A photosystem I (PS I) complex containing plastoquinone-9 (PQ-9) but devoid of F₆₅₀, F₆₇₀, and F₇₃₀ was isolated and characterized from a mutant strain of Synechococcus sp. PCC 7002 in which the menB and rubA genes were insertionally inactivated. In isolated PS I trimers, the decay of P₇₀₀− measured in the near-IR and the decay of A₁− measured in the near-UV were found to be biphasic, with (averaged) room temperature lifetimes of 12 and 350 μs. The decay-associated spectra of both kinetic phases are characteristic of the oxidized minus reduced difference spectrum of a semiquinone, consistent with charge recombination between P₇₀₀⁺ and PQ-9−. The amplitude of the flash-induced absorbance changes in both the near-IR and the near-UV show that approximately one-half of the A₁ binding sites are either empty or nonfunctional. A spin-polarized chlorophyll triplet is observed by time-resolved EPR, and it is attributed to the 3P₇₀₀ product of P₇₀₀−A₀⁺ recombination via the Tₐ spin level in those PS I complexes that do not contain a functional quinone. In those A₁ sites that are occupied, the P₇₀₀−Q− polarization pattern indicates that PQ-9 is oriented in a similar manner to that in the menB mutant. When excess 9,10-anthraquinone is added in vitro, it displaces PQ-9 and occupies the A₁ binding site more readily than in the menB mutant. This can be explained by a greater accessibility to the A₁ site in the menB rubA mutant due to the absence of F₆₅₀ and the stromal ridge polypeptides. The relatively low binding affinity of 9,10-anthraquinone allows it to be readily removed from the A₁ site by washing. However, all A₁ sites are shown to bind napthoquinones with high affinity and thus are proven to be functionally competent in quinone binding. The ability to readily displace PQ-9 from the A₁ site makes the menB rubA mutant ideal for introducing novel quinones, particularly anthraquinones, into PS I.

Photopsystem I is a multisubunit, pigment-protein complex that is found in the membranes of plants, algae, and cyanobacteria and that mediates the light-induced transfer of electrons from plastocyanin/cytochrome c₆ to ferredoxin/flavodoxin. According to current understanding, light-induced charge separation results in the oxidation of the primary electron donor P₇₀₀ (Eₘ⁺+430 mV), a chlorophyll a/a′ heterodimer located on the luminal (inner) side of the membrane, and the reduction of the primary electron acceptor A₁ (Eₘ− approximately −1000 mV), a chlorophyll a monomer located in the interior of the membrane. The electron is passed to A₁ (Eₘ− approximately −800 mV), an alkyl-substituted menadione (2-methyl-1,4-naphthalediencarbonitrile) to F₆₅₀ (Eₘ−705 mV), an interpolyptide (4Fe-4S) cluster; and finally to F₆₇₀ (Eₘ−520 mV) and F₇₃₀ (Eₘ−580 mV), which are (4Fe-4S) clusters bound to the extrinsic subunit PsaC located on the stromal (cytoplasmic) side of the membrane. In most organisms, including Synechocystis sp. PCC 6803, the quinone in the A₁ site is phylloquinone (2-methyl-3-phytyl-1,4-naphthoquinone), but in Euglena gracilis and Anacystis nidulans, the quinone is 5′-monohydroxyphylloquinone (1), and in the red alga Cyanidium caldarium it is menaquinone-4 (MQ-4)² (2).

Our approach to studying structural and functional relationships involving A₁ is to replace the native quinone in PS I with quinones that have different thermodynamic and structural properties but are still able to mediate electron transfer from A₁ to the Fe/S clusters. The replacement of the quinone can be accomplished either in vitro using chemical extraction and reconstitution protocols (3) or in vivo using genetic approaches (4). In the latter method, the mena or menB genes (5–7) or the menD or menE genes (8), which code for enzymes in the phylloquinone (PhQ) biosynthetic pathway, have been interrupted in Synechocystis sp. PCC 6803. In the absence of PhQ, PS I recruits plastochinone-9 (PQ-9), which is normally associated with PS II, into the A₁ site. When present in the quinone binding site of PS II, PQ-9 has a midpoint potential of 68 mV (9) to 80 mV (10), but when PQ-9 occupies the A₁ site of PS I, it has an estimated midpoint potential of −670 mV (11). PQ-9 can be displaced both in vivo (7) and in vitro (12) by a variety of...
substituted naphthoquinones, including authentic phylloquinone, thus allowing detailed spectroscopic analyses of this essential cofactor.

In the experiments described in this paper, we extend our studies of PSI complexes that contain PQ-9 in the A1 site to a mutant in which the Fe/S clusters F_b, F_p, and F_c are also missing. This was achieved in Synechococcus sp. PCC 7002 by interrupting the *menB* gene, which codes for 1,4-dihydroxy-2-naphthoate synthase (5–7, 11), as well as the *rubA* gene, which codes for a membrane-bound rubredoxin (13, 14). We isolated PSI complexes from the resulting *menB* rubA mutant and evaluated the kinetics of charge recombination between P700+ and PQ-9−. The structural and functional properties of PQ-9− correspond to those in the *menB* mutant. We additionally show that these PSI complexes can reversibly incorporate 9,10-anthraquinone into the A1 binding site more efficiently than in the *menB* mutant.

**MATERIALS AND METHODS**

**Construction of the *menB* and *menB rubA* Mutants—**The *menB* mutant was constructed in *Synechocystis* sp. PCC 6803 as described previously (6). Wild type and mutant strains of *Synechococcus* sp. PCC 7002 were grown as described previously (14, 15). A DNA fragment containing the *menB* gene was cloned and sequenced from the genome of *Synechococcus* sp. PCC 7002 (GenBank™ accession number AY563042). This *menB* gene was identified by the high sequence similarity (89%) of its product with that of *slr1127* (*menB*) of *Synechocystis* sp. PCC 6803. The *acoC1* gene, which was derived from plasmid pMS266 after restriction digestion with PstI and which contains gentamicin resistance, was inserted into the unique PstI site of the *menB* coding region. This construct was used to transform cells of the kanamycin-resistant *rubA* mutant of *Synechococcus* sp. PCC 7002 as described in Ref. 14. Full segregation of the *menB::acoC1* and *menB::alleles was verified by PCR analysis.

