Recruitment of a Foreign Quinone into the A₁ Site of Photosystem I

ALTERED KINETICS OF ELECTRON TRANSFER IN PHYLLOQUINONE BIOSYNTHETIC PATHWAY MUTANTS STUDIED BY TIME-RESOLVED OPTICAL, EPR, AND ELECTROMETRIC TECHNIQUES*

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The overall goal of these studies is to replace phylloquinone (vitamin K₁; 2-methyl-3-phytyl-1,4-naphthoquinone) in the A₁ site of photosystem I with a foreign quinone of different redox and/or kinetic properties but still capable of forward electron transfer to the FeS² clusters. In two previous papers, we described the construction and physiology of phylloquinone biosynthetic mutants in Synechocystis sp. PCC 6803 (1), and we reported EPR and electron nuclear double resonance studies that implied the presence of a foreign quinone, termed Q, in the A₁ site of PS I (2). To summarize briefly, the interruption of the menA and menB genes, which code for dihydroxyynaphthoic acid synthase and phytyltransferase, respectively, results in the termination of phylloquinone biosynthesis as judged by high pressure liquid chromatography/mass spectroscopy (HPLC/MS) and gas chromatography/mass spectroscopy of pigment extracts from isolated PS I complexes. However, the menA and menB mutant strains grow photoautotrophically under low light intensities, and isolated PS I complexes are capable of sustaining high rates of steady-state electron transfer from cytochrome c₅₅ to flavodoxin. HPLC and HPLC/MS studies show that quantitative amounts of plastoquinone-9 are present in PS I complexes isolated from the menA and menB mutants, whereas plastoquinone-9 is present only in vanishingly small amounts in PS I complexes isolated from the wild type. EPR studies indicate that Q⁻ has a considerably larger g anisotropy than the native phylloquinone, consistent with the presence of a 1-ring benzoquinone rather than a 2-ring menaquinone. In addition, the prominent hyperfine splittings due to the 2-methyl group in phylloquinone are absent in the CW and transient EPR spectrum of Q⁻. Spin-echo and transient EPR studies show that Q⁻ is located at the same distance from P700⁺ and with the same orientation with respect to the membrane, as phylloquinone in the wild type. Pulsed electron nuclear double resonance studies additionally show features that arise from nearly axially symmetric hyperfine couplings tentatively assigned to two methyl groups on Q. These results indicate that in the absence of phylloquinone PS I recruits plastoquinone-9 into the A₁ site of the menA and menB mutants and that plastoquinone-9 functions as an efficient elec-

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The abbreviations used are: FeS, iron-sulfur cluster; either F₅₅, F₅₆, or F₅₇; PS I, photosystem I; PS II, photosystem II; Chl, chlorophyll; Q, quinone in the A₁ site of the menA/B mutants; P700, chlorophyll dimer that represents the primary electron donor; DCPIP, 2,6-dichlorophenolindolophenol; β-DM, n-dodecyl-β-D-maltoside; DMF, dimethylformamide; CW, continuous wave; NHE, normal hydrogen electrode; HPLC, high pressure liquid chromatography; MS, mass spectroscopy.

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tron cofactor from A₀ to the FeS clusters.

The premise of the present study is that since plastocyanin-9 and phylloquinone have different one-electron reduction potentials in dimethylformamide, they likely possess different reduction potentials and hence different kinetic properties in the A₁ site. The kinetics of forward electron transfer from A₁ to the FeS clusters in wild-type PS I have been well documented using transient EPR spectroscopy and time-resolved UV spectroscopy. Both methods are consistent in showing a forward electron transfer time of ~280 ns in spinach and cyanobacterial PS I complexes (3–6). Photovoltage measurements of oriented cyanobacterial PS I complexes reveal an electronic phase with a similar forward electron transfer time that was ascribed the A₁ → Fₓ transition (7). Optical studies reveal an additional component with a forward electron transfer time of ~10 ns in detergent-treated samples but not in whole cells (5). In the absence of detergent, the fast phase represents only 30% of the total contribution, leading to the speculation that in the native membranes the forward transfer time is the same in spinach and cyanobacteria.

This third paper in the menA/menB series focuses on the kinetics of electron transfer in PS I complexes from the menA and menB mutants of Synechocystis sp. PCC 6803. Since electron transfer rates are sensitive to changes in Gibbs free energy as well as to alterations in distances and reorganization energies among donor and acceptor pairs, the replacement of phylloquinone would be expected to translate to a change in the rate of electron transfer through A₁. The analysis is simplified by the findings that the orientation of Q" and the distance between Q" and P700⁺ in the mutants are the same as for phylloquinone in the wild type (2). Similarly, the reorganization energy is not expected to differ significantly given that the replacement quinones are structural analogs of phylloquinone. We find that the rate of forward electron transfer from Q" to Fₓ is slowed by a factor of ~100–1000 compared with the wild type. The forward electron transfer kinetics allows us to measure a light-induced difference spectrum of Q minus Q in the UV. Based on the behavior of plastocyanin-9 and phylloquinone in organic solvents, and based on rate versus free energy relationships derived from electron transfer theory, we estimate the redox potential of plastocyanin-9 in the A₁ site.

