Electron Transfer from the A_{1A} and A_{1B} sites to a Tethered Pt Nanoparticle Requires the FeS Clusters for Suppression of the Recombination Channel

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Abstract

In this work, a previously described model of electron withdrawal from the A_{1A}/A_{1B} sites of Photosystem I (PS I) was tested using a dihydrogen-producing PS I-NQ(CH₂)₁₅S-Pt nanoconstruct. According to this model, the rate of electron transfer from A_{1A}/A_{1B} to a tethered Pt nanoparticle is kinetically unfavorable relative to the rate of forward electron transfer to the FeS clusters. Dihydrogen is produced only when an external donor rapidly reduces P_{700}^{+} , thereby suppressing the recombination channel and allowing the electron in the FeS clusters to proceed via uphill electron transfer through the A_{1A}/A_{1B} quinones to the Pt nanoparticle. We tested this model by sequentially removing the FeS clusters, F_B , F_A , and F_X , and determining the concentration of cytochrome c_6 (Cyt c_6) at which the backreaction was outcompeted and dihydrogen production was observed. P_{700} - F_A cores were generated in a *menB* insertionally inactivated strain by removing F_B with HgCl₂; P_{700} - F_X cores were generated in a *menB psaC* insertionally inactivated strain that lacks F_A and F_B , and P_{700} - A_1 cores were generated in a *menB rubA* insertionally inactivated strain that lacks F_X , F_A and F_B . Quinone incorporation was measured using transient electron paramagnetic resonance spectroscopy and time resolved optical spectroscopy. Cyt c_6 was titrated into each of these PS I preparations and the kinetics of P_{700}^{+} reduction were measured. A similar experiment was carried out on PS I-NQ(CH₂)₁₅S-Pt nanoconstructs assembled from these PS I preparations. This study showed that the concentration of Cyt c_6 needed to produce dihydrogen was comparable to that needed to suppress the backreaction. We conclude that the FeS clusters serve to ‘park’ the electron and thereby extend the duration of the charge-separated state; however, in doing so, the redox advantage of removing the electron at A_{1A}/A_{1B} is lost.

1. Introduction

The Photosystem I (PS I) reaction center is a multi-subunit, pigment-protein complex found in plants and cyanobacteria that, upon illumination, generates and maintains a long-lived charge-separated state (1, 2). Its core consists of a heterodimer of the PsaA and PsaB proteins in which the electron transfer cofactors are arranged in two pseudo-C2 symmetric functionally active branches denoted A and B (3-5), spanning the width of the thylakoid membrane (6). The ~100 chlorophylls that form the antenna system in cyanobacterial PS I serve to absorb light and transfer energy to the primary donor (P_{700}). The kinetics and pathway of the initial charge separation are not firmly established and several different models have been proposed recently (7-9). However, in all of them charge separation between P_{700} , a chlorophyll (Chl) a'/a special pair (Chl a' is the 13² epimer of Chl a) and either of the chlorophyll acceptors A_{0A} or A_{0B} is complete within a few ps or less. The state $P_{700}^+A_0^-$ is stable for only ~20 ns (10) and to increase the lifetime of the charge separation, the electron is transferred along a chain of cofactors that includes phylloquinone (A_{1A}/A_{1B}) and three [4Fe-4S] clusters (F_X , F_A , and F_B). The latter two clusters are bound to the PsaC subunit that is located on the stromal side of the membrane. The final charge-separated state, $P_{700}^+F_B^-$, is stable for ~65 ms, which is sufficient time for the electron to be transferred to a soluble [2Fe-2S] ferredoxin (6, 11, 12). **Figure 1** provides a comprehensive view of the electron transfer kinetics in PS I.

PS I has a number of properties that make it a promising candidate for use as a photosensitizer in the production of solar biofuels. Not only does it have a high quantum yield of charge separation, estimated to be >0.98 (13), but it is exceptionally stable to high light and high temperatures. Additionally, the midpoint potential of the final

acceptor, F_B , is sufficiently negative to reduce protons to dihydrogen in the presence of a suitable catalyst (1).

Greenbaum and colleagues were the first to exploit this capability by depositing Ru, Os, and Pt catalysts on thylakoid membranes and on PS I particles and observing dihydrogen production on exposure to light (14-20). More recently, a number of groups have successfully coupled both enzymatic and inorganic catalysts to PS I for dihydrogen production (21-31). All of these attempts focused on obtaining the electron from the reduced F_B cluster at the terminus of the transfer chain. However, Gibbs free energy is lost as the electron progresses from A_0 to F_B (**Figure 1**); hence, if the electron could be extracted earlier in the electron transfer chain, it could be obtained at a more reducing potential. For example, direct electron transfer from the A_{1B} quinone would allow the electron to be available at a midpoint potential of -845 mV (5).

Terasaki and coworkers were the first to pioneer a system of electron withdrawal from the A_1 sites for use in a biophotosensor (32). In their work, PS I was treated with diethyl ether to remove phyloquinone from the A_{1A} and A_{1B} sites, which were then reconstituted with a synthesized naphthoquinone derivative. However, chemical extraction removes all of the carotenoids and most of the Chls (33-39), which leads to a decrease in the optical cross-section for photon capture. Additionally, the lack of carotenoids renders the reaction center susceptible to oxidative damage. Thus, we attempted to reconstitute the A_{1A} and A_{1B} sites in a larger and more robust system containing all of the carotenoid and Chl molecules by using the *menB* strain of PS I.