**PQ-9− and Chlorophyll Analysis—**Cells of *Synechococcus* sp. PCC 6803 or *Synechococcus* sp. PCC 7002 were broken using a French pressure cell operated at 4 °C at 120 megapascals. Thylakoid membranes were prepared in 150 mM sodium Tris buffer, pH 8.3, containing 0.2% Triton X-100 and 0.5 mM P700−. Sodium ascorbate and 1.6-dichlorophenolindophenol were added to final concentrations of 2 mM and 5 μM, respectively. β-DM was added to a final concentration of 0.04% (w/v) to reduce light scattering. A differential extinction coefficient of 8000 M−1 cm−1 at 820 nm was used for calculations of P700+/P700 concentration.

**Time-resolved Optical Spectroscopy in the Visible—**Optical studies in the UV were conducted using a pulse probe spectrometer described in Ref. 21. Measuring flashes were supplied by a xenon lamp and selected by a 1/4 meter monochrometer incorporating a 10-cm × 10-cm interference grating blazed at 350 nm. The bandwidth of the measuring flash was 5 nm, and the data were recorded in 5-nm intervals from 400 to 600 nm. Excitation flashes were provided by a Q-switched, Nd:YAG laser (Brilliant®, Quantel S. A., Les Ulis, France) equipped with an optical 0.04% P700− indicator (Vibrant Arrow 355, type II crystal) tuned to 685 nm. The photodiodes were protected with cyan subtractive dichroic filters (Edmund H52-536). The sample was placed in a 10 × 10-mm quartz cuvette perpendicularly to the direction of the excitation flash. An identical sample was placed in a 10 × 10-mm quartz cuvette in the reference beam. Each data point represents the average of 16 measurements taken at a flash intensity of 3.4 × 1016 photons cm−2 s−1. An extinction coefficient of 1.59 × 104 M−1 cm−1 is used for calculations of [Car] in *Synechocystis* sp. P1 may differ considerably.

**Data Analysis—**Multieponential fits of the optical kinetic data were performed using the Marquardt algorithm in Igor Pro version 3.14 (WaveMetrics Inc., Lake Oswego, OR) running on a Macintosh computer. For global analyses in the visible region, individual kinetics were analyzed first. The results of these analyses were used for fitting the whole set of data to global lifetimes, and the best solution was chosen based on the analysis of χ2, standard errors of the parameters, and the residuals. In several instances, several closely spaced kinetic components were required to fit the data at the longer times. A stretched multieponential fitting routine was employed in such cases (24); the stretch parameter, β, assumes a value between 0 and 1. This equation represents a robust solution of a general equation for kinetics with a distributed time constant; in the case when β = 1, the equation turns into a sum of simple exponentials.

**PQ-9− Exchange into the menB and menB rubA Mutants—**A 100-fold molar excess of 9,10-anthraquinone (10 μM of 0.034 mM 9,10-anthraquinone in Me2SO) was added to PSI complexes (150 μM in Tris-HCl buffer pH 8.3 containing 0.2% Triton X-100) isolated from the *menB* and *menB rubA* mutant strains of *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7002, respectively. The incubation was carried out at room temperature (2–4 h) with vigorous stirring. In an additional step, the PSI complexes were washed twice by ultrafiltration with 150 μl of buffer solution to remove the excess 9,10-anthraquinone as well as exchanged PQ-9−. The washed PSI complexes were resuspended in 150 μl of buffer and stored at −80 °C.

**Time-resolved Optical Spectroscopy in the Near-IR—**Optical studies in the near-IR were conducted using a laboratory-built, time-resolved spectrophotometer. The high frequency roll-off amplifier described in Ref. 20 was not used to ensure resolution of the saturating pulse phases in the submicrosecond range. A tunable titanium-sapphire laser (Schwartz Electro-Optics, Orlando, FL) was pumped at 532 nm by using a 5-watt, frequency-doubled CW YAG laser (Millenia® Series; Spectra Physics) and provided the 820-nm measuring beam. The actinic flash was provided by a frequency-doubled, Nd:YAG laser (Quanta-Ray DCR-11, Spectra Physics). For most studies, the excitation flash intensity was adjusted to ~2 μJ cm−2, which is just sufficient to saturate P700+ under the conditions employed. For studies of the dependence of the signal intensity on the excitation flash energy, the energy was varied from 1 μJ cm−2 to 80 μJ cm−2. The flash energy was adjusted by the timing of the Q-switch and by the use of neutral density filters. PSI complexes were diluted under anaerobic conditions with 50 mM Tris-HCl buffer, pH 8.5. A final concentration of 0.034 mM P700−. Sodium ascorbate and 1.6-dichlorophenolindophenol were added to final concentrations of 2 mM and 5 μM, respectively. β-DM was added to a final concentration of 0.04% (w/v) to reduce light scattering. A differential extinction coefficient of 8000 M−1 cm−1 at 820 nm was used for calculations of P700+/P700 concentration.