MATERIALS AND METHODS

Optical Kinetic Spectroscopy in the Near IR Region—Optical absorbance changes in the near IR were measured using a laboratory-built spectrophotometer (8). To ensure resolution of kinetics in the microseconds time domain, a high frequency roll-off amplifier described in the series focuses on the EPR spectroscopy of dimethylformamide was used. The sample cuvette was 1 cm. Each data point represents the average of eight measurements, taken with a flash spacing of 20 s to allow complete reduction of P700⁺ by the external donor. A background measurement was taken similarly except that the sample was shielded from the detecting flash to allow for correction of the actinic flash artifact. The absorbance change shown represents the difference between the two measurements. The sample cuvette contained the PS I trimers isolated from the menB mutant at 10 μg/ml Chl, 25 μm Triton-HCl, pH 8.3, 10 μm sodium ascorbate, 4 μm DCPIP, and 0.03% β-DM.

Electron Transfer Kinetics of A₁ Mutants

Optical properties of the near IR were measured using a Bruker ESP 300E spectrometer equipped with a TM₁₂₀ cylindrical resonator (Bruker ER4103). A frequency-doubled Nd-YAG laser (Spectra Physics DCL) provided the excitation flash at a wavelength of 532 nm and an energy of 14 mJ. The computer was configured to capture and average the data and to flash the laser at 10-s intervals. The data represent an average of 64 flashes. The flat cell (Wilmad WG-813-4) contained the PS I trimers isolated from the menA or menB mutants at 400 μg/ml Chl, 25 μm Triton-HCl, pH 8.3, 10 mM sodium ascorbate, 4 μM DCPIP, and 0.03% β-DM.

Electron Spin Polarized Transient EPR Spectroscopy (Direct Detection)—Room temperature-transient EPR experiments were carried out using a setup described in detail elsewhere (10). The samples were pumped continuously through a flat cell mounted in a Varian rectangular cavity (20 cm) equipped with a rough-surfaced glass window that scatters the laser light to provide a more even distribution of light intensity in the cell. Time/magnetic field data sets were collected using direct detection by measuring light-induced transients at fixed magnetic field positions over an appropriate spectral region. Decay-associated spectra were then generated by fitting the transients with a kinetic function and plotting the amplitude(s) against the magnetic field as described (3, 4).

Transient EPR Spectroscopy with Field Modulation Detection—The same setup and conditions were also used to collect time/field data sets shown in Fig. 7 but with field modulation and lock-in detection. By using direct detection, the decay of the spin polarization limits the accessible time range to times shorter than a few microseconds. By using field modulation, the spectrometer has a rise time of ~50 μs but a much higher sensitivity so that slow forward electron transfer and charge recombination can be monitored.

Photovoltage Measurements—Measurements of transmembrane electric potential difference generation by PS I-containing proteoliposomes adsorbed onto the surface of azole-impregnated colloidion film were done at room temperature as described elsewhere (11). The instrument rise time was 200 ns. Saturating light flashes were provided by a frequency-doubled Quantel Nd:YAG laser (λ = 532 nm; pulse half-width, 15 ns; flash energy, 20 mJ).

Analysis of Kinetic Data—The multieponential fits of optical and EPR kinetics were performed by the Marquardt algorithm in Igor Pro version 3.14 (WaveMetrics, Inc., Lake Oswego, OR) and further processed in Igor Pro 3.14 (WaveMetrics, Inc., Lake Oswego, OR). Actinic flashes were supplied using a frequency-doubled Nd-YAG laser (Spectra Physics) operating at 532 nm at a flash energy of 1.4 mJ. Each kinetic trace represents 16 averages within the digital oscilloscope. The sample cuvette (10 μl) containing 1.0 ml of PS I complexes isolated in 25 mM Tris, pH 8.3 with 0.04% β-DM, 10 mM sodium ascorbate, and 4 μM DCPIP.

