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Phylloquinone biosynthetic pathway

by

Thomas Wade Johnson

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

Major: Biochemistry
Major Professor: Parag R. Chitnis

Iowa State University
Ames, Iowa
2000

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This is to certify that the Doctoral dissertation of

Thomáys Wade Johnson

has met the dissertation requirements of Iowa State University

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Major Professor

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For the Major Program

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For the Graduate College
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ABSTRACT

Photosynthesis converts solar energy into chemical energy to drive the biological processes on our planet. Photosystem I (PSI) is one of the two reaction centers of oxygenic photosynthesis in cyanobacteria and higher plants. PSI is a multisubunit membrane bound protein complex that catalyzes the photooxidation of plastocyanin in the thylakoid lumen and the photoreduction of ferredoxin in the cytoplasm. The PsaA/PsaB subunits form the heterodimeric core that harbors the primary electron donor P700 and acceptors A0, A1, and FX. Two electron acceptors, Fx and Fb, occupy PsaC one of three peripheral proteins bound to PSI. The secondary electron acceptor, A1, contains a phylloquinone, a molecule utilized only in PSI. The object of this dissertation is to probe phylloquinone biosynthesis and the bioenergetic aspects of phylloquinone, plastoquinone, and other naphthoquinones in the A1 site of PSI.

To investigate phylloquinone’s role in PSI, we substituted other naphthoquinones and plastoquinone the A1 site to observe the physiological changes of the cells and energetic changes in PSI. Menaquinone biosynthetic genes have been identified in *Escherichia coli* with the phylloquinone homologs elucidated from the *Synechocystis* sp. PCC 6803 genome. Four phylloquinone biosynthetic genes in *Synechocystis* have been mutationally disabled, *mnaA*, *menB*, *menD*, and *menE*, leading to a complete loss of phylloquinone in the cells. The A1 site of the mutant contains plastoquinone, allowing a reduced electron transfer in PSI rate. The orientation and distance of the A1 quinone to P700 is the same as wild type. Physiological changes in the mutants include decreased growth rate, oxygen evolution, and chlorophyll content. Addition of the direct product of naphthoate synthase (*menB*), 2-carboxy-1,4-naphthoquinone, restored phylloquinone content of the PSI complexes to the wild-type levels. Mutant cells also use free phylloquinone from the growth medium to displace plastoquinone in PSI. Addition of vitamin K3, 1,4-naphthoquinone, 2-hydroxy-1,4-naphthoquinone, or 1,2-naphthoquinone allowed the cells to synthesize phytolated naphthoquinones, which were inserted into the A1 site in a fraction of the PSI complexes. The results in this dissertation determined the biosynthetic pathway of phylloquinone and alternative phytolated quinones in the A1 site are capable of electron transfer from A0 to FX.
CHAPTER 1. GENERAL INTRODUCTION

Photosynthesis is an extremely important process that converts solar energy into chemical energy, thereby fueling the living world directly or indirectly. In oxygenic photosynthesis, cyanobacteria and the chloroplasts of higher plants and algae sequester light through photosynthesis to release oxygen from water and produce carbohydrates from carbon dioxide reduction. In the light driven reactions of photosynthesis, photosystem I (PSI) and photosystem II (PSII) capture photons to utilize the energy for electron transfer from water to NADP⁺ through three membrane bound complexes: PSII, cytochrome b₆f and PSI. This process produces NADPH and a proton gradient across the thylakoid membranes that is used by ATP synthase to produce ATP. These products, ATP and NADPH, are used to reduce carbon dioxide in the production of carbohydrates.

Photosystem I (PSI) is a well-characterized photosynthetic reaction center in cyanobacteria and higher plants (Golbeck, 1993; Nitschke and Rutherford, 1991; Nugent, 1996). PSI catalyzes the photooxidation of plastocyanin in the thylakoid lumen and the photoreduction of ferredoxin in the cyanobacterial cytoplasm (or chloroplast stroma). PSI contains at least eleven proteins, with PsaA and PsaB as a heterodimeric core. Surrounding PsaA/PsaB are 6 small subunit proteins, that serve various identified or unknown functions (Chitnis, 1996). In addition to light-harvesting chlorophyll a (Chl a) molecules, PsaA/PsaB is imbedded with the P700 reaction center and a series of cofactors (A₀, A₁, Fₓ) that act as intermediate electron acceptors and provide a charge separation from the initial P700 reaction center (Brettel, 1997). P700 is comprised of a dimer of Chl a molecules. The electron acceptor cofactors include a molecule of chlorophyll a, A₀, phylloquinone, A₁ and a [4Fe-4S] iron sulfur protein, Fₓ. PSI also contains three overlapping peripheral proteins (PsaC, PsaD and PsaE). PsaC is a prerequisite for PsaD and PsaE binding and contains the two terminal electron transfer [4Fe-4S] iron sulfur clusters Fₐ and Fₜ (Yu et al., 1993; Antonkine et al., 2000; Yu et al., 1995). Fₐ and Fₜ act as an electron shuttle from the PsaA/PsaB core cofactors to docked ferredoxin. PsaD provides a ferredoxin docking site (Kruip et al., 1997; Chitnis et al., 1996), with PsaE assisting in the interaction of PSI with ferredoxin (Xu et al., 1994).
The secondary electron acceptor site, $A_1$, contains phylloquinone (PhQ, vitamin $K_1$, 2-methyl-3-phytyl-1,4-naphthoquinone). There are two bound molecules of phylloquinone per molecule of P700 in PSI (Malkin, 1986; Schoeder and Lockau, 1986; Takahashi et al., 1985; Biggins and Mathis, 1988). This molecule acts as an intermediate in the electron transfer from the primary acceptor $A_0$, a chlorophyll $a$ to $F_x$, an iron-sulfur cluster (Kumazaki et al., 1994; Brettel, 1988). The $A_0$ to $F_x$ electron transfer is an estimated 250 mV (Semenov et al., 2000) to 320 mV (Brettel, 1997) event. PhQ acts as both a physical and energetic bridge for the electron between the primary acceptor and the terminal iron sulfur clusters.

From the literature, several of non-native quinones were inserted into cyanobacterial PSI with varied and contradictory amounts of success towards $A_1$ reduction. Phylloquinone can be extracted from PSI with a dry or water saturated diethyl ether solution (Itoh et al., 1987; Itoh and Iwaki, 1989) or by a hexane containing 0.3% methanol (Rustandi et al., 1990; Setif et al., 1987). Both of these methods remove a significant amount of antenna Chl. caroteniods and lipids. Both extraction procedures blocked the electron from traveling past $A_0$ (Biggins and Mathis, 1988; Itoh et al., 1987). Iwaki and Itoh reported then function of PhQ could be replaced by a quinone of an appropriate redox potential (Iwaki and Itoh, 1994). However, these compounds did not restore reduction of the terminal iron sulfur clusters. Biggins suggested structural constraints imposed by an alkyl tail in addition to energetic considerations (Biggins, 1990). Forward electron transfer to $F_x$ was only achieved with quinone compounds that had a hydrophobic side chain that determined the orientation of the quinone in the $A_1$ site. Discrepancy between these results could have arrived from a combination of the harsh PhQ extraction procedures and the lack of resolution of the instruments.

The genes encoding the synthesis of phylloquinone have not been described in cyanobacteria. However, the biosynthetic genes of menaquinone, a phylloquinone-like molecule, have been cloned in other bacteria, such as *Escherichia coli*. (Palaniappan et al., 1992; Sharma et al., 1992; Sharma et al., 1993; Daruwala et al., 1996; Sharma et al., 1996; Suvarna et al., 1998). The nucleotide sequence of the *Synechocystis* sp. PCC 6803 genome shows the existence of homologues of the *menA*, *menB*, *menC*, *menD*, *menE*, and *menF*
(entC), and menG genes which code for enzymes involved in menaquinone biosynthesis in other bacteria. It is likely that the phylloquinone biosynthetic pathway is similar to menaquinone, given that the difference between the molecules is a mostly saturated C-20 phytol tail and an unsaturated C-45 a nine unit isoprenoid like side chain, respectively. The phytol transferase enzyme, MenA, has low homology compared to the menaquinone analog. The MenB enzyme, that closes the second ring in the naphthoquinone, is the last step in the construction of the naphthoquinone head group with the following two steps consisting of attaching the phytol tail and a C-2 methylation of naphthoquinone. The menD and menE genes encode for enzymes towards the beginning of the PhQ biosynthetic pathway.

With the current understanding of how phylloquinone is utilized in PSI and the existence of homologs of the menaquinone biosynthetic genes in Synechocystis sp. PCC 6803, we propose to systematically disable genes in the biosynthetic pathway of phylloquinone to achieve two goals:

1) Create a series phylloquinone-less mutants, thus confirming the biosynthetic pathway.

2) Monitor the physiological changes within the cell and the energetic changes within PSI of the phylloquinone-less mutants. We would then be able to supplement non-native quinones to probe the structural and energetic requirements of the A₁ site of PSI.

1. Organization of Dissertation

This dissertation contains four manuscripts and a literature review of phylloquinone synthesis and function in PSI. To confirm the genes involved in proposed phylloquinone biosynthetic pathway, the menA, menB, menD, and menE genes were mutationally disabled. This work has been divided into two manuscripts. The first half of this work concerning the menD and menE gene disruptions, which are towards the beginning of the PhQ pathway, has been summarized in the manuscript "The menD and menE homologues in cyanobacteria code for essential enzymes in phylloquinone biosynthetic pathway" to be submitted to the Plant Physiology. The work covering the menA and menB gene disruptions is titled "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" and is published in the Journal of Biological Chemistry, 275.
The four gene disruption mutations revealed that plastoquinone-9 occupies the A₁ site of PSI in place of the absent phyiloquinone (Semenov et al., 2000; Zybaïlov et al., 2000; Johnson et al., 2000). The next logical step is to insert alternative naphthoquinones into the A₁ site to monitor the physiological adaptations of the cells and the energetic changes within PSI. This study has been described in the manuscript "Recruitment of foreign quinones in the A₁ site of cyanobacterial photosystem I complex. IV. In vivo replacement of the native quinone in the phyiloquinone-less mutants of Synechocystis sp. PCC 6803 by externally supplied naphthoquinones" to be submitted to Journal of Biological Chemistry. Further investigation of the addition of 1,2-naphthoquinone to the menB mutant cells was performed and organized in the manuscript "Recruitment of foreign quinones in the A₁ site of cyanobacterial photosystem I V in vivo replacement of the native quinone in the phyiloquinone-less mutants of Synechocystis sp. PCC 6803 by 1,2-naphthoquinone". Following will be a general conclusions section highlighting the developments of the research. For future graduate students who will be expanding this research, there is a section of protocols and procedures that were developed or adapted specifically for these experiments and conditions. The remaining sections are summaries of second author papers published or in preparation. The appendices will refer the reader to related topics concerning the phyiloquinone less mutants and experiments that are beyond the scope of this dissertation.

2. References


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CHAPTER 2. PHYLLOQUINONE BIOSYNTHESIS AND BIOENERGETICS OF QUINONES IN THE A₁ SITE OF PHOTOSYSTEM I

1. Format of review

This review highlights two related topics: phylloquinone (PhQ, vitamin K₁ (K), 2-methyl-1,4-naphthoquinone) biosynthesis and the energetic properties of phylloquinone in photosystem I (PSI). To adequately describe and place each subject in context, several secondary foci will be addressed. PhQ. and related compounds, in bacteria, plants, and animals are described for production and functions within the organisms. Plastoquinone (PQ) will also be described, since this molecule can mimic some of the functions of phylloquinone. The genes involved in the biosynthetic pathway of phylloquinone will be compared to menaquinone (vitamin K₂) biosynthesis genes in bacteria and higher plants, given the structural similarities between the molecules. Phylloquinone is also a critical cofactor in PSI, hence the PSI complex and its subunit components are described. How PSI interacts with the electron donor and acceptor proteins is also mentioned. A brief description of the entire photosynthetic electron shuttling process from photosystem II (PSII) through Cyt b₆f through PSI will be given. The photosynthetic energy relationship of PSI is described in greater detail, with a focus on phylloquinone.

2. The use of phylloquinone in bacteria, plants, and animals

Phylloquinone or vitamin K is a fat soluble essential nutrient found in vegetables and certain oils. Fig 2.3 (Booth and Suttie. 1998). The recommended dietary allowance (RDA) of vitamin K is 1 µg/(kg body wt • d) with the average American consumption exceeding the RDA at 300-500 µg/d (Monsen. 1989). Green leafy vegetables such as collard greens, spinach, and salad greens contain 40-50% of the daily intake of vitamin K. Oils like canola and soybean make an appreciable but minor contribution to the total vitamin K intake, at close to 15% (Bolton-Smith et al., 2000). Menaquinone, referred to as vitamin K₂, contributes a relatively small amount to satisfy the human requirement for the vitamin (Reedstrom and Suttie, 1995). Both molecules are absorbed in the gut for utilization by the body. Regardless of the structural similarities between the two, menaquinone cannot be converted into phylloquinone, at least not in rats (Ronden et al., 1998).
Vitamin K, Figure 2.1 and Figure 2.3, is a well known molecule that is essential for blood clotting (Voet and Voet, 1995). In vertebrates and invertebrates, the only known use for phylloquinone is as a cofactor for a single enzymatic reaction: the conversion of glutamic acid to γ-carboxyglutamic acid (Gla) in select proteins (Furie et al., 1999). Vitamin K is involved in activating several blood clotting proteins (factor VII, factor IX, and factor X) (Dam, 1935) and creating the γ-carboxyglutamic acid which then acts as a metal binding amino acid side chain that confers chelating properties to proteins (Sperling et al., 1978). These metal chelating proteins derive a structural rigidity or conformational change upon addition of magnesium or calcium ions. As an example, a conotoxin derived from a venomous cone snail contains the polypeptide conantokin G (Rigby et al., 1997). This inactive sleeper peptide contains 17 residues with 5 γ-carboxyglutamic acid residues spaced every three to four amino acids. Upon addition of Mg²⁺ or Ca²⁺, the polypeptide forms an α-helix and the Gla side chains orient into a linear array with each ion being chelated by two adjacent carboxyl groups from different residues. The molecule is then toxic.

Recently the vitamin K-dependant carboxylase (carboxylase) was purified and cloned (Wu et al., 1991). Carboxylase is a single chain protein of 758 amino acids (94 kD) and is dominated by hydrophobic amino acids. The enzyme has at least five known functional properties: a carboxylase active site, an epoxidase active site, a substrate recognition site, a polypeptide stimulation site for carboxylase and epoxidase activity, and a phylloquinone binding site. Each of the properties is employed in the mechanism for γ-carboxylation.

Carboxylation of glutamic acids is a multistep process in which the premodified amino acid is recognized and modified by carboxylase with a cocontaminant cycling of phylloquinone from they hydroquinone to epoxide state, Figure 2.1. The native state phylloquinone is first reduced to the active hydroquinone by a separate enzyme, vitamin K epoxide reductase (Suttie, 1985). Then phyllohydroquinone is converted, by the carboxylase, to the alkoxide which acts as a strong base for the removal of the γ-proton on specific glutamic acid residues creating an anion and releasing hydroxide. Subsequently, CO₂ is added to the anionic carbon creating the γ-carboxyglutamic acid side chain. The activated vitamin K species convert into vitamin K epoxide. The epoxide is reduced back
into the phyllohydroquinone by vitamin K epoxide reductase (Wallin and Martin, 1987). A class of vitamin K antagonists, such as warfarin, were discovered as anticoagulation agents that inhibit the conversion of the epoxide back into the phyllohydroquinone (Campbell and Link, 1941).

![Diagram of vitamin K function](image)

Figure 2.1 Function of phylloquinone in vitamin K-dependant carboxylase. Vitamin K (PhQ) acts as a strong base in the alkoxide form of the molecule, removing a γ-proton from glutamic acid. The resulting carbanion is then carboxylated to form γ-carboxyglutamic acid. The resulting vitamin K epoxide is recycled. From Ref. (Furie et al., 1999)

Vitamin K is not synthesized naturally in animals but extracted in the digestive system from consumed green leafy parts of plants. The function of vitamin K is significantly different in animals from in plants and photosynthetic bacteria, with the only known use being an electron transfer intermediate cofactor of PSI.

PSI along with photosystem II (PSII), the cytochrome b6f complex (Cyt b6f), and ATP synthase are the protein complexes imbedded in the thylakoid membranes of cyanobacteria and chloroplasts of higher plants. PSI is the last segment of the electron transport pathway that reduces ferredoxin for the production of NADPH. Previous segments
in the electron transport chain oxidize water to dioxygen thereby creating a proton gradient which is ultimately used to produce ATP by ATP synthase.

3. The use of plastoquinone in living systems

Plastoquinone (PQ, 4,5-methyl-3-isoprenyl-1,4-benzoquinone), Figure 2.2. is a dimethyl substituted p-benzoquinone with a nine unit isopreniod tail that imparts a high degree of hydrophobicity. It is present in the inner and outer envelope membrane of chloroplasts (Roshchina, 1979) and within the thylakoid membranes of cyanobacteria (Tiemann et al., 1979). PQ undergoes a two electron (and two proton) reduction to the dihydroplastoquinol by PSII. PQ links PSII to the cytb\(\alpha\)f complex. Reduced plastoquinone then diffuses through the membrane until it encounters the Cyt \(b\alpha\)f complex binding site. This will be described in greater detail in section 6. It will also be shown in later chapters that plastoquinone mimics phylloquinone functions in PSI of the phylloquinone-less generated mutant.

![Plastoquinone](image)

Figure 2.2 Structure of plastoquinone.

4. Genes involved in phylloquinone biosynthesis

The biosynthesis of phylloquinone has not been described before in cyanobacteria. However, the biosynthetic pathway of an analog compound menaquinone has been determined. Figure 2.3. The differences between the molecules resides in the "tail" with phylloquinone having a mostly saturated side chain and menaquinone an unsaturated alkyl group. Given the similarity of the molecules and that there is no known function for menaquinone in cyanobacteria, most of the genes describing menaquinone biosynthesis should also describe phylloquinone biosynthesis. In \(E\)sherichia Coli as with some other
bacteria. vitamin K₂ (menaquinone) is used during fumarate reduction in anaerobic respiration (Unden and Bongaerts, 1997). Since several partial and full genomes are known, there is now a method for doing a direct gene comparison among organisms. Also described are strategies used to choose which genes are disrupted in the phylloquinone biosynthetic pathway.

![Figure 2.3 Structures of phylloquinone and menaquinone.](image)

In the biosynthesis of menaquinone (Figure 2.3), the 2-methyl group is derived from L-methionine (Threlfall, 1971; Kaipling et al., 1984) and the aromatic ring and the C-4 quinone carbonyl have been shown to be derived from shikimic acid, a precursor of chorismate (Cox and Gibson, 1966; Campbell et al., 1971). The three remaining carbons (C-1 through C-3) of the naphthalene ring are from 2-oxoglutaric acid (Campbell et al., 1971). Both quinone oxygens are derived from water, not from molecular oxygen (Snyder and Rapoport, 1970). The phytyl tail synthesis was thought to be generated from Acetyl-CoA through the mevalonate pathway to form geranylgeranyl pyrophosphate (Rohmer et al., 1993). This molecule is then hydrogenated to phytyl pyrophosphate with NADPH as the contributing electron donor (Schultz et al., 1985). The synthesis of the phylloquinone phytyl tail and the menaquinone isoprenoid tail is treated in greater detail in Ricardo Reategui’s Masters thesis entitled “Phylloquinone in the photosynthetic activity of Synechocystis sp. PCC 6803” (Reategui, 1998). Given that both molecules are closely related in structure and synthesis, it is likely that the sources of atoms that comprise phylloquinone are similar to menaquinone.
The genes encoding the synthesis of phylloquinone have not been described in cyanobacteria. However, the biosynthetic genes of menaquinone have been cloned in other bacteria, such as *E. coli*. (Palaniappan et al., 1992; Sharma et al., 1992; Sharma et al., 1993; Daruwala et al., 1996; Sharma et al., 1996; Suvarna et al., 1998). It is likely that the phylloquinone biosynthetic pathway is similar to menaquinone, given that the difference between the molecules is the phytol tail and isoprenoid side chain, respectively. With this exception, the synthesis of the naphthalene nucleus in phylloquinone and menaquinone is expected to include similar steps. The genome database for *Synechocystis* sp. PCC 6803 (Kaneko et al., 1996) 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* (DHNA synthase) and *menA* (identified as a "menaquinone biosynthesis protein"). Possible homologs of *menC* and ORF241 (the DHNA thioesterase) have also been identified in database searches. This proposed pathway is illustrated in Figure 2.4. This review will focus on the likely dedicated steps to phylloquinone synthesis: isochorismate to phylloquinone, involving genes *menD*, *menC*, *menE*, *menB*, *menA*, and *menG*. The *menF* gene is not likely to be a dedicated step to phylloquinone biosynthesis given that isochorismate also participates in the synthesis of serine and enterochelin (www.genome.ad.jp, Website address).