The *menB* gene encodes 1,4-dihydroxy-2-naphthoyl-CoA synthetase, a necessary enzyme in the phyloquinone biosynthesis pathway (40, 41). When the *menB* gene is

inactivated, phyloquinone is not synthesized, and the A_{1A} and A_{1B} sites are occupied by plastoquinone-9 (PQ-9) (42-46), a mobile electron carrier that shuttles electrons from Photosystem II to the cytochrome *b₆f* complex and transports protons from the stroma to the lumen. This substituted benzoquinone binds loosely to the A_{1A} and A_{1B} sites of PS I and can be readily displaced with a variety of substituted naphthoquinones (45, 47, 48). We took advantage of this ability to displace PQ-9, to introduce a menaquinone with a head group identical to that of the native phyloquinone, but with a carbon chain long enough to reach the surface of the protein. When a Pt nanoparticle is bound to the thiolated end of the substituted menaquinone and the resulting PS I-NQ(CH₂)₁₅S-Pt nanoconstruct is illuminated, it evolves dihydrogen in the presence of a sacrificial electron donor (31).

In a previous publication (31) we proposed a hypothesis for how this system might function. In the model proposed, the rate of electron transfer to the attached nanoparticle is kinetically unfavorable relative to the rate of forward electron transfer from A_{1A}⁻ and A_{1B}⁻ to the FeS clusters (20 ns and 200 ns) and to the rate of charge recombination from the FeS⁻ clusters to P₇₀₀⁺ (~65 ns). Under these conditions, the electron in the FeS clusters ultimately recombines with P₇₀₀⁺, and no dihydrogen is produced. However, if charge recombination is suppressed by reducing P₇₀₀⁺ rapidly with an external donor, the electron is transferred instead to the Pt nanoparticle. In essence, the FeS clusters serve to ‘park’ the electron, thereby extending the lifetime of the charge-separated state and making it possible for an external donor to reduce P₇₀₀⁺. The key to dihydrogen production, then, is the suppression of the charge recombination channel.

If this model is correct, then faster charge recombination times from earlier acceptors should require higher concentrations of Cyt c_6 to produce dihydrogen. In this work, we test the proposed model by sequentially removing the FeS clusters F_B (P_{700} - F_A cores), F_A/F_B (P_{700} - F_X cores), and F_X , F_A/F_B (P_{700} - A_1 cores) from PS I and measuring the backreaction kinetics and dihydrogen production as a function of Cyt c_6 concentration for each sample.

2. Materials and Methods

2.1 Cell growth and PS I purification

Strains used for the purification of PS I include the wild type (WT) strain, the *menB* insertionally inactivated strain of *Synechocystis sp.* PCC 6803 (hereafter *menB* strain), and a *menB rubA* insertionally inactivated strain of *Synechococcus sp.* PCC 7002 (hereafter *menB rubA* strain). The *Synechocystis sp.* PCC 6803 *menB* gene was disrupted with the *aadA* gene conferring spectinomycin resistance. The *Synechococcus sp.* PCC 7002 *menB rubA* strain was created by the insertional inactivation of *menB* and *rubA* by antibiotic resistance genes for gentamicin resistance (*aacC1*) and for kanamycin resistance (*aphII*), respectively. These strains were grown and PS I was purified as described previously (42, 49, 50). Cells were harvested by centrifugation, broken by three passes through an M-110EH-30 microfluidizer processor (Microfluidics). Cell debris was removed via low speed centrifugation ($2000 \times g$) and the resulting supernatant was centrifuged at $158,000 \times g$ for 1 h to pellet the thylakoid membranes. Membranes were solubilized with 1% (w/v) β -dodecylmaltoside (β -DM) for 1 h at 4°C in the dark. The PS I trimers were purified on a 5 to 20% sucrose density gradient in 50 mM Tris-HCl buffer (pH 8.3), 0.05% (w/v) β -DM, subsequently pelleted, and suspended in 50 mM Tris-HCl buffer (pH 8.3) containing 20% (w/v) glycerol and 0.05% (w/v) β -DM. The *menB rubA* strain did not produce PS I trimers; hence, PS I monomers were collected from the first gradient, dialyzed, and the contaminating PS II was removed using a DEAE-sepharose anion exchange column (Sigma) with 50 mM Tris-HCl buffer (pH 8.0), 0.05% β -DM. PS I monomers were eluted at 75-100 mM NaCl and PS II was eluted at 200 mM NaCl. The

purity of the fractions was confirmed by fluorescence emission spectroscopy at 77 K (data not shown).

