:

Since the first report of streptavidin as a platform for bio-hybrid catalysts (Wilson and Whitesides 1978), there have been a tremendous amount of progress in the field of protein based hybrid organometallic catalysts. Until now, most of these catalysts are focused on synthetic organic chemistry transformation, more specifically using protein scaffolds as a source for stereo-selection in hydrogenation and sulfoxidation of organic molecules (Creus et al. 2008; Dürrenberger et al. 2011; Pordea et al. 2008; Skander et al. 2004). Here, we applied the same approach to a reaction- the reduction of protons to hydrogen- that is not stereo-specific. However, we hypothesized that shielding the catalyst from the aqueous environment would increase its efficiency by preventing unwanted side reactions. Further, the protein scaffold could potentially provide second sphere and long-range interactions to augment the intrinsic reactivity of the center. In addition, the mechanism of action of these catalysts is thought to involve a thermodynamically unstable but catalytically active form of the diiron site. The natural hydrogenase utilizes specific interactions to the inorganic ligands to stabilize this
enthalpic conformation. In future studies, we aim to exploit an unique advantage of the bio-hybrid systems over other supramolecular mimics of hydrogenases, as protein-based system such as the streptavidin based one, present the unique opportunity to optimize activity through a so-called chemigenetic approach. Both the protein scaffold and the ligand component can be exchanged and optimized independently, by mutating the catalytic center as well as by introducing specific mutations in the protein scaffold. Exploiting the tolerance of biotin-streptavidin binding to modifications of the valeric acid chain, these complexes can be biotinylated and introduced into streptavidin with little loss in binding affinity. Native-like secondary shell interactions can also be introduced with much ease as compared to purely organometallic complexes. For example, its worth mentioning that the bridgehead atom of the biomimetic complex used in this study is a carbon; where as its reported to be a nitrogen atom in the natural enzyme. This bridge-head nitrogen atom is believed to be involved in proton shuttling during proton reduction (Mulder et al. 2011). Synthesizing the corresponding N-containing organometallic complex turns out to be much harder, but similar kind of proton shuttling groups, for example histidine residues, can be introduced using site directed mutagenesis in close proximity of the biomimetic active site to rescue the offset of replacing bridgehead nitrogen by carbon. To facilitate photocatalysis, photosensitizers can be introduced to the close proximity of the active site using covalent attachment. Further facilitating the development of applications and its use in devices, streptavidin-based catalysts are unique for their robustness, unparalleled in a biological/proteinogenic environment.
DE NOVO DESIGN OF AN ARTIFICIAL BIS[4Fe-4S] BINDING PROTEIN

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My contributions included verification of [4Fe-4S] cluster concentrations and percent incorporation as well as editing of the manuscript. See Appendix I for permission details)

Abstract:

In nature, protein subunits containing multiple iron-sulfur clusters often mediate the delivery of reducing equivalents from metabolic pathways to the active site of redox proteins. The de novo design of redox active proteins should include the engineering of a conduit for the delivery of electrons to and from the active site, in which multiple redox
active centers are arranged in a controlled manner. Here, we describe a designed three-helix protein, DSD-bis[4Fe-4S], that coordinates two iron-sulfur clusters within its hydrophobic core. The design exploits the pseudo two-fold symmetry of the protein scaffold, DSD, which is a homodimeric three-helix bundle. Starting from the sequence of the parent peptide, we mutated eight leucine residues per dimer in the hydrophobic core to cysteine to provide the first coordination sphere for cubane-type iron-sulfur clusters. Incorporation of two clusters per dimer is readily achieved by in situ reconstitution and imparts increased stability to thermal denaturation compared to that of the apo form of the peptide as assessed by circular dichroism-monitored thermal denaturation. The presence of [4Fe-4S] clusters in intact proteins is confirmed by UV-vis spectroscopy, gel filtration, analytical ultracentrifugation, and electron paramagnetic resonance spectroscopy. Pulsed electron-electron double-resonance experiments have detected a magnetic dipole interaction between the two clusters $\sim 0.7$ MHz, which is consistent with the expected intercluster distance of 29–34 Å. Taken together, our data demonstrate the successful design of an artificial multi-iron-sulfur cluster protein with evidence of cluster-cluster interaction. The design principles implemented here can be extended to the design of multicluster molecular wires.

Introduction:

[Fe-S] cluster proteins make up one of the most abundant and functionally pliable classes of proteins: they carry out a wide variety of biological functions, including catalysis, oxygen sensing, and electron transfer (Beinert et al. 1997; Lill 2009). The chemical properties of the [Fe-S] clusters, such as low redox potential (with the exception of high-potential iron-sulfur proteins, HIPIP) and high oxygen sensitivity, their prevalence, and their structural and functional flexibility, suggest that they
emerged during early evolution (Eck and Dayhoff 1966; Meyer 2008). Most frequently, [Fe-S] clusters act as donors or acceptors of electrons over a wide range of potentials, and they are organized in protein-embedded redox chains that function as relays for the transfer of electrons from soluble protein partners to catalytic centers in complex, often multiunit, proteins (Fontecilla-Camps et al. 2007; Hinchliffe and Sazanov 2005; Jordan et al. 2001; Peters et al. 1998; Sazanov and Hinchliffe 2006).