Assuming that phylloquinone biosynthesis is comparable to menaquinone biosynthesis, the starting molecule for the naphthoquinone nucleus is isochorismate. Figure 2.4. The first committed step (SHCHC synthase) involves the loss of pyruvate and carbon dioxide with the attachment of 2-oxoglutarate to the benzene ring. The next two steps involve stripping of the aromatic ring of the hydroxyl group (o-succinyl benzoic acid synthase) and subsequent attaching a CoA to the aromatic carboxylate group (o-succinylbenzoate:CoA synthetase). The succinyl group provides the C-2 through C-4 carbons in the formation of the second aromatic ring which is catalyzed by DHNA synthase with subsequent removal of the CoA by a thioesterase (ORF241). At this point the naphthoquinone head group of phylloquinone is essentially formed. The remaining step involves attaching a phytol group in the C-3 position (phytyl transferase) with loss of
pyrophosphate and carbon dioxide from the pyrophosphate-phytyl group and carboxylate, respectively. Finally the demethylphylloquinone is methylated the C-2 position (demethylphylloquinone methyltransferase).

Figure 2.4 Proposed biosynthetic pathway of phylloquinone biosynthesis in *Synechocystis* sp. PCC 6803.

The search for the proteins involved in the phylloquinone biosynthetic pathway in *Synechocystis* sp. PCC 6803 starts with the identified menaquinone producing genes. The genes encoding enzymes involved in the conversion of the chorismate to menaquinone have been cloned in *E. coli* (Palaniappan et al., 1992; Sharma et al., 1992; Sharma et al., 1993; Daruwala et al., 1996; Sharma et al., 1996; Suvarna et al., 1998; Wu et al., 1992) and *Bacillus subtilis* (Driscoll and Taber, 1992; Hill et al., 1990; Palaniappan et al., 1994; Taber
et al., 1981). Specific genes involved in the pathway have been determined in spinach (Kaipling et al., 1984) and *Bacillus stearothermophilus* (Koike-Takeshita et al., 1997). Menaquinone biosynthetic genes have also been identified by homology in a variety other genomes presented in Table 2.1: *Haemophilus Influenzae* and *Arabidopsis Thaliana* (www.genome.ad.jp. Website address). Each gene in the PhQ biosynthetic pathway has been examined individually for domain/active site homologies between *Synechocystis* sp. PCC 6803 and four other organisms ranging from bacteria to higher plants. The phylogenetic tree has been determined for each of them; it suggests the evolutionary ties among organisms, see Figures 2.5-2.10.

Table 2.1. Percent identity and percent homology of enzymes of proposed homologous gene in the phylloquinone biosynthetic pathway of *Synechocystis* sp. PCC 6803 to menaquinone genes in four other organisms: *Escherichia coli, Bacillus Subtilis, Haemophilus Influenza*, and *Arabidopsis Thaliana*.

<table>
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<th>Gene</th>
<th><em>Escherichia coli</em></th>
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<th><em>Haemophilus Influenza</em></th>
<th><em>Arabidopsis Thaliana</em></th>
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<td>28 / 42</td>
<td>25 / 45</td>
<td>*</td>
<td>25 / 43</td>
</tr>
<tr>
<td>menA</td>
<td>26 / 44</td>
<td>20 / 42</td>
<td>24 / 41</td>
<td>30 / 46</td>
</tr>
<tr>
<td>menG</td>
<td>30 / 50</td>
<td>33 / 51</td>
<td>*</td>
<td>32 / 44</td>
</tr>
</tbody>
</table>

* No homologous proteins identified.

As suggested by the homology comparisons with other organisms, the *menD, menC, menE, orf241, and menB* genes of *Synechocystis* sp. PCC 6803 are thought to be involved in 1,4-dihydroxy-2-naphthoate synthesis with the product of a *menA* homologue catalyzing the addition of phytol chain. The product of the phytol transferase, 2-phytyl-1,4-naphthoquinone, requires a methylation step to complete the phylloquinone biosynthesis. The gene originally identified as *gerC2* (sll1653, *menG*) in the *Synechocystis* sp. PCC 6803...
database codes for the 2-phytyl-1,4-naphthoquinone methyl transferase enzyme that catalyzes this reaction (Shen and Bryant, 2000).

In general, the phylogenetic trees in Figures 2.5 through 2.10. indicate in the biosynthetic pathway that Synechocystis (Syn) and Bacillus Subtilis (BSU) tend to be paired first. E. Coli (Eco) and Haemophilus Influeza (HIN) also tend form a first pair throughout the pathway. Those two first pairs (Syn/BSU and Eco/HIN) are then usually joined before Arabidopsis Thaliana (Art) intersects. For many of the steps Syn is more closely conserved with other bacteria and Art shows the most deviation in protein alignment, for obvious reasons. Nearly all Art proteins are significantly longer than the bacterial proteins. This is probably attributed to Art containing introns. This reduces the homology of the Art proteins to the bacterial. Some discussions will be given to likely common active sites or domains within an alignment.

The menD (SHCHC synthase) and menC (o-succinyl benzoic acid synthase) genes are the first two genes coding for enzymes that are likely involved in phylloquinone biosynthesis. The enzyme SHCHC synthase product is 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate (SHCHC) with the subsequent step of o-succinyl benzoic acid synthase producing o-succinyl benzoic acid. Both menD genes from E. coli (Palaniappan et al., 1992) and Bacillus subtilis (Palaniappan et al., 1994) have been cloned. The menC gene has also been cloned in both E. coli (Sharma et al., 1993) and Bacillus subtilis (Hill et al., 1990; Guest, 1977). Comparisons with Syn the SHCHC synthase show a steady 42-52% homology and slightly less at 37-46% for the menC gene product. Table 2.1. Protein alignments for SHCHC synthase. Figure 2.5, show a highly conserved N-terminus domain that is likely to be the active site of the enzyme. Both pairs of organisms (Syn/BSU and Eco/HIN) show additional homologies within each pair. This domain could be part of the active site or a possible substrate binding site for 2-oxogluterate which is consumed in the reaction with pyruvate and carbon dioxide released as side products. Removed from Figure 2.5 is the first 400 amino acids on the N-terminus of Art. The o-succinyl benzoic acid synthase (menC) has no well defined identical domains that can be considered an active site. Figure 2.6. Yet, there are several sets of residues that are have complete identity towards the C-terminus end. Given that the reaction involves only a loss of a hydroxyl from the substrate
Figure 2.5 Homology and phylogenetic tree of the **menD** gene. Abbreviation for organism: Art2 is *Arabidopsis Thaliana*, BSU is *Bacillus Subtilis*, Eco is *E. Coli*, HIN is *Haemophilus Influenza*, and Syn is *Synechocystis* sp. PCC 6803.
Figure 2.6 Homology and phylogenetic tree of the menC gene. Abbreviation for organism: Art is Arabidopsis Thaliana, BSU2 is Bacillus Subtilis, Eco is E. Coli, HIN is Haemophilus Influenza, and Syn is Synechocystis sp. PCC 6803.
without a cofactor suggests that a smaller active site is plausible. Again Art has had the remaining 300 residues removed from the C-terminus end. The only change between protein alignments with and without the truncated protein is seen in the phylogenetic tree, with Art aligning more closely with Eco/HIN. To determine if genes are coding for enzymes in the phylloquinone biosynthetic pathway, the menD gene was mutationally disabled (Johnson et al., 2000a). The menD gene was chosen over the menC gene because it had a higher homology and identity between Syn and Eco. Table 2.1.

The largest average enzyme in the pathway is o-succinyl benzoate:CoA synthetase which is encoded by menE. Figure 2.7. The homology comparison to the other organisms is low compared to the other protein homologies in this pathway. Unlike menC which also has a low homology, in menE there is a C-terminal region of identity that could be the active site domain. Many of the other residues in this domain have more highly conserved hydrophobic side chains and possible proline kinks or turns in the 350 – 400 amino acid region of Eco. The enzyme performs a relatively complex task of attaching a CoA while utilizing the energy of hydrolysis of ATP to AMP. This suggests that there may be several domains that perform different aspects of the enzyme function. The Art protein is again longer than the homologous bacterial proteins, but only by approximately 100 amino acids. Given that in Art additional amino acids are contained in 4-8 residue stretches within the alignment of the other proteins, these additional residues could be additional loops or helices contained within the protein or possible introns. From the identification of this gene in both Eco (Sharma et al., 1996) and BSU (Driscoll and Taber, 1992), the menE gene was chosen for gene disruption in Syn (Johnson et al., 2000a).

The menB gene product (DHNA synthase) is the most conserved protein within the biosynthetic pathway of phylloquinone. Table 2.1. Art has the lowest identity at 54% which possibly can be attributed to an intron in the N-terminal region. Figure 2.8. The overall homology of bacterial proteins is 80% and upon visual inspection the homology of Art is similar, once the excess residues (possible introns) are removed. The C-terminus half is very highly conserved. Given the cross organism homology, the second ring closure can be considered a critical step turning a substituted benzene ring into a naphthoquinone. DHNA synthase is the only step that does not produce byproducts or consume energy as it
Figure 2.7 Homology and phylogenetic tree of the *menE* gene. Abbreviation for organism: Art2 is *Arabidopsis Thaliana*. BSU is *Bacillus Subtilis*. Eco is *E. Coli*. HIN is *Haemophilus Influenza*, and Syn is *Synechocystis* sp. PCC 6803.
Figure 2.8 Homology and phylogenetic tree of the *menB* gene. Abbreviation for organism: 
Art is *Arabidopsis Thaliana*, BSU is *Bacillus Subtilis*, Eco is *E. Coli*, HIN is *Haemophilus Influenza*, and Syn is *Synechocystis* sp. PCC 6803.
functions. This suggests that the enzyme mechanism is quite refined, hence imparting a high degree of homology. DHNA meets all the requirements of fitting into the A₁ site, except the tail. For these reasons, this step was the first chosen to be mutationally disabled in Syn (Johnson et al., 2000b; Reategui et al., 1998; Zybailov et al., 2000). This disallows the production of the head group of phylloquinone. By disabling the function of DHNA synthase there are two possible outcomes for the A₁: remain empty or find a substitute molecule to act as an electron intermediate. Interestingly, plastoquinone occupies and functions within the A₁ site of this mutant. All gene disruptions (menB, menD, and menE) in the phylloquinone biosynthetic pathway of Synechocystis have the same result. Given the homology of the genes and that plastoquinone is a possible natural rescue molecule, future analysis of the phylloquinone biosynthetic pathway in higher plants such as Arabidopsis suggests that the menB gene is a logical place to start.

Given the little knowledge gathered concerning orf241, we will only really acknowledge that it is probably in the menaquinone/phylloquinone biosynthetic pathway and that the homology is relatively low among organisms. It is thought to be a thioesterase which releases CoA from DHNA-CoA.

The DHNA phytol transferase encoded from menA in Syn. is clearly an important step. While low, the homologies are surprisingly similar given that menA product in Syn is transferring a mostly C-20 saturated phytol tail and in other bacterial organisms the menA product is attaching an unsaturated C-40 group to naphthoquinone. Figure 2.9. This step differentiates the production of phylloquinone and menaquinone. The synthesized DHNA meets all the requirements of fitting into the A₁ site, except the tail, which led to the author’s question, “was the tail necessary for A₁ function or was the naphthoquinone head group the only requirement?” Therefore, this step was the first chosen along with menB to be mutationally disabled in Syn (Johnson et al., 2000b; Reategui et al., 1998; Zybailov et al., 2000). It was found that DHNA was not incorporated into the A₁ site, instead plastoquinone was utilized similar to gene disruptions earlier in the pathway. Therefore, the head group and alkyl tail are both requirements for stable interaction.

The menG methyl transferase, is the most illusive of the genes in the pathway to identify decisively. Methylation is a common reaction in cells and is performed by many
Figure 2.9 Homology and phylogenetic tree of the menA gene. Abbreviation for organism: Art is Arabidopsis Thaliana, BSU is Bacillus Subtilis, Eco2 is E. Coli. HIN is Haemophilus Influenza, and Syn is Synechocystis sp. PCC 6803.
enzymes on a variety of substrates (Voet and Voet, 1995). Finding the specific enzyme responsible for demethylphyloquinone methylation by homologies would be time consuming and not necessarily conclusive. It is also possible that another enzyme may perform the same function. The homologies amount organisms are comparable to other genes. However, no comparable domains for the possible active site are found. Figure 2.10. Yet, in Syn the gene, sll1653, has been identified and mutationally disabled (Shen and Bryant, 2000). The result of this mutant is that only demethylphyloquinone is produced with a complete lack of phyloquinone. This confirms not only the gene responsible for this enzymatic reaction, but that there is no alternative pathway for demethylphyloquinone methylation. Demethylphyloquinone is apparently utilized in PSI in place of phyloquinone and preferentially over plastoquinone. However, demethylphyloquinone in the A1 site reveals some kinetic differences have been observed and are being fully explored (Shen and Bryant, 2000).

Homologous genes involved in phyloquinone biosynthesis of Synechocystis sp. PCC 6803 have been identified. The regions of homology within organisms vary significantly among genes. Several of the genes in which high homology between Synechocystis sp PCC 6803 and E. coli, which have been cloned, have been disrupted. Five genes (menA, menB, menD, menE, and menG) of the seven in the pathway have been have been disrupted.

5. Physical description of Photosystem I

Detailed biochemical, biophysical, and molecular biological studies have shown that the PSI complex of cyanobacteria is structurally and functionally equivalent to that of higher plants (Golbeck, 1994; Chitnis, 1996; Nexhushtai et al., 1996; Malkin, 1996). However, the greater stability of the cyanobacterial complex (Tsiotis et al., 1993), an improving X-ray structure that has reached atomic level resolution (Schubert et al., 1997; Krauss et al., 1996; Klukas et al., 1999a; Klukas et al., 1999b), as well as the relative ease of genetic manipulations in cyanobacteria (Thiel, 1994) are reasons for studying the cyanobacterial model complex. The fundamental organization of most of the photochemical electron transport components is similar in the Type I (PSI, green bacterial RCs) and Type II
<table>
<thead>
<tr>
<th>Gene</th>
<th>Consensus</th>
<th>Syn_\text{menG}.t</th>
<th>BSU_\text{menG}.t</th>
<th>Ecoi \text{menG}</th>
<th>Arab_\text{menG}.t</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Syn_\text{menG}.t</td>
<td></td>
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<td></td>
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<tr>
<td>BSU_\text{menG}.t</td>
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<tr>
<td>Ecoi \text{menG}</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Arab_\text{menG}.t</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 2.10** Homology and phylogenetic tree of the \textit{menG} gene. Abbreviation for organism: Arab is \textit{Arabidopsis Thaliana}, BSU is \textit{Bacillus Subtilis}, Ecoi is \textit{E. Coli}, and Syn is \textit{Synechocystis} sp. PCC 6803.
(PSII, purple bacterial RC) reaction centers (Schubert et al., 1998). A comparison of the structure of PSI (presently at a resolution of ~3 Å (Summers, 1980) with that of the bacterial RC (at a resolution of ~2.3 Å (Lancaster and Michel, 1999)) shows that the organization of the primary donor (special pair), accessory Chls, primary acceptor, and secondary quinone acceptor is arranged in a manner similar to the equivalent cofactors in the purple bacterial. Figure 2.11.

PSI is a membrane-bound multisubunit protein complex that catalyzes the photooxidation of plastocyanin in the thylakoid lumen and the photoreduction of ferredoxin in the cyanobacterial cytoplasm (or chloroplast stroma) (Manna and Chitnis, 1998; Malkin, 1996). Purified cyanobacterial PSI complexes contain at least eleven proteins: core proteins (PsaA and PsaB), six integral membrane proteins (PsaF, PsaI, PsaJ, PsaK, and PsaL), and three peripheral proteins (PsaC, PsaD, PsaE). PsaM is an integral membrane protein found only in cyanobacteria. There are three other proteins contained in chloroplasts: one stromal peripheral protein (PsaH), one lumenal protein (PsaN), and one integral membrane protein (PsaG). The genes and characteristics of the PSI proteins are shown in Table 2.2.

The PsaA and PsaB proteins form the hydrophobic core of PSI. The proteins are similar in mass (~82 kDa) and homology. Because of the similar sizes, the PsaA and PsaB proteins usually co-migrate as a diffuse band in a SDS-PAGE gel. The primary sequences of the proteins from various organisms contain higher than 78% and 76% amino acid identities for PsaA and PsaB, respectively (Sun et al., 1998). A direct comparison of PsaA and PsaB primary sequences to each other shows a 42% amino acid identity and an additional 15% conservative replacement (Sun, 1999). PsaA and PsaB may have evolved by gene duplication, this is supported from the similar structural features.

Structurally, PsaA and PsaB have a total of 22 transmembrane helices, arranged around a pseudo-2-fold axis of symmetry (Schubert et al., 1997; Krauss et al., 1996). The cofactors involved with electron transfer from the primary reaction center P700 to Fx are located in ten transmembrane helices within the central region of the PsaA/PsaB subunits (five helices from each subunit), see Fig. 2.11. The helices farther away provide stability to the central core of helices and interact with other PSI subunits (Xu et al., 1994a; Chitnis et al., 1993a; Chitnis et al., 1995; Chitnis et al., 1997).
Table 2.2 Polypeptide subunits of PSI. The molecular mass is determined from primary sequences of *Synechocystis* sp. PCC 6803 and apparent mass observed from PAGE is in parenthesis. *PsaG*, *PsaH*, and *PsaN* molecular masses are the range from higher plants.

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Gene</th>
<th>Mass</th>
<th>Properties</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>PsaA</td>
<td><em>PsaA</em></td>
<td>82.9 (66)</td>
<td>11 transmembrane, 3 stromal and 1 luminal surface helices each; together, they bind ~100 Chl a, P700 Chl a dimer, 12-16 b-carotene, 2 PhQ, and one [4Fe-4S] cluster (Fx)</td>
<td>Light-harvesting; charge separation; electron transfer photoprotection; charge separation</td>
</tr>
<tr>
<td>PsaB</td>
<td><em>PsaB</em></td>
<td>81.2 (66)</td>
<td>Peripheral on stromal (cytoplasmic) side; binds two [4Fe-4S] clusters (FA, FB); 2 single-turn a-helices</td>
<td>Terminal acceptors; donates electrons to ferredoxin</td>
</tr>
<tr>
<td>PsaC</td>
<td><em>PsaC</em></td>
<td>8.8 (8)</td>
<td>Peripheral on stromal (cytoplasmic) side; 1 a-helix</td>
<td>Ferredoxin-docking; assembly of PsaC; normal EPR properties of FA and FB</td>
</tr>
<tr>
<td>PsaD</td>
<td><em>PsaD</em></td>
<td>15.6 (17.7)</td>
<td>Peripheral on stromal (cytoplasmic) side; b-barrel structure</td>
<td>Facilitates interaction with ferredoxin; essential for cyclic electron transport</td>
</tr>
<tr>
<td>PsaE</td>
<td><em>PsaE</em></td>
<td>8.1 (8.8)</td>
<td>2 transmembrane helices; large exposure on luminal side; may bind 1-3 Chl a</td>
<td>Plastocyanin-docking for fast electron transfer; stabilize core antenna?</td>
</tr>
<tr>
<td>PsaF</td>
<td><em>PsaF</em></td>
<td>15.7 (15.8)</td>
<td>2 putative transmembrane helices; only in chloroplasts</td>
<td>Interaction with LHCl</td>
</tr>
<tr>
<td>PsaG</td>
<td><em>PsaG</em></td>
<td>10-10.8</td>
<td>Peripheral on stromal side; only in chloroplasts</td>
<td>?</td>
</tr>
<tr>
<td>PsaH</td>
<td><em>PsaH</em></td>
<td>10.2-11</td>
<td>1 transmembrane helix</td>
<td>Normal organization of PsaL</td>
</tr>
<tr>
<td>PsaI</td>
<td><em>PsaI</em></td>
<td>4.4 (3.4)</td>
<td>1 transmembrane helix</td>
<td>Normal organization of PsaF</td>
</tr>
<tr>
<td>PsaJ</td>
<td><em>PsaJ</em></td>
<td>4.5 (3.0)</td>
<td>1 transmembrane helix</td>
<td>Interaction with LHCl; stabilize core antenna?</td>
</tr>
<tr>
<td>PsaK</td>
<td><em>PsaK</em></td>
<td>8.6 (5.1)</td>
<td>2 transmembrane helices; may bind 1-2 Chl a</td>
<td>PSI trimerization; stabilize core antenna?</td>
</tr>
<tr>
<td>PsaL</td>
<td><em>PsaL</em></td>
<td>16.6 (14.3)</td>
<td>2 transmembrane helices; may bind 2-4 Chl a</td>
<td>Stabilize trimer in cooperation with PsaK?</td>
</tr>
<tr>
<td>PsaM</td>
<td><em>PsaM</em></td>
<td>3.4 (2.8)</td>
<td>1 transmembrane helix; not in chloroplasts</td>
<td>?</td>
</tr>
<tr>
<td>PsaN</td>
<td><em>PsaN</em></td>
<td>9</td>
<td>Peripheral on luminal side; only in chloroplasts</td>
<td></td>
</tr>
</tbody>
</table>

Of the remaining membrane bound subunits PsaL is of note. The function of PsaL was determined by inactivation of the PsaL gene. Trimeric PSI complexes cannot be obtained from the PsaL-deficient mutants of *Synechocystis* sp. PCC 6803, hence PSI is in the monomeric form. PsaL is also accessible to proteolytic digestion in the monomers but not in the PSI trimers from wild type membranes (Chitnis et al., 1993b; Chitnis and Chitnis.
1993). It was concluded that PsaL is responsible for the formation of trimers, which has been confirmed in another PsaL-less mutant of cyanobacterium *Synechococcus* sp. PCC 7002 (Schluchter, 1996). Typically the analysis of purified PSI complexes is done only on the trimers, since this form is what is commonly found *in vivo*.

