Recruitment of a Foreign Quinone into the $A_1$ Site of Photosystem I

II. STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF PHYLLOQUINONE BIOSYNTHETIC PATHWAY MUTANTS BY ELECTRON PARAMAGNETIC RESONANCE AND ELECTRON-NUCLEAR DOUBLE RESONANCE SPECTROSCOPY

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Electron paramagnetic resonance (EPR) and electron-nuclear double resonance studies of the photosystem (PS) I quinone acceptor, $A_1$, in phylloquinone biosynthetic pathway mutants are described. Room temperature continuous wave EPR measurements at X-band of whole cells of men$A$ and men$B$ interruption mutants show a transient reduction and oxidation of an organic radical with a $g$-value and anisotropy characteristic of a quinone. In PS I complexes, the continuous wave EPR spectrum of the photoaccumulated $Q^-$ radical, measured at Q-band, and the electron spin-polarized transient EPR spectra of the radical pair $P700^+ Q^-$, measured at X-, Q-, and W-bands, show three prominent features: (i) $Q^-$ has a larger $g$-anisotropy than native phylloquinone, (ii) $Q^-$ does not display the prominent methyl hyperfine couplings attributed to the 2-methyl group of phylloquinone, and (iii) the orientation of $Q^-$ in the $A_1$ site as derived from the spin polarization is that of native phylloquinone in the wild type. Electron spin echo modulation experiments on $P700^+ Q^-$ show that the dipolar coupling in the radical pair is the same as in native PS I, i.e. the distance between $P700^+$ and $Q^-$ (25.3 ± 0.3 Å) is the same as between $P700^+$ and $A_1^-$ in the wild type. Pulsed electron-nuclear double resonance studies show two sets of resolved spectral features with nearly axially symmetric hyperfine couplings. They are tentatively assigned to the two methyl groups of the recruited plastocyanine-9, and their difference indicates a strong inequivalence among the two groups when in the $A_1$ site. These results show that $Q$ (i) functions in accepting an electron from $A_0^-$ and in passing the electron forward to the iron-sulfur clusters, (ii) occupies the $A_1$ site with an orientation similar to that of phylloquinone in the wild type, and (iii) has spectroscopic properties consistent with its identity as plastocyanine-9.

Light-induced charge separation in all well characterized photosynthetic reaction centers (RCs) proceeds via a common multistep electron transfer process to a stabilized, charge-separated radical pair state $P^- Q^-$ consisting of an oxidized (bacterio)chlorophyll donor and a reduced quinone acceptor (whether the green sulfur bacterial RC and the heliobacterial RC contain a quinone acceptor is still controversial). Two types of RCs can be distinguished according to the electron acceptors and electron transfer pathways subsequent to the first quinone acceptor. A series of iron-sulfur clusters with electron transfer essentially perpendicular to the membrane characterize Type I RCs (PS I, green sulfur bacteria, and heliobacteria), whereas a second quinone acceptor $Q_b$ and electron transfer parallel to the membrane from the first quinone acceptor $Q_A$ characterize Type II RCs (PS II and the RC of purple bacteria). The first quinone is therefore the interface either between electron transfer involving organic cofactors and electron transfer involving iron-sulfur clusters (Type I) or between pure electron transfer and coupled electron/proton transfer involving a second organic cofactor (Type II).

Compared with the binding sites for $Q_A$ and $Q_b$ in bacterial RCs, the binding sites for phylloquinone in PS I are poorly defined. Transient EPR studies of the radical pair $P^- A_1^-$ (1, 2) show that phylloquinone is oriented with its carbonyl bonds parallel to the vector joining $P700^+$ and $A_1^-$. Measurement of the dipolar coupling between the $P700^+$ and $A_1^-$ radical pair yields the distance between these two radicals as 25.4 ± 0.3 Å (3–5). A spin echo study of $P700^+ A_1^-$ in single crystals of PS I (6) determined an angle of 27 ± 5° between the diagonal axis between $P700^+$ and $A_1^-$ and the crystallographic $c$ axis (membrane normal). Transient EPR studies of the same PS I crystals (7) provided more detailed information on the quinone orientation and, consistent with the spin echo result, found the angular range to be 25–30°. The orientation of the other axis in the quinone plane is less certain (7, 8). For photoaccumulated $A_1^-$ in oriented membranes, the quinone plane is found to be nearly perpendicular to the membrane (76°) (9). Recent transient Q-band EPR of $P700^+ A_1^-$ in PS I single crystals specify an angle of 65 ± 20° between the quinone plane and the plane made up of the crystalline $c$ axis (membrane normal) and the quinone carbonyl bond direction. This is in good agreement with the
most recent x-ray structure (10, 11). Although the x-ray structure is not sufficiently resolved to identify the amino acids in the binding pocket, electron spin echo envelope modulation studies (12) and a model based on all available structural data (8) suggest that a tryptophan residue may be close to the semiquinone radical in PS I (12).