The most common type of [Fe-S] cluster, the [4Fe-4S] cubane-like structure, is generally coordinated by four cysteines in a variety of protein folds, often in a combination of loops and secondary structure elements. A common fold is that of the ferredoxins, small soluble electron carrier proteins that can contain one or two clusters coordinated by four conserved cysteines each (Krishna et al. 2006; Meyer 2008). The two-cluster ferredoxin-like fold is also found in the PsaC subunit of Photosystem I (Antonkine and Golbeck 2006) and in hydrogenases as part of the accessory iron-sulfur cluster binding domain, which regulates long-range electron transfer to and from the active site (H-cluster) (Dubini et al. 2009; Mulder et al. 2011; Nicolet et al. 1999; Peters et al. 1998).

Over the years, a substantial amount of scientific effort has been dedicated to elucidating the structure-function relationships of different clusters with their protein counterparts in natural and de novo-designed model systems. Model proteins designed to coordinate an iron-sulfur cluster generally utilize loop regions to accommodate the cluster, with a sequence and topology resembling those found in most of the natural proteins (Antonkine et al. 2009; Antonkine et al. 2007; Kennedy and Gibney 2002; Scott and Biggins 1997). Computational approaches have been used to position an idealized cluster into a designed four-helix coiled coil (Grzyb et al. 2012; Grzyb et al. 2010) or into the structure of natural proteins (Coldren et al. 1997). These models were designed to
contain a single iron–sulfur cluster, either isolated within the protein scaffold or in the proximity of a second cofactor (Gibney et al. 1996; Laplaza and Holm 2001). When functioning as electron conduits, however, iron-sulfur clusters are generally arranged in chains of two or more (Pontecilla-Camps et al. 2007; Hinchliffe and Sazanov 2005; Jordan et al. 2001; Peters et al. 1998; Sazanov and Hinchliffe 2006).

As a first step toward constructing bioinspired functional redox proteins encompassing an electron transfer domain and a catalytic domain (Faiella et al. 2013; Roy et al. 2012), we have engineered a model system that contains two [4Fe-4S] clusters embedded in the hydrophobic core of a de novo-designed helical three-helix bundle. We present here the design, synthesis, and characterization of this model system, DSD-bis[4Fe-4S], demonstrating for the first time the incorporation of two clusters at a defined distance. This approach can be extended to the design of other systems, in which the intercluster distance can be modulated by exploiting the geometric properties of α-helical coiled coils (Crick 1953; Grzyb et al. 2010; Woolfson et al. 2012).

Materials and Methods:

Chemicals

Chemicals were purchased from Sigma-Aldrich; Fmoc-protected amino acids for solid-phase peptide synthesis were purchased from Novabiochem. All reactions were performed in an inert environment using a Schlenk line or in a glovebox (Coy Scientific), unless otherwise noted. All buffers and solutions were degassed extensively by being purged with N₂ prior to their transfer to the glovebox.
Protein Design

Models of DSD-4Cys and DSD-bis[4Fe-4S] were built in MacPyMol (DeLano 2007) starting from the coordinates of DSD [Protein Data Bank (PDB) entry 1G6U]. Leucines 9, 12, 22, and 43 in DSD were mutated to cysteine, and side chain orientations were selected among the backbone-dependent favorable rotamers in MacPyMol. The disembodied [4Fe-4S] cluster and four cysteines that form the primary coordination sphere were obtained from Thermotoga maritima tryptophanyl tRNA synthase (PDB entry 2G36) and manually docked onto DSD-4Cys using pairwise alignment in MacPyMol. Finally, new bonds were created using the Build function. The models of DSD-4Cys, DSD-bis[4Fe-4S], and the original DSD structure were analyzed with Q-SiteFinder (Laurie and Jackson 2005), which identifies and measures the volume of cavities using PDB entries as input.

Peptide Synthesis and Purification

DSD-4Cys, which is comprised of 49 amino acids, was synthesized on a Liberty microwave-assisted solid phase peptide synthesizer (CEM). Peptide synthesis was performed using a standard Fmoc/t-Bu/Trt protecting strategy on a Rink amide resin; the N-terminus of the peptide was acetylated. Cleavage from the resin and simultaneous removal of side chain protecting groups was achieved by treatment with 94% TFA, 2.5% water, 2.5% EDT, and 1% TIS. The crude peptide was purified by high-performance liquid chromatography on a C-18 semipreparative column (Vydac, 1 cm × 25 cm), using gradient elution with solvent system A (100% water and 0.1% TFA) and solvent system B (95% acetonitrile, 5% water, and 0.1% TFA) at a flow rate of 4 mL/min. Peptide purity was confirmed by reinjecting appropriate fractions on an analytical C-18 column; the molecular mass of the peptide was verified by matrix-assisted desorption ionization
time-of-flight (MALDI-TOF) mass spectrometry. Peptides used for further experiments were >99% pure.