RESULTS

P700⁺ Recombination Kinetics, Optical and EPR Spectroscopy—In PS I trimers isolated from the wild type with β-DM, the reduction of P700⁺ is multiphasic after a saturating flash (8). When measured by optical spectroscopy in the near-IR and in the absence of external electron acceptors, the majority of P700⁺ is reduced with lifetimes of ~86 and 12 ms in an ~10:1
Electron Transfer Kinetics of A1 Mutants

Fig. 1. P700+ reduction kinetics in PS I complexes isolated from the menB mutant. A, flash-induced optical transient measured at 810 nm after a single flash. Sample conditions are as follows: 50 μg/ml Chl in 25 mM Tris, pH 8.3, 0.04% β-DM, 10 mM ascorbate, and 4 μM DCPIP in a 1 x 1-cm fluorescence cuvette. Excitation wavelength was 532 nm, and excitation energy was 1.4 mJ. A 300-MHz bandwidth was used in the preamplifier to recover kinetics in the microsecond time range. B, flash-induced EPR transient measured at 3485 G magnetic field position (average of 64 traces recorded at 10 s intervals between flashes). Sample conditions are as follows: 400 μg/ml Chl in 25 mM Tris, pH 8.3, 0.04% β-DM, 10 mM ascorbate, and 4 μM DCPIP in an EPR flat cell. Excitation wavelength was 532 nm, and excitation energy was 14 mJ. Time is plotted on a logarithmic scale in which a deviation from the horizontal represents a kinetic phase. The computer-generated exponential fits are shown as solid lines. Results of the exponential fits are displayed as fit curves (broken line) with the lifetimes of each phase depicted by an arrow. Each individual component is plotted with vertical offset relative to the next component (with a longer lifetime) or the base line, the offset being equal to the amplitude of the latter component. The relative contributions of each kinetic phase can be judged by the intersection of the fit line with the abscissa.

The absorption ratio (minor microsecond kinetic phases are present due to back reactions from earlier acceptors in damaged reaction centers). There also exists a long lived kinetic phase of P700+ reduction due to direct reduction of P700+ by reduced DCPIP that represents ~20% of the total absorbance change in PS I trimers isolated from the menB mutant, the reduction of P700+ is also multiphasic after a saturating flash (Fig. 1A). When measured in the absence of external electron acceptors, P700+ is reduced with lifetimes of ~2.6 and 7 ms in a 0.63:0.37 ratio (minor microsecond kinetic phases are similarly present due to back reactions from earlier acceptors in damaged reaction centers). There also exists a minor long lived kinetic phase of P700+ reduction due to direct reduction of P700+ by reduced DCPIP that represents 7% of the total absorbance change. The menA mutant showed nearly identical kinetics (data not shown). When measured in the same reaction medium using time-resolved EPR spectroscopy with field modulation detection (Fig. 1B), the majority of P700+ in the menB mutant is reduced with a lifetime of 3.2 ms, a value in good agreement with the optical study. Since the signal-to-noise limits the precision of these measurements, the single kinetic phase found in the EPR measurement may correspond to a convolution of the 2.6- and 7-ms lifetimes found in the optical experiment. The EPR study also shows a long lived kinetic phase of P700+ reduction that corresponds to ~13% of the total spin concentration. The menA mutant showed similar kinetics (data not shown). If we consider only the 3.2-ms kinetic phase, then the charge recombination of the electron acceptor(s) with P700+ is ~25 times faster in the menA and menB mutants than in the wild type.

Global Multieponential Analysis of Optical Spectra in the Blue Region—Fig. 2A depicts spectra obtained in the blue region by global multieponential analysis of kinetics in the absence of methyl viologen in PS I complexes from the menB mutant. Three discrete kinetic components are present. The component fitted to the slowest kinetic phase approximated by a base line (checked boxes) corresponds to the spectrum (P700+ minus P700) and represents a population of P700+ reduced by the external electron donor, DCPIP. In these reaction centers, the electron has already escaped from FeS− to an external acceptor, probably molecular oxygen. The 3.2-ms kinetic phase (solid squares) corresponds to the spectrum of (P700+ FeS− minus P700 FeS) and represents a close match to the kinetics of P700+ relaxation measured optically and by EPR (Fig. 1, A and B). The fastest kinetic phase is the 13-μs kinetic event (solid circles), which will be discussed in detail later. Fig. 2B depicts spectra obtained by global multieponential analysis of kinetics in the presence of methyl viologen. Three discrete kinetic components are also present. The component fitted to the slowest kinetic phase (open squares) corresponds to the spectrum of (P700+ minus P700), and it represents the entire population of P700+ reduced by the external electron donor, DCPIP. The spectrum of the 18-μs component (open circles) resembles that of the 13-μs component in the absence of methyl viologen (this component will be discussed later). An additional third component, with a lifetime of 744 μs (solid diamonds), is present with a broad bleaching from 400 to 500 nm characteristic of an S → Fe charge-transfer transition and corresponds to the spectrum of FeS− minus FeS. It is not possible to identify unambiguously the FeS cluster giving rise to this absorbance change because the oxidized minus reduced difference spectra of Fb, Fb, and Fa are nearly identical. However, the electron is likely in equilibrium between Fb and Fa, and the kinetics likely represents the forward electron donation from the terminal electron acceptor FeS− to methyl viologen.