**PQ-9− Exchange into the *Synechococcus* sp. PCC 6803—**The PSI complexes was isolated from the wild type, the *menB* mutant, and the *menB rubA* mutant were obtained using a Bruker ECS-106 spectrometer equipped with an Oxford temperature controller and cryostat. The instrument conditions for *F_b* and *F_p* were as follows: microwave power, 20 milliwatts; temperature, 15 K; and modulation amplitude, 10 G. The sample was suspended to 0.6 mg/ml Chl in 50 mM Tris buffer, pH 8.3, containing 10 mM sodium ascorbate and 30 μM 2, standard errors of the parameters, and the

![Image](https://example.com/image.png)
enzyme cleavage sites in wild type and the menB rubA mutant are shown in a Bruker ER 056 QMV microwave bridge, equipped with a home-built modulation amplitude, 32 G. The sample was suspended to 0.6 mg/ml as follows: microwave power, 80 milliwatts; temperature, 6 K; and system operating at the second harmonic (533 nm) and a repetition rate of 32 G. The samples were illuminated using a Spectra Physics Nd-YAG laser and 2,6-dichlorophenol-indophenol. The instrument conditions for the FX,F A, and F B iron-sulfur clusters were similarly not detected when PS I complexes were treated with sodium hydrosulfite.

Flash-induced Absorbance Changes in the Near-IR and UV Regions—Fig. 2 (top) shows decay kinetics measured at 820 nm in PS I complexes isolated from the menB rubA mutant. Under the conditions employed, the absorbance change after a saturating flash corresponds primarily to the decay of P700°. The best fit to the data results in kinetic phases with lifetimes (stretch parameters) of 1.9 μs (0.92), 10.3 μs (0.97), and 315 μs (0.74). The longest kinetic phase represents the major compo-

FIG. 1. Restriction maps of the menB gene in wild type and the menB rubA mutant in Synechococcus sp. PCC 7002. Restriction enzyme cleavage sites in wild type and the menB rubA mutant are shown in a and b, respectively. Primers used to amplify and clone the menB gene are shown in black short arrows. PCR analysis on the rubA mutant (lane 1) and the menB rubA mutant (lane 2) to verify the segregation of the menB and menB::aacC alleles is shown in c.

2,6-dichlorophenol-indophenol. The instrument conditions for FX were as follows: microwave power, 80 milliwatts; temperature, 6 K; and modulation amplitude, 32 G. The sample was suspended to 0.6 mg/ml Chl in 100 mM glycine buffer, pH 10.0, containing 10 mM sodium hydrosulfite.

Transient EPR Spectroscopy at X-band and Q-Band—Low temperature, X-band (9-GHz) transient EPR experiments were performed with a laboratory-built spectrometer using a Bruker ER046 XKT microwave bridge equipped with an ER-4118X-MD-5W1 dielectric ring resonator and using an Oxford CF935 helium gas flow cryostat (25). The loaded Q value for this dielectric ring resonator was about 3000, equivalent to a rise time of τr = Q/(2π×τm) = 50 ns. Q-band (35-GHz) transient EPR spectra of the samples were measured with the same set-up except that a Bruker ER 056 QMV microwave bridge, equipped with a home-built cylindrical resonator, was used. All samples contained 1 mM sodium ascorbate as an external electron donor and were frozen in the dark. The samples were illuminated using a Spectra Physics Nd:YAG laser system operating at the second harmonic (533 nm) and a repetition rate of 10 Hz.

RESULTS

Construction of the menB rubA Mutant in Synechococcus sp. PCC 7002—The menB gene of Synechococcus sp. PCC 7002 was inactivated by inserting the aacC1 gene, conferring gentamicin resistance, from plasmid pMS266 into the unique PstI site within the coding sequence of the gene (Fig. 1, a and b). After transformation of this construction into the rubA mutant of Synechococcus sp. PCC 7002, segregation of the menB::aacC1 and menB alleles in the rubA mutant strain was analyzed by PCR. As expected for the parental strain (Fig. 1a), the PCR using the designed primers resulted in a product of 1.0 kb (Fig. 1c, lane 1). In the transformed rubA strain, however, the 1.0-kb product is absent, and a new product of 2.1 kb was detected (Fig. 1c, lane 2). The difference in the sizes of the PCR products from the parental and transformant strains corresponds to the size of the inserted 1.1-kb gentamicin resistance cartridge (Fig. 1, a and b). The data show that the transformed rubA mutant strain is homozygous for the menB::aacC1 allele.

HPLC Pigment Analysis of Synechococcus sp. PCC 7002—Authentic PhQ and PQ-9 elute at 22 and 36 min, respectively, using the HPLC protocol described under “Materials and Methods.” When the pigment extracts from whole cells and PS I complexes from wild-type Synechococcus sp. PCC 7002 were analyzed, no UV-absorbing compounds were detected at 22 min. Further examination of the chromatogram led to the discovery of a new peak eluting at 14 min that showed an intense UV absorption with maxima at 248, 263, 270, and 332 nm. The spectrum is consistent with a 1,4-naphthoquinoid compound having alkyl substitutions at the C2 and C3 positions (16). Mass spectroscopic analysis showed that the compound has an m/z of 444 as opposed to an m/z of 450 for PhQ. This difference is most easily explained by the presence of a geranylgeranyl tail with four fully unsaturated isoprenoid units, which is characteristic of MQ-4. Thus, Synechococcus sp. PCC 7002 probably synthesizes 2-methyl-3-all-trans-tetraisopropenyl-1,4-naphthaledione (MQ-4) instead of 2-methyl-3-(3,7,11,15-tetramethyl-2-hexadecenyl)-1,4-naphthaledione (PhQ). The transient EPR spectra of wild-type PS I complexes isolated from Synechocystis sp. PCC 6803 and Synechococcus sp. PCC 7002 were identical at both X- and Q-bands, indicating that the difference in the degree of unsaturation of the C2 tail has no detectable influence on the orientation of the 1,4-naphthaledinedione head group in the A1 site (data not shown).