In the accompanying paper (13), we describe the genetic and physiological characteristics of menA and menB interruption mutants in Synechocystis sp. PCC 6803. The menA gene product, naphtho synthase, and the menB gene product, phytly transferase, were proposed to be involved in the biosynthesis of phylloquinone and were therefore targeted for deletion mutagenesis. Our goal was to disallow the biosynthesis of phylloquinone and produce an empty A1 site, which could then be reconstituted with a variety of quinones. We found that photoautotrophic growth under low light intensities and steady-state rates of flavodoxin reduction were relatively unaffected by the absence of phylloquinone. To account for these results, we proposed that a foreign quinone, Q₁, had been recruited into the A1 site of PS I the menA and menB mutants. This conclusion was predicated on the finding that plastoquinone-9 could be half the rate of H₂O grown cells when fully adapted. Cells grown in the cells were pelleted and reinoculated to

FIG. 1. Molecular structures of phylloquinone (vitamin K₃, 2-methyl-3-phytyl-1,4-naphthoquinone) and plastoquinone-9. A common numbering of the quinone ring positions is chosen in the text, with the carbonyl group next to the hydrocarbon chain (in position 3) placed in position 4.

color, but they did not divide. Preparation of PS I Trimmers—PS I trimers from the wild type and from the menA and menB mutants were isolated from sucrose gradients (13). The PS I complexes were stored in 20% glycerol containing buffer at −80°C prior to use.

X-band CW EPR Spectroscopy—Whole-cell EPR experiments were carried out using a Bruker ER300E spectrometer equipped with a TMₓ₀ cavity. Mutant and wild type cells of Synechocystis sp. PCC 6803 were grown photomixotrophically to early exponential phase, collected by centrifugation at room temperature, and resuspended in growth medium to 400 μg ml⁻¹ Chl. The protocol for studying the time-dependence at 95% D₂O and A₁₂₅ is a modification of the method described in Refs. 14 and 16. Live cells were placed in a 10-ml reservoir that was placed in a closed circuit by tubing and a microprocessor-controlled peristaltic pump (ISCO WIZ) to a aqueous flat cell (Wilmad WG-813-Q). The reservoir, tubing, and TM₁₁₀ cavity were maintained in complete darkness to ensure that the cells were dark-adapted. A 200-watt mercury-xenon lamp provided controlled illumination by use of a UniBlitz shutter and shutter driver/trigger (model T132). The EPR computer was programmed to begin data acquisition at t = 0, open the shutter at t = 0.2 s, close the shutter at t = 2.7 s, and pump a fresh sample into the flat cell at t = 4.0 s. This protocol was repeated 20 times at 10-s intervals at a given magnetic field to obtain data with an adequate signal to noise ratio. The magnetic field was stepped 0.5 G, and the parameters repeated until all data had been obtained over different magnetic field settings. The data were downloaded to a Power Macintosh G3 computer and plotted as three-dimensional and two-dimensional data sets using Transform 2.0 (Spyglass, Inc.).

Q-band CW EPR Spectroscopy—Photoaccumulation experiments at Q-band (34 GHz) were carried out using a Bruker ER300E spectrometer equipped with an ER 5106 QT-W1 resonator with a port for in-cavity illumination. Cryogenic temperatures were maintained with an ER4118CV liquid nitrogen cryostat and controlled with an ER4121 temperature control unit. The microwave frequency was measured with a Hewlett-Packard 5352B frequency counter, and the magnetic field was measured with a Bruker ER035M NMR gaussmeter. The magnetic field was calibrated using γ-bispidphenylene-β-phenylallyl complexed 1:1 with benzene. Photoaccumulation protocols on wild type and mutant PS I complexes were carried out in a manner similar to that described in Ref. 15. Prior to the measurement, the pH of the sample was adjusted to 10.0 with glycine buffer, and sodium dithionite was added to a final concentration of 50 mM. After incubation for 20 min in the dark, the sample was placed into the resonator, the temperature adjusted to 205 K, and a background spectrum was recorded. The sample was illuminated with a 20-mW He-Ne laser at 630 nm for 40 min, the laser was turned off, and the light-induced record was recorded. The spectra reported represent the difference between the two measurements. EPR spectral simulations were carried out on a Power Macintosh G3 computer using a Windows 3.1 emulator (SoftWindows 3.0, Insignia Solutions, Santa Clara, CA) and SimFonia software (Bruker Analytik) and imported into IGOR Pro 3.1 (WaveMetrics, Inc., Lake Oswego, OR).