The larger absorbance change of the 3.2-ms phase in the absence of methyl viologen (Fig. 2A) compared with the slow phase in the presence of methyl viologen (Fig. 2B) is derived from the additional contribution of a reduced electron acceptor. Assuming that the spectrum of the 3.2-ms component in the absence of methyl viologen corresponds to the absorbance changes brought about by P700+ plus a reduced acceptor, whereas the spectrum of the slow kinetic component in the presence of methyl viologen corresponds to the absorbance changes of P700+ alone, the difference will correspond to the spectrum of the reduced acceptor. The resulting spectrum, presented in Fig. 2C, is similar to that of the 744-μs kinetic phase in the presence of methyl viologen (Fig. 2B, solid diamonds), showing a broad bleaching from 400 to 500 nm characteristic of an S → Fe charge-transfer transition. The amplitude of the absorbance change is lower than in Fig. 2B (solid diamonds) because a fraction of the reaction centers has already lost the electron from the FeS− clusters. The electron that back-reacts with P700+ with a 3.2-μs lifetime is therefore located in a FeS cluster, although for reasons mentioned above, it is not possible to differentiate between FeS and Fb. The menA mutant showed similar results (data not shown).

Forward Electron Transfer, Decay of the Electron Spin Po-
FIG. 2. Global decomposition of optical kinetic spectra of menB mutant in the blue region. A, spectrum of flash-induced optical transient measured in the absence of methyl viologen. Solid squares represent a component with a 3.2-ms lifetime; checked boxes represent a long lived component (sensitive to DCPIP concentration); solid circles represent a component with a 13-μs lifetime. B, spectrum of flash-induced optical transient measured in the presence of 100 μM methyl viologen. Open squares represent a long lived component (sensitive to DCPIP concentration); solid diamonds represent a component with a 744-μs lifetime; open circles represent a component with an 18-μs lifetime. C, difference between A (solid squares) and B (open squares). The contribution of the spectrum in A (checked boxes) was ignored; hence, the difference spectrum underestimates the amount of FeS cluster reduced on a flash (see text). Sample conditions are as follows: PS I complex isolated from menB mutant at 10 μg/ml Chl in 25 mM Tris, pH 8.3, 0.04% β-DM, 10 mM ascorbate and 4 μM DCPIP.

Electron spin polarized EPR spectra of the wild-type, menA, and menB mutants at room temperature. Top trace, native PS I. The two sequential spin polarized spectra have been extracted from the complete time/field data set as described in detail (4). The solid curve corresponds to the radical pair P700⁻ A¹⁺, whereas the emissive spectrum is due to P700⁻ FeS⁻. Middle and lower traces, menA and menB mutants. The curves are decay-associated spectra extracted from the complete time/field data sets. In the mutants only one kinetic component is observed which we assign to P700⁻ Q⁺. The data were collected using direct detection as described under "Experimental Procedures" and are plotted with absorption (A) in the positive direction and emission (E) in the negative direction.

time to an emissive contribution at later times. We have shown that in samples devoid of Fₓ and Fᵧ, the late signal is retained indicating that the electron transfer proceeds via Fₓ (4). However, it is not possible to identify unambiguously the FeS cluster involved in the radical pair giving the emissive spectrum in intact samples because its contribution is spread over a large spectral range and because of fast relaxation at room temperature (see Ref. 12 for discussion). The subsequent decay of the emissive spectrum with a time constant of 1.5 μs represents the relaxation of the spin-polarized signal. This represents the limit on the time resolution of the spin polarization method.

For the menA (Figs. 3, middle, and 4, middle) and menB (Figs. 3, bottom, and 4, bottom) mutants, the onset of the laser flash results in the appearance of a spectrum that we assign to the state P700⁻ Q⁺, where Q is tentatively identified as plastoquinone-9 in the A₁ site. This spectrum does not show any indication of singlet-triplet mixing in the precursor state, and its rise time is governed by the response time of the spectrometer. Thus, we conclude that electron transfer to Q occurs on a time scale of less than ~0.5 ns. Compared with the wild type, the spectrum of P700⁻ Q⁺ decays more slowly than that of P700⁻ A¹⁺, and there is no indication of P700⁻ FeS⁻ during the 1.5-μs decay of the spin polarization pattern. Thus, we can place a conservative lower limit on the lifetime of P700⁻ Q⁺ at 2–3 μs. This result indicates that electron transfer from A₁ to Fₓ is slowed by a factor of at least 10, from ~280 ns in the wild type to ≥2–3 μs in the menA and menB mutants; one kinetic phase will be shown to be ~300 μs when measured directly in
the field modulation transient EPR experiment described later.