Direct Incorporation of the \([4\text{Fe}-4\text{S}]\) Cluster into the Synthesized Peptides. All the reactions were performed in an anaerobic chamber (Coy Scientific) under an inert atmosphere. Cluster incorporation was performed following an established protocol (Antonkine et al. 2007). Briefly, a 120 \(\mu\)M solution of DSD-4Cys peptide in 100 mM Tris-HCl (pH 8.5) and 2 M urea was treated with 0.8% (v/v) BME for 2 h. To this solution were added iron(III) chloride (FeCl\(_3\)) and a freshly prepared solution of sodium sulfide (Na2S) in water in 30 min intervals to a final concentration of 30 mM. The resulting dark brown solution was incubated overnight at 4 °C under N\(_2\). The reaction mixture was then passed through a PD10 G25 desalting column (GE Healthcare) pre-equilibrated with 50 mM Tris-HCl buffer (pH 7.5) to exclude all nonprotein, low-molecular mass contaminants and salts. Appropriate fractions were collected in 1 mL increments and subjected to characterization.

**UV–Vis Spectroscopy**

UV–vis spectra were recorded on a Perkin-Elmer Lambda-35 spectrophotometer under anaerobic conditions using a sealed cuvette with a path length of 1 cm.

**Gel Filtration**

The oligomerization states of DSD-4Cys and DSD-bis[4Fe-4S] were determined on a G-25 gel filtration column using an Agilent Technologies 1260 Insight FPLC system. The apo peptide was treated with a 10-fold molar excess of DTT at 4 °C to prevent disulfide formation. DSD-bis[4Fe-4S] was kept under anaerobic conditions prior to injection. The
assignment of the elution peak to the dimer was confirmed by analytical ultracentrifugation (Supporting Information).

**Circular Dichroism (CD) Spectroscopy**

CD spectroscopy of the apo and holo peptides was conducted on a JASCO J-815 spectropolarimeter. Spectra were recorded from 240 to 190 nm in 1 nm increments at protein concentrations of 0.050 and 0.025 mM in 10 mM Tris-HCl (pH 7.5) using a quartz cuvette with a path length of 1 mm; the final spectra are the average of three data sets. Thermal unfolding curves were monitored by following the CD signal at 222 nm as a function of temperature from 4 to 96 °C. Holo DSD-bis[4Fe-4S] peptide samples were handled in an airtight cuvette to exclude air during the course of the experiment. CD spectra of DSD-4Cys were recorded in the presence of excess reducing agent (TCEP) to prevent oxidation of the cysteine side chains.

**Electron Paramagnetic Resonance (EPR) Spectroscopy**

Appropriate fractions from the PD10 column were concentrated using a centrifuge concentrator with a molecular mass cutoff of 3000 Da (GE Healthcare). The pH values of the samples were then adjusted to pH 10 by addition of 1 M glycine buffer, and the samples were reduced by addition of 100 mM Na₂S₂O₄ in 1 M glycine buffer (pH 10). Aliquots (150 µL) of the reduced samples were transferred to quartz EPR tubes, flash-frozen by immersion in liquid nitrogen, and stored at 77 K until EPR characterization.

Continuous wave (CW) EPR experiments were performed on the X-band EPR spectrometer Elexys E500 (Bruker). Pulsed EPR experiments were performed on a home-built broadband (2–18 U 26–40 GHz) pulsed EPR spectrometer at microwave
(mw) frequencies of ~9.6 GHz. The measurement temperature was 15 K. Detailed experimental conditions are given in the figure captions.

**Cluster Quantification**

For each preparation, the percentage yield of cluster incorporation was estimated by UV-vis, using an $\varepsilon$ of 5500 M$^{-1}$ cm$^{-1}$ per protein and an $\varepsilon$ of 16000 M$^{-1}$ cm$^{-1}$ per cluster at 410 nm; depending on preparations, the yield of incorporation ranged between 80 and 100% (Hoppe et al. 2011). Cluster incorporation was assessed quantitatively by measuring independently the concentration of iron and of peptide in identical samples. First, DSD-bis[4Fe-4S] samples were further purified by anion exchange chromatography on a Q-Sepharose FF column (GE Healthcare), using 100 mM Tris (pH 8.5) as equilibration buffer and 100 mM NaCl and 100 mM Tris (pH 8.5) as elution buffer. The samples were split into two portions: one was used to measure the peptide concentration by amino acid analysis (Proteomics Department, University of California, Davis, CA), and the second was used to determine the iron concentration by a ferrozine assay (Carter 1971). Incorporation of the EPR active, reduced cluster was determined by EPR spin quantification. Briefly, a concentrated DSD-bis[4Fe-4S] sample was divided into two parts, one of which was submitted for peptide concentration determination by amino acid analysis. The second portion was prepared for EPR spectroscopy as described above and subjected to spin quantification by comparing the double integral of the CW EPR spectrum of [4Fe-4S]$^+$ recorded at 7 K with the spectrum of 5 mM Cu(II) nitrate recorded at 21 K [at lower temperatures, the Cu(II) EPR signal was saturated even at the lowest accessible mw power of 0.2 $\mu$W]. The estimated concentration of [4Fe-4S]$^+$ was then compared with the DSD protein concentration evaluated using amino acid analysis for the same sample (accounting for dilutions).
Results and Discussion:

**Design of the Model Peptide**

DSD-bis[4Fe-4S] was designed by using as a scaffold a dimeric three-helix bundle originally designed to probe domain swapping (domain-swapped dimer, DSD, PDB entry 1G6U). In DSD, two identical peptides each form a helical hairpin, in which one of the helices is approximately twice the other in length. The long helices from two monomers pair up in an antiparallel manner, while the two shorter helices dock against the dimer formed by the long helices. An approximate 2-fold symmetry axis is located between the two short helices (Figure 67). The fold is stabilized by a leucine-rich hydrophobic core and by salt bridges positioned at the helix–helix interfaces to impart specificity.

The arrangement of the two monomers in DSD is reminiscent of the internal pseudo 2-fold symmetry found in two-cluster ferredoxins, which have long been thought to derive from gene duplication events and domain swapping (Eck and Dayhoff 1966). Ferredoxins comprise two repeats of a 29-residue sequence and adopt the so-called

**Figure 67 – Design of DSD-bis[4Fe4S].** (Top) The crystal structure of DSD (PDB ID: 2G6U) serves as a starting point. The [4Fe4S] cluster from *Thermotoga maritima*, including the four cysteines comprising its first coordination sphere, was docked in the core of DSD. Appropriate core leucine residues in the hydrophobic core were mutated to cysteines. The first coordination sphere of DSD-bis[4Fe4S] displays an excellent agreement with the natural coordination sphere of the cluster. (Bottom) Sequences of DSD and DSD-4Cys; mutations to the DSD sequence in DSD-4Cys are highlighted in bold. A tryptophan was inserted at the C terminal to facilitate concentration assessment.
ferredoxin fold defined by a βαβαβ secondary structure. In the two-cluster bacterial ferredoxins, each of the two symmetric clusters is coordinated by three sequentially contiguous cysteines contained in the loop, between the helix and the second β-sheet. A fourth distal cysteine, which is located in the helix of the second sequence repeat, completes the first coordination sphere of the cluster. The sequence motif is CXXCXXC...C. Mirroring this arrangement, we designed a binding site in which three of the coordinating cysteines belong to a monomer, while the fourth is provided by the second monomer. DSD-bis[4Fe-4S], however, differs from the ferredoxins both in its highly helical secondary structure and in its dimeric oligomerization state. To ensure a correct selection of cysteine rotamers, we docked a natural [4Fe-4S] cluster and its primary coordination sphere into the hydrophobic core of DSD. This “metal-first” approach has been successfully used to insert iron-sulfur clusters into natural and designed proteins (Coldren et al. 1997; Grzyb et al. 2010). We referred to the cluster found in *T. maritima* tryptophanyl tRNA synthase (PDB entry 2G36) (Han et al. 2010), which has been used by Grzyb et al. as a starting point to model a symmetrized cluster (Grzyb et al. 2010). The cluster is coordinated through a C...C...CXXC sequence motif, in which the CXXC residues are part of a helix, as assessed by backbone dihedral angles, while the two additional cysteines are found at the base of interhelix loops and are in a nonhelical conformation. The distances between the backbone atoms of the cysteines are compatible with the regular three-helix coiled coil geometry of DSD. Accordingly, once we overlaid the CXXC motif with the LXXL motif that forms the core of DSD, the Cα atoms of the two remaining cysteines overlapped with the Cα atoms of leucines forming the core of native DSD. Several core positions corresponding to different layers within the coiled coil were possible matches for the geometry of the four-cysteine motif; we selected the alignment that results in minimal clashes and supports the correct rotamers.
for the cysteine side chains (see Materials and Methods). We evaluated the impact of four Leu to Cys mutations and of cluster insertion on the integrity of the peptide scaffold by analyzing the models with Q-SiteFinder (Laurie and Jackson 2005) and comparing the results to the X-ray structure of DSD (Figure 72 of the Supporting Information). This analysis revealed the presence of a 58 Å³ cavity in the core of DSD-4Cys corresponding to the cluster binding site, and smaller pockets to the side. This central cavity is completely occupied by the [4Fe-4S] cluster in the model of DSD-bis[4Fe-4S], which presents only the small pockets to the side. DSD-bis[4Fe-4S] resembles the original DSD structure, which contains no cavities in the core, and identical side pockets.

The cluster binding site is located at one end of the bundle; because of the symmetry of the scaffold, the site is replicated in the second half (Figure 69). The sequence of the monomeric peptide contains four Leu to Cys mutations, three of which are contiguous in the sequence and a fourth one that is distal; we have named the peptide DSD-4Cys. The peptide was synthesized by solid phase peptide synthesis (SPPS) and purified as described in Materials and Methods.