Time-resolved EPR and ENDOR Spectroscopy—All samples contained 1 mM sodium ascorbate and 50 μM phenazine methosulfate as external redox agents and were frozen in the dark for the low temperature experiments. The W-band (94 GHz) transient EPR spectra were measured using a Bruker E680 spectrometer. Illumination was accomplished with a frequency-doubled Nd-YAG laser using an optical fiber fed into the sample capillary and ending directly above the PS I sample. Q-band (35 GHz) transient EPR spectra were measured in a set-up as described earlier (see, for example, Ref. 17), except that a Bruker ER 056 QMV microwave bridge equipped with a home-built cylindrical resonator and an Oxford CF935 liquid helium cryostat was used. The transient and pulsed X-band EPR measurements were performed using the experimental setup described in Ref. 6. Two microwave (mw) pulses with a variable pulse spacing, τ, were applied to the radical pair P700⁻ Q⁻ at a time t, following the laser flash. Electron spin echo (ESE) amplitude modulation curves were obtained by collecting the amplitude of the echo detected at time t = τ after the second mw pulse as a function of τ. The mw pulse lengths were set to 8 ns for the first pulse and 16 ns for the second pulse, resulting in wobbles of angles of about 255 and 130°, respectively, with a mw pulse field B₀ = 0.8 mT. The external magnetic field B₀ and the detection time T were adjusted to yield maximum off-phase echo intensity. The echo modulation was recorded at 512 points with 8-ns increments in τ, and 64 traces were averaged to increase the signal-to-noise ratio. Pulsed ENDOR studies of the P700⁻ Q⁻ state were performed using the same experimental setup as the pulsed EPR experiments using a Bruker
Results

In Vivo Detection of a Quinone in the A₁ Site by X-band EPR—The semiquinone anion radical of A₁ can be observed transiently in whole cells of *Synechococcus* sp. PCC 7002 as a light-induced resonance centered at *g* = 2.0049 and with a *H*$_{pp}$ (peak-to-peak) of 10 G (14, 16). The ability to detect a quinone radical in *vivo* at room temperature thus provides a useful method to determine whether a quinone is present or absent in the A₁ binding site of the menA and menB mutants. When whole menA cells of *Synechocystis* sp. PCC 6803 are dark-adapted and illuminated for 2.5 s, a plot of time versus magnetic field versus amplitude depicts two independent radicals undergoing time-dependent, nearly reciprocal patterns of oxidation and reduction (Fig. 2). Whole cells of the menB mutant show a similar time course (data not shown). The radical attributed to P700$^+$ is the highly symmetrical resonance with maxima and minima at 3482 and 3490 G, and the radical attributed to a foreign quinone, termed Q$^-$, is the anisotropic resonance visible around 3475 G as the low field shoulder (Fig. 2, arrow). The time course is rationalized by a transient rate limitation in the utilization of NADPH, which causes electrons to back up, reducing, in sequence, F$_{A}$, F$_{B}$, F$_{X}$, and finally the quinone in the A₁ site. At a certain point in time, the rate limitation is relieved, the reduced acceptors pass their electrons forward, and P700$^+$ once again is allowed to accumulate due to the availability of oxidized acceptors beyond A$_{2}$. Were the foreign quinone, Q$^-$, not able to pass electrons forward in the menA and menB mutants, then both the quinone and P700$^+$ would remain reduced in the light.

The resonances from P700$^+$ and Q$^-$ can be better distinguished when the plot is oriented so that the time axis is normal to the plane of the paper. In this depiction, the P700$^+$ radical that develops on initial illumination is identified in Fig. 3 (top, dotted line) as the highly symmetrical resonance centered at *g* = 2.0023 with an *H*$_{pp}$ of 7.8 G. The spectrum of Q$^-$ denoted by the arrow in Fig. 2 is the highly anisotropic resonance in Fig. 3 (top, solid line), with a crossover at *g* = 2.0042 and an *H*$_{pp}$ of 10.5 G. On cessation of illumination, the highly symmetrical resonance in Fig. 3 (top, dashed line) is derived again from P700$^+$. The kinetics of P700$^+$ and Q$^-$ can be resolved by measuring the former at the Q$^-$ zero crossing point of 3484.5 G, and the latter at the P700$^+$ zero crossing point of 3475 G. After an initial instrument-limited rise, P700$^+$ undergoes a time-dependent decay that is roughly mirrored as a reciprocal rise in Q$^-$ (Fig. 3, bottom). After reaching a maximum at 0.5 s of illumination, the slower decay of Q$^-$ is roughly mirrored as a reciprocal rise in P700$^+$. Measurements made at 3486 G, which is on the low field shoulder of the 10.5-G-wide Q$^-$ radical and for which the contribution from P700$^+$ is negligible, confirm the kinetic measurement made at 3475 G (zero crossing point of P700$^+$ at *g* = 2.00232).