The *menB psaC* deletion strain of *Synechococcus* sp. PCC 7002 (hereafter *menB psaC* strain) was constructed by knock-out with antibiotic resistance genes for gentamicin (*aacCI*) and kanamycin (*aphII*) into the *psaC* and *menB* genes, respectively, using a PS I-less background strain as described previously (51-53) (see supplemental information for details). Cells were grown in A⁺ media, supplemented with gentamicin, kanamycin, and 0.1% (w/v) glycerol, under low light conditions (12 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Cells were harvested and broken with three passes through an M-110EH-30 microfluidizer processor (Microfluidics). Cell debris was removed via low speed centrifugation (2000 $\times g$) and the resulting supernatant was spun at 158,000 $\times g$ for 1 h to pellet the thylakoid membranes. The membranes were solubilized with 1% (w/v) β -DM for 1 h at 4°C in the dark, and the resulting solution was partly purified over a 5-20% sucrose density gradient in 50 mM Tris-HCl buffer (pH 8.3), 0.05% (w/v) β -DM. Because no PS I trimers were present, PS I monomers were collected, dialyzed, and the contaminating PS II was removed using a DEAE-sepharose anion exchange column (Sigma) with 50 mM Tris-HCl buffer (pH 8.0), 0.05% β -DM. PS I monomers were eluted at 75-100 mM NaCl and PS II was eluted at 200 mM NaCl. The purity of the fractions was confirmed by fluorescence emission spectroscopy at 77 K (data not shown).

2.2 Removal of the F_B Cluster

F_B was removed by incubation of PS I with HgCl_2 under conditions similar to those of a previously described method (54-59). A 0.8 mg/mL Chl solution of *menB* PS I

in 50 mM Tris-HCl buffer (pH 8.3) containing 0.05% (w/v) β -DM was incubated with 1 mM HgCl_2 for 24 h at 4°C. The residual HgCl_2 was removed by ultrafiltration using a 50 kDa cutoff membrane (Amicon) in the dark at 4°C. The buffer, 50 mM Tris-HCl (pH 8.3), was exchanged after two, three, and 14 h. The final concentrate was suspended in 50 mM Tris-HCl buffer (pH 8.3) 0.05% (w/v) β -DM, 20% (w/v) glycerol, and stored at –80°C until used.

2.3 Synthesis of 1-[15-(3-methyl-1,4-naphthoquinone-2-yl)]pentadecyl disulfide

1-[15-(3-methyl-1,4-naphthoquinone-2-yl)]pentadecyl disulfide, $(\text{NQ}(\text{CH}_2)_{15}\text{S})_2$, was synthesized and purified in as previously described (31, 32). Briefly, menadione was reacted for 2 h with ω -bromohexadecanoic acid, silver nitrate and ammonium persulfate. The resulting product was reacted with thiourea for 24 h, and then with tetraethylenepentamine for an additional 24 h. The final product was characterized by 400 MHz ^1H -NMR, Fourier transform infrared spectroscopy, and mass spectrometry. More complete details can be found in the supplemental information.

2.4 Synthesis of Pt Nanoparticles

Pt nanoparticles were synthesized as described previously (60). One milliliter of 23.7 mM mercaptosuccinic acid was added to 10 mL of 3.38 mM hexachloroplatinic acid and stirred vigorously. The reaction was initiated by adding 5 mL of 67.6 mM sodium borohydride to the solution. The reaction was considered complete when the solution turned dark brown. The resulting particles were characterized via transmission electron microscopy and found to be ~5 nm in diameter (data not shown).

2.5 Quinone Incorporation

Incorporation of various quinones into PS I complexes from the various *menB* strains was carried out as described previously (31, 43). A 100-fold excess of the target quinone was added to a solution of PS I for 16-24 h. Excess quinone was removed by washing twice with 50 mM Tris-HCl (pH 8.3), containing 0.05% (w/v) β -DM using a 50 kDa ultrafiltration membrane (Amicon).

2.6 Time-Resolved Optical Studies at 830 nm

Electron transfer kinetics in the PS I complexes were monitored by transient absorbance changes at 830 nm following a single-turnover laser flash at 532 nm using a laboratory-built, dual-beam spectrometer as described previously (61). The measuring beam was provided by a 830 nm laser diode (Crystalaser, model number DL830-100-O). Samples were placed in a 10 mm $\times$ 4 mm quartz cuvette which was oriented such that the optical path length was 10 mm. The measuring and reference beam intensities were balanced using a variable density optical filter wheel. The difference signal was amplified with a differential amplifier (Thor Labs PDB460A) and processed using a 12-bit, 200 MS/s, NI-5124 PCI card (National Instruments). The samples contained 50 μ g/mL Chl, Tris-HCl (pH 8.3), 10 μ M 2,6-dichlorophenolindophenol (DCPIP), 5 mM sodium ascorbate, and 0.05% (w/v) β -DM.

2.7 CW X-Band EPR of $HgCl_2$ Treated Samples

CW X-band measurements were carried out was using an ELEXSYS EPR spectrometer equipped with a Super X CW-EPR microwave bridge and a high Q

resonator (Bruker BioSpin Corp, Billerica, MA). The sample was cooled using an ESR 900 cryostat (Oxford Instruments, Austin, TX). Excitation was provided by a Coherent Verdi-V5 laser (500 mW at 532 nm) passed through a 3 X beam expander (Oriel Instruments, Irvine, CA). Samples were measured at a concentration of 0.8 mg/mL Chl PS I and treated with 10 μ M DCPIP and 5 mM ascorbate, dark adapted for 45 minutes, and frozen in the dark before analysis.