![Diagram of PSI cofactors](image)

**Figure 2.11** Arrangement of the PSI cofactors. Distances are depicted in angstroms. The cofactors are identified as the primary donor P700 (a Chl *a* dimer), the primary acceptor A₀ (a Chl *a* monomer), phylloquinone Q₅, and the three iron-sulfur clusters, Fₓ, Fₐ, and Fₜ. From Ref. (Klukas et al., 1999b).

The core proteins PsaA/PsaB bind and contain numerous cofactors. The electron carriers from P700 to Fₓ and 12-16 B-carotenes are bound within the heterodimer. The P700 reaction center is a dimer of Chl *a* and *a' *molecules with a histidine from each subunit coordinating to one Chl *a* (Fromme et al., 1994). There are two symmetrical Chl monomers identified as the A₀ primary electron acceptor, adjacent to P700. The phylloquinones in the secondary electron acceptor site A₁ have been identified in the crystal structure (Klukas et al., 1999a) and orientation of the quinones further was refined by EPR techniques (Bittl et al., 1997; Van der Est et al., 1997). The final electron acceptor is a [4Fe-4S] iron sulfur cluster Fₓ that is located on the pseudo-2-fold symmetrical axis on the stromal side. Fig. 2.11. It is coordinated by both PsaA and PsaB with conserved cysteines. The β-carotene molecules are not well defined within the crystal structure of PSI.
The PSI complex carries its integral core antenna system consisting of ~100 antenna Chl \( a \) molecules. In the crystal structure of PSI at 4 Å resolution, 89 chlorophyll \( a \) molecules have been identified (Schubert et al., 1997). Six of those chlorophylls are cofactors of the electron transfer chain with the remaining 83 constituting the core antenna system. Most of these antenna chlorophylls are ligated to histidines from conserved amino acids within PsaA and PsaB. It is also thought that PsaL, PsaK, and PsaF may also coordinate antenna chlorophyll (Schubert et al., 1997).

The overall organization of the peripheral subunits attached to PsaA/PsaB shows PsaC oriented on top of the \( C_2 \) psuedo-rotation of axis of PsaA/PsaB with PsaD overlapping PsaC towards the PSI trimeric \( C_3 \) axis and PsaE also overlapping PsaC away from the PSI trimeric \( C_3 \) axis, Figure 2.12 (Klukas et al., 1999b). Little of PsaC protein seems to be solvent exposed, being surrounded by PsaD and PsaE. PsaC is a prerequisite for PsaD and PsaE binding (Mannan et al., 1994; Yu et al., 1995b). The peripheral protein formation is not necessary for stable PsaA and PsaB assembly in cyanobacteria, but is in other organisms like \( C. \) reinhardtii (Takahashi et al., 1991).

Figure 2.12 Stromal ridge of PSI shown form the stromal side onto the membrane plane. PsaC is depicted as light grey, PsaD in vertical lines, and PsaE in horizontal lines with the proposed ferredoxin binding site being the diagonal lines. Adapted from Ref. (Klukas et al., 1999b)
The peripheral proteins PsaC, PsaD, and PsaE are critical in shuttling the electron from \( F_X \) in the PsaA and PsaB heterodimer core to soluble ferredoxin. PsaC contains the two terminal electron transfer \([4\text{Fe}-4\text{S}]\) iron sulfur clusters \( F_A \) and \( F_B \) (Yu et al., 1993; Antonkine et al., 2000; Yu et al., 1995a). \( F_A \) and \( F_B \) act as an electron sink from the PsaA/PsaB core cofactors to docked ferredoxin. PsaD provides a ferredoxin docking site (Kruip et al., 1997; Chitnis et al., 1996). Inactivation of PsaD in \textit{Synechocystis} indicated that PsaD is essential for efficient function of cyanobacterial PSI and for stable assembly of PsaE (Chitnis et al., 1989). The PsaD:ferredoxin docking involves electrostatic interactions between the basic PsaD protein and electronegative surfaces of ferredoxin, Figure 2.12 (Lelong et al., 1994). PsaE assisting in the interaction of PSI with ferredoxin (Xu et al., 1994b). Membranes of the PsaE-less mutant were severely deficient in the reduction of ferredoxin. It is suggested that both PsaD and PsaE together form a suitable docking site for ferredoxin.

The \( A_1 \) site of PSI is of particular interest in this review. In the crystal structure, phylloquinone is fairly well identified in the location of PSI but does not include the interaction of amino acids (Klukas et al., 1999a). However, a highly conserved primary amino acid sequence in both PsaA and PsaB containing the peptides \( A_{686}^\text{LFSGRGYWQELIE}_{698} \) and \( B_{686}^\text{LISWREGYWQELIE}_{698} \) in the connections between transmembrane helices \( m \) and \( m' \) and peripheral helices \( n \) and \( n' \) are proposed to bind phylloquinone (Schubert et al., 1997). Derivations of this fragment peptide are also partially conserved in PshA of the P800 reaction center of \textit{heliobacterium} and PscA of the P840 reaction center of green sulfur bacterium \textit{Chlorobrium}. The protein-phylloquinone interaction, by electron spin echo envelope modulation (ESEEM), suggests that there are two protein nitrogen nuclei coupled to the phyllosemiquinone radical \( A_1^- \) (Hanley et al., 1997). One nitrogen is thought to be from an indole tryptophan and the other from a histidine, glutamine, or asparagine. Thus, the conserved amino acids \( A_{W693}/A_{Q694} \) or \( B_{W668}/B_{Q669} \) may bind phylloquinone. On the cyanobacterium \textit{Anabaena variabilis} (Rigby et al., 1996) and \textit{Synechococcus} (Klughammer et al., 1999), ENDOR and related techniques determined that each quinone oxygen contained a hydrogen bond. However, positive identification of the proton donor has not been established. Point mutations have been performed on various amino acids within and near the \( A_1 \) binding site to determine
their interaction with phylloquinone (Xu and Chitnis, 2000). However, final analysis has not been compiled.

Further localization of quinones within the A₁ site of *Synechocystis* sp. PCC 6803 has been determined by non-crystallographic techniques. Pulsed EPR has determined that the distance from the reduced phylloquinone in the A₁ site to P700⁻ is 25.4 Å and 27 ± 5° out of line from P700 to Fₓ (Bittl and Zech, 1997). The vector of the carbonyl oxygens in phylloquinone point at P700 (Zech et al., 1997) with the carbonyls oriented 63° from the membrane plane (MacMillan et al., 1997). However, 1,4-naphthoquinone contained within the A₁ site is oriented by 90° with the carbonyl oxygens nearly perpendicular to P700 (Zech et al., 1997). This indicates that the alkyl ‘tail’ of the quinone is important for proper orientation within the A₁ site.

6. A general summary of the bioenergetics of PSII through ATPase

Photosynthesis is a complex series of steps that converts solar energy into the high energy form of cofactors (Ort et al., 1996). In cyanobacteria and higher plants, two separate complexes trap photons of light creating a charge separation initiating a electron transfer through the protein. Several additional complexes either shuttle the generated electrons or siphon off a portion of the potential energy to channel it into the high energy form of a cofactor. Most of the complexes and electron transports are lipid bound in the thylakoid membranes in cyanobacteria or chloroplasts. The soluble proteins act as the electron transport agents between the lipid bound protein complexes.

The initial event is the absorption of a photon by a Chl molecule in the light harvesting complex II (LHC II). The energy is transferred as an exciton among the Chls to the P680 reaction center (RC). Within the P680 a charge separation occurs and the high energy electron is then transferred into the electron transfer chain. This is composed of a series of intermediate pheophytin electron receptors and the QA and QB site, both of which are occupied with plastoquinone (PQ). PQ is a lipid soluble molecule that serves as a mobile carrier between PSII and the Cyt b₆f complex. The QB site is essentially a loading dock for PQ. Two electrons from two turnovers of P680 reduce the PQ, which through binding two protons from the stroma, creates PQH₂. The PQH₂ has a lower affinity to the QB site, thus dissociates into the thylakoid membrane. The Cyt b₆f complex performs many functions.
starting with stripping the electrons from the plastoquinol. In the process, the protons are dumped into the lumen. Plastoquinone is then released from the Cyt b₆f complex to be recycled. A second proton discharging site is the oxygen evolving complex (OEC) which is attached to the luminal side of PSII. The OEC generates dioxygen from water using the four electrons for the reduction of P680 with the four proton being released into the lumen. This, in turn creates a proton gradient between the lumen and stroma. The generated electrons are transferred through Cyt b₆f to reduce plastocyanin. The oxidized P700⁺ is reduced by the electron provided from the plastocyanin. The energy from a second photon is used to excite the P700 RC electron of PSI so that it can enter the second of the electron transport chains. P700 is the RC that is the primary electron donor in PSI. The PSI electron moves across the membrane through a series of prosthetic groups to a docked ferredoxin. The reduced ferredoxin transfers the electron via ferredoxin-NADP⁺ generating NADPH or back to the Cyt b₆f complex, depending on energy needs. The previously generated proton gradient drives ATP synthesis while cocontaminantly releasing protons back into the stroma. The specific energetic mechanisms of PSI will be further discussed.

7. Photosystem I energetic schemes

After absorption of a photon by an antenna Chl and migration of the exciton to the P700 trap, primary charge separation occurs between the primary reactants P700 and A₀, leading to a short-lived P700⁻ A₀⁺ radical pair. This charge-separated state is stabilized by rapid, secondary electron transfer events through the intermediary acceptors A₁ and Fₓ to the terminal Fₐ/Fₐ acceptors. This stabilization allows the soluble electron transport proteins plastocyanin (or cyt c₆) and soluble [2Fe-2S] ferredoxin to interact with the primary donor and terminal acceptors, respectively, in diffusion-limited processes. The final step in non-cyclic electron transport is the reduction of NADP⁺ by reduced ferredoxin, catalyzed by the enzyme ferredoxin:NADP⁺ oxido-reductase (Knaff, 1996). An indispensable review by Klaus Brettel covers in brilliant detail electron transfer within PSI, therefore this review will be restrained to post 1997 publications, except for applicable background material (Brettel, 1997).

In *Synechocystis* sp. PCC 6803 photon of light absorbed by an antenna Chl undergoes a complex equilibrium among the bulk Chl and red-absorbing Chls before it is
trapped by the P700 reaction center in approximately 20-25 ps kinetics (Hastings et al., 1994; Savikhin et al., 1999). Recent work suggests that the formation of the initial radical pair of (P700$^+$$A_0^-$) is 9-10 ps (Savikhin et al., 2000), which is slower than the 1-3 ps. as suggested by Brettel earlier in a review (Brettel, 1997). The reoxidation of $A_0$ and presumed reduction of $A_1$ occurs with 50 ps (Hecks et al., 1994).

Direct reduction of $A_1$ has not been well described due to, in part, of inadequately fast spectroscopic instruments that operate in the blue region at which PhQ absorbs. Flash absorption kinetics monitoring PhQ at 380 nm show that the PhQ reduction was faster than the 5 ns time resolution (Brettel, 1988).

The reoxidation of the $A_1$ phylloquinone is in the tens and hundreds of ns time range depending on the experiment (Brettel, 1997). Determining the reoxidation is further complicated by the organism, variations in preparation of the PSI particles, and the state of the iron-sulfur clusters. For both spinach and *Synechocystis*, in general the reoxidation of PhQ takes place at around 200 ns, with a 15-25 ns component that may be either a heterogeneity in the sample or a possible fast redox equilibrium between $A_1$ and $F_x$. However, more recently Joliot has developed an *in vivo* technique for monitoring the PSI PhQ and determined that for *Chlorella sorokininia* there is a biphasic oxidation of PhQ with times of 18 and 160 ns (Joliot and Joliot, 1999). He attributes the two phases to two possible hypotheses “(1) Photosystem I reaction centers are present under two conformational states which differ by the reoxidation rate of $A_1$. (2) The two phylloquinones corresponding to the two branches of the PS I heterodimer are involved in the electron transfer.” (Joliot and Joliot, 1999). Hypotheses 1 suggests that there is a fast (18 ns) equilibrium between $A_1$ and $F_x$ and the slow phase (160 ns) being attributed to the electron being bled off onto $F_A$ and $F_B$. Evidence to argue against hypothesis 1 includes extraction of $F_A$ and $F_B$ does not remove the 160 ns phase. Support for hypothesis 2 includes the similar amplitudes of the two phases implies that the rates of electron transfer from P700 to each PhQ is roughly equal. The two different rate constants measured both $A_1^+$ oxidation suggests some asymmetry between PhQ and $F_X$. This however may not be concluded until site directed mutants of the $A_1$ site generated in *Synechocystis* and other organisms are completely analyzed (by this lab and others).
Figure 2.12 Energetics of PSI. The back reaction rates in the wild type refer to conditions where the succeeding electron acceptor has been removed either biochemically or genetically. The values in bold represent the dominant kinetic phase. The back reaction pathways are depicted as direct to P700 for the sake of clarity; actual pathway likely proceeds, at least in part back though the electron acceptor chain. Figure from Ref. (Semenov et al., 2000).

The electron transfer rates from $A_1$ to $F_X$ and between $F_A$ and $F_B$ are complex. Given the similar spectroscopic properties of the clusters, discerning the electron transfer rates among the sites are an ongoing process. A variety of studies have determined that the sequence of forward electron transfer was $A_1$ to $F_X$ to $(F_A F_B)$ (Brettel, 1997). Recently evidence by Golbeck has suggested that the correct arrangement of the iron-sulfur clusters is $F_X$ to $F_A$ to $F_B$ (Shinkarev et al., 2000). This was in support that $F_B$ was found to be the iron-sulfur cluster that interacts with flavodoxin and ferredoxin (Vassiliev et al., 1998).

One last topic with the electron transfer within PSI concerns charge recombination kinetics between a secondary electron acceptor site and the P700$^-$ RC. As given in Fig. 2.11, the dotted lines that connect a secondary site with P700 is the back reaction time reversing through the acceptor chain. The primary pair P700$^- A_0^-$ decays with a half time in the order of 30 ns, forming two products: a singlet ground state of P700 and a triplet state
$^3$P700. The $^3$P700 then decays to the ground state on a $\mu$s time scale (Golbeck and Bryant, 1991). The A₁ site back reacts at about 100 $\mu$s when the subsequent iron-sulfur cluster acceptors are either removed or prereduced (Brettel, 1990). The backreaction of the terminal iron-sulfur clusters is the most pertinent of the recombination kinetics with regards to the rest of the dissertation. Normally the $F_AF_B$ back reaction in the wild type is close to 80 ms and 12 ms (Vassiliev et al., 1997). The biphasic decay is considered to be a result of the PSI complexes being in different confirmations. The microsecond decay is attributed to damaged reaction centers. However, this backreaction rate from $F_AF_B$ is sensitive to the quinone in the A₁ site (Johnson et al., 2000b). Hence, this is an effective spectroscopic tool for probing the electron intermediates within PSI.

8. Alternative quinones in the A₁ site of PSI

The latter part of dissertation concerns replacing phylloquinone with alternative quinones into the A₁ site *in vivo*. Previous work from the late 1980's and early 1990's places the quinones into PSI *in vitro* upon extraction with organic solvents. It has been shown that phylloquinone can be extracted from PSI with a dry or water saturated diethyl ether solution (Itoh et al., 1987; Itoh and Iwaki, 1989) or by a hexane containing 0.3% methanol (Rustandi et al., 1990; Setif et al., 1987). However, approximately half of the antenna Chl and all the carotenoids are also removed. Both procedures demonstrated that charge separation was essentially blocked at the level of A₀ (P700"A₀") (Biggins and Mathis, 1988; Itoh et al., 1987). There were a variety of studies focusing on the reconstitution of electron transfer in phylloquinone depleted PSI by incubating with non-native quinones. It has been shown that many benzoquinones, naphthoquinones, anthraquinones, and even anthrones and fluorenones can be inserted into A₁ and accept electrons from A₀ in the phylloquinone depleted cells (Rustandi et al., 1992; Iwaki and Itoh, 1994b). Iwaki and Itoh reported the function of the A₁ site could be reactivated by a quinone of an appropriate redox potential (Iwaki and Itoh, 1994a). This included reduction of the iron-sulfur clusters by monitoring the absorbance changes of $F_AF_B^-$ and P700" at 470 nm and 430 nm, respectively. However, these results conflicted with other quinone reconstitution experiments where the phylloquinone was removed by a different organic solvent mixture (Biggins, 1990). Work by Biggins (Biggins, 1990) and Thurnauer (Rustandi et al., 1992)
showed by both optical and EPR experiments that the assortment of benzo-, naphtho-, and anthro-quinones used in the reconstitution experiments integrated into the A₁ site with varying degrees of success and only the quinones with a "tail" actually passed electrons onto the iron-sulfur clusters. Time resolved EPR also showed that upon reconstitution with phylloquinone a charge separation of P700^FeS⁻ was restored (Sieckman et al., 1991). The remaining quinones (Q = duroquinone, deuterated duroquinone, and 1,4-naphthoquinone) contained a charge separation of P700^Q⁻ with the electron being blocked from proceeding further. Biggins suggested structural constraints imposed by an alkyl tail in addition to energetic considerations (Biggins, 1990). Discrepancy between these results could have arrived from a combination of the harsh PhQ extraction procedures and the lack of resolution of the instruments. This was one motivation for the author to attempt to remove phylloquinone from PSI in vivo. Then in vivo or in vitro PSI complexes, alternative quinones can be reconstituted into the A₁ site of PSI complexes that have not been damaged or modified by organic solvents.

9. Summary

Phylloquinone plays very specific roles in biological processes, yet critical ones. A lack of vitamin K in an animal would have a lethal result from the inability to clot blood. But the use of phylloquinone as a regulatory cofactor for the carboxylase reaction, converting specific glutamic acid residues to γ-carboxyglutamic acid, simplifies the regulation of the whole pathway. Additionally, the phylloquinone is consumed either directly or indirectly in adequate levels in the diet of the animals. Within plants phylloquinone has only one known use as an intermediate electron acceptor in PSI. How phylloquinone became the evolutionary target as a specific electron transfer agent is unknown. Why plastoquinone is not used or has not evolved as a replacement for phylloquinone within PSI is also unknown. The author muses that phylloquinone is being used over plastoquinone in PSI as a possible response to some previous evolutionary pressure. Once the stress was past, PSI was quite optimized for phylloquinone, both in the electron intermediate head group and the binding of the shorter mostly unsaturated tail.
10. References


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CHAPTER 3. THE menD AND menE HOMOLOGUES IN CYANOBACTERIA CODE FOR ESSENTIAL ENZYMES IN PHYLLOQUINONE BIOSYNTHETIC PATHWAY

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Summary

The genome of the cyanobacterium *Synechocystis* sp. PCC 6803 contains homologues of the *Escherichia coli* genes that encode enzymes of the menaquinone biosynthetic pathway. We inactivated the homologues of menD and menE, which code for 2-succinyl-6-hydroxyl-2,4-cyclohexadiene-1-carboxylate (SHCHC)³ synthase and O-succinylbenzoic acid-CoA ligase, respectively. The membranes of mutant strains do not contain phylloquinone. In Photosystem I complexes, the only known cyanobacterial enzyme that utilizes phylloquinone in the wild type cells, contain plastoquinone-9 (PQ) in the mutant cells. The menD and menE mutants grow photoautotrophically under 40 μE·m⁻²·s⁻¹ light intensity, with doubling times longer than the wild type. However, the menD mutant and wild type have similar doubling times under heterotrophic growth conditions, with menE remaining slower. Low temperature fluorescence show that the ratio of photosystem I to photosystem II is reduced in the menE and increased in menD relative to wild type. Electron transport activities are reduced in both mutants relative to wild type. Photoaccumulated EPR and 810 nm optical backreaction studies of mutant PSI trimers indicate that PQ is contained.

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¹ Abbreviations: PS I, photosystem I; PS II, photosystem II; EPR, electron paramagnetic resonance; DPIP, 2,6-dichlorophenolindophenol; Tris, PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PhQ, phylloquinone; PQ Plastoquinone
and active within the A₁ site. In summary, we demonstrate that the \textit{menD} and \textit{menE}
homologues in cyanobacteria code for enzymes in phylloquinone biosynthetic pathway. We
propose that there is no bypass for the SHCHC synthase and O-succinylbenzoic acid-CoA
ligase in the PhQ biosynthetic pathway and that PQ is recruited as a cofactor into the A₁ site
of PSI.