**Forward Electron Transfer, Optical Absorbance Changes in the Near-UV**—We found it possible to measure accurately forward electron transfer from Q\textsuperscript{A} to the FeS clusters in the UV using a flash-detection, pump-probe spectrophotometer (13). Absorption changes were determined in the menA mutant from 245 to 330 nm at time points 5 μs, 100 μs, and 5 ms after an actinic flash. The light-minus-dark difference spectrum recorded 5 μs after the flash shows a maximum at 310 nm, a cross-over at 280 nm, and a minimum of 255 nm, as well as a shoulder at 290–295 nm (Fig. 5A, circles). The difference spectra at 100 μs (Fig. 5A, squares) and 5 ms (Fig. 5A, triangles) after the flash resemble the spectrum at 5 μs, implying that all kinetic phases are derived from the same species. This spectrum is strikingly similar to the plastosemiquinone-9 anion radical minus plastoquinone-9 difference spectrum in methanol (Fig. 5B, checked circles), which shows a maximum at 315 nm, a crossover at 280 nm, and a minimum of 255 nm as well as a shoulder at 280–300 nm (14). The reader should note that the absolute value of the absorbance change of the trough on the short wavelength (blue) side is larger than the absolute value of the absorbance change of the peak on the long wavelength side in both the Q\textsuperscript{2}/Q (Fig. 5A, circles) and the plastoquinone anion radical minus plastoquinone-9 (Fig. 5B, checked circles) difference spectra. The spectrum of Q\textsuperscript{2}/Q is also strikingly similar to the Q\textsubscript{A} minus Q\textsubscript{A} difference spectrum in deoxycholate-isolated PS II particles (15), except that the latter is shifted to the red about 15 nm (Fig. 5B, checked squares). It is noteworthy that the spectrum in Fig. 5A does not resemble that of wild-type PS I, in which the flash-induced difference spectrum (with phyloquinone in the A\textsubscript{1} site) shows a peak centered at 380 nm and a crossover of ~325 nm (5). It is also interesting that the Q\textsuperscript{2}/Q difference spectrum differs from the PQH/PQ difference spectrum, in which the peak occurs at 295 nm, and in which the absolute value of the extinction coefficient at 250 nm is over twice that at 300 nm (14). This indicates that the plastoquinone radical in the A\textsubscript{1} site remains unprotonated during its measured lifetime. The light-minus-dark difference spectrum of Q\textsuperscript{2}/Q therefore supports the assignment of Q as plastoquinone-9 in the A\textsubscript{1} site (1, 2).

The kinetics of the absorbance change at the 310 nm maximum of the menA mutant are depicted in Fig. 6A. The decay kinetics are (at least) biphasic, and the lifetime of the fast phase is estimated to be 17.6 μs. The slow phase is difficult to fit precisely due to the limited number of data points. Nevertheless, the extrapolated absorbance at the onset of the flash indicates that ~60% of Q\textsuperscript{−} decays within 100 μs. Assuming that 100 chlorophyll molecules are associated with each PS I monomer, then at a chlorophyll concentration of 10 μg/ml, the P700 concentration in this sample would be 112 nM. The flash-induced absorption change of Q\textsuperscript{2}/Q at the peak maximum of 315 nm (Fig. 5A, circles) would correspond to 0.94 mOD measured 5 μs after the flash. The differential extinction coefficient of PQ\textsuperscript{−}/PQ at the peak in the UV is reported as 13,000 M\textsuperscript{−1} cm\textsuperscript{−1} in solution (14), and this value has also been used for QA in PS II (15). By using this value and the absorption difference at the onset of the flash gives a total of 72 nM of Q undergoing light-induced reduction. Assuming an equimolar concentration of plastoquinone-9 in the A\textsubscript{1} site with P700, this would correspond to 64% of the redox-active Q. However, the difference spectrum is recorded 5 μs after the flash, and given that the lifetime of the fast kinetic phase is 17.6 μs, the absorption change of Q\textsuperscript{2}/Q at the onset of the flash can be estimated as 1.04 mOD. Hence 80 nm, or 71% of the redox-active Q, is associated with forward electron transfer. This estimation suffers from uncertainty in the extinction coefficient of plastoqui-
none-9 in the A₁ site and in the estimate of the absorbance at the onset of the flash. Nevertheless, the calculation shows that the majority of electrons that are transferred from A₁ to the FeS clusters are mediated by plastoquinone-9. This rules out a significant electron bypass of the quinone at room temperature in the menA and menB mutants.

Forward Electron Transfer Kinetics, Electrochromic Bandshift at 490 nm.—The global multiexponential analysis of the flash-induced changes in the absorption spectra of PS I complexes from the menB mutant revealed a 13-µs kinetic event in the absence of methyl viologen with a derivative-shaped spectrum centered at 460 nm (Fig. 2A, solid circles connected with dotted line). This spectrum is characteristic of an electrochromic shift of a pigment that occurs in response to electron transfer. The same electrochromic shift can be seen in the presence of methyl viologen, except that the extracted lifetime is 18 µs and the amplitude is higher (Fig. 2B, open circles with dotted line). The longer lifetime may be instrument-related; to obtain a reasonable signal-to-noise, the rise time (1/e) of the amplifier was limited to 10 µs, which would tend to underestimate the initial amplitude in Fig. 2A. The electrochromic shift also shows a slower kinetic phase in the absence (Fig. 2B) and presence (data not shown) of methyl viologen. The kinetics in the near-millisecond time domain are complicated by absorbance changes at 490 nm due to the decay of P₇₀₀⁺, and no further attempt was made to separate their relative contributions. Similar kinetic phases were measured in the menA mutant (data not shown).