The relevance of this analysis to the menA and menB mutants is 2-fold: (i) the *g*-value and *g*-anisotropy of the transiently produced Q$^-$ is consistent with its identification as a semiquinone anion radical, and (ii) the transiently produced radical shows that Q is capable of not only accepting electrons from A$_{2}$ via P700$^+$ but also discharging electrons by forward electron transfer after a suitable period of adaptation. Hence, the foreign quinone, Q, is dynamic and participates in forward electron transfer to the iron-sulfur clusters under physiological conditions.

**CW EPR Spectroscopy at Q-band of Photoaccumulated A₁**—

The *g*-anisotropy derived from high field CW EPR studies of photoaccumulated PS I complexes contains information about the degree of aromaticity of the semiquinone anion radical. To extract the *g*-tensor, we measured the EPR spectrum of the photoaccumulated Q$^-$ radical in the wild type and in the menA and menB mutants at 34 GHz (Q-band), a frequency in which the nuclear-electron hyperfine couplings no longer dominate the spectrum. Fig. 4 (solid line) shows the spectrum of wild type PS I complexes isolated from *Synechocystis* sp. PCC 6803 grown in H$_2$O. At 34 GHz, the field-dependent *g*-anisotropy dominates the spectrum of A$_{1}^-$ (18), allowing the turning points of *g*$_{xx}$ = 2.0062 and *g*$_{zz}$ = 2.0021 to be partially resolved. The *g*$_{xx}$ component of the tensor is obscured by the four hyperfine lines resulting from the high spin density at the carbon position 2 of the phyllosemiquinone radical with the methyl group attached. To better resolve the *g*-anisotropy, PS I complexes were isolated from *Synechococcus* sp. PCC 6803 grown in 92% D$_2$O, the highest concentration that permitted growth.

**FIG. 2.** Magnetic field versus time and Q$^-$ versus peak-to-peak field for P700$^+$ in whole cells of the menA mutant strain. Dark-adapted cells were illuminated for 2.5 s, and the intensity was plotted as a function of time at a given magnetic field. EPR conditions were as follows: microwave frequency, 9.7698 GHz; microwave power, 50 mW; modulation frequency, 100 kHz; modulation amplitude, 1 G; receiver gain, $4 \times 10^3$; conversion time, 327 ms; time constant, 10 ms. The arrow indicates the low field shoulder discussed in the text.
As shown in Fig. 4 (solid line), the suppression of the hyperfine couplings results in a narrowing of the line widths of the g-components, with the consequence that \( g_{xx}, g_{yy}, \) and \( g_{zz} \) are almost completely resolved. Satisfactory simulation of the spectrum is obtained with the g-tensor principal values \( g_{xx} = 2.0062, g_{yy} = 2.0051, \) and \( g_{zz} = 2.0022 \) and line widths of 2.4, 3.7, and 2.4 G, respectively. This is in good agreement with previous determinations of the \( A_1^+ \) tensor from 34 GHz (Q-band) studies of deuterated PS I complexes from \( \text{Synechococcus} \) sp. PCC 7002 (15), from 95 GHz (W-band) studies of in non-deuterated PS I complexes from \( \text{Synechococcus lividus} \) (2), and from 283 GHz studies of nondeuterated PS I complexes from \( \text{Synechocystis} \) sp. PCC 6803 membrane fragments (9). Because the simulated spectrum (Fig. 4, dashed line) matches the experimental spectrum even in the high field region quite well, the amount of contaminating \( A_0^- \) or \( P700^+ \) appears to be minimal in the deuterated sample. The nondeuterated spectrum was simulated (Fig. 4, dashed line) using the g-tensor extracted from the deuterated \( A_1^- \) spectrum and a hyperfine A-tensor with principal values of 9.0, 12.9, and 9.0 MHz (19). The major deviations from the simulated spectrum appear principally in the mid- and high field regions and therefore include a minor contribution from \( A_0^- \) and/or \( P700^- \).