\textbf{Introduction}

Phylloquinone, \textit{(PhQ, vitamin K₁, 2-methyl-3-phytyl-1,4-naphthoquinone)}, in
animals is used as a cofactor for a single known enzymatic reaction, which converts specific
 glutamic acid residues to \(\gamma\)-carboxyglutamic acid (Gla) by carboxylase \(1\). The Gla residue
has the ability to chelate metals and impart structural changes in proteins upon exposure to
calcium. Typically several Gla residues within a protein are clustered together and form a
complex network of calcium chelated to multiple Gla residues \(2\). For example, these
specific structural changes are responsible for activating several blood clotting factor
proteins (VII, IX, X) and activating conantokin neuroactive peptides found in poisonous
snails \(3\). The utilized phylloquinone is not synthesized by the animals but absorbed in the
gut by consuming primarily green leafy parts of plants \(4\).

Phylloquinone’s only known purpose in plants and cyanobacteria is to act as an
electron acceptor in photosystem I (PSI). PSI is a photosynthetic reaction center (RC) that
catalyzes the photooxidation of plastocyanin in the thylakoid lumen and the photoreduction
of ferredoxin in the cyanobacterial cytoplasm (or chloroplast stroma) \(5,6\). PSI is a
multisubunit protein, with PsaA and PsaB as a heterodimeric core surrounded by 6 small
proteins \(7\). In addition to light-harvesting chlorophyll \(\alpha\) molecules imbedded in PsaA/PsaB,
there is the P700 RC, a dimer of Chl, and a series of cofactors (A₀, A₁, Fₓ). PSI also contains
three stromal peripheral proteins; PsaD and PsaE acts as a ferredoxin docking site and PsaC
contains two [4Fe-4S] iron-sulfur clusters (F\textsubscript{A} and F\textsubscript{B}). These cofactors act as electron
acceptors which provide a charge separation from P700\textsuperscript{+} and act as an electron shuttle from
the P700 on the luminal side of the membrane to the terminal iron-sulfur clusters F\textsubscript{A} and F\textsubscript{B}
on cytoplasmic side.

The secondary electron acceptor site. A₁, contains phylloquinone. This molecule acts
as an intermediate in the electron transfer from the primary acceptor A₀, a chlorophyll \(\alpha\)
molecule, to \( F_X \), a \([4\text{Fe}-4\text{S}]\) iron-sulfur cluster. From isolated PSI complexes, there are two bound molecules of phylloquinone per molecule of P700 (8-11); however only one molecule is considered to actually participate in the electron transfer (8,9,12). Although phylloquinone is an important photosynthetic cofactor, the biosynthetic route has not been determined for cyanobacteria.

Many prokaryotes contain the metabolic pathway for phylloquinone biosynthesis. Fig. 1. In some bacteria, menaquinone (vitamin K\(_2\)) is used during fumarate reduction in anaerobic respiration (13,14). The genes encoding many enzymes involved in the conversion of the chorismate to menaquinone have been cloned in \textit{Escherichia coli}. (15-20). \textit{Bacillus subtilis} (21-24), and in \textit{Bacillus stearothermophilus} (25). Menaquinone differs from phylloquinone in having a partially unsaturated C-40 side chain rather than a mostly saturated C-20 phytol side chain, respectively. Therefore, we expect the synthesis of the naphthalene rings to involve similar steps. The genome of \textit{Synechocystis} sp. PCC 6803 (26) contains homologs for several genes that encode enzymes in menaquinone biosynthesis: \textit{menF} (\textit{entO}) (isochorismate synthase), \textit{menD} (2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate (SHCHC) synthase), \textit{menE} (O-succinylbenzoic acid-CoA ligase). \textit{menB} (DHNA synthase) and \textit{menA} (phytol transferase). Possible homologs of \textit{menC} and ORF241 (the DHNA thioesterase) are also present in the genome. Previously we have shown phylloquinone is not synthesized in the cells by mutationally disabling the \textit{menA} and \textit{menB} homologs, therefore those genes are involved in phylloquinone biosynthesis (27).

Two open reading frames sll0603 and slr0492 in \textit{Synechocystis} sp. PCC 6803 genome code for proteins that have 28\% and 29\% sequence identity to the deduced amino acid sequences of the SHCHC synthase (\textit{menD} gene) and O-succinylbenzoic acid-CoA ligase (\textit{menE} gene) enzymes of \textit{Arabidopsis Thaliana}, respectively. Therefore, we propose that the \textit{menD} and \textit{menE} genes code for the enzymes in the beginning of the phylloquinone biosynthetic pathway. In this paper we describe generation and characteristics of the mutant strains and properties of the PSI complexes in them. We also show that the A\(_1\) site of PSI is occupied by plastoquinone.
Material and Methods

Generation of the menD and menE mutant strains of Synechocystis sp. PCC 6803

To generate a recombinant DNA construction for inactivation of the menD gene, a 1.8 kb DNA fragment was amplified from Synechocystis sp. PCC 6803 genomic DNA by polymerase chain reaction using primer menDSN1 (5'-GGATCAAGCTTTCAGCAG-3') and menDSN2 (5'-ACCTCAGCCTAGGTAATGGC-3'). The 1.8 kb fragment containing the full menD gene and flanking sequences was cloned at EcoRV blunt end in pBluescript II KS(+) and validity of the insert was confirmed by sequencing. The chloramphenicol resistance gene (CAT) from pUC4C was cloned at a unique EcoRV site within menD coding region. The resultant plasmid was designated menDC and used for transformation of the wild-type strain of Synechocystis sp. PCC 6803. Recombinants were selected on BGl 1 plates containing chloramphenicol (20 μg/ml) and isolation of segregated mutants was performed according to the previously published methods(28). Recombinant clones were confirmed for insertional inactivation of menD gene by PCR. For PCR confirmation primers menDSN3 (5'-ACCGCCGTTATTGTTGAGC-3') and menDSN4 (5'-GGTGGTGACATCTTTAATTGTC-3') were used.

To generate a recombinant DNA construction for inactivation of the menE gene, a 996 bp DNA fragment was amplified from Synechocystis sp. PCC 6803 genomic DNA by polymerase chain reaction using primer menESN3 (5'-TAATGTTGGCAACAGCAGTGG-3') and menESN4 (5'-AGGGTGGAAACATCATTCCC-3') and was cloned at EcoRV blunt end in pBluescript II KS(+). This clone, designated as menEC, was digested with MscI enzyme which has two sites within menE coding region. A 260 bp region was replaced by inserting the chloramphenicol resistance gene at blunt end. The chloramphenicol resistance gene (CAT) from pUC4C was cloned at MscI and SmaI. The resultant plasmid was designated as menEC3 and used for transformation of the wild-type strain of Synechocystis sp. PCC 6803. Recombinants were selected on BGl 1 plates containing Chloramphenicol (20 μg/ml) and isolation of segregated mutants was performed according as above. Recombinant clones were confirmed for insertional inactivation of menE gene by PCR using menSNE3 and menSN4 primers.
DNA isolation, PCR, and Southern blotting

Genomic DNA from *Synechocystis* sp. PCC 6803 was prepared as described (29). 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.

Cyanobacterial strains and growth

A glucose-tolerant strain of *Synechocystis* sp. PCC 6803 was used as the wild type strain. The wild-type, *menD* and *menE* mutant cells were grown in the BG-11 medium, with 5mM glucose and chloramphenicol antibiotic for the mutants (30). Agar plates for the growth of the stock cells were kept at low light intensity (2 to 10 µE m⁻² s⁻¹). Liquid cultures of the wild type and mutant strain were grown heterotrophically under normal light conditions (40 to 60 µE m⁻² s⁻¹) and were bubbled with sterile filtered air. Cell growth was monitored by measuring the optical density at 730 nm (A₇₃₀) with a Shimadzu spectrophotometer. Cells from liquid cultures in the late exponential phase of growth (A₇₃₀ = 0.8-1.2) were harvested by centrifugation at 5000 x g for 15 min. Growth on a large scale was achieved by starting a 3 l culture at 0.050 A₇₃₀ and adding 2.5 mM glucose final concentration in addition to chloramphenicol for the mutants.

Growth rates of the wild-type and mutant cells

Cyanobacterial cultures in the late exponential phase were pelleted by centrifugation, washed twice with BG11 medium, and suspended in BG11 and chloramphenicol at approximately 10 A₇₃₀ concentration. For estimating growth rates, the cultures were grown in 6 well culture plates with 8-ml liquid medium in each well. All cultures were adjusted to the same initial cell density (A₇₃₀ = 0.08 - 0.1). Final concentrations of glucose and DCMU were 5mM. and 10µM. respectively. The cells were shaken on an orbital shaker at 110 rpm under a bank of fluorescent lights at normal light conditions (40 to 60 µE m⁻² s⁻¹).

Isolation of Thylakoid Membranes and PS I Complexes

Thylakoid membranes were prepared from cells as described by Sun et al. (31). The thylakoid membranes were pelleted by centrifugation at 50,000 x g for 90 min and were resuspended in SMN buffer (0.4 M sucrose, 10mM MOPS, 10mM NaCl) for storage. Chlorophyll was extracted from thylakoid membranes and PSI trimers with 80% acetone
and determined according to (32). For the isolation of PS I complexes, thylakoid membranes were incubated in SMN buffer with 20 mM CaCl₂ for 0.5 to 1.0 hr at room temperature in the dark to enhance trimerization of PSI. To the mixture, n-dodecyl-β-D-maltoside (DM) was added to a final concentration of 1.5% (w/v) of chlorophyll amount and incubated in the dark on ice with occasional gentle mixing for 0.5 to 1.5 hrs. The non-solubilized material was removed by centrifugation at 10,000 x g for 15 minutes. The trimeric and monomeric PS I particles and PSII were separated by centrifugation in 10 to 30% (w/v) sucrose gradients with 0.04% DM in 10 mM MOPS, pH 7.0.

**Chlorophyll Analysis and Oxygen Evolution Measurements**

Chlorophyll was extracted from whole cells and thylakoids with 100% methanol. Chlorophyll concentrations were determined according to (33). Oxygen evolution measurements were performed using a Clark-type electrode as described in (34). Cells were prepared by starting cultures at the same time and grown photomixotrophically with 5mM glucose for two subcultures. The final liquid culture was made without addition of glucose and grown until mid log growth phase (0.4 - 0.6 Δ730) and then analyzed. The temperature of the electrode chamber was maintained at 25°C by a circulating water bath. Cells were pelleted and washed twice with BG11 and resuspended in 40 mM HEPES/NaOH, pH 7.0 buffer in a concentration of 8 - 10 Δ730 ml⁻¹. Measurements were done on 5 A of cells. Whole-chain electron transport (H₂O to CO₂) measurements were determined after the addition of 5 mM NaHCO₃; oxygen evolution mediated by PS II only was determined after addition of 4 mM p-benzoquinone (BQ). Light intensity was 1840 μE m⁻² s⁻¹.

**77 K Fluorescence Emission Spectra**

The low temperature fluorescence emission spectra were measured using a SLM 8000C spectrofluorometer as described (35). Cells from the exponential phase of growth were harvested by pelleting and resuspended in 25 mM HEPES/NaOH, pH 7.0 buffer. Cells (5 μg Chl.) were diluted in 25 mM HEPES/NaOH, pH 7.0 to a final volume of 30 μl and dark adapted for 30 minutes on ice. To the solution, 70 μl of neat glycerol was added, mixed, prior to quickly freezing in liquid nitrogen. The excitation wavelength was 435 nm. The excitation slit width was set at 4 nm and the emission slit width was set at 2 nm. The emission was scanned from 600 nm to 800 nm twice and averaged.
**Analysis of phylloquinone using HPLC and mass spectrometry**

On an equal chlorophyll basis, the samples were prepared and quinones were analyzed as previously described (36), with the following changes. Membranes containing 0.025 mg chlorophyll were centrifuged at 10000 x g for 60 min and the supernatant was removed. PSI trimers were concentrated by a Centracon and then lyophilized to dryness. The pigments were sequentially extracted with 1 ml methanol, 1 ml 1:1 (v/v) methanol:acetone, and 1 ml acetone, and the three extracts were combined. The resulting solution was concentrated by vacuum at 4°C in the dark to dryness. Dry pigment samples were stored at -80°C until used. The pigments were resuspended in a mixture of 1:1 methanol:isopropanol at ca. 0.8 mg Chl ml\(^{-1}\). HPLC separations were monitored with photodiode array UV-visible detection using a Hewlett Packard (Agilent Technologies, Palo Alto, CA) model 1100 quaternary pump and model G1316A photodiode array detector. Sample injections (35 μL) were made on a 4.6 mm × 25 cm Ultrasphere C\(_{18}\) column (4.6mm × 250mm) with 5 μm packing (Beckman Instruments, Palo Alto, CA) using a gradient elution at (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 1.0 mL/min. The column was washed after each run (from 100% B to 100% A at 10 min. 0.5 mL/min. 100% A for 5 min at 1.0 mL/min.). 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 post-column flow splitter delivered column eluent to the electrospray ion source at 10 μL min\(^{-1}\).

**Spectroscopic characterization of photosystem I complexes**

Optical kinetic spectroscopy of P700 in purified PSI trimers was performed as described in (37). Q-band EPR Spectroscopy was used to detect photoaccumulated A\(_1\) signal in PS I Trimers as described in (38).

**Results**

**Genotype of the mutant strains**

The genotypes of the men\(D^-\) mutants strain were confirmed by PCR amplification of the appropriate genomic loci and then sequenced. In Fig. 2A (left panel), the genomic region
of menD is shown for the wild type and mutant. A 1.1 kb chloramphenicol resistance cartridge was inserted in the EcoRV site of the menD gene. Using primers within the menD coding sequence containing the EcoRV site, PCR amplification of the menD locus of the wild type produced the expected 990 bp fragment (Fig. 2A, right panel). PCR amplification of the menD mutant locus produced the 2.1 kB fragment, accounting for the integration of the chloramphenicol-resistance cartridge. There was no amplification of the 990 bp fragments in the mutant strain indicating complete segregation of the mutant allele. Southern blot hybridization analysis of the menD mutant confirmed complete segregation in the mutant strain (data not shown).

Insertional inactivation of the menE gene was verified by PCR amplification of the menE locus and by DNA sequencing. The left panel of Fig. 2B shows the restriction map of the wild type and mutant menE gene. Using primers within the menE coding sequence, PCR amplification of the menE locus of the wild type produced the expected 980 bp fragment. A 260 bp fragment of the menE gene was deleted and replaced with a 1.2 Kb chloramphenicol cassette. PCR amplification of the mutant strain produced the expected 1.9 Kb fragment. The wild-type 980 bp fragment was not observed in the mutant. These results indicate that segregation of the alleles had occurred completely in the menE mutant.

Absence of phylloquinone in the menD' and menE' mutant strains

The phylloquinone content of the thylakoid membranes and PSI trimers of the menD' and menE' mutants was determined using HPLC with a photodiode array UV-Visible detector. By co-injecting standards and by interpreting the UV-Visible spectra, chlorophyll a was identified at 19.0 min and β-carotene was identified at 28.5 min. With this solvent gradient, phylloquinone standard elutes at 20.7 min and shows the distinctive doublet peak at 248 nm and 269 nm. The 254 nm chromatogram of the solvent extracted pigments from PSI trimer complexes of the mutant (menE') indicate plastoquinone with an elution time 29.7 min and peak at 256 nm (Fig. 3). In the PSI extracted pigments only plastoquinone was observed in the menD' and menE' mutants (Fig. 3). However, the wild type PSI pigments contains only phylloquinone and lack plastoquinone. To determine if phylloquinone was present elsewhere, the thylakoid membranes were analyzed. The menD' and menE' mutant thylakoid membrane solvent extracted pigments also show an absence of phylloquinone (Fig
4. inset and Fig. 4. respectively). Inset of Fig. 4. wild type pigments contained both the phylloquinone and plastoquinone peak. In the wild type, phylloquinone is contributed from PSI and plastoquinone from PSII and Cytb6f which are also thylakoid membrane bound complexes that utilize plastoquinone as a lipid soluble electron transporter (39).

Calculating the concentrations of chlorophyll to quinone for wild type and mutant yields a ratio close to the known approximately 50:1 Chl:PhQ (40). The wild type has a ratio of 42:1 ± 3.6 Chl:PhQ while the menD' and menE' mutants have a slightly higher ratio of 51:1 ± 2.1 and 54:1 ± 4.1 Chl:PQ, respectively. The higher ratio could indicate that a small number of the mutant A1 sites are without a quinone.

Wild type showed a minute amount (<2%) of plastoquinone in the extracted pigments of the purified trimers. Wild type PSI trimers that are washed with 3-10 volumes of 10 mM MOPS and 0.04% DM and concentrated do not contain plastoquinone, yet have stiochiometric quantities of phylloquinone (data not shown). Therefore, the detected plastoquinone is probably attributed being captured in the lipid membranes and detergent during PSI purification from the plastoquinone rich thylakoid membranes.

UV-Visible detection has a minimum detection limit of phylloquinone for the mutants of approximately 15% that of wild type (27). To achieve smaller detection limits LC-MS was also used since it has several orders of magnitude greater sensitivity (41). Phylloquinone and plastoquinone eluted at the same times as with the UV-Visible detector with the molecular ion at \( m/z = 450 \) and \( m/z = 748 \), respectively. Pigments extracted from both thylakoid membranes and from purified PSI trimers of the menD' and menE' mutants show only plastoquinone. Phylloquinone was not detected in either membrane or PSI trimer preparation (data not shown).

However, this indicates that plastoquinone is only present in the mutant trimers, not actually being utilized by PSI in electron transport. We address this by directly probing the A1 site by monitoring the back reaction kinetics from the iron sulfur clusters to P700' and photoaccumulation EPR.

**EPR spectroscopy of photoaccumulated A1**

The EPR spectra of the photoaccumulated quinones have significant differences between the wild type and mutants (Fig. 6). In both mutants, the prominent phylloquinone
methyl hyperfine lines are missing and there is a larger g-anisotropy of Q' than in phylloquinone. menE shown. As shown previously, these changes in the spectrum are consistent with a loss of phylloquinone, which is replaced with plastoquinone, in the A1 site (38). Not only is plastoquinone found in stiocheometric amounts in PSI, but it appears to be able to be reduced by A0.

$P700^-$ optical recombination kinetics

In PSI trimers isolated from the wild type, the reduction of the P700$^-$ RC is multiphasic after a saturating flash (37,42). When measured by 810 nm near-IR optical spectroscopy and in the absence of external electron acceptors, the typical wild type spectrum shows a majority of the P700$^-$ being reduced with a lifetime of 86 ms and a minor 12 ms phase. The biphasic decay is considered to be a result of the PSI complexes being in different confirmations (12,22). The microsecond decay is attributed to damaged reaction centers. There is also a long-lived (>1200 ms) kinetic phase of P700$^-$ reduction from direct reduction of the P700$^-$ by reduced DCPIP that contributes to 5 – 9 % of the total absorbance change. In PSI trimers isolated from the menD$^-$ and menE$^-$ mutants, the reduction of P700$^-$ is also multiphasic. Fig. 5. When menE$^-$ is measured in the absence of external electron acceptor, P700$^-$ is reduced with lifetimes of approximately 2.6 ms and 10 ms in a similar ratio to the wild type. Lifetimes of the menD$^-$ mutant strain is similar, at 2.7 ms and 9.9 ms. Both mutants have a relative contribution of the 3 ms to 10 ms phases of 65:30. The mutants also exhibit a minor long-lived kinetic phase attributed to electron donation of DCPIP to the oxidized P700$^+$. The kinetic back reaction phases for both the menD$^-$ and menE$^-$ mutants are consistent with plastoquinone being in the A1 site of PSI and acting as an electron intermediate from A0 to Fx (37).

Growth of the mutant strains

Exchanging plastoquinone from phylloquinone in PSI has cellular effects primarily involved in growth rates and high light sensitivity.

Focusing on the phenotype of the menD$^-$ and menE$^-$ mutant strain of whole cells reveals interesting differences to the wild type strain. Under normal light intensity conditions (40 - 60 µE m$^{-2}$ s$^{-1}$), the menE$^-$ mutant cells grew photoautotrophically but a slower rate than wild type cells. The 80 h doubling time of the menE$^-$ is significantly slower.
than the 26 h wild type (Table 1). Yet menD' mutant was only slightly slower at 30 h. At higher light levels (120 – 160 µE) the mutant menE is incapable of growing. However, menD' mutant does grow at the same high light. Photomixotrophic growth rates of the mutants were determined in the presence of 5 mM glucose, which allows both respiration and photosynthesis to provide energy for growth. The 14 h wild type doubling time was 42 h for menE' and 15 h for menD' mutant strains. Photoheterotrophic respiration with 10 µM DCMU to inhibit PSII, shows doubling times of both menD' and menE' mutants were similar to those of the wild type strain. Only the photosynthetic energy conversion has changed leaving respiration to function normally. Clearly menD' mutant strains growth rates behaved more like wild type than a phylloquinone deficient mutant (27). These studies indicate that the mutant is capable of photoautotrophic growth with high light sensitivity in menE' mutant was alleviated by inhibiting PSII activity. However, the menD' mutant seemed to display more wild-type phenotype resulting from differences in photosynthetic electron transfer rates and the ratio of PSI to PSII, described below.

