

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

I. GENETIC AND PHYSIOLOGICAL CHARACTERIZATION OF PHYLLOQUINONE BIOSYNTHETIC PATHWAY MUTANTS IN *SYNECHOCYSTIS* SP. PCC 6803*

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Genes encoding enzymes of the biosynthetic pathway leading to phyloquinone, the secondary electron acceptor of photosystem (PS) I, were identified in *Synechocystis* sp. PCC 6803 by comparison with genes encoding enzymes of the menaquinone biosynthetic pathway in *Escherichia coli*. Targeted inactivation of the *menA* and *menB* genes, which code for phytyl transferase and 1,4-dihydroxy-2-naphthoate synthase, respectively, prevented the synthesis of phyloquinone, thereby confirming the participation of these two gene products in the biosynthetic pathway. The *menA* and *menB* mutants grow photoautotrophically under low light conditions (20 $\mu\text{E m}^{-2} \text{s}^{-1}$), with doubling times twice that of the wild type, but they are unable to grow under high light conditions (120 $\mu\text{E m}^{-2} \text{s}^{-1}$). The *menA* and *menB* mutants grow photoheterotrophically on media supplemented with glucose under low light conditions, with doubling times similar to that of the wild type, but they are unable to grow under high light conditions unless atrazine is present to inhibit PS II activity. The level of active PS II per cell in the *menA* and *menB* mutant strains is identical to that of the wild type, but the level of active PS I is about 50–60% that of the wild type as assayed by low temperature fluorescence, P700 photoactivity, and electron transfer rates. PS I complexes isolated from the *menA* and *menB* mutant strains contain the full complement of polypeptides, show photoreduction of F_A and F_B at 15 K, and support 82–84% of the wild type rate of electron transfer from cytochrome *c*₆ to flavodoxin. HPLC analyses show high levels of plastoquinone-9 in PS I complexes from the *menA* and *menB* mutants but not from the wild type. We propose that in the absence of phyloquinone, PS I recruits plastoquinone-9 into the A₁ site, where it functions as an efficient cofactor in electron transfer from A₀ to the iron-sulfur clusters.

All well characterized photosynthetic reaction centers are known to contain a bound quinone molecule that participates in the early stages of photochemical charge separation and stabilization (1–3). Type II reaction centers, such as photosystem (PS)¹ II or those present in the purple nonsulfur bacteria, contain a bound benzoquinone or naphthoquinone as the secondary electron acceptor. Type I reaction centers, such as PS I of cyanobacteria and green plants, contain a bound menaquinone, usually phyloquinone (vitamin K₁, 2-methyl-3-phytyl-1,4-naphthoquinone), or less commonly, 5'-monohydroxyphyloquinone, as the secondary electron acceptor (4). (Whether green sulfur bacteria and heliobacteria, which have a PS I-like reaction center, contain a similar bound quinone is still under active investigation.) Two molecules of phyloquinone can be extracted per molecule of P700 from isolated PS I complexes (5–9); however, only one molecule of phyloquinone is considered to participate as an intermediate in electron transfer from A₀ to F_X (5, 6, 10).

One strategy to disallow A₁ function is to inactivate genes that code for enzymes involved in the proposed pathway of phyloquinone biosynthesis. Many prokaryotes, as well as chloroplasts, contain the metabolic pathway for phyloquinone (vitamin K₁) biosynthesis. In several bacteria, vitamin K₂ (menaquinone) is used during fumarate reduction in anaerobic respiration (11). The genes encoding the enzymes involved in the conversion of chorismate to menaquinone have been cloned in *Escherichia coli* (12–14) and *Bacillus subtilis* (15, 16). Although the route of phyloquinone biosynthesis has not been described in cyanobacteria, the pathway is likely to be similar to the pathway of menaquinone biosynthesis in other bacteria. Menaquinone differs from phyloquinone by the presence of a partly unsaturated, predominantly C-40 side chain rather than a mostly saturated, C-20 phytol side chain. With this exception, the synthesis of the naphthalene nucleus in phyloquinone and menaquinone is expected to include similar steps (Fig. 1). The genome data base for *Synechocystis* sp. PCC 6803 (17) contains homologs for several genes that encode enzymes for menaquinone biosynthesis: *menF* (*entC*) (isochorismate synthase), *menD* (2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate synthase), *menE* (*O*-succinylbenzoic acid-CoA ligase), *menB* (dihydroxynaphthoate synthase) and *menA* (identified as “menaquinone biosynthesis protein,” but probably a phytyl transferase). Possible homologs of *menC* and ORF241 (the

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¹ The abbreviations used are: PS, photosystem; EPR, electron paramagnetic resonance; HPLC, high performance liquid chromatography; MS, mass spectroscopy; PCR, polymerase chain reaction; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; bp, base pair(s); kb, kilobase; Chl, chlorophyll; Vis, visual light.

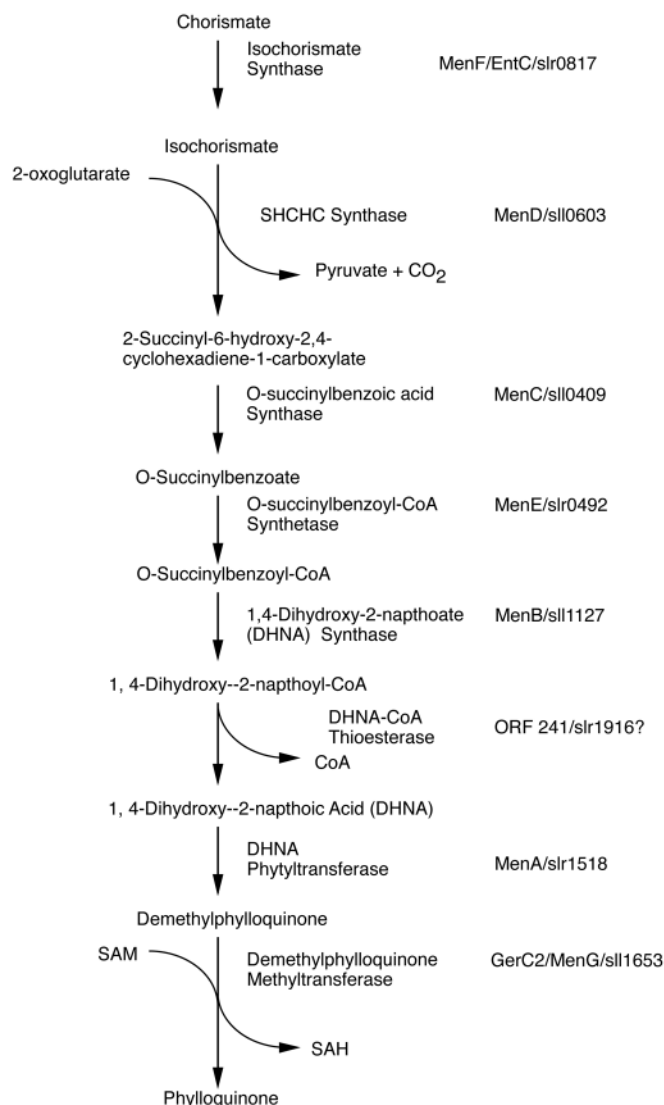


FIG. 1. Proposed biosynthetic pathway of phylloquinone biosynthesis in *Synechocystis* sp. PCC 6803. The gene products responsible for the biosynthesis of menaquinone were initially described in *E. coli* (see Ref. 14). The homologs of these genes that have been identified in the genome sequence of *Synechocystis* sp. PCC 6803 are indicated. SAM, S-adenosyl methionine; SAH, S-adenosyl homocysteine.