Assembly of DSD-bis[4Fe-4S]

We reconstituted the clusters into the apo DSD-4Cys peptide using a well-established in situ synthetic procedure under anaerobic conditions (Antonkine et al. 2007). Briefly, the reconstitution reaction was conducted with ferric chloride and sodium sulfide in aqueous buffer (pH 8.5) in the presence of 2-mercaptoethanol and 2 M urea that serves to partially unfold DSD-4Cys, permitting access to the cysteines located in the hydrophobic core. Cluster incorporation is entropically favorable and proceeds by ligand exchange between the four preorganized cysteines and the more basic 2-
mercaptoethanol that initially ligate the cluster (Antonkine et al. 2007; Beinert 2000; Koay et al. 2008).

The UV−vis spectrum of the resulting DSD-bis[4Fe-4S] protein shows broad absorptions at 415 and 360 nm, which arise from sulfur to iron charge transfer excitations in oxidized [4Fe-4S] clusters; the absorption at 415 nm disappears upon reduction with sodium dithionite (Figure 68). The positions of the maxima are red-shifted compared with those of inorganic [4Fe-4S] clusters, consistent with a cluster shielded from the aqueous environment. These spectral features and redox-dependent behavior are typical of iron-sulfur cluster proteins (Jin et al. 2008; Sweeney and Rabinowitz 1980). The ratio of absorbance at 410 nm to that at 280 nm is 0.76, within the range of 0.7–0.8 usually observed in natural proteins (Jin et al. 2008; Sweeney and Rabinowitz 1980). On the basis of these values, we estimate the peptide/cluster stoichiometry to be 1/1, consistent with a dimeric peptide coordinating two clusters. Analysis by MALDI of a preparation made at low peptide concentrations showed a broad
peak corresponding to the mass of DSD-bis[4Fe-4S], two peptides, and two cubane clusters (Figure 77 of the Supporting Information).

The level of incorporation evaluated on a more concentrated preparation using the ferrozine assay was 74%, corresponding to an estimated population of at least 55% of the dimers containing two clusters.

We further characterized the redox behavior of DSD-bis[4Fe-4S] by cyclic voltammetry (Figure 76 of the Supporting Information). In the presence of 4 mM neomycin as a stabilizer, we observed a cathodic wave at -478 mV versus the standard hydrogen electrode, which is in the range observed for natural cubane-type iron–sulfur clusters (Koay et al. 2008). However, the absence of an anodic wave indicates the electrochemical irreversibility of the process under these conditions.

**Protein Folding and Stability**

We verified that DSD-4Cys and DSD-bis[4Fe-4S] fold into dimers, as observed in the structure of DSD, by gel filtration, monitoring elution at wavelengths of 220, 280, and 410 nm. The two proteins have identical elution profiles dominated by a single peak at 30 min, consistent with the presence of a single species with the molecular mass of a dimer (Supporting Information). Furthermore, sedimentation velocity experiments by analytical ultracentrifugation of DSD-4Cys yielded an apparent molecular mass (12.4 kDa) in excellent agreement with that expected for a dimer (10.6 kDa) at a loading concentration of 100 µM, thus indicating that the protein forms a stable dimer in the micromolar range (Supporting Information). The sedimentation data confirm our assignment of the peaks observed in the gel filtration elution profile to dimers.

The impact of mutations on the structure of DSD in apo DSD-4Cys and in reconstituted DSD-bis[4Fe-4S] was verified by CD spectroscopy. Analysis of the models
of DSD-4Cys and DSD-bis[4Fe-4S] shows that exchanging the hydrophobic side chain of leucine with the smaller, polar side chain of cysteine results in a polar cavity in the core of the bundle. The dimensions of the cavity calculated using Q-SiteFinder are compatible with the cubane iron-sulfur cluster; further, coordination by the cysteines to the iron-sulfur cluster stabilizes the fold and replaces hydrophobic interactions.

The secondary structure content of apo DSD-4Cys and of reconstituted DSD-bis[4Fe-4S] was evaluated by analyzing the corresponding CD spectra. Like that of DSD, both spectra contain bands at 190, 208, and 222 nm typical of α-helical proteins. The spectra are indistinguishable, indicating that the secondary structure of the protein is not distorted significantly upon insertion of the [4Fe-4S] cluster. The visible portion of the CD spectrum of DSD-bis[4Fe-4S] is dominated by a complex feature, comprising a narrow negative signal at approximately 360 nm and a broader signal centered at 560 nm, which arises from the [4Fe-4S] clusters and resembles the spectra of ferredoxins (Bertini et al.; Sweeney and Rabinowitz 1980). Such a signature for DSD-bis[4Fe-4S] corroborates the evidence of the successful incorporation of the cluster.