When the pigment extracts from whole cells of the menB rubA mutant were analyzed, no UV-absorbing material eluted at 14 min. This indicates that the menB homolog indeed encodes 1,4-dihydroxyxynaphthoate synthase in Synechococcus sp. PCC 7002 and that it is required for the synthesis of MQ-4. The absence of MQ-4 was confirmed in pigment extracts from PS I complexes; however, a peak appeared at 36 min with an m/z of 748 characteristic of PQ-9. This observation is in agreement with a previous study in which the interruption of PhQ biosynthesis in Synechocystis sp. PCC 6803 results in the incorporation of PQ-9 into the PS I (6). These results indicate that the interruption of 1,4-dihydroxy-2-naphthoate synthase in Synechococcus sp. PCC 7002 similarly results in the incorporation of PQ-9 into PS I.

Low Temperature CW EPR Spectroscopy in PS I Complexes from the menB rubA Mutant—The FX, F A, and F B iron-sulfur clusters were not detected in PS I complexes isolated from the menB rubA mutant when measured by low temperature CW X-band EPR spectroscopy (data not shown). The iron-sulfur clusters were similarly not detected when PS I complexes were treated with sodium hydrosulfite at pH 10.0 in an attempt to reduce FA° and F B; additionally, no spectrum was obtained by illuminating the same sample during freezing in an attempt to photoaccumulate FX°. These results are identical to those for PS I complexes isolated from the rubA mutant and indicate that all three (4Fe-4S) clusters are missing from the PS I complexes of the menB rubA mutant as expected (13, 14).
ponent to the overall signal amplitude (~75%), and the stretch parameter indicates that this value encompasses a broader distribution of lifetimes than the faster components. Fig. 2 (bottom) shows decay kinetics measured at 315 nm in PS I complexes isolated from the menB rubA mutant. In this spectral region, the flash-induced absorbance change corresponds primarily to the decay of PQ-9. The best fit to the data results in kinetic phases with lifetimes (stretch parameters) of 15 μs (1.00), 392 μs (1.00), and a long lived residual. (Due to the time resolution of the spectrometer, it could not be determined whether the 1.9-μs kinetic phase measured in the near-IR is also present in the near-UV.) The similarity in the lifetimes of the two slower kinetic components in the near-IR and near-UV suggests that these two kinetic phases can be assigned to the same process, namely charge recombination between P700 and PQ-9. The total absorbance change (excluding the unassigned 1.9-μs component) in the near-IR (Fig. 2, top) corresponds to 223 nM P700+, which is equivalent to 251 Chl/P700 given that the total Chl a content in the sample was 56 μg (50 μg/ml Chl a). Similarly, the total absorption change in the near-UV (Fig. 2, bottom) corresponds to 46 nM PQ-9−, which is equivalent to 239 Chl/P700, given that the total Chl a content in the sample was 11 μM (10 μg/ml Chl a). Because cyanobacterial PS I trimers contain 96 Chl/P700, 40–45% of the expected flash-induced absorbance change in the menB rubA mutant can be accounted for by long lived charge separation between P700 and PQ-9. Thus, the near-IR and the near-UV spectral data are in agreement that less than one-half of the quinone binding sites on the redox-active chain(s) of electron transfer cofactors contain functional PQ-9. Either the remaining quinone binding sites are empty or the sites contain quinones that are not functional in electron transfer.

Decay-associated Spectra in the Near-UV and Visible Region—Fig. 3 (top) shows the point-by-point difference spectrum from 250 to 340 nm of flash-induced absorbance changes taken at 10 μs, 100 μs, and 1 ms after a saturating flash. All three spectra have a similar derivative shape with a peak at 316 nm, a crossover at 287 nm, and a trough at 264 nm and are consistent with the difference spectrum of PQ-9−/PQ-9 (11). However, when compared with the sum of the spectra from the two kinetic phases measured in the menB mutant, (Fig. 3, top, dotted line), the amplitude of the sum of the spectra from the three kinetic phases in the menB rubA mutant is lower due to the large number of unoccupied or nonfunctional quinone binding sites, and the peak maximum is red-shifted by ~7 nm. The latter change may reflect either a structural difference in the
environment of PQ-9 due to the absence of the $F_X$ cluster and the stromal ridge proteins ($PsaC$, $PsaD$, and $PsaE$) or to a change in the electronic properties of the semiquinone radical due to the absence of the net $(-2)$ charge on the oxidized $F_X$ cluster.

Fig. 3 (bottom) depicts a global fit of the flash-induced absorbance changes in the 380–600-nm visible region for PS I complexes isolated from the $menB$ rubA mutant. The 9-μs kinetic phase may represent an admixture of two components. One component, which has a peak at 530 nm, a positive-going shoulder at 490 nm, a crossover at 478 nm, and weaker set of bleachings at 460 and 440 nm, probably represents the decay of a carotenoid triplet (27). The total absorbance change at 530 nm corresponds to 40 nM 3Car, which is equivalent to 280 Chl/3Car given that the Chl content in the sample was 11 μM (10 g/ml). This represents $-0.33$ 3Car/P700. Wild-type PS I complexes show a flash-induced absorbance change that represents less than $0.08$ 3Car/P700 (data not shown), which would suggest an increased generation of 3Car in PS I complexes from the $menB$ rubA mutant. However, the bleaching at 430 nm (Fig. 3, bottom) is too large to be accounted for solely by a triplet-minus-singlet spectrum of a carotenoid (27). The other component may therefore represent a contribution from P700/P700, which is expected based on the existence of a similar decay component with a 10–15 μs lifetime in the near-IR and near-UV (Fig. 2, top and bottom). The 585-μs kinetic phase (Fig. 3, bottom) represents a P700/P700$^-$ difference spectrum that is probably derived from charge recombination between P700$^-$ and PQ-9. Its lifetime is somewhat longer lived than the 315- and 392-μs kinetic phases observed in the near-IR and the near-UV regions, respectively, although this relatively minor difference may be a consequence of the spectral decomposition procedure.

Time-resolved EPR Observation of a 3P700 Product from P700$^-$PQ-9 Recombination—Time-resolved (TR) EPR has proved to be the method of choice for the determination of sufficiently long lived ($\geq$10-ns) intermediate states during the course of primary processes in PS I (28). Compared with CW EPR of photoaccumulated, reduced electron acceptors, TR EPR has the advantage that the functional, charge-separated state can be studied in real time. Below, the appearance of the radical ion pair state P700$^-$Q together with that of the 3P700 recombination product is described.