DHNA thioesterase) have also been identified in our data base searches. We propose that *menF/entC*, *menD*, *menE*, and *menB* of *Synechocystis* sp. PCC 6803 are involved in 1,4-dihydroxy-2-naphthoate synthesis, whereas the product of a *menA* homologue catalyzes the addition of phytol chain. The product of the phytol transferase, 2-phytyl-1,4-naphthoquinone, requires a methylation step to become phylloquinone. The gene originally identified as *gerC2* (sll1653) in the *Synechocystis* sp. PCC 6803 data base probably codes for the 2-phytyl-1,4-naphthoquinone methyl transferase enzyme that catalyzes this reaction.

There is no known function for menaquinone in cyanobacteria except to provide a precursor for phylloquinone biosynthesis. PS II and respiration require plastoquinone-9, which is a benzoquinone derivative that is synthesized by an independent pathway (18). There are two expected consequences of an interruption in the menaquinone pathway: 1) the participation of *men* genes in the biosynthetic pathway of phylloquinone in cyanobacteria would be confirmed; and 2) the A_1 site should be empty, thereby allowing a test of the requirement of phylloqui-

none in electron transfer from A_1 to F_X . We initially generated mutants in which the *menA* and *menB* genes in *Synechocystis* sp. PCC 6803 have been inactivated by targeted mutagenesis. These two mutations were selected because they would additionally allow us to determine whether the phytol chain is essential for function. If the phytol chain is dispensable, then the *menA* deletion mutant may allow the head group 1,4-dihydroxy-2-naphthoate to be incorporated into the A_1 site. In this paper, we describe the construction, as well as the genetic and functional characterization, of *menA* and *menB* mutant strains of *Synechocystis* sp. PCC 6803. We propose that in the absence of phylloquinone, the A_1 site does not remain empty. Instead, we suggest that PS I recruits plastoquinone-9 into the A_1 site, and this quinone supports high efficiency electron transfer from A_0 to the iron-sulfur clusters.

EXPERIMENTAL PROCEDURES

Growth of *Synechocystis* sp. PCC 6803—Wild type *Synechocystis* sp. PCC 6803 cells were grown in medium BG-11 (19). The *menA* and *menB* mutant strains were grown in medium BG-11-TES supplemented with 5 mM glucose and the appropriate antibiotic. Agar plates for the growth of mutant strains were kept at low light intensity ($2\text{--}5\ \mu\text{E m}^{-2}\text{ s}^{-1}$); liquid cultures of wild type and mutant strains were grown under reduced light conditions ($10\text{--}20\ \mu\text{E m}^{-2}\text{ s}^{-1}$) in the presence of 5 mM glucose. Cell growth in liquid cultures was monitored by measuring the absorbance at 730 nm using a Cary-14 spectrophotometer that had been modified for computerized data acquisition by On-Line Instruments, Inc. (Bogart, GA). Cells from liquid starter cultures in the late exponential phase of growth ($A_{730} = 0.8\text{--}1.2$) were harvested by centrifugation and were washed once with BG-11 medium. All cultures were adjusted to the same initial cell density ($A_{730} = 0.1$) for growth experiments and bubbled with air as described (20).

Generation of the *menA* and *menB* Mutant Strains of *Synechocystis* sp. PCC 6803—To generate a recombinant DNA construction for inactivation of the *menA* gene, two DNA fragments were amplified from *Synechocystis* sp. PCC 6803 genomic DNA by polymerase chain reaction (Fig. 2A). The *Pst*I restriction sites were incorporated in both fragments, whereas the *Apa*I restriction site was also added to the downstream fragment. The first amplification product was digested with *Eag*I and *Pst*I, whereas the second fragment was digested with *Pst*I and *Apa*I restriction enzymes. The fragments were ligated with the pBlue-script SK (Stratagene) vector that had been digested with *Eag*I and *Apa*I. The kanamycin resistance gene from pUC4K was cloned in the *Pst*I site; this yielded recombinant plasmid pRRR that contained replacement of a 442-bp part of the *menA* gene by the resistance marker. Transformation of the wild type strain of *Synechocystis* sp. PCC 6803 and isolation of segregated mutants was performed according to previously published methods (21).

To generate a recombinant DNA construction for inactivating the *menB* gene, two 1.0-kb fragments from upstream and downstream of the *menB* gene were amplified by PCR (Fig. 2B). The oligonucleotide primers included unique restriction sites (*Spe*I and *Eco*RI in the upstream fragment and *Eco*RI and *Xho*I sites in the downstream fragment). The amplified fragments were cloned into pBluescript (Stratagene), and a 2.0-kb *Eco*RI fragment containing the streptomycin/spectinomycin resistance cassette of the Ω element of the recombinant plasmid pHP45 Ω (22) was inserted into the newly created unique *Eco*RI site. The resultant plasmid was designated pRRB and was used to produce the *menB* mutant strain according to previously described methods (21).

DNA Isolation, PCR, and Southern Blotting—Genomic DNA from *Synechocystis* sp. PCC 6803 was prepared as described (20). Hybridization probes were generated with the DIG High-Prime DNA labeling system (Roche Molecular Biochemicals). Hybridization and detection were performed according to the manufacturer's protocols.

Chlorophyll Analysis and Oxygen Evolution Measurements—Chlorophyll was extracted from whole cells and thylakoids with 100% methanol, and chlorophyll concentrations were determined as described (23). Oxygen evolution measurements were performed using a Clark-type electrode as described (24). The temperature of the electrode chamber was maintained at 30 °C by a circulating water bath. Cells were resuspended in 25 mM HEPES/NaOH, pH 7.0, buffer at a final concentration of $1.0\ A_{730}\text{ ml}^{-1}$. Whole-chain electron transport (H_2O to CO_2) measurements were determined after the addition of 5 mM NaHCO_3 . Oxygen evolution mediated by PS II alone was determined after addition of 0.5