To further evaluate the impact of modifications on DSD in apo DSD-4Cys and in reconstituted DSD-bis[4Fe-4S], we have investigated their thermal denaturation by monitoring the decrease in the magnitude of the helical signal at 222 nm as a function of temperature. A comparison of the melting curves (see Figure 69) reveals conspicuous differences in stability. The thermal denaturation of DSD-bis[4Fe-4S] is irreversible, as partial denaturation results in a loss of the cofactor, thus precluding a rigorous thermodynamic analysis aimed at extrapolating folding parameters: the following discussion is thus limited to comparison of denaturation profiles and apparent melting points (T_m).
We chose DSD as a starting scaffold because of its remarkable thermal stability. DSD retains >90% of its helical structure at temperatures as high as 94 °C; a complete unfolding transition was observed only by subjecting DSD to thermal denaturation in the presence of 3 M guanidinium chloride (Ogihara et al. 2001). Not surprisingly, substituting eight leucines per dimer with cysteines in the hydrophobic core of DSD-4Cys results in a loss of stability. In contrast to that of DSD, the thermal denaturation curve of DSD-4Cys shows unfolding even in the absence of guanidinium chloride; the protein, however, is still very stable, with an apparent Tm of 50 °C. Incorporation of the cluster into DSD-4Cys restores the thermal stability: as shown in Figure 69, DSD-bis[4Fe-4S] is more than 90% folded at 80 °C; the apparent Tm is 87 °C. As the temperature increases above 80 °C, though, the protein undergoes rapid unfolding and loses the cluster, as evident from the observation of an iron precipitate. The apparent Tm observed for DSD-bis[4Fe-4S] is remarkable for a protein of this size. Further, the trend in stability observed across the Tm series (DSD, DSD- bis[4Fe-4S], and DSD-4Cys)
reflects the packing integrity of the cores as estimated by PocketFinder (see above). Loss of stability is observed in DSD-4Cys, which contains unfilled pockets in the core.

**EPR Spectroscopy**

The CW EPR spectrum obtained for the DSD-bis[4Fe-4S] sample reduced with dithionite (shown in Figure 70a) exhibits principal g values of 1.879 (gₓ), 1.943 (gᵧ), and 2.058 (gᶻ), with a gₐₐₐ of 1.965, and is similar to the spectra of [4Fe-4S]⁺ clusters described in the literature (Koay et al. 2008), which confirms the presence of intact [4Fe-4S] clusters bound to DSD. The temperature dependence of the EPR amplitude (Figure 70b) shows a nearly exclusive population of the S = 1/2 ground state at the temperatures below 15 K. The temperature dependence presented in Figure 70b is typical of [4Fe-4S]⁺ clusters and can be used to obtain information about the energies and multiplicities of the excited states (Papaeftymiou et al. 1980). Spin quantification of the reduced, EPR active clusters revealed that the [4Fe-4S]⁺/protein concentration ratio is ~56%. In contrast, cluster quantification on a different sample conducted using the ferrozine assay to assess iron content yielded a level of incorporation of 73.5%. Such a difference could be explained by the challenge of completely reducing the clusters in the EPR sample.

To obtain additional proof of the pairwise binding of the [4Fe-4S] clusters to the DSD protein, we used the pulsed electron–electron double-resonance (ELDOR) technique that detects a magnetic dipole interaction between the paramagnetic centers (Milov et al. 1998). Pulsed ELDOR and related techniques are routinely used to determine the distances between paramagnetic centers (spin-labels, native radicals, and/or metal centers) in biological systems (Astashkin et al. 2012; Astashkin et al. 2006; Gordon-Grossman et al. 2011; Jeschke 2012; Lovett et al. 2009; Schiemann and Prisner
In particular, pulsed ELDOR was employed to study the electron transfer systems containing [Fe-S] clusters and to obtain information about the relative spatial arrangement of the electron transfer carriers and the electronic structure of the [Fe-S] clusters themselves (Astashkin et al. 2006; Elsaesser et al. 2005; Elsasser et al. 2002).

In the pulsed ELDOR experiment, the pumping mw frequency, $v_{\text{pump}}$, was set in resonance with the maximum of the ESE field sweep spectrum ($g_z$ position). The observation frequency, $v_{\text{obs}}$, was 93 MHz higher, which corresponds to the observation EPR position shifted by $\sim$3.3 mT toward $g_z$ (Figure 71a). Because the characteristic width of the EPR spectrum ($\sim$17 mT) is much greater than the pumping mw excitation width ($\sim$1.5 mT for the 12 ns pumping mw pulse), the ELDOR effect was expected to be very small, especially taking into account the incomplete pairing of the EPR active $[4\text{Fe-4S}]^{+}$ clusters and possible (and unpredictable) orientational selectivity effects. With a distance
between the [4Fe-4S] cluster binding sites of ∼30 Å in the model, the dipole interaction constant was not expected to be very large, less than ∼4 MHz (see below). Therefore, we have used in our experiments the three-pulse ELDOR sequence (based on the two-pulse observation sequence) (Milov et al. 1998), which offers somewhat better sensitivity than the four-pulse sequence (Martin et al. 1998). The pulsed ELDOR measurements were performed at 15 K, where the population of the excited states is still negligible (see Figure 70b), but the longitudinal relaxation time is already short enough to allow the measurements with a reasonably fast pulse repetition rate of 500 Hz.