Fig. 4 (top) compares the low temperature (80 K), TR EPR spectra of PS I complexes from the $menB$ and $menB$ rubA mutants, both with PQ-9 in the A$_1$ binding site. The wide field sweep spectrum with the characteristic spin polarization pattern, A/E/E/A/A/E, is readily assigned to the $^{3}$P700 state. The
Electron Transfer in A1 Mutants of Photosystem I

is blocked, and \(^3\)P700 recombination becomes the predominant decay channel. Similarly, PS I complexes with a singly or doubly reduced quinone yield a \(^3\)P700 recombination product. Analogous triplet formation by primary radical pair recombination is also observed in PS II complexes when the quinone is either missing or reduced in the Q\(_A\) site (see Ref. 31). In all of these \(^3\)Chl spectra, the observed spin polarization pattern is incompatible with any intersystem crossing (ISC) process. In the latter case, the spin polarization pattern is a consequence of spin selectivity with respect to the zero field states, and a distinctly different pattern is observed.

In addition to the wide field scan triplet contribution, both spectra in Fig. 4 also show a narrow signal in the center (with an E/A/E polarization pattern), which is due to the \(^3\)P700 \(^\cdot\) state that is populated by forward electron transfer from the P700\(^\cdot\) state in competition with \(^3\)P700 recombination. Relative to the signal amplitude of the P700\(^\cdot\) state, the \(^3\)P700 signal in Fig. 4 (top) is found to be large for the \(\text{menB rubA}\) mutant (solid line) but not detectable for the \(\text{menB}\) mutant (broken line). The \(^3\)P700 signal can thus serve as an indicator for the quinone occupancy of the A\(_1\) binding site in the respective sample.

The flash-induced absorbance changes in the near-IR as well as in the near-UV (Figs. 2 and 3) show for the case of the \(\text{menB rubA}\) mutant that long lived charge separation (i.e. P700\(^\cdot\) \(^\cdot\) formation) occurs in only about one-half of the PS I complexes. The appearance of the recombinant \(^3\)P700 triplet spectra in the transient EPR experiment of the \(\text{menB rubA}\) mutant indicates that a significant part of the other half of the PS I complexes follows the pathway to \(^3\)P700 recombination. In these reaction centers, either (i) the PQ-9 molecule was not incorporated into the A\(_1\) binding site, (ii) the PQ-9 molecule was lost from the A\(_1\) binding site during purification and sample preparation, or (iii) the empty or filled A\(_1\) binding site is not functional in electron transfer past A\(_0\) due to modified properties. Under all of these conditions, the \(^3\)P700 triplet would appear as a recombination product from the primary P700\(^\cdot\)A\(_1\) radical pair state. The radical pair recombines in nanoseconds and thus would not be detectable in our flash-induced absorbance measurements due to the rise time of the spectrometer. As shown below, all A\(_1\) sites in these samples will be shown to be functionally competent, since they can be filled with various naphthoquinones to such an extent that the \(^3\)P700 triplet contribution becomes undetectable.

The appearance of the \(^3\)P700 spectra in Fig. 4 suggests that the removal of the stromal subunits and the \(F_x\) cluster may alter the binding affinity of quinones in the A\(_1\) site (14). Note, however, that empty but otherwise unmodified quinone binding sites can be distinguished from those with altered properties, since the former can be reconstituted with externally provided quinones (see below).

Fig. 4 (bottom) demonstrates that at a later time observation window, a different triplet polarization pattern is observed, with altered zero field splitting parameters and a predominantly E/A/E/A/E/A polarization pattern. Such spectra have been reported previously for PS I (32, 33) and more recently for purple bacteria (34) and are attributed to a long lived \(^3\)Car triplet state. The polarization pattern identifies it as being populated by triplet-triplet energy transfer from a preceding \(^3\)Chl triplet state, which has the characteristic spin polarization, E/E/A/E/A/A, associated with an intramolecular intersystem crossing process. Under our experimental conditions with light intensities near the saturation limit, excitation energy that cannot be trapped by the reaction center eventually decays via a competing intersystem crossing channel to \(^3\)Chl in the antenna system. This triplet state is then efficiently quenched.
by 3Car as observed optically (27) or by EPR (32–34). The same kind of triplet polarization patterns and competing excitation trapping processes were also observed in a recent study of PS I with a Met to Leu mutation of the axial ligand to the Mg\(^{2+}\) of the primary electron acceptor A\(_0\) (21). Note that evidence for a related 3Car signal contribution is also available in the decay-associated spectra of Fig. 3. Similarly, the experimental conditions in the previous studies involved light intensities near saturation.

**Time-resolved EPR Spectra of the P700\(^{+}\)Q\(^{-}\) Radical Pair**—The P700\(^{+}\)Q\(^{-}\) radical pair state spectra (5), which in Fig. 4 appear as the sharp, central features, will now be described. In Fig. 5, the X-band (top) and Q-band (bottom) spectra of the respective P700\(^{+}\)Q\(^{-}\) state are compared on an expanded magnetic field scale. The spectral patterns of the PS I complexes from the menB and menB rubA mutants do not show any significant differences at either microwave frequency. However, the lower signal-to-noise ratio for the menB rubA mutant spectra is consistent with a lower signal amplitude, which correlates with the appearance of a larger 3P700 contribution (see Fig. 4). Equally, the flash-induced absorbance changes in the near-IR as well as in the near-UV (Figs. 2 and 3) indicated that charge separation to the P700\(^{+}\)Q\(^{-}\) state occurs in only about 50% of the PS I complexes. The Q-band spectrum in
Fig. 5 (bottom) exhibits good g-tensor resolution. In this case, the polarization pattern is most sensitive to the orientation of the quinone head group. Since there are no significant differences between the spectra of the menB mutant (5) and the menB rubA mutant (Fig. 5), it is concluded that the absence of the Fx cluster does not disturb the orientation of the quinone head group in the A1 site. The orientation of the quinone is also insensitive to its chemical identity, as shown by the similar polarization patterns of the PS I complexes from the wild type and the rubA mutant, which contain PhQ, and from the menB (5) and menB rubA (this paper) mutant strains, which contain PQ-9.