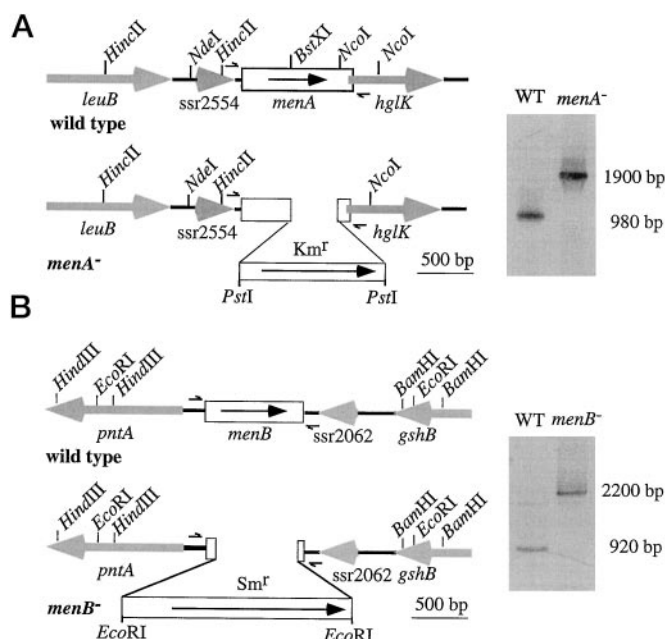


FIG. 2. Construction and verification of the *menA* and *menB* mutant strains of *Synechocystis* sp. PCC 6803. *A*, inactivation of the *menA* gene. *Left panel*, restriction maps of the genomic regions surrounding the *menA* gene in the wild type and mutant strain. The *small arrows* indicate the position of the PCR primers used to amplify the *menA* coding sequence. *Right panel*, electrophoretic analysis of the DNA fragments amplified from the genomic DNA of the wild type and *menA* mutant strains. *B*, inactivation of the *menB* gene. *Left panel*, restriction maps of the genomic regions surrounding the *menB* gene in the wild type and mutant strains. The *small arrows* indicate the position of the PCR primers used to amplify the *menB* coding sequence. *Right panel*, electrophoretic analysis of the DNA fragments amplified from the genomic DNA of the wild type and *menB* mutant strains.

mm $K_3Fe(CN)_6$ and 0.1 mM 2,5-dimethyl-*p*-benzoquinone.

Isolation of Thylakoid Membranes and PS I Particles—Thylakoid membranes were prepared from cells in the late exponential growth phase as described (20). Cells were broken by two passages through a French pressure cell at 20,000 lb in⁻² at 4 °C. The thylakoid membranes were pelleted by centrifugation at 50,000 × *g* for 45 min. The thylakoid membranes were resuspended in buffer (50 mM HEPES/NaOH, pH 8.0, 5 mM $MgCl_2$, 10 mM $CaCl_2$, 0.5% (v/v) dimethyl sulfoxide, and 15% (v/v) glycerol) for storage and/or in 50 mM Tris/HCl, pH 8.0, for further PS I particle preparations. For the isolation of PS I complexes, thylakoid membranes were solubilized in 1% (w/v) *n*-dodecyl- β -D-maltoside for 2–4 h at 4 °C. The trimeric and monomeric PS I particles were separated by centrifugation on 5–20% (w/v) sucrose gradients with 0.03% *n*-dodecyl- β -D-maltoside in 50 mM Tris, pH 8.0. Further purification was achieved by a second centrifugation on sucrose gradients in 50 mM Tris, pH 8.0, in the absence of *n*-dodecyl- β -D-maltoside (25).

SDS-Polyacrylamide Gel Electrophoresis Analysis—The methods used for SDS-polyacrylamide gel electrophoresis were identical to those previously described (24). To resolve the subunit compositions of the PS I preparations from the *Synechocystis* sp. PCC 6803 wild type strain and the *menA* and *menB* mutants, the Tricine/Tris discontinuous buffer system was used (26). A 16% (w/v) acrylamide gel containing 6 M urea was used as the separating gel. The resolved proteins were visualized by silver staining.

77 K Fluorescence Emission Spectra—Low temperature fluorescence emission spectra were measured using a SLM 8000C spectrofluorometer as described (24). Cells from the exponential phase of growth were harvested and resuspended in 25 mM HEPES/NaOH buffer, pH 7.0. Cells were diluted in 25 mM HEPES/NaOH, pH 7.0, containing 60% (v/v) glycerol to a concentration of 1.0×10^7 cells ml⁻¹ prior to freezing in liquid nitrogen. The excitation wavelength was 440 nm. The excitation slit width was set at 4 nm, and the emission slit width was set at 2 nm.

PS I Activity Measurements—Steady-state rates of electron transfer for isolated PS I complexes were measured using cytochrome *c*₆ as electron donor and flavodoxin as electron acceptor as described (10). The measurement of P700 photooxidation in whole cyanobacterial cells was performed as described (27).

Electron Paramagnetic Resonance (EPR) Spectroscopy of *F_A* and *F_B*—EPR studies were performed using a Bruker ECS-106 X-band spectrometer and a standard-mode resonator (ST 8615) equipped with a slotted port for light entry. Cryogenic temperatures were maintained with a liquid helium cryostat and an ITC-4 temperature controller (Oxford Instruments, Oxford UK). The microwave frequency was measured with a Hewlett-Packard 5340A frequency counter, and the magnetic field was calibrated using α,α' -diphenyl- β -picryl hydrazyl as the standard. Sample temperatures were monitored by a calibrated thermocouple located 3 mm beneath the bottom of the quartz sample tube and referenced to liquid N₂. Samples were illuminated with a 150 W xenon arc source (Oriol, Stratford, CT, model 66057) passed through 5 cm of water and a heat-absorbing color filter to remove the near-infrared light. Samples used for EPR measurements contained 1 mg Chl ml⁻¹, 1 mM sodium ascorbate, 4 μ M 2,6-dichlorophenol-indophenol in 50 mM Tris, pH 8.3.

Analysis of Phylloquinone Using HPLC-UV/Vis and Mass Spectrometry—Membranes containing 0.1 mg of chlorophyll were centrifuged at 1000 × *g* for 60 min, and the supernatant was removed. The membrane pigments were sequentially extracted with 1 ml of methanol, 1 ml of 1:1 (v/v) methanol:acetone, and 1 ml of acetone, and the three extracts were combined. The resulting solution was concentrated by vacuum at 10 °C in the dark to approximately 0.8 mg of Chl ml⁻¹. Chromatography with UV/Vis detection was performed on an ISCO dual pump HPLC system (Lincoln, NE). The pumps were operated by ISCO Chemresearch version 2.4.4 software, UV/Vis detection was performed with an ISCO V4 absorbance detector set at 255 nm, and data collection and processing were done using JCL6000 version 26 software (Jones Chromatography Limited, Mid-Glamorgan, UK). HPLC separations were also monitored with photodiode array UV-visible detection using a Hewlett-Packard (Palo Alto, CA) model 1100 quaternary pump and model G1316A photodiode array detector. Sample injections (20 μ l) were made on a 4.6-mm × 25-cm Ultrasphere C₁₈ column (4.6 × 250 mm) with 5 μ m packing (Beckman Instruments, Palo Alto, CA), using gradient elution (solvent A, methanol; solvent B, isopropanol; 100% A from 0–10 min to 3% A/97% B at 30 min, hold until 40 min) at 0.5 ml min⁻¹. A solution of phylloquinone (40 mM) was prepared in absolute ethanol and kept at –20 °C as a standard for calibration. Extracts were also analyzed by LC/MS using a Perseptive Biosystems Mariner time-of-flight mass spectrometer using electrospray ionization in negative mode with a needle potential of –3500 V and a nozzle potential of –80 V. A postcolumn flow splitter delivered column eluent to the electrospray ion source at 10 μ l min⁻¹. Gas chromatography/MS analyses were performed using a Hewlett-Packard 5972 mass spectrometer coupled to a Hewlett-Packard 5890 gas chromatograph. Splitless injections of 1.0 μ l were made onto a 30-m DB-5 column (J & W Scientific, Folsom, CA) using helium (35 cm s⁻¹) as the carrier gas. The column was programmed from 100 to 300 °C at a rate of 6 °C per min. Data were acquired in both full scanning mode and using selected ion monitoring of *m/z* = 450 for trace detection of phylloquinone.