The Q-band EPR spectra of photoaccumulated \( Q^- \) in PS I complexes isolated from the \( \text{menB and menA} \) mutants of \( \text{Synechocystis} \) sp. PCC 6803 grown in \( \text{H}_2\text{O} \) are shown in Fig. 4 (solid lines). In both mutants, the four prominent hyperfine lines are missing, and the g-tensor of \( Q^- \) appears more anisotropic than that of \( A_1^- \) in the wild type. Attempts to simulate the spectrum of \( Q^- \) were complicated by a large contamination with a second radical, probably \( A_0^- \) and/or \( P700^- \). Using the g-tensor for \( Q^- \) extracted from transient EPR studies (see below), the spectrum was accurately represented by principal values \( g_{xx} = 2.0067, g_{yy} = 2.0051, \) and \( g_{zz} = 2.0022 \), with an error of 2 in the last digit, and line widths of 6 G for each value (Fig. 4, dotted line). The difference between the actual and simulated spectrum of \( Q^- \) yields a roughly symmetrical radical with a g-value of approximately 2.0030 and a line width of approximately 12–15.
been photoaccumulated the menA and menB mutants. Indeed, the larger $g$-anisotropy (also seen in the transient spin-polarized EPR spectra; see Figs. 5 and 6, below) can be further rationalized in terms of differences in the delocalization of unpaired electron density over plastoquinone-9 and phyloquinone. Because benzoquinone derivatives, such as plastoquinone-9, have a single aromatic ring, compared with the two rings of naphthoquinone derivatives, such as phyloquinone, the spin density on their carbonyl oxygens is higher, and they exhibit larger $g$-anisotropies. Thus, with plastoquinone-9 incorporated into the A$_1$ site, an increase in the $g$-anisotropy compared with native PS I would be expected.

**Time-resolved EPR Spectroscopy at X-, Q-, and W-bands—**

The spin polarization patterns derived from transient EPR spectra of the functional radical pair state P700$^+$ Q$^-$ contain information about the relative orientation of the species involved, as well as about the influence of the protein environment and electron transfer kinetics on the magnetic properties and spin dynamics. For native PS I in frozen solution, spectra of the state P700$^+$ A$_1$ are observed from the RC fraction in which cyclic electron transfer to A$_1$ takes place. At temperatures below ~200 K, this fraction accounts for about 1/3 of the centers, whereas stable charge separation between P700 and F$_A$/F$_B$ occurs in the remaining 2/3 (see Ref. 20 for review).

In Figs. 5 and 6, the spin-polarized transient EPR spectra of PS I complexes from the menA and menB mutants are compared with the wild type at X-, Q-, and W-bands (9, 35, and 94 GHz, respectively). The spectra of the two mutants are depicted as **solid lines**, and those of the wild type are depicted as **dashed curves**. In the spectrum of the wild type the low field features are due to A$_1^-$, whereas the high field region is dominated by P700$^+$. For the mutants, the high field sides of the spectra are very similar to the corresponding wild type spectra, whereas there is a clear shift of the features on the low field side, particularly at the W-band. The partially resolved hyperfine couplings to the methyl group in position 2 of the phylloquinone (Fig. 1), discussed above with Fig. 4, also result in the feature on the central absorptive maximum of the spin-polarized X-band and Q-band spectra of the wild type (Figs. 5 and 6, dashed lines). Again, these features are absent in the spectra of the menA and menB mutants (**solid lines**).

In general, the overall spectral width increases as the microwave frequency is increased, i.e. in proceeding from X-band (Figs. 5 and 6, **bottom**) to W-band (Figs. 5 and 6, **top**), because the components of the quinone g-tensor are more highly resolved at higher field/frequency. The features on the low field side of the transient EPR spectra are shifted downfield compared with the wild type, and the magnitude of the shift increases with increasing microwave frequency. This behavior is in agreement with the CW EPR finding at Q-band (Fig. 4) that Q in the menA and menB mutants has a larger $g$-anisotropy than phyloquinone, and additionally, it demonstrates that Q is actively involved in the electron transfer and is not simply a low quantum yield trap.

The emissive and absorptive (Figs. 5 and 6, E and A, respectively) components of the high field and low field regions of the spectra have equal intensity because the radical pair P700$^+$ A$_1$ is generated from a singlet precursor on a picosecond time scale. If the lifetime of the precursor is sufficiently long that appreciable singlet-triplet mixing takes place, i.e. longer than about 0.5 ns, net polarization develops on each of the radicals (21–23). No net polarization of this type is visible in the spectra of the menA and menB mutants; thus we can conclude that the electron transfer to Q occurs on a time scale less than roughly 0.5 ns. If the A$_1$ site in the mutants were vacant or forward electron transfer were slow, charge recombination from A$_0$
would generate a spin-polarized EPR spectrum of $^3\text{P}_700$ (24–26). Thus, the observation of a weakly coupled radical pair spectrum and no significant contribution from the triplet state of the donor $^3\text{P}_700$ leads to the conclusion that efficient forward electron transfer past $A_0$ occurs in the mutants and suggests that the $A_1$ binding site is fully occupied.