Incorporation of 9,10-Anthraquinone into the A1 Site of PS I Complexes from the menB rubA Mutant—PQ-9 is readily displaced from the A1 site of PS I for the menB mutant when native PhQ or substituted 1,4-naphthoquinones are added in vivo to the growth medium (4, 7) or when they are added in vitro to isolated PS I complexes (5, 6, 12, 35). If the A1 site in the menB rubA mutant is more accessible to solvent or if it is not as structurally confined by the presence of the stromal subunits, then one may expect that facilitated displacement of PQ-9 and/or displacement by structurally more dissimilar quinones might become feasible. To demonstrate the feasibility of this approach, replacement studies using 9,10-anthraquinone (AQ) are described below; additionally, differences in the behavior of the quinones in PS I complexes of the menB and menB rubA mutant strains are emphasized.

Fig. 6 shows a comparison of the X-band (top) and Q-band (bottom) spectra of the transient radical pair state P700⁻Q⁻ for the following set of PS I complexes: wild type with Q = PhQ (A); menB rubA mutant with Q = PQ-9 recruited into the A1 site (B); menB mutant with AQ added in vitro (with only partial replacement of PQ-9) (C); menB rubA mutant with AQ added in vitro (D); and organic solvent-extracted wild-type PS I with Q = AQ in the A1 site (E). The TR EPR spectra of all samples exhibit the same overall polarization pattern as wild-type PS I (i.e., E/A/E (where E represents emission and A is absorption) at X-band and E/A/A/E/A at Q-band). Spectral decomposition of trace C indicates that only a partial substitution (~30%) of PQ-9 by AQ is achieved in PS I complexes from the menB mutant. In contrast, a comparison between traces D and E indicates that nearly complete substitution of PQ-9 by AQ is achieved in the complexes isolated from the menB rubA mutant, as judged by the identical spectral pattern. The higher degree of incorporation in the latter may be attributed to a greater accessibility of the A1 binding site in the absence of Fx and/or the stromal ridge subunits PsaC, PsaD, and PsaE. The g-tensor parameters of the incorporated 9,10-anthraquinone (\(g_{xx} = 2.0058, g_{yy} = 2.0049, g_{zz} = 2.0022\)) were extracted by simulation of the Q-band radical pair spectrum (dashed line in trace D of Fig. 6). Note that, compared with the simulated spectrum (dashed line), the measured spectrum (solid line) differs by a net absorptive polarization contribution (additional details are available in the accompanying paper (36)).

The ability of AQ to fill the available A1 sites depends upon the sample preparation protocol used (e.g., incubation temperature, additional washing after incubation, etc.). Partial information about the AQ occupancy is obtained by a comparison of the relative signal amplitudes of the P700⁻Q⁻ and the P700 states for samples prepared under different conditions (Figs. 7 and 8). To have comparable signal amplitudes for the P700⁻Q⁻ spectra, the narrow field scan spectrum (as in Fig. 6) is shown in compressed form (full line) as an insert in the center of the wide field scan triplet spectrum (broken line). Before commenting further on the results, it is worth mentioning that the signal amplitudes of each of the states are provided, by necessity, in arbitrary units. Note also that the signal amplitudes (P700⁻Q⁻ and P700) are not only proportional to the respective state population, but they are also determined by the degree of spin polarization acquired in the light excitation process, and they are measured under very different experimental conditions (microwave power, cavity tuning, signal averaging conditions, etc.). Therefore, absolute spin quantitation, which is even a problem under ideal conditions, is simply impossible in this case. However, to the extent that the respective spin polarization mechanism remains the same for the different samples studied and experimental conditions were kept identical for all P700⁻A1 radical pair measurements and
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The narrow A1 sites are able to accept a sufficiently tightly binding quinone not shown). This raises the following questions of whether all high binding affinity are introduced. Essentially all of the empty A1 sites can be filled with functional quinones.

In Fig. 7, the various PS I samples in which AQ replacement has occurred are compared. When the sample is subjected to the washing procedure after AQ replacement, the relative contribution of the P700+ state pattern is clearly changed in favor of the P700 state. This alters the spin polarization patterns observed in the P700 state. The experimental control is that the P700 spectra increases from A to B or C; the latter have nearly the same ratio.

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**Fig. 7.** Comparison of the relative amplitudes of the wide field scan spectra of the P700 state (broken line; experimental conditions the same as for Fig. 4, top) and the narrow field scan P700+Q- spectrum (full line shown as an insert in the center) with AQ in the A1 site of PS I complexes from three different samples. A, menB rubA mutant with AQ exchanged in the A1 site and subsequently washed; B, same as A but without washing; C, solvent-extracted PS I with AQ reconstituted in the A1 site. The narrow P700+Q- state patterns are measured under the same conditions as the spectra in Fig. 4 and then added as an insert in the middle of the wide field scan triplet spectra (to assure reliable amplitude comparisons for each of the signals). Note that the ratio of the relative amplitudes of the P700+Q- versus the P700 spectra increases from A to B or C; the latter have nearly the same ratio.

**Fig. 8.** Similar comparison as in Fig. 7 but for PS I complexes of the menB rubA mutant with different quinones in the A1 site: PQ-9 (A) and 2-CH3-1,4-naphthoquinone (B). The ratio of the relative spectral amplitudes of the P700+Q- versus P700 state with PQ-9 (A) is about the same as with AQ in Fig. 7, B and C, but the ratio increases substantially when NQs of sufficiently high binding affinity are introduced. Essentially all of the empty A1 sites can be filled with functional quinones.