RESULTS

Analysis of the Genotype of the *menA* and *menB* Mutant Strains—The genotypes of the *menA* and *menB* mutant strains were confirmed by Southern blot hybridization analyses and by PCR amplification of the appropriate genomic loci. The *left panel* of Fig. 2A shows restriction maps of the genomic regions surrounding the *menA* gene in the wild type and mutant strains. A 440-bp fragment in the *menA* gene was deleted and replaced by a 1.3-kb kanamycin resistance cartridge encoding the *aphII* gene. Using primers flanking the coding sequence (Fig. 2A, *small arrows*), PCR amplification of the *menA* locus of the wild type produced the expected fragment of 980 bp (Fig. 2A, *right panel*). PCR amplification of the *menA* locus of the mutant strain produced the expected 1.9-kb fragment (Fig. 2A, *right panel*). Because no amplification of the 980-bp fragment occurred when DNA from the mutant strain was used as a template, this result indicates that the wild type and mutant *menA* alleles had fully segregated. Southern blot hybridization analyses were also performed, and these experiments confirmed that the *menA* gene was interrupted as expected and that the *menA* mutant strain was homozygous (data not shown).

Insertional inactivation of the *menB* gene was also verified

TABLE I
Physiological characteristics of the *Synechocystis* sp. PCC 6803 wild type and the *menA* and *menB* strains

Values are the average of four independent experiments (n = 4).

Strain	Wild type	<i>menA</i>	<i>menB</i>
Photoautotrophic growth doubling time (h)			
Low light	34 ± 1.2	73 ± 3.4	71 ± 2.8
High light	16 ± 1.4	NM ^a	NM
Photomixotrophic growth doubling time (h)			
Low light	17 ± 1.1	22 ± 1.8	24 ± 2.3
High light	12 ± 0.8	NM	NM
Photoheterotrophic growth doubling time (h)			
Low light	22 ± 1.6	25 ± 2.1	26 ± 1.8
High light	24 ± 2.2	29 ± 2.6	31 ± 3.2
Oxygen evolution ^b (μmol O ₂ /A ₇₃₀ hl)			
Whole chain	1260 ± 76	787 ± 49	718 ± 57
PS II-mediated	1430 ± 84	1408 ± 106	1387 ± 93
Chlorophyll content ^b (μg Chl/A ₇₃₀ ml)	4.21 ± 0.18	3.36 ± 0.23	3.43 ± 0.32

^a NM, not measurable.

^b Cells were grown in BG11 plus 5 mM glucose under reduced light intensity; units are with reference to absorbance of the whole cells at 730 nm.

by both Southern blot hybridization analysis and by PCR amplification of the *menB* locus from the mutant strain. As shown in the left panel of Fig. 2B, most of the *menB* gene was deleted and replaced with a 2-kb spectinomycin resistance cartridge. Using primers flanking the coding sequence (Fig. 2B, small arrows), PCR amplification of the *menB* locus of the wild type produced the expected fragment of 920 bp (Fig. 2B, right panel). However, PCR amplification of this locus in the mutant strain produced a 2.2-kb DNA fragment (Fig. 2B, right panel), and no 930-bp fragment was observed. These results indicate that the *menB* mutant is homozygous and that full segregation of alleles had occurred. The PCR amplification results were confirmed by Southern blot hybridization analyses, which demonstrated that the *menB* mutant strain was homozygous and that the *menB* gene had been insertionally inactivated as shown in the left panel of Fig. 2B.

Analysis of the Phenotype of the *menA* and *menB* Mutant Strains—We focused initially on the phenotypic analysis of whole cells of the *menA* and *menB* mutant strains. Photoautotrophic growth rates of the *menA* and *menB* mutants were measured in cells grown in BG-11-TES medium, which contains minerals and bicarbonate as the sole carbon source. Under low light intensity conditions (20 μE m⁻² s⁻¹), the *menA* and *menB* mutant cells grew photoautotrophically but at a slower rate than the wild type cells. The 73-h doubling time of the *menA* mutant cells and the 71-h doubling time of the *menB* mutant cells were more than twice the 34-h doubling time of the wild type cells (Table I). Under high light intensity conditions (120 μE m⁻² s⁻¹), the doubling time of the wild type cells decreased to 16 h, but surprisingly, the *menA* and *menB* mutant cells showed no measurable growth. Photomixotrophic growth rates of the *menA* and *menB* mutants were determined in the presence of 5 mM glucose, which allows both respiration and photosynthesis to provide energy for growth. Under low light intensity conditions, the 17-h doubling time of the wild type was one-half that under photoautotrophic conditions, and the 22–24-h doubling times of the *menA* and *menB* mutants were fractionally greater than that of the wild type (Table I). Under high light intensity conditions, the wild type cells had a doubling time of 12 h, but again, the *menA* and *menB* mutant cells showed no measurable growth. Photoheterotrophic growth rates of the *menA* and *menB* mutants were determined in the

presence of glucose to allow respiration but with 20 μM atrazine to inhibit PS II function. Under both low and high light intensity conditions, the doubling times of the *menA* and *menB* mutants were slightly greater than those of the wild type strain (Table I). These studies show that the *menA* and *menB* mutants are capable of photoautotrophic growth only under low light intensities. However, the high light sensitivity of the *menA* and *menB* mutants can be alleviated by inhibiting PS II activity.