The experimental ELDOR trace is presented in Figure 71 (solid trace). It exhibits the oscillations with a high frequency of ∼15 MHz (∼70 ns period), which represent a residual electron spin echo envelope modulation (ESEEM) at the Zeeman frequency of 1H. Such a residual ESEEM is often observed in ELDOR experiments with an insufficient frequency separation between ν_{obs} and ν_{pmp} (Jeschke 2012). In addition, a fast-damping low-frequency oscillation is observed with the first minimum at a τ’ of ∼700 ns (where τ’ is the time interval between the first observation pulse and the pumping pulse). This oscillation arises from the dipole interaction between the [4Fe-4S]+ clusters, which follows from the facts that the [4Fe-4S]+ clusters exist only as a protein-bound species and no such oscillation was observed for the samples with low [4Fe-4S]+/protein concentration ratios, resulting in a negligible probability of pair formation.

The characteristic frequency of the low-frequency oscillation assigned to the dipole interaction between the clusters is only ∼0.7 MHz (as estimated from the position of the first minimum). In a point dipolar approximation, this dipole interaction would correspond to a distance of ∼42 Å, significantly longer than the structurally reasonable distance of ∼30–34 Å. The dashed trace in Figure 71 shows the simulation (using a simple point dipolar model and neglecting any possible orientational selectivity) for the
The simulated pairwise effect is superimposed on the exponential decay from the uniformly distributed spins.

To explain this discrepancy, one has to take into account that the [4Fe-4S]$^+$ clusters represent mixed valence systems, with the total dipole interaction being a weighted sum of the contributions of the interactions between the individual ions (Astashkin et al. 2006; Elsaesser et al. 2005; Elsasser et al. 2002). Therefore, representing the whole cluster by a single point dipole is not appropriate, and the spins of the individual ions and the way they are coupled together have to be considered explicitly. The spin coupling scheme for the [4Fe-4S]$^+$ cluster was described elsewhere (Torres et al. 2003). Briefly, the total spin $S$ of 1/2 results from antiferromagnetic coupling of two pairs of ferromagnetically coupled ions, Fe(II)–Fe(II) ($S = 4$) and Fe(II)–Fe(III) ($S = 9/2$). The spin projection factors that determine the statistical weights of individual dipole
interactions are \(-4/3\) for the ions of the Fe(II)–Fe(II) pair and \(11/6\) for the ions of the Fe(II)–Fe(III) pair (the iron ions in this pair are actually equivalent and have a charge of 2.5). The dipole interaction between two \([4\text{Fe}-4\text{S}]^+\) clusters depends on their relative orientation. To make a simple estimate of the overall range of the possible dipole interaction constants, we will use the fact that the distance between the clusters (\(\sim 30\) Å) is \(\sim 1\) order of magnitude greater than the cluster size (cube side of \(\sim 2.5\) Å) and will write for the dipole interaction constant (Elsaesser et al. 2005; Martin et al. 1998)

\[
D \approx \sum_{i,j} K_i K_j \frac{g_i g_j \beta^2}{R_{ij}^3}
\]

Equation 4

where \(K_i\) and \(K_j\) are the spin projection factors of Fe ions in the different clusters, \(g_i\) and \(g_j\) are their \(g\) values, respectively, \(\beta\) is the Bohr magneton, \(R_{ij}\) is the distance between ions \(i\) and \(j\), and all of the \(\mathbf{R}_{ij}\) vectors are thought to be collinear. Then, with the minimal and maximal \(R_{ij}\) values equal to \(\sim 30\) and \(35\) Å, respectively, one can easily estimate the minimal and maximal possible \(D\) values: \(D_{\text{min}} \approx 0.4\) MHz, and \(D_{\text{max}} \approx 5.2\) MHz. The values close to \(D_{\text{min}}\) are realized for the clusters oriented in such a way that the Fe(II)–Fe(II) pair of one cluster is the closest to the other cluster. The values close to \(D_{\text{max}}\) are realized when Fe(II)–Fe(III) pairs of the clusters are closest to each other (at 30 Å), while their Fe(II)–Fe(II) pairs are at a maximal separation (35 Å).

The observed ELDOR oscillation frequency of \(\sim 0.7\) MHz is closer to the lower limit of the estimated range of dipole interactions, which indicates that the clusters are approximately oriented by the Fe(II)–Fe(II) pairs toward each other. These considerations demonstrate that the interpretation of the observed dipole interaction
between mixed-valence systems in terms of distance requires additional information about the internal electronic structure and relative orientation of the centers.

Conclusions:

In this work, we exploited the pseudo-2-fold symmetry of DSD, a *de novo*-designed dimeric three-helix bundle, to generate a model protein that contains two iron-sulfur clusters in the hydrophobic core. Starting with the primary coordination sphere of a natural [4Fe-4S] cuboidal cluster, we replaced leucines in the three-helix bundle with coordinating cysteines in the appropriate side chain rotamers to satisfy cluster coordination. The substitutions result in the formation of a hydrophilic cavity in the protein core, which is then filled by insertion of the cluster. Our data show that the mutations are structurally conservative, resulting in highly helical dimeric proteins in the apo and holo states. The folding stability of the proteins is found to increase in the following order: DSD-4Cys < DSD-bis[4Fe-4S] < DSD (mirroring the trend in core hydrophobicity and packing). Further, we showed that DSD- bis[4Fe-4S] contains two intact cubane iron-sulfur clusters located at the intended distance. EPR and ELDOR data are consistent with the expected distance of 29–34 Å, derived from the model.