2.8 Transient EPR Spectroscopy.

Time/field transient EPR data sets were measured using a modified ESP 300E spectrometer equipped with an ER 042 MRH X-band microwave bridge and a Flexline ER 4118 X-MD-5W1 dielectric resonator (Bruker BioSpin Corp., Billerica, MS). A Vibrant 355II laser (Opotek, Inc., Carlsbad, CA) running at 10 Hz and a wavelength of 532 nm provided single-turnover flashes at 20 mJ per pulse. The temperature was controlled using a CF935 continuous flow cryostat (Oxford Instruments, Austin, TX). The transient EPR signal was collected in direct detection mode with a home-built broadband amplifier (bandwidth >500 MHz), and digitized using a 500 MHz bandwidth, 8-bit, 2GS/s, Model CS85G PCI card (GaGe Instruments). The software for data acquisition was written in-house using LabView (National Instruments). Samples were pretreated with 1 mM sodium ascorbate and adapted to the dark for 45 minutes followed by freezing in darkness with liquid nitrogen. The data was analyzed using IGOR (Wavemetrics, Portland, OR).

2.9 Nanoconstruct Assembly and Dihydrogen Analysis.

The NQ(CH₂)₁₅S–Pt adduct was constructed as described previously (31). Briefly, 2 mL of ~0.2 μ M Pt nanoparticles were incubated with 100 μ L of a 20 mM solution of (NQ(CH₂)₁₅S)₂ in the dark vigorously stirring the mixture for ~7 days. The nanoconstruct was assembled by 16–24 h incubation of 75 μ L of the NQ(CH₂)₁₅S–Pt adduct with PS I at a final concentration of 50 μ g/mL Chl (6.5 μ M P₇₀₀), in 50 mM Tris-HCl buffer (pH 8.3) containing 0.05% (w/v) β -DM to a final volume of 250 μ L. After assembly, soluble electron donors were added in the dark to final concentrations of 10 μ M DCPIP, 5 mM ascorbate, and up to 200 μ M Cyt *c*₆. Prior to illumination, the headspace of the assembled nanoconstruct was purged in the dark for 10 min using ultrapure argon. The samples were exposed to continuous illumination using a 100 W xenon arc lamp (996 μ mol of photons m⁻² s⁻¹). The temperature was maintained using a clear polycarbonate flask filled with doubly distilled H₂O to remove infrared radiation. A total of 200 μ L of headspace was removed at regular intervals with an airtight locking syringe and analyzed with a Shimadzu GC-8A gas chromatograph equipped with a ShinCarbon 80/100 column (2 m $\times$ 2 mm) and thermal conductivity detector (detector current of 100 mA) with ultrapure N₂ as the carrier gas (flow rate of 0.75 mL/min). Samples that produced no dihydrogen were measured over the course of 48 h of illumination.

3. Results

3.1 CW EPR Studies of Hg-Treated PS I

The terminal FeS cluster, F_B , has been shown to be quantitatively and selectively removed from PS I by treatment with $HgCl_2$ (54, 55, 57-59). While the mechanism is not fully understood, it is thought that Hg interacts with the sulfur bonds, causing the $[4Fe-4S]$ cluster to disintegrate. The efficiency of cluster removal can be assessed using low-temperature CW EPR spectroscopy, wherein illumination of a frozen PS I sample promotes a single electron from P_{700} to either the F_A or F_B cluster. In wild type PS I, F_A and F_B are reduced according to their relative redox potentials, and resonances associated with F_A and F_B occur in a $\sim 4:1$ ratio. In the case in which F_B is missing or inactive, only the rhombic resonances associated with F_A^- would be expected.

The CW EPR data on Hg-treated *menB* PS I complexes are shown in **Figure 2**. In the *menB* strain (top), resonances associated with F_A (2.05, 1.95, and 1.86) and F_B (2.07, 1.93, 1.88) are visible (62). However, upon treatment with $HgCl_2$ (bottom), the F_B resonances disappear and only resonances from F_A are observed. Additionally, one sees a shift in the g -value for the high field trough of F_A from 1.86 in the control PS I complexes to 1.84 in the Hg-treated preparation consistent with published results (54). Both of these observations indicate that F_B is missing in the Hg-treated PS I complexes.

3.2 Transient EPR Spectroscopy

Transient EPR (TrEPR) spectroscopy at low temperatures has been used extensively to study the light-induced radical pair $P_{700}^{\bullet+} A_{1A}^{\bullet-}$ in PS I (references (63, 64) provide reviews). The TrEPR spectrum of $P_{700}^{\bullet+} A_{1A}^{\bullet-}$ is spin-polarized and is sensitive to