Complementation of *menA* and *menB* Mutant Strains with Wild Type Genes—To determine whether secondary mutations account for the observed high light sensitivity phenotype, we complemented the *menA* and *menB* mutant strains with the corresponding wild type genes. The *menA* and *menB* genes and their flanking regions were amplified by PCR and used for transformation of the corresponding mutant strains. After transformation of the mutant strains with the appropriate DNA fragment, the plates were incubated under nonselective, photoautotrophic conditions at an intermediate light intensity of 40 μE m⁻² s⁻¹. At this light intensity, the *menA* and *menB* mutant cells could grow under photoautotrophic, photomixotrophic, and photoheterotrophic conditions (data not shown). After 3 days, the plates were shifted to high light intensity (160 μE m⁻² s⁻¹), and colonies appeared after about 2 weeks. After restreaking under high light intensity growth conditions to ensure that segregation had occurred, selected independent transformants were tested for loss of antibiotic resistance and subjected to PCR analysis to verify that the *menA* or *menB* locus had been transformed to the wild type genotype. Using this approach, antibiotic-sensitive, complemented strains of the *menA* mutant (AWT) and *menB* mutant (BWT) were isolated. These transformants, which were tolerant of high light intensities, had growth rates and photosynthesis rates (measured by oxygen evolution) that were similar to those of the wild type (data not shown) and distinctly different from the *menA* and *menB* mutant strains. It was also possible to isolate suppressor mutants by omitting the transforming DNA. However, these suppressor mutants arose at a much lower frequency than was observed in the transformation experiments, retained their antibiotic resistance, and had growth and electron transport properties that differentiated these strains from the wild type (data not shown). Analysis of these suppressor mutants is in progress. We conclude that the phenotype observed for the *menA* and *menB* mutant strains is entirely due to the inactivation of the targeted genes.

Electron Transfer Rates in Whole Cells—We next explored the possibility that the high light sensitivity of the *menA* and *menB* mutant strains arises from a result of an imbalance between PS I and PS II electron transfer rates. The activities of PS I and PS II can be measured in the *menA* and *menB* mutant strains by whole chain and partial chain electron transfer assays. Whole-chain electron transfer from water to bicarbonate was measured in cells grown photomixotrophically under low light conditions. On an equivalent cell number basis, the rates of oxygen evolution in the *menA* and *menB* mutants were 62 and 57%, respectively, those of the wild type strains (Table I). This result was as surprising as the ability of the mutants to grow photoautotrophically; if phylloquinone is absent, then room temperature electron transfer to NADP⁺ should not occur (28, 29). Because their PS II activities are unaffected, these results indicate that the decreased, whole-chain, electron transport activity of the *menA* and *menB* mutant cells is due to a PS I-related defect. This could be the result of a lower amount of PS I per cell, an impairment in PS I function, or both.

Relative Content of Active PS I Per Cell—Fig. 3 shows the 77 K fluorescence emission spectra of whole cells on an equal cell number basis for the wild type strain and for the *menA* and

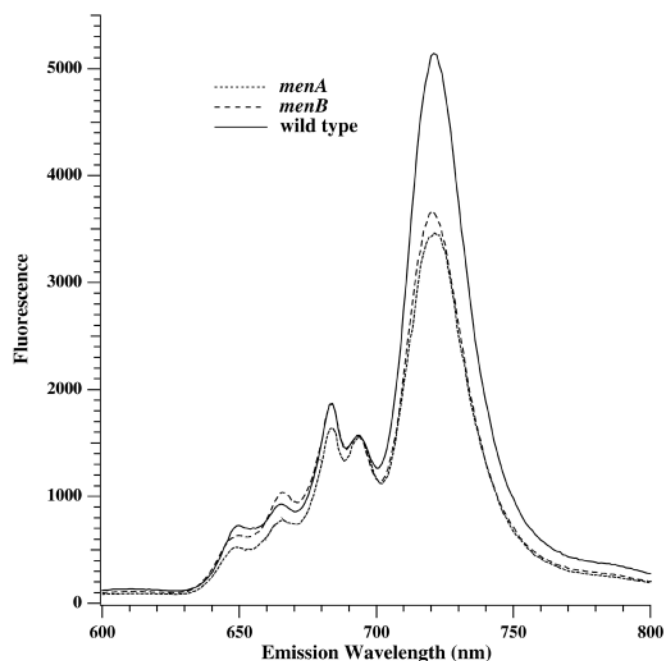


FIG. 3. Fluorescence emission spectra at 77 K of whole cells from *Synechocystis* sp. PCC 6803 wild type and the *menA* and *menB* mutant strains. Spectra were recorded at the same cell density ($1.0 A_{730} \text{ ml}^{-1}$). Each spectrum was the average of five measurements. The excitation wavelength was 440 nm, which excites mostly chlorophyll. PS II and its accessory pigments exhibit emission maxima at 685 and 695 nm; PS I has a maximum emission at 721 nm.

menB mutants. In the *menA* and *menB* mutant cells, the PS II-chlorophyll fluorescence emission at 685 and 695 nm (30) shows no obvious differences in intensity from the wild type cells. However, the PS I-chlorophyll fluorescence emission at 721 nm was reduced in intensity in the two mutant strains relative to the wild type (Fig. 3). This result indicates that cells of the two mutant strains contain the same amount of PS II per cell as the wild type, but less PS I per cell than the wild type.

The absolute PS I content of whole cells can be determined by the light-induced absorbance increase at 832 nm due to the oxidation of P700 (27, 31). On the basis of equal cell numbers, both the *menA* and *menB* mutant cells were found to contain 50–60% of the photooxidizable P700 of the wild type cells (data not shown). In *Synechocystis* sp. PCC 6803, approximately 100 Chl are associated with PS I, and approximately 60 Chl are associated with PS II. If the smaller amount of photooxidizable P700 is due to fewer PS I complexes per cell, then the chlorophyll content should be lower in the *menA* and *menB* mutant cells than in the wild type cells. Table I shows that, on an equivalent cell number basis, the chlorophyll content of the *menA* and *menB* mutant cells is significantly lower than that of the wild type cells. The reduced contents of PS I per cell in the mutant strains are therefore responsible, at least in part, for the lower whole-chain electron transfer rates in the *menA* and *menB* mutant cells.

Polypeptide Composition of Isolated PS I Complexes—PS I complexes were solubilized from thylakoid membranes using *n*-dodecyl- β ,D-maltoside and purified by ultracentrifugation on two successive sucrose gradients. PS I isolated from the wild type is 15% monomeric and 85% trimeric, whereas PS I isolated from the *menA* and *menB* mutants is 35% monomeric and 65% trimeric. Only PS I trimers were used in these studies. Analysis by SDS-polyacrylamide gel electrophoresis of the PS I trimers isolated from the mutant and wild type showed no differences in the polypeptide composition (data not shown). Also, no degradation fragments of PS I proteins were detected in the PS I

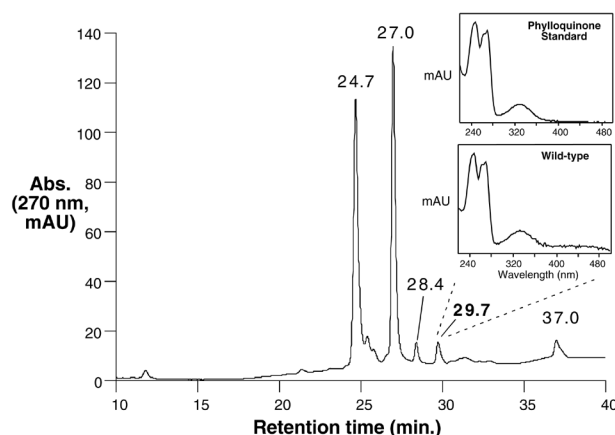


FIG. 4. HPLC profiles of pigment extracts from lyophilized PS I complexes of the wild type strain of *Synechocystis* sp. PCC 6803. The pigments were separated on a 5.0 μm Ultrasphere C_{18} reverse phase column. The detection wavelength was 270 nm. The extract from the wild type shows a peak that co-elutes with authentic phylloquinone at 29.7 min. *Top inset*, UV/Vis spectrum of authentic phylloquinone. *Bottom inset*, near-UV/Vis spectrum of HPLC peak that elutes at 29.7 min.

trimers. These results show that the interruption of the phylloquinone biosynthetic pathway has no effect on the protein complement of PS I.