In wild-type PS I, the light-minus-dark difference spectrum shows a positive-going absorption band from 440 to 500 nm with a shape that resembles the red shift of a pigment centered at about 470 nm (5). A similar set of absorption changes were recently measured in mutant cells of Chlorella sorokiniana that lack PS II (16). These features were attributed in the wild type to an electrochromic red shift of an absorption band of a carotenoid that is induced by A₁. Similarly, the spectrum of the ~18-µs component measured in menB mutant most probably represents an electrochromic bandshift due induced by Q⁻ on a nearby carotenoid. The kinetics of the flash-induced carotenoid bandshift therefore represent an indirect, but reliable, method to measure the oxidation kinetics of the plastosemiquinone anion Q⁻ in the visible region.

Photovoltage Measurements on PS I Complexes in Proteoliposomes—Excitation of oriented PS I complexes with a single turnover flash leads to the generation of a transmembrane electric potential difference from which the forward electron transfer rates and dielectrically weighted transmembrane distances can be measured (7, 11, 17). PS I complexes were incorporated into proteoliposomes, and the flash-induced response corresponded to the negative charging of the proteoliposome interior. For wild-type PS I in the absence of an external electron donor to P₇₀₀⁺, the photoelectric response due to charge separation between P₇₀₀ and the terminal electron acceptors, F₇₃/F₃₀₆, occurs within the ~0.2-µs rise time of the instrument (8). Thus, the 20- and 200-ns kinetic phases of forward electron transfer between A₁ and Fₓ in wild-type PS I are not resolved using our instrumentation. However, for PS I complexes isolated from the menB mutant (Fig. 6C, inset) and menA mutant (data not shown), the photoelectric response shows an instrument-limited rise followed by a slower rise in the submillisecond time range. Decomposition of these kinetics, presented in Fig. 6C, reveals components with lifetimes of ~11.4 and 306 µs, and equal relative contributions (~10%) to the overall photoelectric response. The decomposition also includes an offset that represents a longer lived component in the low millisecond time range. The lifetime of the fast kinetic phase is in reasonable agreement with the measurements of
Q$^-$ oxidation at 315 nm and the carotenoid bandshift at 485 nm. The slow kinetic phase is also probably related to the oxidation of Q$^-$, although the optical data at 315 and 485 nm are compromised by the presence of millisecond lifetime P700$^-$/P700 changes at these wavelengths. We assign these two components to vectorial electron transfer from Q$^-$ to FeS of 300 $\mu$s and a recombination lifetime of 5 ms. The offset at long times is due to reduction of P700$^+$ by an exogenous donor in centers in which the transferred electron is lost from FeS.

**Discussion**

The results from time-resolved optical, electrometric, and EPR techniques support the identity of Q in the menA and menB mutants as plastoquinone-9 and show that despite the altered forward and back electron transfer kinetics, plastoquinone-9 functions as an efficient electron cofactor in PS I. To accomplish forward electron transfer, it is necessary that the midpoint potential of the A$_1$ site be sufficiently reducing to transfer electrons to the acceptor flavodoxin and at a rate that outcompetes the inherent back reaction of reduced plastoquinone-9 with P700$^+$. We estimate the midpoint potential of Q$^-$/Q using two approaches as follows: a comparison of the redox properties of plastoquinone-9 and phylloquinone in organic solvents, and a consideration of rate versus free energy relationships from electron transfer theory.

Phyloquinone has a reported $E_{1/2}$ of $-465$ mV (versus NHE) in dimethylformamide (DMF) (21), whereas plastoquinone-9 has a reported $E_{1/2}$ of $-369$ mV (versus NHE) in DMF (22). If the A$_1$ site has a polarity similar to that of DMF, then plastoquinone-9 would be +96 mV more oxidizing than native phyloquinone in the A$_1$ site. However, this is only a crude estimate, and it should be possible to refine this value using the concept of "acceptor number." Jaworski and colleagues (18) showed that the redox potential of a quinone undergoing the first electron reduction in organic solvent is related to the electrophilic properties of the solvent. This work was based on a formulation by Gutman (19) of an acceptor number, a dimensionless number that expresses the acceptor properties of a given solvent relative to that of SbCl$_5$. The central idea is that $E_{1/2}$ values of different quinones show smaller differences to one another in solvents with low acceptor numbers and higher differences to one another in solvents with high acceptor numbers. This solvent effect is quantitatively described by the empirical Equation 1:

$$E_{1/2} = E_{1/2}^0 + \alpha (AN)$$

(Eq. 1)