For native PS I, simulations of the polarization patterns of $\text{P}^+\text{A}_1^-$ (1, 2, 7, 17) show that $A_1$ is oriented such that the carbonyl bonds of the phylloquinone head group are parallel to the vector joining $\text{P}_700^-$ and $A_1^-$. Qualitatively, this orientation of the quinone is reflected in the emissive feature on the low field edge of the spectra shown in Figs. 5 and 6 (dashed lines). For the mutants, simulations (not shown) using the same parameters as found for wild type PS I (17) but with a value of $g_{xx} = 2.0067$ for the acceptor gives excellent agreement with the experimental polarization patterns at all three microwave frequencies. Thus we can conclude that the acceptor is bound at the $A_1$ site. Recently, ESE experiments have been used to determine the distance between $\text{P}_700^-$ and $A_1^-$ in native PS I (3–5) and to demonstrate that the distance remains the same for a number of nonnative quinones used in $A_1$ extraction/substitution experiments (4, 29). The ESE of a weakly coupled spin correlated radical pair is phase shifted by 90° compared with that of a single radical and shows deep amplitude modulations as a function of the pulse spacing (30). The modulation frequency is selectively determined by the spin-spin coupling, the dipolar part of which yields the distance between the radicals. The out-of-phase ESE amplitude modulation curves from the mutants and native PS I are compared in Fig. 7. The solid curves are from the mutants $\text{menA}$ (Fig. 7, top) and $\text{menB}$ (bottom), whereas the result for the wild type is shown as a dashed curve for reference. Comparison of the echo modulation curves shows that the dominant frequency components from both mutants and the wild type are virtually identical.

The values for the dipolar and exchange coupling parameters can be determined quantitatively by comparison of the experimental echo modulations with numerical simulations calculated using the correlated coupled radical pair model. An important feature of such simulations is that the anisotropies of the $g$-tensor and hyperfine splittings do not influence the out-of-phase echo modulation significantly and can be safely ignored (5, 31, 32). Simulation of the ESE modulation curves in Fig. 7 yields dipolar coupling constants, $D = -172 \pm 4 \mu\text{Tesla}$ for both mutant samples. This value is identical within error to that ($D = -170 \pm 4 \mu\text{Tesla}$) obtained for the wild type sample (29). In all three samples, the isotropic exchange coupling is extremely weak, and only an upper limit of $J = 1.0 \pm 0.8 \mu\text{Tesla}$ can be given. This small value is consistent with the
Pulsed ENDOR Spectroscopy—CW EPR spectroscopy used in photoaccumulation and transient studies is capable of resolving information on the aromaticity of the foreign quinone in its binding site. Fig. 8 shows the pulsed ENDOR spectra of the \( \text{P700}^+ \) state in native PS I complexes from \( \text{menA} \) (solid curve, top), \( \text{menB} \) (solid curve, bottom), and wild type (dashed curves) at 80 K. Experimental conditions are described under “Experimental Procedures.”

The recruited quinone acceptor in the mutants is indeed bound to the native A\(_1\) site. This corresponds to the outer wings of the spectra. Shown is a Davies type ENDOR pulse sequence; temperature, 80 K.

The spectral ranges assigned to the CH\(_3\) hyperfine couplings in \( \text{Q}^- \) are shaded. The vertical dashed lines at about 8.3 and 21.1 MHz in the traces of \( \text{menA} \) and \( \text{menB} \) indicate the outer wings of the spectra. These lines are assigned to the CH\(_3\) of plastoquinone-9 in frozen solution and 11.6 MHz for plastoquinone-9 in liquid and in frozen alcoholic solution, as well as for the reduced primary acceptor QA in PS II (34). The invariance of the CH\(_3\) groups for the reduced primary acceptor QA in PS II (34) is very similar, indicating substantial asymmetry in the dense distribution of plastoquinone-9 in the A\(_1\) binding site.

The second is that the ENDOR spectra of the \( \text{P700}^+ \) state in \( \text{menA} \) (top) and \( \text{menB} \) (middle) and, for comparison, the \( \text{P700}^- \) state in native PS I complexes from \( \text{Synechococcus elongatus} \) (bottom). The first observation is that \( \text{menA} \) and \( \text{menB} \) spectra are identical within the available signal to noise ratio. The second is that spectra are drastically different from the \( \text{P700}^- \) A\(_1\) spectrum taken in \( S. \ elongatus \). This is shown by the clearly resolved lines of an (almost) axially symmetric tensor in the range of 8.6–10.6 MHz and 18.9–20.9 MHz in the wild type PS I complex. These lines are assigned to the 2-CH\(_3\) group of phylloquinone, with \( \Lambda_{\parallel} = 12.8 \text{ MHz} \) and \( \Lambda_{\perp} = 9.0 \text{ MHz} \) (19). The pulsed ENDOR studies in both the light-induced (transient) \( \text{P700}^- \) A\(_1\) state and on photoaccumulated A\(_1\) agree (33), thereby bolstering confidence in the ability of the latter to measure the quinone in the active A\(_1\) site.