Absence of Phylloquinone in the *menA* and *menB* Mutant Strains—The phylloquinone content of the PS I trimers was determined using HPLC with photodiode array UV-visible detection. As shown in Fig. 4, multiple peaks are present in the 254-nm chromatogram of the solvent extracts from PS I complexes of wild type cells. By co-injecting standards and by interpreting the UV-visible spectra, chlorophyll *a* was identified at 24.7 and 27.0 min (the former is missing the phytol tail), a polar carotenoid (probably monohydroxylated but otherwise uncharacterized) was identified at 28.4 min, and β -carotene was identified at 37.0 min. Phylloquinone was identified by co-elution at 29.7 min with an authentic phylloquinone standard (Fig. 4, *top inset*) and by its UV-visible spectrum (Fig. 4, *bottom inset*). As shown in Fig. 5, virtually identical chromatograms were obtained for solvent extracts of PS I complexes from the *menA* and *menB* mutants except that the phylloquinone peak at 29.7 min is missing. As expected, phylloquinone was present in the membrane pigment extracts of the complemented AWT and BWT strains at levels comparable to the wild type (data not shown, but similar to Fig. 4). Based on a phylloquinone calibration curve, the minimum detection limit for phylloquinone using HPLC was estimated to be approximately 15% of the wild type levels.

Gas chromatography/MS is capable of separating and detecting nonpolar benzoquinones, ubiquinones, and naphthoquinones, provided their molecular masses are less than about 600 Da. Using total ion current for detection, the crude solvent extract from wild type PS I complexes showed a peak with a retention time of approximately 8 min, which matched that of authentic phylloquinone (data not shown). The molecular ion at $m/z = 450$ confirmed the identification of this molecule as phylloquinone. Sensitive selected-ion-monitoring analyses did not find a detectable amount of phylloquinone in solvent extracts of PS I complexes isolated from either the *menA* or *menB* strains. The limit of detection using selected-ion-monitoring was determined from the calibration curve to correspond to approximately 0.1% of the wild type level. Because there are approximately 100 Chl/P700 in cyanobacterial PS I complexes, the *menA* and *menB* mutant strains thus contain <0.02 phylloquinones/P700.

Presence of Plastoquinone-9 in the *menA* and *menB* Mutant

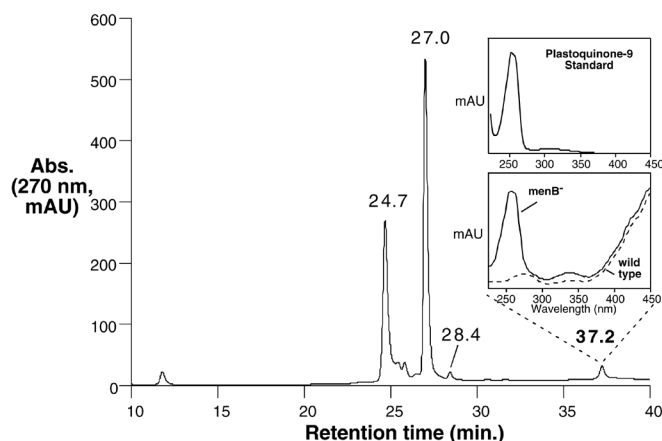


FIG. 5. HPLC profiles analysis of pigment extracts from lyophilized PS I complexes of the *menB* mutant strain of *Synechocystis* sp. PCC 6803. The pigments were separated on a 5.0- μ m Ultrasphere C₁₈ reverse phase column. The peak at 37.2 min in the wild type co-elutes with β -carotene, and shows a spectrum in the visible identical to β -carotene. The peak at 37.2 min in the *menB* mutant shows an additional UV-absorbing component (bottom inset) that co-elutes with plastoquinone-9 and that shows a UV spectrum similar to plastoquinone-9 (top inset) and an m/e of 748 (not shown). The LC/MS analysis of the *menA* mutant was similar.

Strains—The idea that a foreign quinone might be present in the A₁ site first came about when we discovered that a quinone-like EPR signal is present in whole cells of the *menA* and *menB* mutants (see the accompanying paper (33)). To determine the identity of this quinone, solvent extracts of PS I trimers from the *menA* and *menB* mutants were analyzed by HPLC using photodiode array UV-visible detection. The search was initially complicated by the absence of new peaks in chromatograms ($\lambda = 270$ nm) from the *menA* and *menB* mutants when compared with the wild type (Fig. 5). We therefore sought evidence of a new component coeluting with another pigment by comparing the UV-visible spectra of peaks in chromatograms of the *menA* and *menB* mutants with the corresponding peaks for the wild type. The only significant difference was in a component that coeluted with β -carotene at 37 min. The difference spectrum of the components eluting at 37 min showed a strong absorbance near 254 nm that was lacking in the wild type (Fig. 5, bottom inset). This is the spectral region in which the biologically occurring benzoquinones, ubiquinones, and naphthoquinones absorb strongly but in which β -carotene has relatively weak absorbance.

We noted that the UV spectrum of the coeluting component was similar to plastoquinone-9, a quinone that is present at a 10-fold higher concentration than phylloquinone in thylakoid membranes (32). Indeed, we found that authentic plastoquinone-9 co-elutes with, and has a UV spectrum that matches, the peak at 37 min (Fig. 5, top inset). Sensitive selected-ion-monitoring analyses of the HPLC eluate at the mass of plastoquinone-9 ($m/e = 748$) showed a peak at this retention time. We consistently found levels of plastoquinone-9 in trimeric PS I complexes from the *menA* and *menB* mutants in amounts similar to phylloquinone in PS I complexes from the wild type. In contrast, we found no plastoquinone-9 or a very small amount in PS I complexes from the wild type. Full-scan HPLC/MS analyses of the mutants showed that none of the other peaks displayed mass spectral characteristics of related naphthoquinones, such as biosynthetic precursors of phylloquinone.