The design approach adopted here replaces the hydrophobic core of a scaffold protein with a sterically compatible metal binding center and has been validated in several systems (Calhoun et al. 2005; Chakraborty et al. 2012; Coldren et al. 1997; Cordova et al. 2007; Der et al. 2012; Ghirlanda 2009; Ghirlanda et al. 2004; Grzyb et al. 2010; Salgado et al. 2010; Shiga et al. 2012; Shinde et al. 2012; Zastrow et al. 2012). Helical systems derived from the coiled coil motif have proven to be particularly adept in accommodating metal binding sites, because the interhelix distances in the core are compatible with the typical bond length in metal–ligand complexes. The regular
structure repeat of the coiled coil lends itself to parametrization and thus to computational design; further, the structure repeat is reflected at the sequence level in a motif of seven amino acids that fold in a full helical turn, named the heptad repeat (Crick 1953). Thus, a metal binding site can be easily moved along the longitudinal axis of the scaffold simply by replicating the sequence motif in a modular fashion. Here, we demonstrated the incorporation of those design principles in a model system that contains two clusters at a fixed distance, estimated to be 29–34 Å. Although this distance is not conducive to efficient electron tunneling, the design principles described here could be extended to modulate intercluster distances by repositioning the motif containing the cluster coordinating cysteines along the longitudinal axis of DSD. Further, this procedure may be used to add additional metal binding sites or extended to other three-helix bundle scaffolds.
Supporting Information:

Figure 72 – a.) Models of DSD-4Cys, b.) DSD-bis[4Fe4s], c.) and X-ray structure of DSD; the pockets existing in each structure are visualized as mesh. Pocket volumes were calculated using Pocket-finder (Laurie and Jackson 2005) as described in Methods.
Figure 73 – Analytical HPLC trace of pure DSD–4Cys. Red line: absorbance at 220nm and black line: absorbance at 280nm

Figure 74 – Analytical ultracentrifugation of DSD–4Cys, Estimated Molecular weight 12.4 kDa.
Figure 75 – Size exclusion chromatography of DSD–4Cys and DSD–Bis[4Fe-4S].

Figure 76 – Cyclic Voltametry of DSD_Bis[4Fe-4S], Black Trace: Raw CV data(left Y axis), Red Trace: Baseline subtracted data (right Y axis)
The \([4\text{Fe}4\text{S}]^+\) clusters represent mixed valence systems, with the total dipole interaction being a weighted sum of the contributions of the interactions between the individual ions. The total spin \(S = 1/2\) results from antiferromagnetic coupling of two pairs of ferromagnetically-coupled ions, \(\text{Fe(II)}–\text{Fe(II)} (S = 4)\) and \(\text{Fe(II)}–\text{Fe(III)} (S = 4)\).
9/2). The spin projection factors that determine the statistical weights of individual dipole interactions are -4/3 for the ions of Fe(II)–Fe(II) pair and 11/6 for the ions of Fe(II)–Fe(III) pair (the iron ions in this pair are actually equivalent and have the charge of 2.5) (Elsasser et al. 2002; Torres et al. 2003).

Cyclic voltammetry

The reduction potential of [4Fe-4S]²⁺/ [4Fe-4S]³⁺ in DSD-bis[4Fe4S] was determined by cyclic voltammetry using a pyrolytic graphite as working electrode, Pt mesh as counter electrode and a Ag/AgCl as reference electrode. No signal was observed from a solution of DSD–Bis[4Fe-4S] cluster in 100 mM NaCl, 100 mM Tris, pH 8.5. However, in presence of 4 mM neomycin as a stabilizer, a cathodic wave was observed at -478mV vs SHE. No anodic wave was observed indicating electrochemical irreversibility of the process under these conditions.

MALDI sample preparation:

Cluster incorporation was carried out as described in the main text. After overnight incubation, the reaction mixture was passed through a G-25, PD10 desalting column pre-equilibrated with 100 mM NH₄HCO₃, pH=7.5. Appropriate fractions were collected and used to prepare MALDI samples anaerobically. Specifically, 2 μL of protein sample were mixed 2 μL of matrix stock (alpha-Cyano-4-hydroxycinnamic acid in 0.1% v/v TFA in water) and dried on a sample plate in the glove box. The plate was transferred in a argon-filled small chamber and transferred out of the glove box for MALDI characterization. MALDI-TOF (Matrix Assisted Laser Desorption Ionization-Time of Flight) spectrometry was carried out using an Applied Biosystems DE-STR MALDI-TOF
instrument under linear/positive ionization mode. Expected masses of individual species are listed within Figure 77.
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APPENDIX I

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Chapter 1: Journal Name: Photosynthesis Research


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