where $\alpha$ is the slope (i.e. the sensitivity to the solvent effect based on Lewis acidity); AN is the acceptor number (which ranges from 0 in hexane to 100 in SbCl$_5$, the reference solvents; benzene is 8.2, DMF is 16, and water is 54.8), and $E_{1/2}^0$ is the intercept (the value of $E_{1/2}$ corresponding to a solvent with AN = 0). An important point is that the semiquinone radical is destabilized in solvents with low acceptor numbers, which leads to a lower redox potential for the first electron reduction. Itoh and co-workers (20) applied Gutman’s ideas to a study of replacement quinone head groups (lacking the phytyl tail) in PS I and estimated the acceptor number for the A$_1$ site to be 4.0, which is similar to the acceptor number for diethyl ether of 3.9. By assuming the redox potential of a given quinone in organic solvent is strictly linearly related to the $E_{1/2}$ value in the A$_1$ site, the following Equation 2 could be derived (20):

$$E_m = 0.69 (E_{1/2}^0 (DMF) - 433 mV)$$

(Eq. 2)

where $E_{1/2}$ is the redox potential in DMF and $E_m$ is the redox potential in the A$_1$ site. Given that phyloquinone has an $E_{1/2}$ of
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The equilibrium constant $K_{eq}$ between Q and $F_X$ is defined in Equation 5 as follows:

$$K_{eq} = k_{forward}/k_{reverse}$$  \hspace{1cm} (Eq. 5)

The equilibrium constant $K_{eq}$ is related to Gibbs free energy according to Equation 6.

$$K_{eq} = 10^{\frac{\Delta G_{\text{rev}}}{2.303 R T}}$$  \hspace{1cm} (Eq. 6)

Hence, the reverse electron transfer rate, $k_{reverse}$, can be expressed in Equation 7 as follows:

$$k_{reverse} = 10^{\frac{-\Delta G_{\text{rev}}}{2.303 R T}}$$  \hspace{1cm} (Eq. 7)

We employ Equation 4 to specify the rate of forward electron transfer in an exergonic reaction and Equation 7 to specify the rate of forward electron transfer in an endergonic reaction. The averaged edge-to-edge distance between $Q_X$ (the two identified quinones in the x-ray crystal structure) and $F_X$ is reported to be 11.3 Å (29). A reorganization energy of 0.7 eV is commonly used for photosynthetic electron transfer reactions (27), although a value of 1.0 eV was recently estimated for $A_1$ and $F_X$ from the temperature dependence of electron transfer (30). The value of $k_{exergonic}$ will therefore depend on the difference in Gibbs free energy between the quinone and the $F_X$ iron-sulfur cluster. This calculation requires knowledge of accurate redox potentials for both cofactors. The $E_{1/2}(Q/Q^\text{+})$ of phylloquinone in wild-type PS I has not been measured directly; however, a value of −810 mV versus NHE has been derived from calculations based on the kinetics of electric field-induced electron transfer rates (31), and a value of less than or equal to −800 mV versus NHE has been calculated based on the P700 triplet yield (32). As mentioned earlier, the considerably higher value of −754 mV has been deduced from the measured $E_{1/2}$ values of phylloquinone and plastoquinone-9 in DMF and an application of Gutman's acceptor number of 4.0 for the $A_1$ site. The $E_{1/2}(F_X/F_X^\text{+})$ of phylloquinone in wild-type PS I has been measured directly; an $E_{1/2}$ of −705 mV was found for $F_X/F_X^\text{+}$ in PS I complexes by electrochemical poising and EPR measurement (33). However, this approach suffers from an uncertainty that the midpoint potential of $F_X$ was determined in the presence of a reduced $F_A$ and $F_X$ and may be underestimated due to an electrostatic effect of nearly reduced acceptors. A considerably higher $E_{1/2}$ of −670 mV has been measured in P700-$F_X$ cores that lack PsaC and, hence, any electrostatic influence from the $F_X$ and $F_X^\text{+}$ clusters. Recently, an equilibrium constant of 73.5 was determined between $F_X$ and $F_A$ in P700-$F_X$ cores by analysis of the back reaction kinetics, which indicates that $F_X$ may be 110 mV more electronreceptive than $F_A$ (34). Since the $E_{1/2}$ of $F_A$ has been measured to be −520 to −540 mV, the $E_{1/2}$ of $F_X$ would be −630 to −650 mV. However, these approaches suffer from an uncertainty whether there is any effect on the midpoint potential of $F_X$ from the removal of PsaC, due either to structural changes or to alterations in solvent accessibility of the iron-sulfur cluster.