Low Temperature Reduction of F_A and F_B—The ability of PS I trimers from the *menA* and *menB* mutants to transfer electrons from P700 to the iron-sulfur clusters was determined at low temperature by EPR spectroscopy. When the samples were

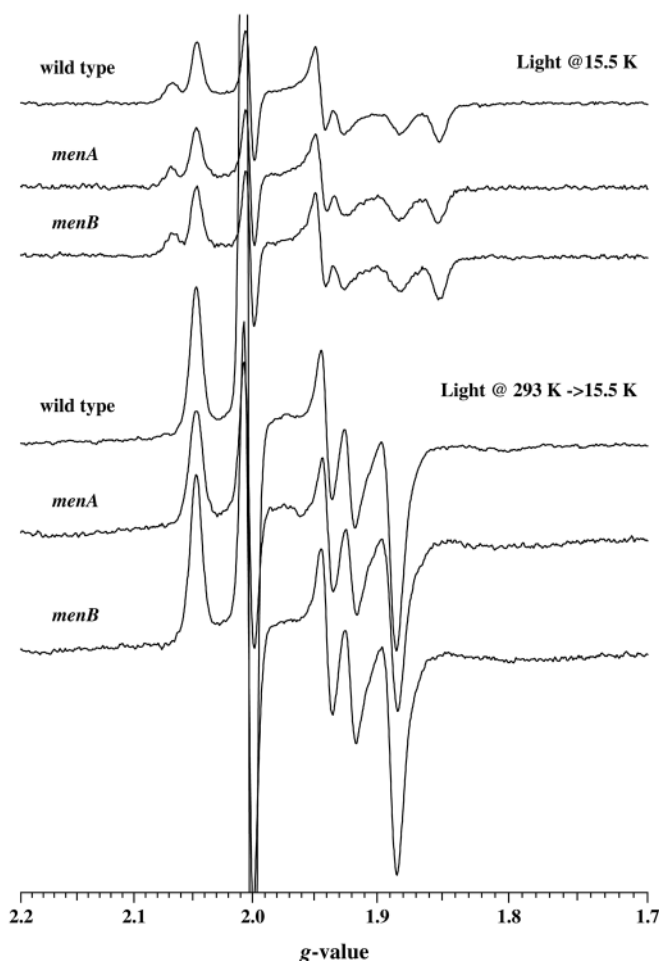


FIG. 6. EPR spectra of F_A and F_B in PS I complexes isolated from the wild type and the *menA* and *menB* mutant strains. The samples were resuspended to a concentration of 800 μ g Chl *a* ml⁻¹ in 25 mM Tris buffer (pH 8.3) with 15% glycerol, 0.02% *n*-dodecyl- β -D-maltoside, 10 mM sodium ascorbate, and 4 μ M 2,6-dichlorophenol-indophenol. The spectra were obtained either upon illumination of the sample frozen in the dark (top three spectra) or upon illumination of the samples during freezing from 293 to 15.5 K (bottom three spectra). Background spectra were recorded in dark-adapted samples frozen to 15.5 K and subtracted from the light-induced spectra. Spectrometer settings were as follows: microwave power, 20 mW; microwave frequency, 9.478 GHz; receiver gain, 6.3×10^4 ; modulation amplitude, 10 G at 100 kHz; magnetic field; center field, 3480 G; scan width, of 1740 G. Results are the average of three scans.

frozen in darkness and illuminated at 15 K, the relative spin concentrations of reduced F_A ($g = 2.05, 1.94, 1.85$) and F_B ($g = 2.07, 1.92, 1.88$) were identical to those for PS I complexes isolated from the wild type (Fig. 6). The ratio of F_A to F_B reduced was also identical in the mutants and the wild type. Thus, the absence of phylloquinone in the A₁ site does not effect low temperature electron transfer from A₀⁻ to the terminal iron-sulfur clusters. When PS I complexes from the *menA* and *menB* mutants were subjected to photoaccumulation conditions by freezing the sample during illumination, F_A and F_B were completely reduced, as shown by the presence of an interaction spectrum (g -values of 2.05, 1.94, 1.92, and 1.88), with total spin concentrations similar to that of the wild type. It should be noted that the quantum yield cannot be determined in these studies because multiple turnovers of the PS I complex occur during continuous illumination. Nevertheless, the quantitative reduction of F_A and F_B does indicate that the entire population of mutant PS I complexes is competent in electron transport.

Flavodoxin Reduction Rates in PS I Complexes—Although

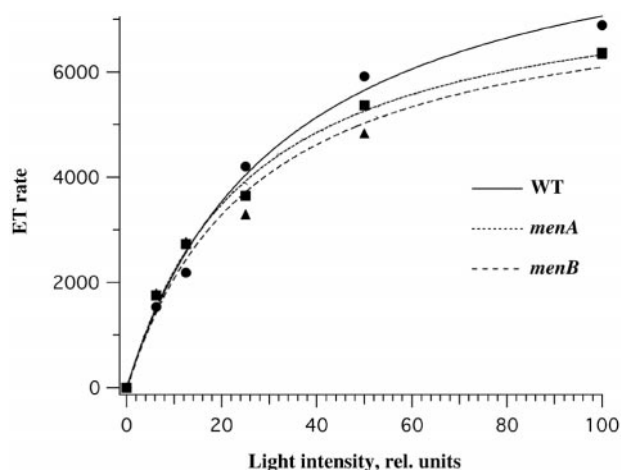


FIG. 7. Steady-state rates of flavodoxin reduction in PS I complexes isolated from the wild type and the *menA* and *menB* mutant strains. Rates of electron transfer in units of $\mu\text{mol mg Chl}^{-1} \text{h}^{-1}$ are plotted as a function of light intensity. The rates at saturating light intensity were determined by least-squares fitting to a double reciprocal plot and by extrapolation to infinite light intensity (not shown).

the lower rates of whole-chain electron transfer in the *menA* and *menB* mutant cells can be explained by a lower PS I content per cell, there still remains the possibility that the efficiency electron transfer in individual PS I complexes is altered by the absence of phylloquinone. Steady-state rates of electron transfer were determined in PS I trimers by measuring the rate of flavodoxin reduction with cytochrome c_6 as electron donor as a function of light intensity (Fig. 7). The rates at saturating light intensity were determined by treating light as a substrate in a Michaelis-Menten kinetic analysis. The maximal rate of flavodoxin reduction that could be sustained was found to be $8460 \mu\text{mol mg Chl}^{-1} \text{h}^{-1}$ in the wild type PS I complexes, $7128 \mu\text{mol mg Chl}^{-1} \text{h}^{-1}$ in the PS I complexes of the *menA* mutant, and $6948 \mu\text{mol mg Chl}^{-1} \text{h}^{-1}$ in the PS I complexes of the *menB* mutant. Assuming 100 Chl per P700 in all PS I complexes, these maximal rates of electron transport correspond to $235 e^- \text{PS I}^{-1} \text{s}^{-1}$ in the wild type, $198 e^- \text{PS I}^{-1} \text{s}^{-1}$ in the *menA* mutant, and $193 e^- \text{PS I}^{-1} \text{s}^{-1}$ in the *menB* mutant. As shown in Fig. 7, the light saturation dependence of the electron transfer rates in the PS I complexes of the *menA* and *menB* mutants strains is similar to that for the wild type complexes, indicating that the relative quantum efficiencies of the PS I complexes are not affected by the mutations. These results show that despite the absence of phylloquinone in the A_1 site, electron transfer throughputs in PS I complexes isolated from the *menA* and *menB* mutants are 82–84% as efficient as in PS I complexes isolated from the wild type strain.