It is clear that this pair of lines is missing in PS I complexes isolated from the \( \text{menA} \) and \( \text{menB} \) mutants (Fig. 8). Instead, two pairs of lines of similar shape arise in the \( \text{menA} \) and \( \text{menB} \) due to an axially symmetric hyperfine coupling tensor. These line pairs in the ranges (i) 9.6–11.4 and 18.0–19.8 MHz and (ii) 12.0–13.4 MHz and 16.2–17.5 MHz correspond to coupling tensors with (i) \( \Lambda_{\parallel} = 9.8 \pm 0.2 \text{ MHz} \) and \( \Lambda_{\perp} = 6.8 \pm 0.2 \text{ MHz} \) and (ii) \( \Lambda_{\parallel} = 5.8 \pm 0.2 \text{ MHz} \) and \( \Lambda_{\perp} = 2.8 \pm 0.2 \text{ MHz} \). The similar shape of the two sets of lines and their similarity to the corresponding shape in the PS I spectrum hints at their assignment to two inequivalent methyl groups in the recruited quinone. Both sets of hyperfine couplings are smaller than those of the 2-CH\(_3\) group of phylloquinone in native PS I, and they differ significantly from each other, indicating substantial asymmetry in the spin density distribution of the recruited quinone. Finally, the ENDOR spectra of the \( \text{menA} \) and \( \text{menB} \) mutants in Fig. 8 show clear spectral wings extending out to 8.3 and 21.1 MHz, respectively. They exhibit the opposite sign in signal intensity compared with the inner signal contributions assigned above to two methyl groups. This opposite sign is most readily associated with an opposite relative sign of the respective hyperfine couplings. This suggests the assignment of the outer wings in the ENDOR spectrum to the H atom of an aromatic C-H fragment in the recruited quinone.
This finding is further supported by the largest coupling of the 
\( \alpha \)-proton, with \( A_{\text{expt}} = -12.8 \text{ MHz} \) assigned to the spectral wings with negative intensity in Fig. 8. The corresponding tensor component was reported as \( A_{\text{expt}} = -9.0 \text{ MHz} \) (and thus significantly smaller) for the plastoquinone-9 radical anion in frozen alcoholic solution (34). A similar increase of the hyperfine coupling of the \( \text{CH}_2 \) group in the corresponding position 2 of phylloquinone is observed between frozen alcoholic solution and \( A_{1}^- \) in native PS I. The average \( \text{CH}_2 \) hyperfine coupling for phylloquinone increases from 7.9 MHz in frozen solution to 10.2 MHz for \( A_{1}^- \) (19). Assuming an equivalent spin density increase at the carbon positions 2 and 6 of the recruited Q, we assign the larger of the two \( \text{CH}_2 \) hyperfine coupling tensors of \( Q^- \) to the group attached to ring position 6 and the smaller \( \text{CH}_3 \) coupling tensor to the one attached to position 5, respectively.

**DISCUSSION**

Although all of the methods presented above provide evidence for a foreign quinone \( Q \) incorporated into the \( A_1 \) binding site, no firm identification of \( Q^- \) is possible on the basis of any one method alone. However, the EPR and ENDOR spectroscopic results presented in this work provide strong support for the proposal that plastoquinone-9 is recruited by the \( \text{menA} \) and \( \text{menB} \) mutants as a substitute for the missing phylloquinone in PS I. Plastoquinone-9 binds in the same \( A_1 \) site with the same orientation, and the specific protein environment induces a similar asymmetry in the spin density distribution. This conclusion is backed up by the finding that plastoquinone-9, but not phylloquinone, is present in PS I complexes isolated from the \( \text{menA} \) and \( \text{menB} \) mutants, whereas phylloquinone, but not plastoquinone-9, is present in PS I isolated from the wild type (13).

The asymmetric spin density distribution found for \( Q \) in the mutants and phylloquinone in native PS I is consistent with an increased spin density at ring position 2 (i.e., between the carbonyl group in position 1 and the hydrocarbon chain in position 3; see Fig. 1). This asymmetric spin density is exactly opposite that for \( Q_A \) in bacterial RCs (see Ref. 35 for a review). Here, a decrease in spin density is observed for the corresponding position 2. The latter is attributed to a stronger H-bond to the carbonyl in position 1 (chain in position 3). This is also concluded from the bacterial RC structure, which shows strong H-bonding to the carbonyl in position 1 of \( Q_A \) from the His residue in the \( \delta \)-helix, which also ligates the nonheme iron. Weaker backbone H-bonding is suggested for the other carbonyl bond of \( Q_A \).