**Fig. 8** compares the relative signals of the P700+Q- and P700 states for quinones with different binding affinities. The PS I complexes from the menB rubA mutant with PQ-9 in the A1 site (Fig. 8A) exhibit essentially the same signal amplitude ratio as when AQ is bound in the A1 site (Fig. 7, B and C). In contrast, the ratio clearly changes in favor of the P700+Q- state signal with quinones, such as 2-CH3-1,4-naphthoquinone (Fig. 8B) or native PhQ (not shown), which have a higher binding affinity for the A1 site, are present. These results show that essentially all A1 sites in the PS I complexes from the menB rubA mutant remain functionally competent in the sense that they can accept electrons when occupied by a suitable quinone acceptor. The experimental control is that the P700 state signal becomes insignificant compared with the P700+Q- state signal.

In the accompanying paper (36), the standard quinone replacement strategy has been used to introduce AQ into the A1 site in PS I. In this protocol, native PhQ is extracted from wild-type PS I complexes with organic solvents (see Ref. 3 for a review), after which AQ is reconstituted into the empty A1 binding site. Identical structural and kinetic properties are found whether AQ is replaced into the solvent-extracted PS I (36) or exchanged into the complexes isolated from the menB rubA mutant (this work). The electron transfer kinetics has been measured in more detail in the case of solvent-extracted AQ reconstituted PS I. The electron transfer rate from AQ to the Fe/S clusters is found to increase in accordance with a more negative redox potential of AQ versus PhQ. Correspondingly, the preceding electron transfer rate from the first radical pair P700+–A0 to the second P700+–AQ is found to slow down sufficiently that spin dynamics can evolve in the P700+–A0 state. This alters the spin polarization patterns observed in the
subsequent radical pair states P700 -AQ- and P700 -[FeS]-. The considerably more bio-compatible AQ incorporation into the PS I complexes of the menB rubA mutant offers the opportunity to compare the observed kinetics of electron transfer in the two cases. This comparison is described and evaluated in the accompanying paper (36).

**DISCUSSION**

Three principal objectives were achieved during the construction and characterization of the menB rubA mutant: (i) identification of the native quinone that occupies the A₁ site in *Synechococcus* sp. PCC 7002; (ii) determination of the room temperature, charge recombination kinetics between P700⁺ and Q- when Q = PhQ and PQ-9; and (iii) incorporation of 9,10-anthraquinone into the A₁ site, followed by studies of the binding, redox, and spectroscopic properties of the resulting PS I complex. Each of these topics is discussed separately in detail below.

The Identity of the Quinone in the A₁ Site of *Synechococcus* sp. PCC 7002—PS I complexes isolated from *Synechococcus* sp. PCC 7002 were shown to contain MQ-4 instead of PhQ, the quinone present in most other cyanobacteria and higher plants. MQ-4 was recently shown to be present in the red alga *C. caldarium* (2) and the early diverging cyanobacterium, *Gloeobacter violaceus* PCC 7421 (37). These results indicate that MQ-4 may be more widely employed in PS I than was previously recognized. MQ-4 differs from PhQ in having a menadione ring. The phytyltransferase enzyme encoded by *menB* rubA from the *Synechococcus* sp. PCC 7002 preferentially binds geranylgeranyl-diphosphate or the analogous enzyme in the different degrees of unsaturation in the C₃ tails, the menadione head group is identically positioned in the A₁ site. Targeted inactivation of the menB homologue in the *Synechococcus* sp. PCC 7002 prevents the synthesis of MQ-4 and results in the incorporation of PQ-9 into the A₁ site. This result, together with the significant similarity in the deduced amino acid sequence with the menB gene product in *Synechocystis* sp. PCC 6803, confirms that the menB gene in *Synechococcus* sp. PCC 7002 codes for 1,4-dihydroxy-2-naphthoate synthase.

**Room Temperature Charge Recombination Kinetics with PQ-9 in the A₁ Site—**The quinone in the A₁ site plays an indispensable role in PS I by linking the transient state P700⁻ A₁ with the stabilized state P700⁻[FeS]⁻. A high quantum yield results from a favorable balance between forward electron transfer from Q⁻ to F₉X and backward electron transfer from F₉X to the primary donor, P700⁺. In PS I complexes isolated from the menB mutant, forward electron transfer from PQ-9⁻ to F₉X was found to be biphasic with lifetimes of ~15 and 250 μs (11). It is shown here that, in PS I complexes from the menB rubA mutant that lacks the Fe/S clusters, backward electron transfer from PQ-9⁻ to the primary donor, P700⁺, is also biphasic, with lifetimes of ~15 μs and 350 μs. If these lifetimes would correspond to the inherent rates of forward and backward electron transfer through the quinone, then the quantum yield of PS I complexes from the menB mutant should be closer to 0.5 than to 1.0. Our optical measurements on the menB mutant show that ~70% of P700 (calculated from Fig. 1 of Ref. 11) and PQ-9 (11) participates in forward electron transfer to the Fe/S clusters on a single turnover flash. Thus, PQ-9 appears to function just at the “tipping point,” where the rate of productive forward electron transfer to the Fe/S clusters just exceeds the rate of nonproductive charge recombination with P700⁺. Thus, a substituted benzoquinone with a slightly more oxidizing midpoint potential would be predicted to favor the backreaction at the expense of the forward reaction. An important caveat in this assessment is that absence of F₉X and the stromal ridge proteins may alter the properties of A₁, thereby resulting in an altered rate of recombination between P700⁺ and A₁⁻. Whether the inherent rate of charge recombination between P700⁺ and A₁⁻ can truly be determined will be examined in a separate publication.