DISCUSSION

Although the phylloquinone biosynthetic pathway in cyanobacteria has not been previously described, the nucleotide sequence of the *Synechocystis* sp. PCC 6803 genome shows the existence of homologues of the *menA*, *menB*, *menC*, *menD*, *menE*, *menF* (*entC*), and *menG* genes, which code for enzymes involved in menaquinone biosynthesis in other bacteria. Because they encode enzymes that function near the end of the biosynthetic pathway, we focused exclusively on the *menA* and *menB* genes in this study. The *menB* gene of *E. coli* codes for 1,4-dihydroxy-2-naphthoic acid synthase, which catalyzes the formation of the two-ring system by converting *o*-succinylbenzoyl-coenzyme A to 1,4-dihydroxy-2-naphthoic acid. Menaquinone differs from phylloquinone by the presence of a partly unsaturated, C-40 isoprenyl tail rather than a mostly satu-

rated, C-20 phytyl side chain attached to the naphthoquinone nucleus. The *menA* gene of *E. coli* codes for 1,4-dihydroxy-2-naphthoate octaprenyl transferase, which catalyzes ligation of the C-40 isoprenyl chain to the C_2 position of the naphthoate moiety. The low degree of identity (17%) in the primary sequences of the MenA proteins of *E. coli* and *Synechococcus* sp. PCC 6803 is consistent with the difference in the substrate specificity of these enzymes.

Because of the structural similarity between menaquinones and phylloquinone, we worked from the premise that the *menA* and *menB* genes code for proteins that function in phylloquinone biosynthesis. To test this premise, we engineered mutants by targeted inactivation of the *menA* and *menB* genes in *Synechocystis* sp. PCC 6803. Southern blot hybridization and PCR analyses were used to confirm the absence of a complete *menA* or *menB* gene in the mutants. HPLC/MS and gas chromatography/MS showed that the membranes of the *menA* and *menB* mutant strains do not contain detectable levels of phylloquinone. Hence, one firm conclusion of this study is that the *menA* and *menB* homologues in the *Synechocystis* sp. PCC 6803 genome code for essential enzymes in the phylloquinone biosynthetic pathway. A corollary to this conclusion is that no other biosynthetic routes to phylloquinone exist beyond naphthoate synthase in *Synechocystis* sp. PCC 6803. The *menA* and *menB* mutants showed similar biochemical and physiological characteristics. Both lacked phylloquinone, and both contained plastoquinone-9 in their PS I complexes. These results further suggest that the phytyl chain of phylloquinone is required for its stable assembly into PS I complexes *in vivo*. These observations confirm the participation of both the *menA* and *menB* gene products in the phylloquinone biosynthesis pathway in *Synechocystis* sp. PCC 6803.

To study their physiology and growth characteristics, as well as the role of phylloquinone in photosynthetic electron transfer, the *menA* and *menB* mutant strains were grown under a variety of conditions. Both mutant strains grew photoautotrophically at low to moderate light intensities (20 and $40 \mu\text{E m}^{-2} \text{s}^{-1}$) but failed to grow either photoautotrophically or photomixotrophically when the light intensity exceeded $100 \mu\text{E m}^{-2} \text{s}^{-1}$. Because photoheterotrophic growth occurs in the mutants at high light intensities when atrazine is present, excess reductant produced by PS II is proposed to be the cause of the failure to grow at high light intensities. Atrazine binds competitively to the Q_B site in PS II, blocks the high rate of damaging reductant and/or oxidant formation, and allows the cells to survive the toxic effect of light and to use glucose as the source of reduced carbon. We have found that although the amount of PS II is unchanged relative to the wild type, the amount of functional PS I per cell in the *menA* and *menB* mutants is approximately 50% lower than in the wild type. The observed phototoxicity may therefore be an indirect effect caused by an imbalance of the rates of electron transport between PS II and PS I. Indeed, we have recently obtained several second-site suppressor mutants that allow a phylloquinone-less mutant to grow photoautotrophically under high light intensity.² Most of these mutants have reduced PS II activity, thereby supporting our postulate that the PS II toxicity is responsible for the inability of the phylloquinone-less mutants to grow under high light intensity. The cause of reduction in the PS I level in the *menA* and *menB* mutant cells could be a decreased rate of assembly or an increased turnover rate of PS I complexes. However, an examination of the degradation rate of the PS I apoproteins in the mutant and wild type strains did not show significant differences (data not shown). We therefore suggest

² P. Chitnis and J. Golbeck, unpublished results.

that the absence of phylloquinone affects the rate of assembly of PS I complexes by influencing one or more steps involved in its biogenesis.

The ability of the *menA* and *menB* mutants to grow at low to moderate light intensities agrees with the finding that the absence of phylloquinone did not abolish room temperature photosynthetic electron transfer activity through PS I complexes isolated from these mutants. The mutant strains contained less PS I than the wild type on a per cell basis; additionally, the steady-state rates of electron transfer from cytochrome c_6 to flavodoxin in PS I complexes from the mutants were high but were 82–84% of the wild type rate. Therefore, the lower rate of whole-chain electron transfer in the mutant cells is a combination of both effects. In PS I complexes isolated from the *menA* and *menB* mutants, electron transfer from P700 to the terminal iron-sulfur clusters is quantitative at cryogenic temperatures. However, in PS I complexes in which phylloquinone has been partially extracted using solvents, the maximum amount of irreversible charge separation after a large number of flashes is independent of the number (0, 1, or 2) of phylloquinone molecules per PS I complex (28). Hence, single turnover optical studies of P700 turnover at room temperature will be necessary to confirm the slightly lower quantum efficiency of PS I electron transfer in the *menA* and *menB* mutants.

The inescapable conclusion from this study is that phylloquinone is not required for efficient electron transfer in PS I at either room or cryogenic temperatures. We considered two explanations for the high rates of PS I activity. One is that the mutant PS I complexes differ from the solvent-extracted PS I complexes in allowing room temperature as well as low temperature electron transfer in the absence of phylloquinone. This bypass may be direct or it may involve a redox-active amino acid in the transmembrane domain of the PsaA and PsaB polypeptides. The second possibility is that a foreign quinone has been recruited into the A_1 site and that it may participate in electron transfer from A_0 to F_X . This quinone, which may be identical to the plastoquinone-9 identified on the basis of the solvent extraction studies described here, may substitute for phylloquinone in the A_1 site, thereby promoting electron transfer to F_X and F_A/F_B . Spectroscopic evidence supporting the presence of plastoquinone-9 in the A_1 site and its participation in forward electron transfer is provided in the second paper of this series (33).

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METABOLISM AND BIOENERGETICS:
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