the orientation of the quinone in the binding site, the chemical structure of the quinone and the nature of its binding to the protein. **Figure 3** shows the X-band TrEPR spectra of PS I complexes from the WT, the *menB psaC* strain (P₇₀₀-F_X cores), and from the *menB rubA* strain (P₇₀₀-A₁ cores). **Figure 3A** shows a comparison of the WT and the P₇₀₀-F_X cores before and after addition of (NQ(CH₂)₁₅S)₂. The corresponding comparison for the P₇₀₀-A₁ cores is shown in **Figure 3B**. There are clear differences in the spectral features before and after addition of the substituted naphthoquinone (dotted and dashed spectra, respectively). The differences in the spectra arise primarily from the different hyperfine couplings in PQ-9 and menaquinone. The two methyl groups associated with the plastoquinone head group have substantial hyperfine interactions, and these overlapping splittings give rise to a broad/featureless spectrum (43, 65). This characteristic spectrum is seen in both the P₇₀₀-F_X cores and the P₇₀₀-A₁ cores prior to (NQ(CH₂)₁₅S)₂ incorporation. However, when a molecule containing a menaquinone head group occupies the binding site, the single methyl group leads to partially resolved hyperfine couplings, most visible as the shoulder on the low-field absorptive feature. The partial delocalization of the electron over two rings of the naphthoquinone head group instead of one ring of the plastoquinone head group, leads to a smaller *g*-anisotropy (66), which also contributes to the spectral differences. However, at X-band this effect is relatively minor. The spectra taken following addition of the quinone are very similar to that of the wild type (solid spectrum) indicating that in both cases the quinone has been successfully incorporated into the A_{1A} (and likely A_{1B}) site in both the P₇₀₀-F_X cores and P₇₀₀-A₁ cores.

In addition to the spectrum of P₇₀₀^{•+}A_{1A}^{•-} a contribution from ³P₇₀₀ is also observed for the P₇₀₀-F_X cores and P₇₀₀-A₁ cores when the spectrum is collected over a

wider field range (data not shown). The triplet background has been removed from the spectra in **Figure 3** by making a linear baseline correction. The spin polarization pattern of the $^3\text{P}_{700}$ spectrum indicates that it is formed by charge recombination and it remains present following incubation of menaquinone into the $\text{P}_{700}\text{-F}_\text{X}$ and $\text{P}_{700}\text{-A}_1$ cores. Thus, we conclude that a fraction of the quinone binding sites in these cores are unable to bind either PQ-9 or menaquinone. However, it is not possible to reliably quantify the size of this fraction from TrEPR data.

3.3 Time Resolved Optical Spectroscopy

Time-resolved optical spectroscopy at 830 nm can be used to monitor the rate of P_{700}^+ reduction either from the electron acceptors or from an external electron donor. At low concentrations of external electron donors, the experiment assesses the charge recombination kinetics from the electron acceptor chain. At sufficiently high concentrations of an external electron donor, the experiment assesses forward electron transfer kinetics from the external donor. We discuss each of the PS I preparations in turn below.

3.3.1 $\text{P}_{700}\text{-F}_\text{A}$ cores from Hg-Treated PS I

Wild type PS I shows the canonical ~65 ms backreaction after a saturating flash, characteristic of charge recombination from the terminal $\text{F}_\text{A}/\text{F}_\text{B}$ cluster(s) (**Figure 4A**). PS I from the *menB* strain, which contains PQ-9 in the $\text{A}_{1\text{A}}$ and $\text{A}_{1\text{B}}$ sites, shows a characteristic 3-4 ms backreaction after a saturating flash (45) (**Figure 4B**). After incubation with $(\text{NQ}(\text{CH}_2)_{15}\text{S})_2$, the 3-4 ms phase is nearly quantitatively replaced with a

~50 ms wild type-like backreaction (31). PS I complexes from the *menB* strain that had been treated with HgCl₂ show a ~2 ms backreaction after a saturating flash (**Figure 4C**) reflecting the absence of the terminal F_B cluster, and addition of (NQ(CH₂)₁₅S)₂ results in a longer kinetic phase with a lifetime of ~25 ms (**Figure 4D**), which shows that plastoquinone-9 can be displaced by the substituted menaquinone. Some fast phase remains, however, which could be due to non-displaced PQ-9 or to PS I cores in which F_A is additionally damaged, the latter resulting in P₇₀₀-F_X cores that show the characteristic ~1-2 ms backreaction. In this sample, we estimate ~50% substituted menaquinone incorporation into the A_{1A} and A_{1B} sites.

The attempted suppression of the P₇₀₀⁺F_A⁻ backreaction in the Hg-treated PS I sample with Cyt *c*₆ is shown in **Figure 5A**. After addition of (NQ(CH₂)₁₅S)₂, suppression of the backreaction occurs only at concentrations of Cyt *c*₆ that exceed 10 μM. This is a higher concentration than the ~1 μM of Cyt *c*₆ needed to suppress the P₇₀₀⁺[F_A/F_B]⁻ backreaction in WT PS I.

3.3.2 P₇₀₀-F_X cores from the *menB* *psaC* strain

The charge recombination kinetics in P₇₀₀⁺F_X⁻ cores from the *menB* *psaC* strain are depicted in **Figure 4E**. Two kinetic phases are present with lifetimes of ~50 μs and 300 μs. A more thorough interpretation of these phases will be forthcoming in a separate article, but for the purpose of this study, we assume that both are due to charge recombination from A_{1A}⁻ and A_{1B}⁻. After addition of (NQ(CH₂)₁₅S)₂, two new phases appear, with lifetimes of 1.4 ms, and ~2 s (**Figure 4F**). Because a menaquinone occupies the A_{1A} and A_{1B} sites, the 1 ms phase is most likely charge recombination from F_X⁻,

while the 2 s phase is forward electron transfer to P_{700}^{+} due to electron loss to dioxygen from the surface-exposed F_X cluster. A small amount of the fast kinetic phase is still present, indicating that either some of the PQ-9 has not been displaced or the sites are unable to accept the substituted menaquinone. Either way, we estimate ~25% naphthoquinone incorporation into the A_{1A} and A_{1B} sites.