**Electron transfer rates in whole cells**

To determine if the differences in growth rates is a product of photosynthetic ability, we measured the activities of PSI and PSII in the mutant strains for whole chain and partial chain electron transfer activity. Whole-chain electron transfer from water to bicarbonate was measured in cells grown photomixotrophically under normal light conditions. On an equivalent cell number basis, the rates of whole chain oxygen evolution was 73% and 77% to the wild type strain for the menD' and menE' mutants, respectively (Table 1.). PSII activity was monitored by adding a soluble quinone electron acceptor, 1,4-benzoquinone. The PSII activity of menE' was 81% of wild type. However, the PSII rates in menD' was significantly reduced (27%). The menD' and menE' mutant chlorophyll levels per cell were 85% and 65% of wild type, respectively. Given the reduced amount in chlorophyll level the electron transport is suitably decreased in whole chain and PSII activity in menE' mutant. However, the relatively high amounts of PSII activity in the mutant may prove to be stressful to the cells as indicated by slower growth rates high light sensitivity. However in the menD' mutant the decreased PSII activity was in balance with PSI resulting in light tolerance and growth rates approaching wild type.
Relative content of PSI per cell

Observing the whole cell 77 K fluorescence emission spectra on an equal chlorophyll basis for the wild type strain and menE mutant reveals a change (Fig. 6). The PSII chlorophyll fluorescence emission at 685 and 695 nm show similar intensities between wild type and the mutant with changes occurring in the 721 nm PSI emission. The PSI to PSII was reduced in the menE mutant (2.2) relative to wild type (3.3). The relative increase of PSII in the mutant produces an excess of reductant overwhelming PSI. At higher light intensities this can prove toxic (27). Yet the menD' mutant has an increased PSI:PSII ratio of 4.0. This supports the reduced PSII activity in whole cells.

Discussion

Previously, latter steps in the phylloquinone biosynthetic pathway (menA, menB) in cyanobacteria has been confirmed by mutationally disabling the genes in the Synechocystis sp. PCC 6803 genome from compared homologue genes which code for enzymes involved in menaquinone biosynthesis in other bacteria (Fig. 1) (27). Disabling the menA and menB genes resulted in a complete loss of phylloquinone. The loss of the menB gene, naphthoate synthase, prevents the second ring closure and menA gene, phytol transferase, disallows phytlylation of the naphthoquinone ring, both critical steps.

Because steps at the end of the phylloquinone biosynthetic pathway have been established, this study focuses on the earlier stages of phylloquinone production. These genes were first determined in E. coli. (15,19) and by analogy the homologs were found in the genome of Synechocystis sp. PCC 6803. The menD' gene codes for the enzyme, 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate (SHCHC) synthase, which catalyzes isochoresmic acid to 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate while concurrently releasing pyruvate and carbon dioxide. Precursor to naphthoate synthase enzyme, the menE gene codes for the enzyme, O-succinylbenzoyl-CoA synthetase, which attaches a CoA to O-succinyl benzoic acid.

To determine if the enzymes encoded by the menD' and menE' genes function in phylloquinone biosynthesis, we engineered mutants by targeted inactivation of the menD and menE genes in Synechocystis sp. PCC 6803. PCR amplification and sequencing were performed on both samples, with southern blot analysis on menD', to confirm the absence of
a complete menD and menE gene in the mutants (Fig. 2). HPLC-MS and HPLC-UV/Vis showed that neither thylakoid membranes (Fig. 4) and purified PSI trimers (Fig. 3) of the menD and menE contained detectable levels of phylloquinone. This concludes that the menD and menE genes in Synechocystis sp. PCC 6803 genome code for essential enzymes in phylloquinone biosynthesis. It also suggests that there are no other biosynthetic routes to producing phylloquinone which circumvent SHCHC synthase or O-succinylbenzoyl-CoA synthetase. Although it has not been confirmed by disabling all the genes in the biosynthetic pathway, we believe that SHCHC synthase is the first enzyme dedicated to phylloquinone biosynthesis. Precursors to the products of SHCHC synthase, chorismate and isochorismate are common molecules employed in not only phylloquinone biosynthesis, but production of ubiquinone, aromatic amino acids, and serine. Therefore a toxic buildup in the cells of these two molecules is unlikely.

Both menD' and menE' mutants contain plastoquinone in place of phylloquinone within the PSI complex. Extracts of the PSI pigments of the mutants show a ratio of Chl:PQ of about 50:1, same as the known Chl:PhQ values from Synechococcus (40). The slight difference in wild type and mutant ratio of Chl:Q may be attributed to a small number of empty or damaged A1 sites in the mutants. Plastoquinone's unsaturated C-40 tail may also not as readily fit into a C-20 phytol pocket in PSI, therefore keeping a portion of the A1 sites free of plastoquinone.

However, actual identification of plastoquinone being utilized by PSI in the A1 site was determined by EPR and monitoring the kinetic back reactions. Photoaccumulated EPR of PSI trimers of the menD' and menE' mutants revealed that the spectrum is identical to the plastoquinone containing menA' and menB' mutants (38). This indicates that the electron is being passed from A0 to A1, reducing PQ to PQ-. To check whether the electron is transferred through the A1 site into the iron-sulfur clusters (F_X, F_A, F_B), we observed the kinetic back reaction by monitoring the reduction of P700-. Observed at 810 nm, the reduction of P700- in the menD' and menE' mutants showed a multiphasic backreaction consistent with an electron originating from F_A/F_B and passing through PQ (37). A mixture of plastoquinone-containing PSI complexes (~3 ms) and a small amount of damaged RCs (~10 ms) was observed (Fig. 5). The contribution of the plastoquinone and damaged RC for
both mutants are similar (31%) to the menA and menB mutants. The mutants contain a greater number of damaged PSI RCs than in the wild type (10%). This suggests that the PSI RCs are operating with plastoquinone in the A1 site, similar to the menA' and menB' mutants.

The imbalance in electron transport rates is seen markedly in the differences in 77 K fluorescent PSI:PSII ratios. Wild type had a 3.3 PSI:PSII ratio with menE lower at 2.1 (Fig. 7). The menE mutant is similar to the menA' and menB' phylloquinone-less mutants in the PSI:PSII ratio and high light sensitivity (27). The resulting physiological changes in growth rates and oxygen evolution are consistent with the menA' and menB' mutants. The menD' mutant has a PSI:PSII of 4.0. The excess PSI is more capable of compensating for the PSII production of reductant.

Stepping back, the whole cells were monitored for whole chain oxygen evolution (water to bicarbonate) and for PSII activity. On an equivalent cell number basis, the menE mutant rates of whole chain oxygen evolution and PSII activity were 77% and 81% of wild type, respectively. Whereas, the menD' has a similar whole chain activity (73%) to menE'. The PSII activity is significantly reduced (27%) to the wild type. The chlorophyll levels per cell of the menE mutant is reduced, 67% of the wild type. Given the lower chlorophyll content of the menE mutant the PSII activity is relatively high compared to the wild type. The menD' mutant, however, has a whole chain activity comparable to PSII activity not three times greater as in the wild type and menE strains. Given plastoquinone reduced efficiency to act as an electron pathway intermediate in PSI (27), menD' has prevented an over production of reductant by decreasing the PSII levels, as indicated by fluorescence. This imbalance is translated into phenotypic changes in the cell.

To study the physiology and growth characteristics of the cells, the menD' and menE mutant strain were grown under several conditions. The photoautotrophic and photomixotrophic growth rate is significantly slower than wild type when grown at normal light levels (40 \( \mu \text{E m}^{-2} \text{s}^{-1} \)). The menE', but not menD', cells are incapable of growing at high light (>120 \( \mu \text{E m}^{-2} \text{s}^{-1} \)). Both mutants have similar doubling times to wild type when grown photoheterotropically with glucose and DCMU to block PSII function. This suggests that slower growth rate and high light phototoxicity in menE' strain is an indirect effect caused by an imbalance of the rates of electron transport between PSI and PSII. The menD' strain
has compensated by adjusting the PSI to PSII levels. The possible secondary site mutation responsible for this adaptation is being investigated.

Both the \textit{menD} and \textit{menE} deletion mutants prevents phylloquinone synthesis. These results parallel the deletion mutations in the \textit{menA} and \textit{menB} genes later in the biosynthetic pathway of phylloquinone (27). Essentially, for the phylloquinone deletion mutants the end result is the same: plastoquinone replaces phylloquinone in the \textit{A1} site and is active although at a reduced rate and cells are light sensitive from a relative increase in PSII activity as seen from oxygen evolution measurements and fluorescent ratios of PSI:PSII.

References


TABLE 1. Physiological Characteristics of the *Synechocystis* sp. PCC 6803 wild-type and *menD* and *menE* mutant strains

<table>
<thead>
<tr>
<th></th>
<th>wild type</th>
<th><em>menD</em></th>
<th><em>menE</em></th>
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<tbody>
<tr>
<td><strong>Doubling Time (hrs)</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>40 μE. m⁻² s⁻¹</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Photoautotrophic Growth</td>
<td>26 ± 3.0</td>
<td>30 ± 1.2</td>
<td>80 ± 8.2</td>
</tr>
<tr>
<td>Photoheterotrophic Growth</td>
<td>14 ± 1.5</td>
<td>15 ± 0.8</td>
<td>42 ± 1.2</td>
</tr>
<tr>
<td>Photoheterotrophic Growth + DCMU</td>
<td>20 ± 2.9</td>
<td>20 ± 1.7</td>
<td>22 ± 1.2</td>
</tr>
</tbody>
</table>

**Photosynthetic Rates**

(μmol O₂/(5 OD₇₃₀•h•l))

<table>
<thead>
<tr>
<th></th>
<th>whole chain</th>
<th>PS II-mediated</th>
</tr>
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<tbody>
<tr>
<td><strong>Whole Chain</strong></td>
<td>2420 ± 180</td>
<td>1770 ± 530</td>
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<tr>
<td><strong>PS II-mediated</strong></td>
<td>7110 ± 560</td>
<td>1960 ± 660</td>
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</table>

(μmol O₂/(Chl•h•l))

<table>
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<tr>
<th></th>
<th>whole chain</th>
<th>PS II-mediated</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Whole Chain</strong></td>
<td>708 ± 62</td>
<td>612 ± 47</td>
</tr>
<tr>
<td><strong>PS II-mediated</strong></td>
<td>2070 ± 130</td>
<td>676 ± 42</td>
</tr>
</tbody>
</table>
FIGURE LEGENDS

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* (19). The homologs of these genes have been identified in the genome sequence of *Synechocystis* sp. PCC 6803 and menA, menB, (36) and menG were confirmed.

FIG. 2. A. Left Panel. The restriction map of the *Synechocystis* sp. PCC 6803 genomic region around the menD wild type (top) and disrupted gene (below). A. Right Panel. Electrophoretic analysis of the DNA fragments amplified by PCR from genomic DNA of the wild type and mutant gene. Molecular weight marker left lane. B. Left Panel. The restriction map of the *Synechocystis* sp. PCC 6803 genomic region around the menE wild type (above) and disrupted gene (bottom). B. Right Panel. Electrophoretic analysis of the DNA fragments amplified by PCR from genomic DNA of the wild type and mutant gene. Molecular weight marker left lane. Each PCR fragment was sequenced to confirm its identity.

FIG. 3. HPLC profiles of pigment extracts from lyophilized PS I complexes of the menE mutant strain of *Synechocystis* sp. PCC 6803. The pigments were separated on a 5.0 μm ultrasphere C18 reverse phase column and detected from 190 nm to 800 nm by a photodiode array. The detection wavelength shown was 254 nm. The inset spectrum from the mutant shows a peak that co-elutes with plastoquinone at 29.7 min.

FIG. 4. HPLC profiles of pigment extracts from lyophilized thylakoid membranes of wild type, menD and menE mutant strain of *Synechocystis* sp. PCC 6803. The pigments were separated on a 5.0 μm ultrasphere C18 reverse phase column and detected from 190 nm to 800 nm by a photodiode array. The detection wavelength shown was 254 nm. The inset chromatograms compare wild type and menD to the full menE chromatogram in the region of the PhQ peak (20.5 min.).

FIG. 5. P700\(^{-}\) reduction kinetics in PS I complexes isolated from menE mutant. Flash induced optical transient measured at 810 nm after a single flash. The sample cuvette (10 mm × 10 mm) contained 3.0 ml of PSI complex at 50 μg/ml Chl suspended in 25 mM Tris, pH 8.3 with 0.04% β-DM, 10 μM sodium ascorbate and 4μM DCPIP. Excitation
wavelength, 542 nm. excitation energy, 1.4 mJ. A 300 MHz bandwidth was used in the preamplifier to recover kinetics in the μs time range.

FIG. 6. Photoaccumulated and Simulated Q-band CW EPR spectra of A₁⁻ and Q in PS I complexes isolated from wild type and menD mutant. Top is compiled spectra and the bottom compares wild-type and menD. The photoaccumulation was carried out for 40 min at 205 K. Instrument settings: microwave power 1 mW. microwave frequency 34.056 GHz. modulation frequency 100 KHz. modulation amplitude 1 G. temperature 205 K. time constant 10 mS. conversion time 10 mS. 100 averaged scans.

FIG. 7. Fluorescence emission spectra at 77 K of whole cells from Synechocystis sp. PCC 6803 wild type and menE mutant strains. Spectra were recorded at the same cell density and normalized relative to PS II. Each spectrum was the average of the four measurements. The excitation wavelength was 435 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 730 nm.
Fig. 1
Fig. 2
Fig. 3
Fig. 4
Fig. 5
Fig. 6
Fig. 7

- **Wild Type**
- **menE**
- **menD**

A.U.

nm

700
650
600

PSII

PSI

15x10^3

10

5

600 650 700 750

nm
CHAPTER 4. 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

A paper published in the Journal of Biological Chemistry.


Summary

Genes encoding enzymes of the biosynthetic pathway leading to phylloquinone, 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 phylloquinone, thereby confirming the participation of these two gene products in the biosynthetic pathway. The menA* and menB* mutants grow photoautotrophically under normal-light conditions (40 μE m⁻² s⁻¹) with doubling times twice that of the wild type, but are unable to grow under high-light conditions (160 μE m⁻² 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 are unable to grow under high-light conditions.

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* The authors thank Bruce Diner for his generous gift of authentic plastoquinone-9

Abbreviations: PS I, photosystem I; PS II, photosystem II; EPR, electron paramagnetic resonance; HPLC, high performance liquid chromatography; GC, gas chromatography; MS, mass spectroscopy; DPIP, 2,6-dichlorophenol-indophenol; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)]ethyl]glycine; DPPH, α,α'-diphenyl-β-picryl hydrazyl; SAM, S-adenosyl methionine; SAH, S-adenosyl homocysteine.
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 to 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 to 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 phylloquinone, PS I recruits plastoquinone-9 into the A_1 site, where it functions as an efficient cofactor in electron transfer from A_0 to the iron-sulfur clusters.

Introduction

All well-characterized photosynthetic reaction centers (RC) are known to contain a bound quinone molecule that participates in the early stages of photochemical charge separation and stabilization (1, 2, 3). Type II reaction centers, such as Photosystem II (PS II) or those present in the purple non-sulfur bacteria, contain a bound benzoquinone or ubiquinone as the secondary electron acceptor. Type I reaction centers, such as Photosystem I (PS I) of cyanobacteria and green plants, contain a bound menaquinone, usually phylloquinone (Vitamin K_1, 2-methyl-3-phytyl-1,4-naphthoquinone) or less-commonly, 5'-monohydroxy-phylloquinone, 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 phylloquinone can be extracted per molecule of P700 from isolated PS I complexes (5, 6, 7, 8, 9); however, only one molecule of phylloquinone is considered to participate as an intermediate in electron transfer from A_0 to F_X (5, 6, 10).

One strategy to disallow A_1 function is to inactivate genes that code for enzymes involved in the proposed pathway of phylloquinone biosynthesis. Many prokaryotes as well as chloroplasts contain the metabolic pathway for phylloquinone (vitamin K_1) biosynthesis. In several bacteria, vitamin K_2 (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, 13, 14) and Bacillus subtilis (15, 16). Although the route of phylloquinone 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 phylloquinone 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 phylloquinone and menaquinone is expected to include similar steps (Figure 1). The genome database 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* (DHNA synthase) and *menA* (identified as ‘menaquinone biosynthesis protein’, but probably a phytol transferase). Possible homologs of *menC* and ORF241 (the DHNA thioesterase) have also been identified in our database 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 database 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; 2) the A₁ site should be empty, thereby allowing a test of the requirement of phylloquinone in electron transfer from A₁ to Fₓ. 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 it 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 A₁ 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 A₁ site does not remain empty. Instead, we suggest that PS I recruits plastoquinone-9 into the A₁ site, and this quinone supports high-efficiency electron transfer from A₀ 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 to 5 μE m⁻² s⁻¹); liquid cultures of wild type and mutant strains were grown under normal light conditions (30 to 50 μE m⁻² s⁻¹) in presence of 5 mM glucose. Cell growth in liquid cultures was monitored by measuring the optical density 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 (OD₇₃₀ = 0.8-1.2) were harvested by centrifugation and were washed once with BG-11 medium. All cultures were adjusted to the same initial cell density (OD₇₃₀ = 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 (Figure 2A). The PstI restriction sites were incorporated in both fragments, whereas the ApaI restriction site was also added to the downstream fragment. The first amplification product was digested with EagI and PstI, whereas the second fragment was digested with PstI and ApaI restriction enzymes. The fragments were ligated with the pBluescript SK (Stratagene) vector that had been digested with EagI and ApaI. The kanamycin-resistance gene from pUC4K was cloned in the PstI site; this yielded recombinant plasmid pRRA 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 (Figure 2B). The oligonucleotide primers included unique restriction sites (SpeI and EcoRI in the upstream fragment and EcoRI and XhoI sites in the downstream fragment). The amplified fragments were cloned into pBluescript (Stratagene), and a 2.0-kb EcoRI fragment containing the streptomycin/ spectinomycin-resistance cassette of the Ω element of the recombinant plasmid pH45Ω (22) was inserted into the newly created unique EcoRI 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 (Boehringer Mannheim-Enzo Diagnostics Inc., Indianapolis, IN). 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 in a final concentration of 1.0 OD730 ml⁻¹. Whole-chain electron transport (H₂O to CO₂) measurements were determined after the addition of 5 mM NaHCO₃. Oxygen evolution mediated by PS II only was determined after addition of 4 mM 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 x g for 45 min. The thylakoid membranes were resuspended in thylakoid buffer (50 mM HEPES/NaOH, pH 8.0; 5 mM MgCl₂, 10 mM CaCl₂, 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 solublized in 1% (w/v) n-dodecyl-β-D-maltoside (DM) for 2 to 4 hrs at 4°C. The trimeric and monomeric PS I particles were separated by centrifugation on 5 to 20% (w/v) sucrose gradients with 0.03% DM 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 DM (25).

**SDS-PAGE 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 (35). 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, pH 7.0 buffer. Cells were diluted in 25 mM HEPES/NaOH, pH 7.0 containing 60% (v/v) glycerol to a concentration of 1.0 OD730 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 c₆ 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).

EPR Spectroscopy of Fₐ and Fₐ Finding

Electron paramagnetic resonance (EPR) studies were performed using a Bruker ECS-106 X-band spectrometer and a standard-mode resonator (ST 8615) which is 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, UK). The microwave frequency was measured with a Hewlett-Packard 5340A frequency counter, and the magnetic field was calibrated using DPPH 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 (Oriel, Stratford, CT, Model 66057) passed through 5 cm of water and a heat-absorbing color filter to remove the near-IR light. Samples used for EPR measurements contained 1 mg Chl ml⁻¹, 1 mM sodium ascorbate, 30 M DCPIP in 50 mM Tris, pH 8.3.

Analysis of phylloquinone using HPLC-UV/Vis and mass spectrometry

Membranes containing 0.1 mg chlorophyll were centrifuged at 1000 x g for 60 min and the supernatant was removed. The membrane pigments were sequentially extracted with 1 ml methanol, 1 ml 1:1 (v/v) methanol:acetone, and 1 ml acetone, and the three extracts were combined. The resulting solution was concentrated by vacuum at 10°C in the dark to ca. 0.8 mg 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 was done using JCL6000 version 26 software (Jones Chromatography Limited, 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 µL) were made on a 4.6 mm × 25 cm Ultrasphere C_{18} column (4.6mm × 250mm) with 5 µm packing (Beckman Instruments, Palto 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 1.0 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 post-column flow splitter delivered column eluent to the electrospray ion source at 10 µL min\(^{-1}\). GC/MS analyses were performed using a Hewlett Packard 5972 mass spectrometer coupled to a Hewlett Packard 5890 gas chromatograph. Split-less injections of 1.0 µL were made onto a 30 meter DB-5 column (J&W Scientific, Folsom, CA) using helium (35 cm sec\(^{-1}\)) as carrier gas. The column was programmed from 100° C to 300°C at a rate of 6° 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 Figure 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 aphl\(_{II}\) gene. Using primers flanking the coding sequence (small arrows in Figure 2A), PCR amplification of the menA locus of the wild type produced the expected fragment of 1070 bp (Figure 2A, right panel). PCR amplification of the menA locus of the mutant strain produced the expected 1.9-kb fragment (Figure 2A, right panel). Since no amplification of the 1070-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 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 Figure 2B, most of the *menB* gene was deleted and replaced with a 2-kb spectinomycin-resistance cartridge. Using primers flanking the coding sequence (small arrows in Figure 2B), PCR amplification of the *menB* locus of the wild type produced the expected fragment of 930 bp (Figure 2B, right panel). However, PCR amplification of this locus in the mutant strain produced a 2.2-kb DNA fragment (Figure 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 Figure 2B (data not shown).