A related issue concerns the similarity in recombination times when PhQ and PQ-9 occupy the A₁ site. The rubA mutant lacks the Fe/S clusters, and 100% of PS I complexes cycle the electron reversibly between P700⁺ and PhQ. Charge recombination between P700⁺ and PhQ⁻ in these P700-A₁ cores is biphasic and occurs with measured lifetimes of ~100 μs and 10 μs at room temperature (13, 14). The menB rubA mutant also lacks the Fe/S clusters, and in those PS I cores that contain a functional PQ-9 (see below), the electron is likewise expected to cycle 100% between P700⁺ and PQ-9. Similarly to the rubA mutant, charge recombination between P700⁺ and PQ-9⁻ in these P700-A₁ cores is biphasic, and the lifetimes are 350 and 12 μs at room temperature. Thus, despite the calculated 135-mV difference in redox potentials of PhQ and PQ-9 in the A₁ site (26), the slow phase of the charge recombination kinetics between P700⁺ and A₁⁻ differs only by a factor of 3–4 when the A₁ site contains PQ-9 rather than PhQ (26). The electron transfer rate is related to the Gibbs free energy difference between donor acceptor pairs in the Frank-Condon term of the Marcus equation. In the wild-type PS I complexes, the midpoint potential of P700/PQ-9 is +430 mV, and the calculated midpoint potential of PhQ/PQ-9 is approximately ~800 mV. Charge recombination therefore dissipates 1230 mV. In the menB mutant, the calculated midpoint potential of PQ/PQ⁻ is ~665 mV; hence, charge recombination dissipates 1095 mV. Because these are similarly large, exothermic values, they may occur near the optimum of the parabolic relationship between rate and Gibbs free energy, and perhaps only a relatively small difference in electron transfer rate should be expected between these two cases.

**Incorporation of 9,10-Anthraquinone into the A₁ Site of PS I Complexes from the menB rubA Mutant—**One difference between the PS I complexes isolated from the menB and menB rubA mutants is that the A₁ binding site is fully occupied by PQ-9 in the former, whereas less than half are occupied in the latter. Another difference is that the addition of AQ to PS I complexes from the menB mutant, at best, results in partial replacement (not more than 30%) of PQ-9 in the quinone binding sites. The addition of AQ to PS I complexes from the menB rubA mutant results in complete displacement (more than 95%) of PQ-9 from the quinone binding sites, although the available A₁ sites are only partially filled before and after the quinone exchange. Note that the addition of 2-CH₃-1,4-naphthoquinone, which has higher affinity for the A₁ site than either PQ-9 or AQ, to PS I complexes from the menB mutant (see Ref. 12) and the menB rubA mutant (this work) yields a nearly complete occupancy of the quinone binding sites. How can this difference be reconciled?

One possibility is that the removal of the Fe/S clusters and the stromal ridge proteins leads to a decrease in the binding affinity of the A₁ sites for PQ-9, and as a result, only a fraction of the sites contain PQ-9. Note that the PS I complexes are suspended in buffer containing the detergent β-DM, and the otherwise-insoluble PQ-9 will have a limited degree of solubility. Another possibility is that removal of the stromal ridge proteins allows the quinone binding site to become more flexi-
ble, thereby allowing a greater incorporation of the larger AQ molecule. Given the location of the A₁ binding site near the beginning of the A₁-jk (B-jk) stromal surface helix and the return to the stromal start of the A₁ (B₁) transmembrane helix, it is feasible that the removal of the stromal ridge proteins Psac, Psbd, and Psae would allow easier access to the external medium and thus the greater chance for quinone loss and/or replacement. Alternatively, the presence of empty quinone binding sites may lead to increased AQ binding because the molecule need only occupy an empty site and not displace a pre-existing benzoquinone, thus making the procedure similar to the quinone incorporation into the PS I particles after organic solvent extraction. Regardless of whether or not the sites are fully occupied, the ability to exchange 9,10-anthraquinone into the PS I cores from the menb rubA mutant indicates that the A₁ binding site is more freely accessible than for PS I complexes from either the menb mutant or the wild type. These properties make PS I cores isolated from the menb rubA mutant ideal for incorporating novel quinones, particularly anthraquinones, into the A₁ site.

During an extension of the quinone exchange protocol, it was found that AQ can be washed out of the PS I complexes (how much depends on the conditions), allowing truly “empty” quinone binding sites to be created in the PS I complexes derived from the menb rubA mutant. Conversely, supplying AQ again results in an increased occupancy of the quinone sites and a decrease in the number of empty sites. It must be emphasized that the presence of empty A₁ sites in PS I complexes from the menb rubA mutant after washing is the result of a relatively low AQ binding affinity to the A₁ site rather than the result of different types or populations of A₁ binding sites. The evidence for this is that an increase (or decrease after sample washing) of the AQ concentration in the solution shifts the AQ binding equilibrium when monitored by TR EPR (see “Results”). Moreover, the fact that strongly binding naphthoquinones can fill all A₁ sites shows that neither in the menb rubA double mutant nor in the solvent-extracted PS I complexes are different or modified nonfunctional A₁ binding sites created.

The possibility of replacing all PQ-9 in the menb rubA mutant with AQ opens another interesting aspect in the study of electron transfer kinetics in comparison with AQ reconstituted in solvent-extracted PS I. In the companion paper (36), it is shown that the more negative redox potential of AQ compared with native PhQ accelerates the A₁ to Fx electron transfer kinetics. Correspondingly, the Aₐ to A₁ electron transfer step is expected to slow down. Indeed, this occurs to such an extent that the electron transfer kinetics become indirectly observable by TR EPR via the influence of the evolving spin dynamics during the lifetime of the primary P700⁺A₁ₕ state on the polarization pattern of the observable P700⁺AQₐₕ state. Thus, the electron transfer kinetics of the A₀ to A₁ electron transfer step can be compared in the presence or absence of the Fx cluster. The first results of such a comparison are described in the companion paper (36).