Table I shows a matrix of the predicted values for the forward rate constant, $k_{exergonic}$, for the three published values of the midpoint potential of $A_1/A_1$, the three published values of the midpoint potential of $F_X/F_X$, and the two published values of the reorganization energy. Assuming a reorganization energy of 0.7 eV, the averaged rate constant of optimum forward electron transfer would be 223 ± 99 ns. This value is similar to the −280-ns lifetime of $A_1$ measured in wild-type PS I complexes (3–7). If a reorganization energy of 1.0 eV is used instead, the averaged rate of optimum forward electron transfer is 1.83 μs, a value considerably slower than that observed experimentally. We next use the measured lifetime of $Q^\text{−}$ in the menA and menB mutants to back-calculate the redox potential for $Q^\text{−}/Q$. Given that the major kinetic phase of forward electron transfer from $Q^\text{−}$ in the menA and menB mutants has a lifetime of −15 μs (Fig. 6, A–C), a careful consideration of Equations 4 and 7 shows that electron transfer must be endergonic (according to Equation 7) between $A_1$ and $F_X$ to accom-
moderate this rate. Table II lists a matrix of the predicted values for the increase in Gibbs free energy between \(A_1\) and \(F_X\) and the calculated redox potential for \(Q^+/Q\), for the three values of the midpoint potential of \(F_{X^+}/F_X\), and the two values of the reorganization energy. The results imply that regardless of whether the reorganization energy is 0.7 or 1.0 eV, electron transfer between \(A_1\) and \(F_X\) is endergonic by 12–95 mV. If we accept a value of \(-705\) mV for the \(F_{X^+}/F_X\) redox couple and a reorganization energy of 0.7, then plastoquinone-9 in the \(A_1\) site of the \(menA\) and \(menB\) mutants will have a midpoint potential of \(-610\) mV. A value of \(-670\) mV for the \(F_{X^+}/F_X\) redox couple leads to a midpoint potential of \(-575\) mV for plastoquinone-9 in the \(A_1\) site, a value which is nearly isopotential with the \(F_B\) iron-sulfur center.

Although the value obtained using electron transfer theory provides an interesting exercise, this approach cannot be used for precise estimations because the uncertainties approach an order of magnitude for the energy and the rate constant. Given the additional uncertainties in the midpoint potentials of the components, in the precise value of the reorganization energies when phylloquinone and plastoquinone-9 occupy the \(A_1\) site, and in the exact edge-to-edge distance between the quinone and the iron-sulfur cluster, the estimated values must be used with caution. Nevertheless, these considerations support the appraisal based on the redox behavior of plastoquinone-9 and phylloquinone in organic solvents in suggesting that the mid-point potential of \(Q^+/Q\) is more oxidizing than the iron-sulfur cluster, \(F_X\) (Fig. 8). If so, then we must ask how a thermodynamically unfavorable reaction can be reconciled with both the high quantum yield of electron transfer observed in the steady-state measurements (1) and the efficient reduction of \(F_{X}/F_B\) observed by optical and EPR spectroscopy (2). The important factor here is that even with an endergonic electron transfer step between \(A_1\) and \(F_X\), overall net electron transfer is exergonic between \(A_1^+\) and \(F_{A}/F_B\) (Fig. 8). A drop in the quantum yield and an inefficiency in \(F_{X}/F_B\) reduction would be expected only if electron transfer between \(A_1^+\) and \(F_X\) were sufficiently slow that it would be outcompeted by the inherent back reaction between \(A_1\) and \(P700^-\). We see no evidence for a back reaction between \(Q^-\) and \(P700^+\) when the terminal iron-sulfur clusters \(F_{A}\) and \(F_B\) are oxidized and available for electron transfer (data not shown). A similar instance of an unfavorable electron transfer with an overall negative change in the free Gibbs energy has been postulated for the electron transfer stage from \(F_X\) via \(F_{A}\) and \(F_{B}\) to ferredoxin (35, 36, 38).

We next compare the calculated values in Table II with the experimental data. The issue is whether the reduction of \(F_{X^+}/F_X\) on a single turnover flash (Fig. 2) corresponds to the amount expected on the basis of chlorophyll concentration. In the following analysis, the term FeS refers to the iron-sulfur cluster that accepts the electron on a single-turnover flash without specifying its identity as \(F_A\) or \(F_B\). Assuming that 100 chlorophyll molecules are associated with each PS I monomer, then at a chlorophyll concentration of 10 \(\mu\)g/ml, the concentration of

| TABLE II | DEDUCTED VALUES FOR \(E_{1/2}^a\) OF \(Q\) |
|----------|-----------------------------------------------|
| \(E_{1/2}^a\) mV | \(\Delta E_{0.19.4}^a\) mV | \(Q/Q\) |
| \(-705\) | +95 | +12 | \(-610\) | \(-693\) |
| \(-670\) | +95 | +12 | \(-575\) | \(-658\) |
| \(-650\) | +95 | +12 | \(-555\) | \(-638\) |
| Mean ± S.D. | \(-580 \pm 28\) | \(-663 \pm 28\) |

*See Ref. 27.*

*See Ref. 30.*

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