**Analysis of the phenotype of the menA<sup>-</sup> and menB<sup>-</sup> mutant strains**

We focused initially on the phenotypic analysis of whole cells of the *menA<sup>-</sup>* and *menB<sup>-</sup>* mutant strains. Photoautotrophic growth rates of the *menA*<sup>-</sup> and *menB*<sup>-</sup> mutants were measured in cells grown in BG11-TES medium, which contains minerals and bicarbonate as the sole carbon source. Under normal-light intensity conditions (40 μE m<sup>-2</sup> s<sup>-1</sup>), the *menA*<sup>-</sup> and *menB*<sup>-</sup> mutant cells grew photoautotrophically but at a slower rate than the wild-type cells. The 86-h doubling time of the *menA* mutant cells and the 77-h doubling time of the *menB* mutant cells were more than twice the 26-h doubling time of the wild-type cells (Table 1).

Under high-light intensity conditions (160 μE m<sup>-2</sup> s<sup>-1</sup>), the doubling time of the wild-type cells was 29-h, but surprisingly the *menA*<sup>-</sup> and *menB*<sup>-</sup> mutant cells showed no measurable growth. Photomixotrophic growth rates of the *menA*<sup>-</sup> and *menB*<sup>-</sup> 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 12-h doubling time of the wild-type was one-half that under photoautotrophic conditions, and the 26 to 15-h doubling times of the *menA*<sup>-</sup> and *menB*<sup>-</sup> mutants were fractionally greater than that of the wild-type (Table 1). Under high-light intensity conditions, the wild-type cells had a doubling time of 23-h, but again the *menA*<sup>-</sup> and *menB*<sup>-</sup> mutant cells showed no measurable growth. Photoheterotrophic growth rates of the *menA*<sup>-</sup> and *menB*<sup>-</sup> mutants were determined in the presence of 20 μM atrazine to inhibit PS II function. Under both low and high light intensity conditions, the doubling times of the *menA*<sup>-</sup> and *menB*<sup>-</sup> mutants were slightly greater than those of the wild-type strain (Table 1). These studies show that the *menA*<sup>-</sup> and *menB*<sup>-</sup> mutants are capable of photoautotrophic growth only under low light intensities.
However, the high-light sensitivity of the \textit{menA} and \textit{menB} mutants can be alleviated by inhibiting PS II activity.

\textit{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 \textit{menA} and \textit{menB} mutant strains with the corresponding wild-type genes. The \textit{menA} and \textit{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 non-selective, photoautotrophic conditions at an intermediate light intensity of 40 $\mu$E m$^{-2}$ s$^{-1}$. At this light intensity, the \textit{menA} and \textit{menB} mutant cells could grow under photoautotrophic, photomixotrophic, and photoheterotrophic conditions (data not shown). After three days, the plates were shifted to high light intensity (160 $\mu$E m$^{-2}$ s$^{-1}$), and colonies appeared after about two 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 \textit{menA} or \textit{menB} locus had been transformed to the wild-type genotype. Using this approach, antibiotic-sensitive, complemented strains of the \textit{menA} mutant (AWT) and \textit{menB} mutant (BWT) were isolated. These transformants, which were tolerant to 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 \textit{menA} and \textit{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 \textit{menA} and \textit{menB} mutant strains is entirely due to the inactivation of the targeted genes.

\textit{Electron transfer rates in whole cells}

We next explored the possibility that the high-light sensitivity of the \textit{menA} and \textit{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 \textit{menA} and \textit{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 \textit{menA} and \textit{menB} mutants were 69% and 49%, respectively, those of the wild type strains (Table 1). This
result was as surprising as the ability of the mutants to grow photoautotrophically: if
phyllquinone were absent, then room temperature electron transfer to NADP$^+$ should not
occur (28, 29). Since their PS II activities are essentially 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

Figure 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 menB$^-$ mutants. In the menA$^-$ and
menB$^-$ mutant cells, the PS II-chlorophyll fluorescence emission at 685 nm 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, 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 (31, 32). On the basis of equal
cell numbers, both the menA$^-$ and menB$^-$ mutant cells were found to contain between 50 and
60% of the photooxidizable P700 of the wild-type cells (data not shown). In Synechocystis
sp. PCC 6803, ca. 100 Chi are associated with PS I and ca. 60 Chi 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 PAGE 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 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 Figure 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 18.0 and 24.0 min (the former is missing the phytol tail), a polar carotenoid (probably monohydroxylated but otherwise uncharacterized) was identified at 22.2 min, and β-carotene was identified at 28.8 min. Phylloquinone was identified by co-elution at 20.7 min with an authentic phylloquinone standard (Figure 4, top inset) and by its UV-visible spectrum (Figure 4 bottom inset). As shown in Figure 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 20.7 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 Figure 4). Based on a phylloquinone calibration curve, the minimum detection limit for phylloquinone using HPLC was estimated to be ca. 15% of the wild-type levels.

GC/MS is capable of separating and detecting non-polar 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 ca. 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 ca. 0.1% of the wild-type level. Since there are ca. 100 Chl/P700 in cyanobacterial PS I complexes, the menA" and menB" mutant strains thus contain < 0.02 phylloquinones per P700.

Presence of plastoquinone-9 in the menA" and menB" mutant 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 companion paper in this series (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 (λ = 270 nm) from the menA' and menB' mutants when compared with the wild-type (Figure 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 29 min. The difference spectrum of the components eluting at 29 min showed a strong absorbance near 254 nm that was lacking in the wild-type (Figure 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 10-fold higher concentration than phylloquinone in thylakoid membranes (34). Indeed, we found that authentic plastoquinone-9 co-elutes with, and has a UV spectrum that matches, the peak at 29 min (Figure 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 none or a very small amount of plastoquinone-9 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 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 (Figure 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 A_i site does not effect low-temperature electron transfer from A_i 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, 1.88), with a 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 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 (Figure 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 $\mu$mol mgChl$^{-1}$ h$^{-1}$ in the wild-type PS I complexes, 7128 $\mu$mol mgChl$^{-1}$ h$^{-1}$ in the PS I complexes of the menA' mutant, and 6948 $\mu$mol mgChl$^{-1}$ 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 $e^{-}$ PS $I^{-1}$ s$^{-1}$ in the wild type, 198 $e^{-}$ PS $I^{-1}$ s$^{-1}$ in the menA' mutant and 193 $e^{-}$ PS $I^{-1}$ s$^{-1}$ in the menB' mutant. As shown in Figure 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, in spite of 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 to 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, and menF 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 bicyclic 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 saturated, C-20 phytol 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 GC/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. One and both contained plastoquinone-9 in their PS I complexes. These results further suggest that the phytol chain of phylloquinone is required for its stable assembly into PS I complexes *in vivo*. These observations confirm the participation of 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 (30 µE m$^{-2}$ s$^{-1}$ and 50 µE m$^{-2}$ s$^{-1}$) but failed to grow either photoautotrophically or photo-mixotrophically when the light intensity exceeded 100 µE m$^{-2}$ 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 while 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 ca. 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 (Chitnis P. and Golbeck, J. unpublished results). 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 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 c₅₅₃ to flavodoxin in PS I complexes from the mutants were high but were 82 to 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 where 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 A₁ site and that it may participate in electron transfer from A₀ to Fₓ. 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 A₁ site, thereby promoting electron
transfer from $F_X$ to $F_A/F_B$. 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).

References
Table 1

Physiological Characteristics of the *Synechocystis* sp. PCC 6803 wild-type and the *menA* and *menB* strains. 

<table>
<thead>
<tr>
<th>Strain</th>
<th>Wild-type</th>
<th>menA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>menB&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Photoautotrophic Growth</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Doubling Time (hrs)</td>
<td></td>
<td></td>
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<tr>
<td>Normal light</td>
<td>26 ± 3.0</td>
<td>86 ± 5.0</td>
<td>77 ± 4.0</td>
</tr>
<tr>
<td>High light</td>
<td>29 ± 1.4</td>
<td>n.m.&lt;sup&gt;b&lt;/sup&gt;</td>
<td>n.m.</td>
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<tr>
<td><strong>Photomixotrophic Growth</strong></td>
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<tr>
<td>Doubling Time (hrs)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Normal light</td>
<td>12 ± 2.1</td>
<td>26 ± 3.8</td>
<td>15 ± 2.3</td>
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<tr>
<td>High light</td>
<td>23 ± 0.8</td>
<td>n.m.</td>
<td>n.m.</td>
</tr>
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<td><strong>Photoheterotrophic Growth</strong></td>
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<tr>
<td>Doubling Time (hrs)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Normal light</td>
<td>18 ± 2.2</td>
<td>19 ± 3.1</td>
<td>20 ± 2.5</td>
</tr>
<tr>
<td>High light</td>
<td>19 ± 2.8</td>
<td>22 ± 3.2</td>
<td>21 ± 2.8</td>
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<td><strong>Oxygen Evolution</strong></td>
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<td>(μmol O₂/OD&lt;sub&gt;730&lt;/sub&gt; h⁻¹)</td>
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<tr>
<td>Whole chain</td>
<td>1077 ± 55</td>
<td>750 ± 71</td>
<td>529 ± 39</td>
</tr>
<tr>
<td>PS II-mediated</td>
<td>1037 ± 70</td>
<td>905 ± 137</td>
<td>993 ± 88</td>
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<tr>
<td><strong>Chlorophyll Content</strong></td>
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<tr>
<td>(μg Chl/OD&lt;sub&gt;730&lt;/sub&gt; ml)</td>
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<tr>
<td></td>
<td>4.21 ± 0.18</td>
<td>3.36 ± 0.23</td>
<td>3.43 ± 0.32</td>
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</tbody>
</table>

<sup>a</sup> Values are the average of four independent experiments (n = 4)

<sup>b</sup> not measurable

<sup>c</sup> Cells were grown in BG11 plus 5 mM glucose under reduced light intensity; units are with reference to optical density of the whole cells at 730 nm.
Figure Legends

**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 *Escherichia coli* (see (23) for review). The homologs of these genes that have been identified in the genome sequence of *Synechocystis* sp. PCC 6803 are indicated. SAM is S-adenosyl methionine and SAH is S-adenosyl homocysteine.

**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.

**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 OD₇₅₀ ml⁻¹). Each spectrum was the average of five measurements. The excitation wavelength is 435 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.

**FIG. 4.** *HPLC profiles of pigment extracts from lyophilized PSI complexes of the wild type strain of* *Synechocystis sp. PCC 6803.* The pigments were separated on a 5.0 μm Ultrasphere C₁₈ 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 minutes. **Inset, Upper.** UV/Vis spectrum of authentic phylloquinone. **Inset, Lower.** Near-UV/Vis spectrum of HPLC peak which elutes at 29.7 min.

**FIG. 5.** *HPLC profiles analysis of pigment extracts from lyophilized PSI complexes of the menB*⁺ *mutant strain of* *Synechocystis sp. PCC 6803.* The pigments were separated on a 5.0 μm 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 (Inset, Lower) which co-elutes with plastoquinone-9, and which shows a UV spectrum similar to plastoquinone-9 (Inset, Upper) and a m/e of 748 (not shown). The LC-MS analysis of the menA mutant was similar.

**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 μg Chl a ml^-1 in 25 mM Tris buffer (pH 8.3) with 15% glycerol, 0.02% DM, 10 mM sodium ascorbate and 4 μM DCPIP. 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: microwave power, 20 mW; microwave frequency, 9.478 GHz; receiver gain, 6.3 x 10^4; modulation amplitude, 10 G at 100 kHz; magnetic field, center field, 3480 G; scan width, 1740 G; 3 scans averaged.

**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 μmol mg Chl^-1 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).
Fig. 1.
Fig. 2.
Fig. 3.
Fig. 4.
Fig. 5.
Fig. 6.
Fig. 7.
CHAPTER 5. RECRUITMENT OF A FOREIGN QUINONE INTO THE A\textsubscript{i} SITE OF PHOTOSYSTEM I

IV. IN VIVO REPLACEMENT OF THE NATIVE QUINONE IN THE PHYLLOQUINONE-LESS MUTANTS OF SYNECHOCYSTIS SP. PCC 6803 BY EXTERNALLY SUPPLIED NAPHTHOQUINONES\textsuperscript{1}

A paper to be submitted to the Journal of Biological Chemistry

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Summary

When the phylloquinone biosynthetic pathway is altered by interruption of the \textit{menA} and \textit{menB} genes in \textit{Synechocystis} sp. PCC 6803 (Johnson, T. W. et al. (2000) \textit{J. Biol. Chem.} 275: 8523-8530; Zybailov, B. et al. (2000) \textit{J. Biol. Chem.} 275: 8531-8538; Semenov et al. (2000) \textit{J. Biol. Chem.} 275, 23429-23438), plastoquinone-9 (PQ)\textsuperscript{3} occupies the A\textsubscript{i} site and functions as an efficient electron transfer cofactor from A\textsubscript{0} to the FeS clusters in Photosystem I. We show here that phylloquinone can be reincorporated into the A\textsubscript{i} site by supplementing the growth medium of \textit{menB}\textsuperscript{'} and \textit{menA}\textsuperscript{'} mutant cells with authentic phylloquinone. The reincorporation also occurs in \textit{menB}\textsuperscript{'} and \textit{menA}\textsuperscript{'} cells that have been treated with protein synthesis inhibitors, as well as in PSI complexes isolated from the \textit{menA}\textsuperscript{'} and \textit{menB}\textsuperscript{'} mutants, indicating that phylloquinone displaces PQ in fully formed PSI complexes. This likely occurs because phylloquinone has a higher binding affinity for the A\textsubscript{i}

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\textit{Abbreviations}: PQ, plastoquinone; plastoquinone-9; PSI, photosystem I; PSII, photosystem II; EPR, electron paramagnetic resonance; HPLC, high performance liquid chromatography; GC, gas chromatography; MS, mass spectroscopy; DPIP, 2,6-dichlorophenol-indophenol; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine;
site than PQ. Phylloquinone is also reincorporated into the A<sub>1</sub> site by supplementing the growth medium of menB<sup>−</sup> mutant cells with 2-carboxy-1,4-naphthoquinone and 2-methyl-1,4-naphthoquinone. Both phylloquinone and demethyl phylloquinone are incorporated into the A<sub>1</sub> site by supplementing the growth medium of menB<sup>−</sup> mutant cells with 1,4-naphthoquinone and 2-hydroxy-1,4-naphthoquinone. This result indicates that the specificity of the phytol transferase enzyme is relatively nonspecific in terms of the groups present at the 2- and 3-positions of the 1,4-naphthoquinone ring. Since phylloquinone and demethyl phylloquinone is present after supplementing the growth medium of menB<sup>−</sup> mutant cells with any of the four naphthoquinones, the phytol tail is considered necessary for displacement of PQ from the A<sub>1</sub> site. These finding open the possibility of incorporating novel quinones into the A<sub>1</sub> site in vivo by supplementing the growth medium of the menB<sup>−</sup> mutant cells with various quinone derivatives.

Introduction

Substituted quinones are frequently employed as electron and proton transfer cofactors in the electron transport chains (1-3). The quinones are comprised of a relatively polar ring, the so-called 'head group', which consists of either a benzoquinone (BQ) or a naphthoquinone (NQ), and a non-polar isoprenoid 'tail' of various chain lengths. Benzoquinones such as plastoquinone-9 and ubiquinone (UQ) function in membrane-bound protein complexes either as fixed or exchangeable electron/proton carriers during photosynthetic and respiratory electron transport. In photosystem II (PSII), PQ functions as a bound one-electron acceptor in the Q<sub>A</sub> site and as an exchangeable 2-electron/2-proton acceptor in the Q<sub>B</sub> site. The reduced PQH<sub>2</sub> is subsequently exchanged from the Q<sub>B</sub> site by an oxidized PQ, diffuses into the membrane, and is oxidized and deprotonated by the cytochrome b<sub>6f</sub> complex. Photosynthetic reaction centers (RCs) of purple bacteria use UQ (e.g. *Rhodobacter sphaeroides*) or menaquinone (e.g. *Rhodosprillum viridis*) in a similar role. In photosystem I (PSI), phylloquinone (PhQ), a substituted NQ with a 20-carbon, largely saturated, phytol tail, functions as bound one-electron acceptor in the A<sub>1</sub> site roughly analogous to that of Q<sub>A</sub> in PSII. There exist two molecules of PhQ in PSI, but to our knowledge, the second quinone does not function in a manner equivalent to the Q<sub>B</sub> quinone in the bacterial RC and PSII. Quinones are therefore extremely versatile; they can function
as the interface between electron transfer involving organic cofactors and electron transfer involving iron-sulfur clusters (PSI), or between pure electron transfer and coupled electron/proton transfer involving a second organic cofactor (PSII). Each quinone displays equilibrium binding and redox properties that are different for each site of interaction (4) that are conferred largely by the protein environment.

To understand the structural determinants that allow PhQ to function with high efficiency in the A, site of PSI, we embarked on a project aimed at biological replacement of the native PhQ with a variety of benzylquinone and naphthoquinone derivatives. Itoh and coworkers, and Biggins and coworkers used organic solvents to extract phylloquinone from lyophilized PSI complexes, and reconstituted the empty A, site with various benzoquinone, naphthoquinone, and anthraquinone head groups (5-9). The extraction procedure removes some of the chlorophyll and most of the carotenoid molecules along with the two molecules of PhQ, leading to the possibility that structural perturbations might have occurred to the PSI complexes. To circumvent this problem, we developed a genetic technique to accomplish the in vivo removal of the native PhQ. In a series of three papers(10-12), we reported the generation and characterization of PQ-less mutants of the cyanobacterium Synechocystis sp. PCC 6803. We accomplished this by interruption mutagenesis of two genes, menA which codes for a phytol transferase, and menB which codes for 1,4-dihydroxy-2-naphthoate (DHNA) synthase. Our strategy was to preclude PhQ production with the intent of creating an empty A, site. To the contrary, we found that the A, site in the mutant PSI complex is occupied by a benzoquinone derivative, PQ. Detailed EPR and optical spectroscopic analyses show that the PQ occupies the site at the same distance from P700+ and with the same orientation as phylloquinone in the wild-type, and functions as an efficient intermediate electron transfer carrier between A0 and Fx. The redox potential of PQ in the A, site is more oxidizing than phylloquinone, rendering electron transfer from Q− to Fx thermodynamically unfavorable. However, forward electron transfer nevertheless occurs because of the large, favorable free energy change fromA0−(3,3),(997,993) to flavodoxin.

In principle, it should be possible to introduce various quinones in the A, site in vivo by utilizing the unmodified phytol transferase and methyl transferase enzymes in the remaining biosynthetic pathway of the menB' mutant cells. In addition, physiological studies
can be performed on living cells and detailed biophysical analyses can be performed on the purified PSI complexes.

**Material and Methods**

**Cyanobacterial Strains and Growth**

A glucose-tolerant strain of *Synechocystis* sp. PCC 6803 was used as the wild type strain. The *menA* mutant strain lacks functional phytol transferase and has been previously described (10). The *menB* strain used here (*menB26*) was derived as a second site suppressor of the *menB18* strain reported earlier (10). Colonies of the *menB18* strain were replica-plated on BG11 plates with 5 mM glucose. One set was grown at ‘normal’ light intensity (40 μE m⁻² s⁻¹) while the other was at ‘high’ light intensity (>100 μE m⁻² s⁻¹). The *menB26* was one of the isolates that could grow at high intensity. This strain showed no changes in the PSI and PSII activities compared to the *menBlS* strain. The purified PSI complexes of the *menB26* and *menB18* strains showed identical optical and EPR characteristics as well as identical protein and pigment composition. The molecular basis for the suppression of the original *menB* phenotype is under investigation. Since the growth of the *menB26* revertant was robust under a variety of different conditions, it was used in the supplementation studies described here. The wild-type, *menA* and *menB26* mutant cells were grown in the BG-11 medium, with kanamycin and spectinomycin added to the media of the *menA* and *menB26* mutant strains, respectively (13). Agar plates for the growth of the stock cells were kept at low light intensity (2 to 10 μE m⁻² s⁻¹); liquid cultures of the wild-type and mutant strains were grown autotrophically under normal light conditions (40 to 60 μE m⁻² s⁻¹). Cell growth in liquid cultures was bubbled with sterile filtered air and was monitored by measuring the optical density at 730 nm with a Shimadzu spectrophotometer (10). Cells from liquid cultures in the late exponential phase of growth (OD₇₃₀ = 0.8-1.2) were harvested by centrifugation at 5000 x g for 15 min.

**Growth Rates of the Wild-Type and Mutant Cells with NQ Supplements**

Cyanobacterial cultures in the late exponential log phase were pelleted by centrifugation, washed twice with BG11 medium, and suspended in BG11 and spectinomycin at approximately 10 A₇₃₀ concentration. For estimating growth rates, the cultures were grown in 6 well plates with 8 ml medium in each well. All cultures were
adjusted to the same initial cell density \((A_{730} = 0.1)\). Final concentrations of naphthoquinone, glucose, and DCMU were 5 \(\mu\)M, 5 mM, and 10 \(\mu\)M, respectively. The cells were shaken on an orbital shaker at 110 rpm. Growth was monitored by measuring absorbance of cultures at 730 nm. Chlorophyll was extracted from whole cells with 100% methanol and the concentration was determined according to (14). The conditions for growing wild-type cells of Synechocystis sp. PCC 6803 in D$_2$O have been described previously (10).