The attempted suppression of the $P_{700}^{+}F_X^{-}$ backreaction in PS I from the *rubA psaC* strain with Cyt c_6 is shown in **Figure 5B**. After addition of $(NQ(CH_2)_{15}S)_2$, suppression of the backreaction occurs only at concentrations of Cyt c_6 exceeding 200 μ M. This is a much higher concentration than the ~10 μ M of Cyt c_6 needed to suppress the $P_{700}^{+}F_A^{-}$ backreaction in the Hg-treated PS I sample lacking F_B .

3.3.3 P_{700} - A_I cores from the *menB rubA* strain

The charge recombination kinetics in $P_{700}^{+}A_I^{-}$ cores from the *menB rubA* strain are shown in **Figure 4G**. In this preparation, charge recombination is biphasic, with lifetimes of 60 μ s and 750 μ s. On addition of $(NQ(CH_2)_{15}S)_2$, the charge recombination kinetics become significantly faster, with lifetimes of 15 μ s and 200 μ s. Titration of the $P_{700}^{+}A_I^{-}$ cores with Cyt c_6 is shown in **Figure 5C**. Even the highest concentrations of Cyt c_6 that we were able to attain, 300 μ M, were unable to outcompete the 15 μ s and 200 μ s backreactions from A_{1A}^{-}/A_{1B}^{-} .

3.4 Dihydrogen Production in the Various PS I Core Preparations

Table 1 shows the rate of dihydrogen production attained in PS I- $NQ(CH_2)_{15}S$ -Pt nanoconstructs prepared from the various PS I cores. In the P_{700} - F_A cores, the onset of

dihydrogen production occurs at 10 μM Cyt c_6 , and the rate increases until a maximum of $0.92 \pm 0.15 \mu\text{mol H}_2 \cdot \text{mg chl}^{-1} \cdot \text{h}^{-1}$ is reached at 200 μM Cyt c_6 . The $\text{P}_{700}\text{-F}_\text{X}$ cores require a significantly higher concentration of Cyt c_6 before any dihydrogen is observed. Only at 200 μM Cyt c_6 was dihydrogen observed, and the rate was $0.11 \pm 0.04 \mu\text{mol H}_2 \cdot \text{mg chl}^{-1} \cdot \text{h}^{-1}$. No dihydrogen was observed in $\text{P}_{700}\text{-A}_1$ cores at any concentration of Cyt c_6 . Additionally, no dihydrogen was observed in control samples that were (i) not illuminated; (ii) lacking soluble electron donors; (iii) lacking the quinone wire; (iv) lacking the Pt nanoparticle; and (vi) when phyloquinone occupies the $\text{A}_{1\text{A}}$ and $\text{A}_{1\text{B}}$ sites.

4. Discussion

Electron transfer among the electron acceptors of PS I is best described by a quasi-equilibrium model (67, 68). The salient feature of this model is that at each step, the rate of forward electron transfer is considerably larger (by several orders of magnitude) than the rate of charge recombination. When quasi-equilibrium is achieved (at about 1 μ s), the electron has a finite probability of residing on each electron transfer cofactor according to its equilibrium constant, which is determined by the change in Gibbs free energy relative to its donor and acceptor pairs. Charge recombination with P_{700}^+ occurs via thermal repopulation from (most likely) the A_{1A}/A_{1B} sites rather than by direct electron transfer to P_{700}^+ from the reduced FeS clusters.

A general diagram for the proposed electron transfer routes in the PS I-NQ(CH₂)₁₅S-Pt nanoconstruct is depicted in **Scheme 1**, where $k_{1/-1}$ is electron transfer to/from the FeS clusters, $k_{2/-2}$ is electron transfer to/from the Pt nanoparticle, k_r is the charge recombination channel, and k_f is the reduction of P_{700}^+ by an external donor. In a previous publication (31), we proposed that k_2 is slower than both k_r and k_1 , and only by eliminating k_r can the electron proceed to the Pt nanoparticle. This was demonstrated by a correlation between the concentration of Cyt c_6 , the rate of P_{700}^+ reduction, and the onset of dihydrogen production. It was shown that dihydrogen was only generated at Cyt c_6 concentrations that reduced P_{700}^+ faster than charge recombination from the $[F_A/F_B]^-$ clusters, *i.e.*, when the backreaction is suppressed by making $k_f > k_r$. It follows that if the terminal acceptors were sequentially removed, this would result in a condition in which k_r is increased, and k_f would also necessarily need to increase for any electron transfer to occur to the Pt nanoparticle.

In the P₇₀₀-F_A core produced by Hg-treatment of the *menB* strain, the absence of the F_B cluster causes the charge recombination time to decrease from ~65 ms in the control to ~20 ms in the treated sample containing (NQ(CH₂)₁₅S)₂. The increase in k_r is not difficult to overcome using reasonable concentrations of Cyt c_6 (10 μ M), which satisfies the condition in which $k_f > k_r$, resulting in suppression of the backreaction. It is only when these conditions are satisfied that the production of dihydrogen occurs. However, suppression of k_r becomes significantly more difficult as additional electron acceptors are removed. The P₇₀₀ F_X-core protein provides an insight into the limits of the conditions in which the backreaction can be out competed with an external donor. In this sample, the rate of charge recombination is increased ~60-fold relative to the WT and only by increasing the concentration of Cyt c_6 by a factor of 20 is $k_f > k_r$. The effect of increasing k_r becomes most apparent in the P₇₀₀A₁-core. Here, k_r has become sufficiently large that the relatively slow diffusion-based chemistry of Cyt c_6 donation cannot result in a condition in which $k_f > k_r$. We therefore conclude that electron extraction from the A₁ sites is contingent on suppression of the charge recombination channel. For forward donation from Cyt c_6 to be able to reduce P₇₀₀⁺, the FeS clusters are required to lengthen the charge-separated state to the ms time range.