If the opposite direction of spin density asymmetry observed for \( A_1 \) in PS I compared with \( Q_A \) in bacterial RCs is attributed to inequivalent H-bonds to the quinone carbonyl, then the preferential H-bond has to be inverted between PS I and the bacterial RC. The present PS I structure (10) supports the concept of a difference in H-bonding to \( A_1 \) compared with \( Q_A \) in purple bacterial RCs. There is a direct correspondence between the transmembrane helices in PS I and bacterial RC, but there are significant differences in the quinone binding region. The \( \delta \)-helix of PS I ends earlier toward the stromal side compared with the corresponding \( \delta \)-helix of the bacterial RC. Moreover, there is no histidine residue in the \( \text{PsaA/B} \) primary sequence in this region. Hence, the strong H-bond donor for \( Q_A \) in the bacterial RC has no analogue in PS I. This may also be one of the reasons for the significantly different orientation and position of \( A_1 \) relative to the rest of PS I (7). On the other hand, sufficiently strong H-bonding to the other carbonyl group of phylloquinone in the \( A_1 \) binding site is neither excluded nor supported by the presently available PS I structure.

O’Malley (36–38) reached similar conclusions on the basis of spin density functional calculations. He was able to explain quantitatively the observed spin densities for \( Q_A \) in purple bacterial RCs (36) versus \( A_1 \) in PS I (37) by assuming a preferential strong H-bond to one of the carbonyl groups and to the opposite one in each of the respective quinone acceptor. Experimental evidence is provided here that the same direction of asymmetric spin density distribution (and correspondingly the same H-bonding pattern) is observed for plastoquinone-9 recruited in the \( A_1 \) site as for phylloquinone in native PS I.

Further comparison can be made to the asymmetric spin density distribution observed for plastoquinone-9 in solution and as \( Q_A \) in PS II. It is interesting to note that an asymmetric spin density distribution has been calculated for plastoquinone-9 in isotropic solution (38), presumably as a result of the inherent asymmetry of the substituents (see Fig. 1). Indeed, a substantial anisotropy has already been observed for plastoquinone-9 in frozen solution (34). Compared with this intrinsic asymmetry for plastoquinone-9 in solution, the asymmetry is found in this work to increase for plastoquinone-9 recruited as \( Q \) in PS I mutants and, in contrast, to decrease for plastoquinone-9 as \( Q_A \) in PS II (34). The latter is consistent with the assumption of an asymmetric H-bonding similar to that established for purple bacterial RCs, see (35). As a consequence, preferential H-bonding appears to be operative in all mentioned RCs. Interestingly, the stronger H-bond concerns opposite carbonyl groups (with respect to the common ring numbering used in Fig. 1) for quinones in the \( A_1 \) site of PS I versus quinones in the \( Q_A \) site of PS II and purple bacterial RCs.

The results raise a number of interesting issues concerning the nature of the \( A_1 \) binding site. First, the fact that the \( A_1 \) site is occupied in the absence of phylloquinone means that under normal conditions there must be strong competition in favor of phylloquinone as compared with plastoquinone-9, both of which are presumed to be present during the biosynthesis and assembly of PS I. Thus, we conclude that the binding affinity of phylloquinone is higher. Given the relatively high concentration of plastoquinone-9 in the membrane, the difference in the binding constants must be quite substantial. One reason for a higher affinity could be structural constraints, i.e., the binding site could be designed to accommodate phylloquinone better than plastoquinone-9. Evidence to support this idea comes from quinone exchange experiments that show that parameters such as hydrophobicity influence the binding affinity of quinones to PS I (39, 40). The structure of the respective quinones may also play a role. The binding of duroquinone (2,3,5,6-tetramethylbenzoquinone) is weaker than predicted by its hydrophobicity (41), and out-of-phase ESE experiments on PS I containing duroquinone suggested a wider distribution of P700+ Q- distances than for P700+ A1- in the native PS I, which could be an indication that the poorer binding is due to steric effects (27). However, the spin polarization patterns of the mutants show that any increase in steric hindrance for plastoquinone-9 compared with phylloquinone is not accompanied by a different orientation of the two acceptors.

Another possible explanation for different binding affinities of phylloquinone and plastoquinone-9 has been given by Zheng and Dismukes (42). For the quinones with a \( \text{CH}_2 \) group at position 2, such as ubiquinone-10 and phylloquinone, the side chain at position 3 is attached perpendicular to the quinone head group plane (dihedral angle \( \alpha \) about 90°), whereas for quinones with a hydrogen at position 2, the side chain is in plane with the head group (dihedral angle \( \alpha \) about 0°) in the ground state. A difference in energy separation between ground state and excited state for opposite side chain to head group conformation of about 6 kcal/mol was estimated, yielding a greater than 10-fold excess of the ground state versus excited state population at room temperature. An
optimized protein structure for the respective quinone ground state conformations has been proposed by Zheng and Dismukes (42) as a possible explanation for the low efficiency of substitution of ubiquinone-10 with plastoquinone-9 in bacterial RCs (43) and plastoquinone-9 by ubiquinone-9 in PS II (44). Because phylloquinone has the same ground state configuration as ubiquinone-9 in PS I, this argument would also explain the selectivity of PS I for phylloquinone.

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