**Isolation of Thylakoid Membranes and PSI Particles**

Thylakoid membranes were prepared from cells as described by (15). The thylakoid membranes were pelleted by centrifugation at 50,000 \(\times\) g for 90 min and were resuspended in SMN buffer (0.4 M sucrose, 10mM MOPS, 10mM NaCl) for storage. Chlorophyll was extracted from thylakoid membranes and PSI trimers with 80% acetone and determined according to (14). For the isolation of PSI complexes, thylakoid membranes were incubated in SMN buffer with 20 mM CaCl$_2$ for 0.5 to 1.0 hr at room temperature in the dark to enhance trimerization of PSI. To the mixture, \(n\)-dodecyl-\(\beta\)-D-maltoside (DM) was added to a final concentration of 1.5% (w/v) and incubated in the dark on ice with occasional gentle mixing for 0.5 to 1.5 hrs. The non-solubilized material was removed by centrifugation at 10,000 \(\times\) g for 15 minutes. The trimeric and monomeric PSI particles and PSII were separated by centrifugation in 10 to 30% (w/v) sucrose gradients with 0.04% DM in 10 mM MOPS, pH 7.0.

**Measurement of Photosystem I Activity**

Steady-state rates of electron transfer for isolated PSI complexes were measured using cytochrome \(c_6\) as electron donor and flavodoxin as electron acceptor as described in (16). The measurement of P700 photooxidation in whole cells was based on the techniques developed by Harbison and adapted to cyanobacteria as described in (17).

**Analysis of Phyloquinone using HPLC and Mass Spectrometry**

PSI samples were prepared and quinones were analyzed on an equal chlorophyll basis, as previously described (10). However, the solvent gradient was modified to a 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 1.0 mL/min. A solution of PhQ (40 mM) was prepared in absolute ethanol and kept at \(-20^\circ\) C as a standard for calibration.
**Phylloquinone in vivo Incorporation**

Cells were grown in 3l flasks of BG11 with Fe and glucose (5mM) to a density of 0.550 to 0.675 at 730nm. For set AG, the cells were then sterile pelleted and resuspended in fresh media with Fe lacking glucose and grown for an additional day. For set 26, no additional preparation was performed other than a decreased amount of glucose (0.6 mM) was added to the media. At time minus 15 min. three antibiotics were added (132 µg/ml chloramphenicol, 30 µg/ml kanomyocin, 75 µg/ml erythromycin) to half of the flasks. At time equal 0 min. 10µM phylloquinone, final concentration, was added. At set time intervals, 400 ml liquid culture was pelleted and resuspended in SMN buffer, with EDTA (5mM) and PMSF (5µM). Thylakoid membrane extractions were performed on a minipreparative scale. In a 2ml screw top vial, 0.250 ml glass beads were added to 1.6 ml of resuspended cells (400 O.D.). A small gap of air (~0.2 ml) was left for mixing. The vials were shaken eight times at 3400 rpm for 30 sec. on a mini-bead beater with a 8 vial adaptor (Biospec. Bartlesville OK). The remainder of the procedure is described in (10). PSI isolation was also done by the above described procedure. Isolated PSI timers were washed to remove sucrose and frozen. HPLC analysis of the pigments was done on an equal chlorophyll basis.

**Q-band EPR Spectroscopy of Photoaccumulated PSI Trimers**

Photoaccumulation experiments were carried out using a Bruker ER300E spectrometer outfitted with an ER 5106 QT-W1 resonator, which is equipped with a port for in-cavity illumination. Cryogenic temperatures were maintained with an ER4118CV liquid nitrogen cryostat, controlled with an ER4121 temperature control unit. The microwave frequency was measured with a Hewlett-Packard 5352B frequency counter and the magnetic field was measured with a Bruker ER035M NMR Gaussmeter. The magnetic field was calibrated using α.α’ bisdiphenylene-β-phenylallyl (BDPA) complexed 1:1 with benzene. Prior to the photoaccumulation, the sample was adjusted to pH 10 with glycine buffer to a final concentration of 233 mM, and sodium dithionite was added to a final concentration of 50 mM. After incubation for 20 min in the dark, the sample was placed into the resonator and the temperature was adjusted to 205 K. The sample was illuminated with a 20mW He-Ne laser at 630 nm for 40 minutes. The dark background was subtracted from the
photoaccumulated spectra. EPR spectral simulations were carried out on a Power Macintosh 7300/200 computer using a Windows 3.1 emulator (SoftWindows 3.0, Insignia Solutions, UK) and SimFonia software (Bruker Analytik GMBH).

Optical Kinetic Spectroscopy in the nIR Region

Optical absorbance changes in the near-IR were measured using a laboratory-built spectrophotometer (12). To assure resolution of kinetics in the microseconds time domain, a high-frequency roll-off amplifier described in the original specifications was not used, and the signal was fed directly into the plug-in (11A33 differential comparator, 100 MHz bandwidth) of the Tektronix DSA601 oscilloscope. The sample cuvette contained the PSI trimers isolated from the menA or menB mutants at 50 μg/ml Chl in 25 mM Tris-HCl, pH 8.3, 10 mM sodium ascorbate, 4 μM DCPIP, and 0.04% n-dodecyl-β-D-maltoside (β-DM).

Results

Growth of Cells with Naphthoquinone Supplements

Growth rates of the wild-type, menA', and menB26' mutants were measured in cells grown in BG-11 medium which contains minerals including bicarbonate (Table 1). Under normal light conditions (40 μE m⁻² s⁻¹), photoautotrophic doubling times for the wild type (26 h) were nearly three times faster than the mutants (menA' 86 h; and menB26', 77 h). The menA' mutant reached stationary growth at ~0.4 O.D, whereas the menB26' mutant and the wild type reached stationary phase at ~0.8 and ~1.0 A, respectively. Photomixotrophic growth that uses both respiration and photosynthesis for energy metabolism was achieved by adding 5 mM glucose. The wild type and menB26' mutant had similar doubling times of 14 h and 15 h, respectively, but the menA' strain grew slower with a doubling time of 26 h. The difference in the growth rates of the menB26' and menA' strains is consistent with the selection procedure used for isolating the former from the original menB18' strain, which grows slower than the wild type strain under photomixotrophic growth conditions. Photoheterotrophic growth conditions included addition of glucose for respiration and of 10 μM DCMU to block PSII activity. The wild-type, menA' and menB26' mutant strains had similar doubling times under these conditions. Therefore, respiratory energy metabolism and cyclic photophosphorylation function normally in the mutants without phylloquinone. At
stationary phase in glucose-containing media, the wild-type and mutant cells attained a culture density between 2.75 and 3.5 A

When the cells attained a culture density of ~0.15 A, a variety of substituted naphthoquinones were added to the growth medium of the wild-type. menA' and menB26' cells to a final concentration of 5 to 10 μM. Supplementing the medium of the wild-type cells produced a wide variety of responses in growth rates. Three naphthoquinone derivates, 5-OH-1,4-naphthoquinone, 2-methyl-5-OH-1,4-naphthoquinone, and 2,3-diCl-1,4-naphthoquinone, are known herbicides (18-20). Indeed, the wild-type cells died or ceased to grow upon addition of these naphthoquinones to the growth medium. Three naphthoquinone derivatives that are identical or closely related to the biosynthetic precursors of phylloquinone were found to slow the growth of the wild-type cells. The addition of 1,4-naphthoquinone, 2-CO2-1,4-naphthoquinone, and 2-methyl-1,4-naphthoquinone (vitamin K3) to the growth medium led to an increase in the doubling times by 28%, 24% and 28%, respectively. Other 2-carboxy naphthoquinone derivatives such as 2-CO2-3,5-naphthoquinone and 2-CO2-3,7- naphthoquinone cells led to an increase in the doubling times of wild-type cells by approximately 32%. Naphthoquinones that provided their own alkyl chain tail (menadione (Vitamin K3) and lacaphol (2-OH-3-prenyl-1,4-naphthoquinone)) also slowed the growth of the wild type. Wild-type doubling times were not affected by addition 2-OH-1,4-naphthoquinone to the growth medium. In general, naphthoquinone derivatives that are analogs or direct products of the naphthoate synthase enzyme appear to stress the wild-type cells and slow their growth, possibly by competing with cellular naphthoquinone for sites in the biosynthetic enzymes or in PSI.

Addition of naphthoquinones that structurally resemble the product of naphthoate synthase (1,4-naphthoquinone, 2-OH-1,4-naphthoquinone, 2-CO2-1,4-naphthoquinone and 2-methyl-1,4-naphthoquinone (vitamin K3)) accelerated the growth rates of the menB26' mutant cells (Table 1). The growth rate of menB26' increased by a factor of 3.7 and 1.8 when supplemented with 2-methyl-1,4-naphthoquinone (vitamin K3) and 1,4-naphthoquinone respectively. Indeed, the doubling time of the former approached that of the wild-type. Supplementing the direct product of naphthoate synthase, 2-CO2-1,4-naphthoquinone improved the growth of menB26' by a factor of 2.1, but the doubling time
was still greater than the wild-type (Table 1). Vitamin K₂, an analog phylloquinone compound with a C-40 unsaturated alkyl group, does decrease the doubling time by almost half. Another compound with a shorter alkyl tail, 2-OH-3-prenyl-1,4-naphthoquinone, also decreases the growth rate.

Interestingly, the addition of naphthoquinones in which the head group does not structurally resemble 1,4-naphthoquinone have marginal effects on growth rates. Other 2-carboxy naphthoquinones (2-CO₂-3.5-naphthoquinone and 2-CO₂-3.7-naphthoquinone) had virtually no effect on the growth rates of the menB26' mutant cells. Thus, the placement of the carbonyl oxygens on the naphthoquinone ring are as important as the presence of a carboxy group for affecting growth, which may be related to the ability of the quinone to become phytylated (Fig. 1). Those naphthoquinones that decreased the doubling times of the menA' and menB26' mutant cells were targeted for further study.

In general, the menA' mutant was either unaffected or apparently stressed by the addition of naphthoquinone derivatives (Table 1). Since the menA' mutant lacks the ability to attach a phytol tail to the naphthoquinone head group, these results would indicate that simple quinones either do not displace plastoquinone-9 from the A₁ site, or alternatively cannot functional as an electron transfer cofactor if present in the A₁ site. Naphthoquinone derivatives that accelerated or did not affect growth rate in the menB26' mutant cells decreased the doubling times of the menA' mutant cells (Table 1). Only 2-OH-naphthoquinone allowed the cells to grow to normal stationary phase, although it did not affect the growth rate. Therefore, a 1,4-naphthoquinone may need to be phytylated before it can assemble and function in the A₁ site of PSI. Since the menA' strain was unable to use externally supplied quinones, all further work was performed using PSI complexes from the menB26' cells that had been grown with supplemented naphthoquinones.

**LC-MS Analysis of Pigment Extracts from PSI Complexes**

The presence of quinones in the extracts of purified PSI trimers was examined using HPLC coupled to a chemical ionization time-of-flight mass spectrometer. By co-injecting standards and by interpreting the spectra at selected masses, chlorophyll a (m/z 892) was identified with a retention time of 19.0 minutes. Phylloquinone (m/z 450) and PQ (m/z 748) had characteristics retention times of 19.9 and 29.1 min. respectively (Fig. 2). Virtually
identical chromatograms were obtained for pigment extracts of the wild-type. menA' and menB26' mutants, except that the phylloquinone peak at 19.9 min was missing in the mutants (Table 2). Quantification by MS is somewhat difficult, given that each molecule has varying ionization characteristics. It is possible to determine the number of counts at a particular molecular weight, thereby calculating the area under the peak. However, without detailed controls and standards this MS data should be taken more as a trend and semi-quantitative. More detailed quantitative data has been calculated from the UV/vis spectra, given that the extinction coefficients are well known for the observed molecules. The amount of PQ in the wild-type trimers appears to be very small (< 1%). The low amount of PQ found in wild-type PSI has not been detected to be involved in electron transfer and is considered to carryover from the thylakoid membrane lipids. PSI complexes isolated from the menB26' mutant cells supplemented with several naphthoquinone derivatives led to the restoration of the phylloquinone peak. The addition of authentic phylloquinone to menB26' mutant cells revealed that the cells could harvest the highly nonpolar quinone from the liquid media and incorporate it into the A1 site at a ratio (> 100:1 PhQ:PQ) similar to the wild-type. Cells are also capable of harvesting menaquinone (m/z 444), the C-40 partially unsaturated tail group, an analog of phylloquinone. There is nearly complete displacement of plastoquinone as determined by MS. The menaquinone elutes earlier (13.3 min) than phylloquinone (20.5 min) in this solvent system. We also monitored a minute (<<1) but detectable amount of phylloquinone (m/z 450) in the pigment extracts that was not found in the neat menaquinone. Supplementing the menB26' mutant cells with the direct product of naphthoate synthase, 2-CO2-1,4-naphthoquinone, resulted in the presence of phylloquinone in the PSI complexes at a ratio greater than 100:1 PhQ:PQ. When the medium was supplemented with 2-methyl-1,4-naphthoquinone (vitamin K3), phylloquinone was found in the PSI complexes but at a ratio (~ 30:1 PhQ:PQ) that was lower than the wild-type. Thus vitamin K3 is probably not incorporated in the phylloquinone biosynthetic pathway as efficiently as is 2-CO2-1,4-naphthoquinone.

Another 'tailed' naphthoquinone used was 2-OH-3-prenyl-1,4-naphthoquinone (lacaphol). The molecule has not been identified in the pigment extracts in the present solvent system. Further investigation identifying the molecule is in progress.
When menB26' cells were grown with 1,4-naphthoquinone, the LC-MS analysis of pigments extracted from isolated PSI complexes showed the presence of phylloquinone (m/z 450). These samples also contained a species with a mass of 436 m/z that probably represents unmethylated 2-phytyl-1,4-naphthoquinone. The NQ/PQ ratio (6:1) was also lower than when the cells were grown with vitamin K, or 2-CO2-1,4-naphthoquinone. The ratio of unmethylated 2-phytyl-naphthoquinone to the methylated phylloquinone (or derivative) is 2 times when the menB26' mutant was supplemented with 1,4-naphthoquinone. This suggests that the rate of methylation is slower than the rate of phytlation, and that the pool of unmethylated derivative is available for assimilation into the A1 site. The presence of demethyl phylloquinone in the PSI complex also shows that the methyl group is not required for the assembly into the A1 site. Phylloquinone was also found in significant amounts in the PSI complexes in the supplementation experiments with 1,4-naphthoquinone. The total phylloquinone derivative (methylated and unmethylated species) to plastoquinone ratios was 6:1 for 1,4-naphthoquinone.

Although 2-OH-naphthoquinone supports an increase in the growth rate of the menB26' mutant, no peaks at m/z 450, corresponding to phylloquinone or m/z 436, corresponding to demethyl phylloquinone, were found. Rather, a new peak at 11 minutes corresponding to a mass of 453 was observed. We have tentatively assigned this peak to a 3-phytyl-1,4-naphthoquinone containing a 2-hydroxyl instead of 2-methyl group. The ratio of 2-hydroxy-3-phytyl-1,4-naphthoquinone to plastoquinone in PSI complexes is approximately 0.10:1. Therefore, plastoquinone is not replaced by the 2-OH derivative of phylloquinone to any significant degree in the mutant PSI complexes. Since the phytly transferase has already been shown to function when a hydrogen or a carboxy group is present at ring position 2, it is not surprising that it also functions when a hydroxy group is present. However, the low amount of 2-hydroxy-3-phytyl-1,4-naphthoquinone in the PSI complexes may indicate that the specificity of the phytly transferase for the 2-hydroxy-1,4-naphthoquinone substrate is low.

**HPLC Analysis of Pigment Extracts**

To confirm the results of the LC-MS analysis of the pigment extracts from PSI complexes, we examined the absorption spectra of the HPLC peaks with a UV-visible range
diode array photospectrometer detector. In short, the distinct absorption spectra of pigments in the chromatograms complement the LC-MS data. However, quantitative data can be determined from the UV-visible data, since the extinction coefficients of the molecules are known. The retention times in the HPLC analysis were consistent and close to those in the LC-MS studies, with slight variations that are attributed to different lengths of the connecting tubes between instruments.

Coelution with other pigments, namely chlorophyll a at 18 minutes and β-carotene at 29 minutes, allowed comparison of the wild-type chromatograms to the phylloquinone-less mutants. Phylloquinone, its derivatives and plastoquinone spectra are shown in Figure 3, and are consistent with the spectra of known standards. Elution and absorption properties of phylloquinone and plastoquinone have been described previously (10). The phylloquinone without methyl group has been identified to elute at 17.7 min. The spectrum of the vitamin K₃ supplement is identical to phylloquinone with absorptions at 248 nm and shoulder at 270 nm. Plastoquinone is only observed in the PSI trimers of phylloquinone-less mutants and some menB26- cells that were grown with naphthoquinone supplements. Plastoquinone could not be detected in any appreciable amount in the wild-type PSI trimers (< 1-2%). In Figure 3, the plastoquinone inset is representative of the plastoquinone spectrum of a singlet at 270 nm.

Phylloquinone and derivatives were identified by a combination of Uv/Visible spectroscopy and mass spectrometry. When available, known standard solutions were used to confirm identifications. Demethylphylloquinone was identified by its mass and similarity to the phylloquinone spectrum. It also appeared before phylloquinone in the chromatogram. The lack of a "greasy" methyl group is attributed to the decreased elution time of 2.3 minutes. The 2-OH-phylloquinone is only identified by its mass, thus tentative in assignment. The hydroxyl group significantly reduced the elution time to 11.0 minutes.

The phylloquinone to plastoquinone ratio of the pigment extracts of PSI trimer complexes varies significantly (Table 3). The wild-type shows a PhQ:PQ ratio of approximately 70:1, which corresponds to less than a 2% amount of PQ in the wild type trimer preparations. Several preparations have detected no plastoquinone in the trimers with other preparations have a plastoquinone content as high as 2% that of phylloquinone. This
suggests that any detected plastoquinone is carried from the lipid membranes over in the preparation of the trimer complexes. Plastoquinone has not been detected to be functioning within the A₁ site of the wild type by any of the experiments presented in this paper. Addition of phylloquinone to the growth media shows that the cells are capable of harvesting and incorporating phylloquinone in PSI in the menB26' mutant strain. The calculated ratio is approximately 60:1 phylloquinone:plastoquinone. As described in the next experiment the amount of phylloquinone within the PSI complexes does change over time. Supplementing whole cells with menaquinone (vitamin K₂) also shows incorporation into the PSI complexes. However, the ratio is not as high (8 MQ:PQ) as phylloquinone incorporation. The difference in displacement is attributed to the menaquinone alkyl group being a C-40 unsaturated "tail" instead of phylloquinone's C-20 phytyl group. Vitamin K, and 1,4-naphthoquinone is also incorporated into PSI after phytylation. The ratios of PhQ:PQ are lower than for directly adding phylloquinone or 2-CO₂-1,4-naphthoquinone. This is thought to result from vitamin K, and 1,4-naphthoquinone being an analogous in structure to 2-CO₂-1,4-naphthoquinone. Both the molecules lack the carboxy group, which may be important in the phytyl transferase mechanism or recognition.

Considering that for most of the supplemented quinones the Chl/Q₇ ratio is close to the published norm (21), the A₁ site is not empty nor contains a non-phytylated quinone. Deviations arise in 1,4-naphthoquinone where it appears that the quinone content has dropped causing an increase in Chl:Q₇ ratio. There is some minor indication by pulsed EPR that a free 1,4-naphthoquinone may be occupied within the A₁ site, which is undergoing further investigation (Stehlik D., personal communication).

P₇₀₀⁰⁻ optical recombination kinetics

To determine if the quinone is within the A₁ site, we probed its activity by observing the kinetic backreaction charge recombination of P700⁻. In PSI trimers isolated from the wild type, the reduction of the P700⁻ RC is multiphasic after a saturating flash (12,22). When measured by 810 nm near-IR optical spectroscopy and in the absence of external electron acceptors, the typical wild type spectrum shows a majority of the P700⁻ being reduced with a lifetime of 60 - 80 ms and an occasional minor faster phase (~10 ms) not seen here (Figure 4). The biphasic decay is considered to be a result of the PSI complexes
being in different confirmations (12.22). The microsecond decay is attributed to earlier damaged reaction centers. There is also a long-lived (>1200 ms) kinetic phase of P700\textsuperscript{−} reduction from direct reduction of the P700\textsuperscript{−} by reduced DCPIP that contributes to 5 - 20 % of the total absorbance change. In PSI trimers isolated from the menB26\textsuperscript{−} mutant, the reduction of P700\textsuperscript{−} is also multiphasic (Figure 4). When menB26\textsuperscript{−} is measured in the absence of external electron acceptor, P700\textsuperscript{−} is reduced with lifetimes of approximately 3.0 ms and 19 ms in a 7:1 ratio. The 3 ms phase has been attributed to plastoquinone in the A\textsubscript{1} site with the electrons originating on the iron sulfur clusters (12). The mutants also exhibit a minor long-lived kinetic phase attributed to electron donation of DCPIP to the oxidized P700\textsuperscript{−} that contribute the remainder of the absorbance change. The kinetic back reaction phases for both the menB26\textsuperscript{−} mutant are consistent with plastoquinone being in the A\textsubscript{1} site of PSI and acting as an electron intermediate from A\textsubscript{0} to F\textsubscript{X} (12).