The question arises as to the reduction potential of the electron when withdrawn from the A_{1A}/A_{1B} sites in these various PS I core preparations. According to the Nernst Equation:

$$E = E^o + \left(\frac{RT}{nF}\right) \ln([Q_r]/[Q_o]) \quad (1)$$

where E is the potential of the electron, $[Q_r]$ is the concentration of the reduced species and $[Q_o]$ is the concentration of the oxidized species.

If we assume quasi-equilibrium between cofactors, the relative concentration of reduced components is a function of the equilibrium constant between each donor/acceptor pair (67, 68):

$$[P^*] \approx \frac{1}{Z}, [A_0^-] \approx \frac{K_{PA_0}}{Z}, [A_1^-] \approx \frac{K_{PA_1}}{Z}, [F_X^-] \approx \frac{K_{PF_X}}{Z}, [F_A^-] \approx \frac{K_{PFA}}{Z}, [F_B^-] \approx \frac{K_{PFB}}{Z}$$

$$Z \approx 1 + K_{PA_0} + K_{PA_1} + K_{PF_X} + K_{PFA} + K_{PFB} \quad (2)$$

where K_{PA_0} is the equilibrium constant between P^* and A_0 , K_{PA_1} is the equilibrium constant between P^* and A_1 , etc. Expanding these terms results in the following:

$$K_{PA_1} \approx K_{PA_0} K_{PA_1}$$

$$K_{PFB} \approx K_{PA_0} K_{PA_1} \dots K_{PFB} \quad (3)$$

If K_{XY} is expressed in terms of the free energy difference, $\Delta G_{XY} = -RT \ln(K_{XY})$, and substituted into equation (2):

$$[A_0^-] \approx \frac{e^{\left[\frac{-\Delta G_{PA_0}}{RT}\right]}}{Z}$$

$$[A_1^-] \approx e^{\left[\frac{-\Delta G_{PA_0} + -\Delta G_{PA_1}}{RT}\right]} / Z \dots$$

$$[F_B^-] \approx e^{\left[\frac{-\Delta G_{PA_0} + -\Delta G_{PA_1} + -\Delta G_{PF_X} + -\Delta G_{PFA} + -\Delta G_{PFB}}{RT}\right]} / Z \quad (4)$$

Using the accepted values (67, 68) of $\Delta G_{PA_0} = -300$ mV, $\Delta G_{A_0A_1} = -250$ mV, $\Delta G_{A_1FX} = -100$ mV, and $\Delta G_{FXFA} = -105$ mV, it follows that at quasi-equilibrium, the concentration of reduced species heavily favors the terminal acceptors. This results in a situation such that for the F_A and F_X cores, respectively:

$$[F_A^-] \gg [F_X^-] > [A_1^-] > [A_0^-]$$

$$[F_X^-] \gg [A_1^-] > [A_0^-]$$

By evaluating the Nernst equation for each cofactor, we are left with a generalized form:

$$E_{tot} = \sum_{Q=P^*}^{F_B} E_Q^o + \left(\frac{RT}{nF}\right) \ln(K_{PQ}/Z), \quad (5)$$

Where E_{tot} is the total redox potential of the electron, E_Q^o is the redox potential of a given cofactor Q , and K_{PQ} is the equilibrium constant between P_{700} and a cofactor Q . Substitution of equation (3) yields

$$E_{tot} = \sum_{Q=P^*}^{F_B} E_Q^o + \left(\frac{RT}{nF}\right) \ln[Q^-] \quad (6)$$

In the case of the F_A core sample, the contribution from the previous acceptors is negligible, and the resulting equation simplifies to:

$$E_{tot} \approx E_{F_A}^o + \left(\frac{RT}{nF}\right) \ln[F_A^-] \quad (7)$$

The same holds true for the F_X core sample wherein:

$$E_{tot} \approx E_{F_X}^o + \left(\frac{RT}{nF}\right) \ln[F_X^-] \quad (8)$$

Thus, in the case of the P_{700} - F_A core protein, the electron has the redox potential of the F_A cluster (~ -520 mV), and in the F_X core protein, the electron has the redox potential of the F_X cluster (~ -700 mV). Therefore, although the FeS clusters serve to extend the lifetime of the charge-separated state, in doing so, the redox advantage of removing the electron at A_{1A}/A_{1B} is lost. The key to obtaining the electron at a lower reduction potential than the FeS clusters lies in finding a fast electron donor to P_{700}^+ that can suppress the faster recombination reaction in the A_{1A}/A_{1B} core preparations.

5. Conclusion

In this study we have provided evidence that suppression of the charge recombination channel is required for electron transfer from A_{1A}/A_{1B} to a tethered Pt nanoparticle that would otherwise be unfavorable due to kinetic constraints. The key is retention of one or more FeS clusters to increase the time of charge separation such that an external electron donor can outcompete the recombination reaction. However, the electron is extracted from A_{1A} and A_{1B} at the potential of the terminal FeS cluster. The description used here is not specific to PS I and applies to any system in which the backreaction takes place via the extended equilibrium mechanism. The results show that although extracting the electron from a low potential acceptor close to the donor can become feasible by extending the lifetime of the charge-separated state using acceptors further from the donor, this comes at the expense of reduction potential. Thus, if a system is to be designed to generate an electron at the low potential of the quinone in the A_{1B} site (~ -845 mV) the electron must be extracted directly from this cofactor by blocking the fast charge recombination with P_{700}^+ (~ 10 μ sec). Only then will the electron be forced to proceed via the thermodynamically favorable but kinetically slower route to the catalytic center tethered to the quinone.

6. Figure Legends

Figure 1. Edge-to-edge distance and midpoint potential of PS I cofactors. Solid lines represent forward electron transfer lifetimes and dashed lines represent charge recombination lifetimes. The A and B branches are depicted as A_{0A}/A_{1A} and A_{0B}/A_{1B} , respectively. It is not known whether A_{0A} and A_{0B} have different reduction potentials; they are separated here for clarity purposes only.

Figure 2. CW-EPR light-minus-dark difference spectra of PS I from the *menB* strain (top) and Hg-treated PS I from the *menB* strain (bottom) at X-band. Spectrometer conditions: microwave power, 20mW; microwave frequency, 100 kHz; modulation amplitude, 20 G; temperature, 15 K; 8 averages.

Figure 3. X-band TrEPR spectra of PS I core complexes at 90K. Comparison of WT PS I with PS I complexes prepared from (A) the *menB psaC* strain, and (B) the *menB rubA* strain before and after incubation with $(\text{NQ}(\text{CH}_2)_{15}\text{S})_2$. A=absorption, and E=emission.

Figure 4. Time-resolved optical kinetics at 830 nm after a saturating laser flash in (A) WT PS I, (B) PS I complexes prepared from the *menB* strain; (C) Hg-treated PS I complexes from the *menB* strain; (D) Hg-treated PS I complexes from the *menB* strain with $(\text{NQ}(\text{CH}_2)_{15}\text{S})_2$; (E) PS I complexes from the *menB psaC* strain; (F) PS I complexes from the *menB psaC* strain incubated with $(\text{NQ}(\text{CH}_2)_{15}\text{S})_2$; (G) PS I complexes from the *menB rubA* strain; and (H) the PS I complexes from the *menB rubA*

strain incubated with $(\text{NQ}(\text{CH}_2)_{15}\text{S})_2$. The data are shown as dots and the fitted spectra are shown as solid lines. The residual of the fit is depicted above each of the plots. The reaction mixture contained PS I at 50 $\mu\text{g/mL}$ Chl, 5 mM sodium ascorbate, 10 μM DCPIP, and 0.05% (w/v) β -DM in 50 mM Tris-HCl buffer, pH 8.3.

Figure 5. P_{700}^{+} dark reduction as a function of Cyt c_6 concentration measured at 830 nm. Samples are PS I complexes from (A) the Hg-treated *menB* strain plus $(\text{NQ}(\text{CH}_2)_{15}\text{S})_2$; (B) the *menB psaC* strain plus $(\text{NQ}(\text{CH}_2)_{15}\text{S})_2$; and (C) the *menB rubA* strain plus $(\text{NQ}(\text{CH}_2)_{15}\text{S})_2$. Samples contained WT PS I at 50 $\mu\text{g/mL}$ Chl, 5 mM sodium ascorbate, and 10 μM DCPIP in 50 mM Tris-HCl buffer, pH 8.3

Scheme 1. Energetic diagram of the PS I nanoconstruct, where $k_{1/-1}$ represents the rate constant to/from all available FeS clusters, $k_{2/-2}$ represents the rate constant to/from Pt-nanoparticle, k_r is the recombination channel, and k_f represents the rate of donation from Cyt c_6

Table 1

[Cyt c_6]	Rate of H ₂ Production ($\mu\text{mol H}_2 \text{ mg}_{\text{chl}}^{-1} \text{ hr}^{-1}$)		
	P ₇₀₀ -F _A cores	P ₇₀₀ -F _X cores	P ₇₀₀ -A ₁ cores
$\leq 2 \mu\text{M}$	0.00 ^{1,2}	0.00 ^{1,2}	0.00 ^{1,2}
10 μM	0.16 $\pm$ 0.07	0.00 ^{1,2}	0.00 ^{1,2}
50 μM	0.37 $\pm$ 0.13	0.00 ^{1,2}	0.00 ^{1,2}
100 μM	0.67 $\pm$ 0.08	0.00 ^{1,1}	0.00 ^{1,2}
200 μM	0.92 $\pm$ 0.15	0.11 $\pm$ 0.04	0.00 ^{1,2}

¹After 48 hours of illumination; ²Limit of detection is 0.034 ($\mu\text{M H}_2 \text{ mg}_{\text{chl}}^{-1} \text{ hr}^{-1}$); ³Phylloquinone in A₁ binding site

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

7. References

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