Monitoring the kinetic back reaction of the quinone supplemented cells indicates whether plastoquinone still remains active in the A\textsubscript{1} site or is replaced by a naphthoquinone (Table 4). The 1,4-naphthoquinone head group in the A\textsubscript{1} site causes a loss or decrease in contribution from the 3 ms phase. Phylloquinone reverts the menB26\textsuperscript{−} PSI complexes back to wild-type spectra. Menaquinone also causes a loss of the 3 ms phase but does not restore wild type spectra. The 60 ms phylloquinone phase is replaced by a 30 ms phase. The difference may result in lower menaquinone to plastoquinone ratio yielding a mixture of two unresolved phases. It may also be attributed to a slightly different orientation of the quinone head group in the pocket given the significantly different alkyl ‘tail’. Addition of the naphthoate synthase product DHNA, shows a complete loss of the 3 ms plastoquinone phase. The PSI complexes appear to be fully functional phylloquinone containing wild type. Both vitamin K\textsubscript{1} and 1,4-naphthoquinone shows a decrease, but not loss, in the phylloquinone contribution, indicating that the phytlated quinone is active in at least a portion of the A\textsubscript{1} sites with plastoquinone occupying the remaining.

Other quinones which have not been located in the PSI pigment extracts also show only plastoquinone in PSI. For 2-CO\textsubscript{2}-3,5-naphthoquinone, 2-CO\textsubscript{2}-3,5-naphthoquinone, and 2-OH-3-prenyl-1,4-naphthoquinone the 3 ms phase is dominate. 2-OH-1,4-naphthoquinone also shows a predominate plastoquinone peak. However, the lower contribution of
plastoquinone with the 2-OH-3-prenyl-1,4-naphthoquinone supplemented cells suggests that there may be partial replacement occurring. The single unit prenyl tail may have contributed to displacement some of the plastoquinone in the A₁ sites.

This technique confirms that not only are certain quinones contained in PSI, but also active within the A₁ site as well. This data coupled with the pigment extract analysis suggests that only the phytlylated quinones can displace plastoquinone. The non-phytylated quinones are not detected in the neither the HPLC or the kinetic back reaction, as indicated by the 3 ms plastoquinone phase.

**CW EPR Spectroscopy at Q-Band of Photoaccumulated A₁**

To further probe the contents of the A₁ site, we photoaccumulated the A₁ signal for Q-band EPR. Plastoquinone has been previously identified in the A₁ site of the menB mutants (12). Figure 5 shows the Q-band EPR spectra of photoaccumulated A₁ in PSI complexes isolated from *menB26* and *menB26* with naphthoquinone supplements. For comparison the PQ containing *menB26* mutant strain and wild type is shown.

Addition of 2-C0₂-1,4-naphthoquinone, vitamin K₃, and phylloquinone supplements to the mutant reveals PhQ⁺ is exclusively photoaccumulated. 1,4-naphthoquinone shows a predominant phylloquinone spectrum, with possible contributions from plastoquinone and demethylphylloquinone. The menaquinone spectrum reveals a 1,4-naphthoquinone head group. EPR spectra showed only PQ⁺ being photoaccumulated of quinone supplements not been identified within PSI by HPLC or kinetic back reaction.

**Phylloquinone in vivo Incorporation Experiment**

We have monitored the rate of incorporation of phylloquinone in the A₁ site over time, to determine whether there was a preferential incorporation of PhQ as a function of PSI assembly or mass diffusion. The phylloquinone-less *menB26* strain was grown to mid-log phase, at which point 10 μM phylloquinone was added to the cultures and antibiotics added to half the cultures. We then observed the incorporation of phylloquinone in the PSI complexes for normal growing cells and cells in which protein synthesis has been inhibited. This will provide a clue as to the stability of phylloquinone in the A₁ site and whether or not phylloquinone diffuses in or is incorporated during PSI assembly. As seen in Figure 6, phylloquinone has completely displaced plastoquinone from PSI in both sets of cultures.
within the first measurement of 30 minutes. Over the course of three days, the level of phylloquinone drops as it is rereplaced by plastoquinone. Both traces, with and without protein inhibiting antibiotics, show a similar trend. Given that the antibiotic containing cultures cannot synthesize new PSI complexes as a process to incorporate phylloquinone, phylloquinone is diffusing in and expelling plastoquinone. The initial incorporation of phylloquinone is very rapid suggesting that the difference in binding stabilities for phylloquinone and plastoquinone within the $A_1$ site are large. However, over time phylloquinone is being replaced by the now native plastoquinone. This probably occurs as a combined process of mass action and catabolism of phylloquinone. Chlorophyll content on a per cell basis does not change significantly, suggesting that the cells are not undergoing substantial changes during the experiment.

**Discussion**

To understand function of phylloquinone in photosynthetic organisms, we generated cyanobacterial mutant strains that are unable to synthesize phylloquinone (10). The PSI complexes of these mutants contain plastoquinone in their $A_1$ site (11). The structure of phylloquinone contains several distinct features that affect their function. The phylloquinone carbonyl oxygens have been found to hydrogen bond to amino acids within the $A_1$ pocket (23), while the whole ring $\pi$ stacks to either a tryptophan or histidine (24). For a naphthoquinone to properly function within the $A_1$ site an alkyl tail must be present in the 3 position on the ring (25). Most significantly, the redox potential of phylloquinone is significantly different in the PSI environment as compared to being free in solution ((12), and references therein). To further probe the structure-function relations of quinones in the $A_1$ site of PSI, we added different naphthoquinone supplements during growth of phylloquinone-less mutants. Once inside the cell, the supplemented quinone could undergo the remaining natural steps in phylloquinone synthesis: attachment of the phytanyl tail by phytanyl transferase (the $menA$ product) and possible methylation by methylase (the product of $menG$). The newly synthesized phylloquinone or derivative competed with and replaced the plastoquinone in the $A_1$ site of the PSI complexes in the mutants.

Much effort has been spent in extracting the phylloquinone from the $A_1$ site to insert alternative quinones *in vitro* (26,27,28,29). These benzo- naphtha- and anthraquinones have
been shown to bind in $A_1$ and to accept electrons from $A_0$ (26,30). But only 1,4-naphthoquinones with a hydrocarbon tail can transfer the electron forward to the [4Fe-4S] cluster $F_X$ (31). From this summary two hypotheses were proposed: the electron transfer is dependent on an appropriate redox potential or that a specific orientation of the quinone is necessary, which is provided by the isoprene tail.

Initially, we studied the growth characteristics of the men$B26^+$ mutant strain and wild type with naphthoquinone supplements (Table 1). Naphthoquinone supplements that were found phytlylated and in PSI decreased the doubling times of the cells. The remaining unutilized naphthoquinones did not significantly change the growth rates. Wild type cell growth rate, however, slowed upon addition of nearly all supplemented quinones. We suggest that there is oxidative stress or competition with analog compounds caused by the exogenous naphthoquinone. Quinones with a 1,4-naphthoquinone moiety are known to be acetolactate synthase inhibitors (19). Acetolactate is used in the synthesis of hydrophobic amino acids and several metabolism pathways (32). Note that for the men$B26^+$ mutant strain the addition of the utilized naphthoquinones does not decrease the doubling time to that of the wild type, but only to the averaged naphthoquinone fed wild type doubling time of 36 ± 7.7 h.

The naphthoquinone compounds used in the present studies can be classified into five categories. First, phylloquinone and menaquinone can be added directly to the growth media and restore phylloquinone or menaquinone in the $A_1$ site, respectively. The cells are apparently capable extracting the two quinones from the media, draw it through the cell membrane and insert it into PSI. Second, compounds those are analogous to the substrate of naphthoate synthase produce phylloquinone, which was incorporated into the $A_1$ site to the exclusion of plastoquinone. Vitamin K$_3$ and 2-CO$_2$-1,4-naphthoquinone are converted into phylloquinone and less than 18% and 6% plastoquinone is left occupying the $A_1$ site, respectively. Third, some naphthoquinones produced phylloquinone derivatives, which competed with plastoquinone for the $A_1$ site. The partial replacement could result from decreased availability due to limiting rates of synthesis or less binding affinity than phylloquinone to the $A_1$ site. 1,4-naphthoquinone produced demethylphyloquinone in addition to phylloquinone. In the fourth set of compounds, the phytlylated naphthoquinone
derivative was not detected in the PSI complexes. These compounds, 2-CO$_2$-3,5-naphthoquinone and 2-CO$_2$-3,7-naphthoquinone, do not affect the growth rates of the cells, are not identified in the A$_1$ site, or affect the activity of PSI. These compounds may not be imported into the cell or are unable to get phytyleted, thereby hindering their assembly into PSI. Consequently, plastoquinone remains active in the A$_1$ site. Alternatively, the phytyleted quinones have lower binding affinity for the A$_1$ site than plastoquinone. This is unlikely, given that the phytyleted quinones would have been identified in thylakoid membrane by LC-MS. Finally, some naphthoquinones were toxic to the cells. 5-OH-1,4-naphthoquinone, 2-Me-5-OH-1,4-naphthoquinone, and 2,3-Cl-1,4-naphthoquinone are known phytotoxlic herbicides (18,33).

Addition of naphthoquinones that resemble the product of naphthoate synthase, exclusively produce phylloquinone, albeit not necessarily to the wild type levels as quantified by HPLC-UV/Visible. Adding 2-CO$_2$-1,4-naphthoquinone, and vitamin K$_3$ to the menB26' mutant generate different levels of phylloquinone found in PSI. Wild type and phylloquinone supplemented mutant cells have a PhQ:PQ ratio of 70:1 and 59:1, respectively. However, the addition of 2-CO$_2$-1,4-naphthoquinone has a lower ratio (16:1 PhQ:PQ). This difference may arise from the difficulty of a relatively water soluble quinone passing through lipid layers or arriving at the location where the next enzymatic step occurs, phytyle transferase. Vitamin K$_3$, which lacks the 2-carboxy group has a ratio of 7:1 PhQ:PQ. The decreased ratio may be accounted for by the lack of the carboxyl group in the 2 position, which is lost as carbon dioxide during the phytyle tail attachment. The methyl group on vitamin K$_3$ suggests that the phytyle transferase can apparently function without the high energy carboxyl group. However, that vitamin K$_3$ produces phylloquinone does reveal methylation is not necessarily the final step in phylloquinone synthesis.

Naphthoquinones that are more dissimilar to the direct product of naphthoate synthase produce phylloquinone, demethylphylloquinone, and derivatives. Addition of 1,4-naphthoquinone to the growth media, produced phylloquinone and demethylphylloquinone (Table 3). The lower ratio of PhQ$_r$:PQ may be accounted by 1,4-naphthoquinone has to be altered by two enzymatic steps instead of just one, as with vitamin K$_3$. The loss of the methyl group may also signal lesser affinities of the naphthoquinone head group for the A$_1$.
site as seen by Iwaki and Itoh with 1,4-naphthoquinone and Vitamin K₃ (2-Me-1,4-naphthoquinone) (34). Demethylphyloquinone was in greater proportion than phylloquinone since it has one less step. We believe that the methylation product phylloquinone is not necessary for function in PSI. This has been also confirmed by genetically disabling the menG gene (methyl transferase) and finding that demethylphyloquinone is operational in the A₁ site (35).

Menaquinone is also incorporated successfully within the A₁ site, albeit at levels lower than phylloquinone. The difference between the two molecules resides in the alkyl side chain. The cost of the C-40 unsaturated prenyl group is a ten fold less ratio of NQ/PQ. However, it does show that a C-40 group, like the C-45 PQ ‘tail’, was also capable of fitting the quinone head group into the A₁ site. The binding pocket that houses the alkyl side chain of the quinones must be fairly accommodating and offers a new line of research: that of the function of the quinone alkyl binding site.

The activity PSI. as monitored by the flash induced P700 back reaction and by photoaccumulated EPR mirrors the content of the A₁ site. Compounds that contained appreciable amounts of phylloquinone or their derivatives show a lack of the characteristic 3 ms back reaction attributed to plastoquinone (Table 4). Vitamin K₃ and 1,4-naphthoquinone supplements, which contained up to 20% plastoquinone, show the 3 ms phase, but at reduced contribution from the normal 70% for menB26'. Compounds not incorporated into PSI show the normal contribution by plastoquinone. This is a quick diagnostic determination of plastoquinone content. It also suggests that 2-OH-3-prenyl-1,4-naphthoquinone may be occupying some of the A₁ sites, given that the 3 ms phase is present by at about half the normal contribution. EPR is less quantitative than P700 measurement, yet still show reduced naphthoquinone signals.

The pulse chase experiment proved to be very valuable in the strategy for the feeding experiments. Phylloquinone was inserted into PSI very rapidly demonstrating that phylloquinone was capable of diffusing into an already constructed PSI complex and displacing plastoquinone. Initially, we did not expect a loss of phylloquinone over time. However the loss was justified by catabolism of phylloquinone and mass action by plastoquinone to kinetically reoccupy the site.
As reported earlier, plastoquinone occupies the A₁ site of PSI in the phylloquinone-less mutants for menB' and menA' of Synechocystis sp. PCC 6803 (10.11). We show that the addition of particular naphthoquinones the cells complete the synthesis by attaching a phytol tail producing phylloquinone and derivatives. These phytolated compounds compete with plastoquinone during PSI assembly and also are capable of replacing the resident plastoquinone in the existing PSI complexes. We used HPLC and LC-MS to detect the presence of phylloquinone analogs in PSI complexes. Inside the PSI complex, the phytolated naphthoquinone derivatives could function as an intermediate in the electron transfer from A₁ to F₅₅. Optical and EPR spectroscopy was used to study activity of the phylloquinone analogs in the PSI complexes. When the foreign quinones replaced plastoquinone partially, proportion of kinetic phases in the P700 reduction kinetics was used to indicate relative contributions of the resident plastoquinone and foreign quinone.

References
32. (www.genome.ad.jp website)
Table 1

Growth of wild-type and mutant strains of *Synechocystis* sp. PCC 6803 with supplemented naphthoquinones

<table>
<thead>
<tr>
<th>Supplement</th>
<th>Doubling Time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild type</td>
</tr>
<tr>
<td>None</td>
<td>26 ± 3.0</td>
</tr>
<tr>
<td>Glucose</td>
<td>14 ± 2.5</td>
</tr>
<tr>
<td>DCMU + Glu</td>
<td>17 ± 2.0</td>
</tr>
<tr>
<td>2-Me-1.4-NQ (Vit K₁)</td>
<td>36 ± 4.0</td>
</tr>
<tr>
<td>menaquinone (Vit K₂)</td>
<td>49 ± 10</td>
</tr>
<tr>
<td>1.4-NQ</td>
<td>34 ± 4.2</td>
</tr>
<tr>
<td>5-OH-NQ</td>
<td>died</td>
</tr>
<tr>
<td>2-Me-5-OH-1.4-NQ</td>
<td>died</td>
</tr>
<tr>
<td>2-OH-NQ</td>
<td>26 ± 3.8</td>
</tr>
<tr>
<td>2-OH-3-prenyl-1.4-NQ</td>
<td>46 ± 4.9</td>
</tr>
<tr>
<td>2,3-Cl-NQ</td>
<td>48*</td>
</tr>
<tr>
<td>2-CO₂-1.4-NQ</td>
<td>38 ± 3.7</td>
</tr>
<tr>
<td>2-CO₂-3,5-NQ</td>
<td>36 ± 5.1</td>
</tr>
<tr>
<td>2-CO₂-3,7-NQ</td>
<td>38 ± 5.0</td>
</tr>
</tbody>
</table>

Light intensity was 50 μE, normal light. Error is in the fit linear slope and was between 5-15%, with an n = 3 or more. Concentrations of naphthoquinones, DCMU, and glucose were 5 μM, 10 μM, and 5 mM, respectively. Initial cell density was 0.10 A at 730 nm. The “none” column is basal growth rate in BG11 medium. * Indicates that the cells doubled once and then died. N.D. = Not done. Numbers without a statistical error were derived from less than three observations.
Table 2

Quinone analysis of the PSI pigment extracts by LC-MS of the wild-type and menB26' mutant with naphthoquinone supplements

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mass Ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DeMePhQ/PQ</td>
</tr>
<tr>
<td>wild-type</td>
<td>nd</td>
</tr>
<tr>
<td>menB26'</td>
<td>nd</td>
</tr>
<tr>
<td>menB26' + PhQ</td>
<td>nd</td>
</tr>
<tr>
<td>menB26' + 2-CO2-1.4-NQ</td>
<td>nd</td>
</tr>
<tr>
<td>menB26' + K3</td>
<td>29 ± 1.3</td>
</tr>
<tr>
<td>menB26' - 1.4-NQ</td>
<td>4 ± 2.7</td>
</tr>
<tr>
<td>menB26' - K2</td>
<td>&gt; 100 (K2 only)</td>
</tr>
</tbody>
</table>

Table: Chromatograms were run on 0.026 mg chlorophyll content of the PSI trimers. Sample injections (35 μL) were made on a 4.6 mm x 25 cm Ultrasphere C18 column (4.6 mm x 250 mm) with 5 μm packing (Beckman) 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 1.0 mL/min. Mass 436, 450, and 748 represents PhQ minus a methyl group (or isomer). PhQ and PQ n = 9, respectively. Mass ratios are based on the intensities of the selected peaks. The peak areas used for the ratios are the m/z ± 1.0 mass units. For a given standard deviation there are 4 to 6 independent results (n = 4 to 6). nd = not detected.
Table 3

Quinone analysis of the PSI pigment extracts by HPLC-UV/vis of the wild-type and menB26' mutant with naphthoquinone supplements

<table>
<thead>
<tr>
<th>Sample</th>
<th>Chl/Qx</th>
<th>PhQ/PQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>42 ± 3</td>
<td>71 ± 40</td>
</tr>
<tr>
<td>menB26'</td>
<td>55 ± 2</td>
<td>---</td>
</tr>
<tr>
<td>menB26' + PhQ</td>
<td>44 ± 4</td>
<td>59 ± 30</td>
</tr>
<tr>
<td>menB26' + 2-CO2-1.4-NQ</td>
<td>45 ± 3</td>
<td>5.9 ± 6.7</td>
</tr>
<tr>
<td>menB26' + K3</td>
<td>50 ± 5</td>
<td>5.4 ± 3.5</td>
</tr>
<tr>
<td>menB26' + 1.4-NQ</td>
<td>250</td>
<td>(4.1)</td>
</tr>
<tr>
<td>menB26' + K2</td>
<td>60 ± 4</td>
<td>8 ± 1.8</td>
</tr>
</tbody>
</table>

Table: Chromatograms were run on 0.026 mg chlorophyll content of the PSI trimers. Sample injections (35 μL) were made on a 4.6 mm x 25 cm Ultrasphere C18 column (4.6 mm x 250 mm) with 5 μm packing (Beckman) 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 1.0 mL/min. PhQ-CH3, PhQ, and PQ n = 9, represents PhQ minus a methyl group. PhQ, and plastoquinone n = 9, respectively. Differences in retention times relative to LC-MS are attributed to slightly different lengths of the connecting tubes. Retention times for the components are PhQ-OH = 11.0 min, demethylPhQ = 17.7 min, PhQ = 19.9 min, PQ = 29.1 min. For a given standard deviation there are 4 to 6 independent results (n = 4 to 6).
Table 4

Flash induced P$_{700}^-$ Back Reaction at measured at 810 nm on PSI trimers

<table>
<thead>
<tr>
<th>Sample</th>
<th>ms back reaction</th>
<th>% of contribution</th>
<th>A$_1$ content</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>62</td>
<td>40</td>
<td>PhQ</td>
</tr>
<tr>
<td>wild type anaerobic</td>
<td>7.2</td>
<td>4</td>
<td>PhQ</td>
</tr>
<tr>
<td>menB26'</td>
<td>2.8 ± 1.5</td>
<td>65 ± 18</td>
<td>PQ</td>
</tr>
<tr>
<td>menB26' + PhQ</td>
<td>10 ± 4.5</td>
<td>20 ± 5</td>
<td>PhQ</td>
</tr>
<tr>
<td>menB26' + K$_2$</td>
<td>32 ± 5.4</td>
<td>20 ± 2</td>
<td>MQ</td>
</tr>
<tr>
<td>menB26' + 2-CO$_2$-1.4-NQ</td>
<td>31 ± 14</td>
<td>9 ± 2</td>
<td>PhQ</td>
</tr>
<tr>
<td>menB26' + K$_3$</td>
<td>2.7 ± 0.9</td>
<td>23 ± 11</td>
<td>PhQ</td>
</tr>
<tr>
<td>menB26' + 1.4-NQ</td>
<td>5.3 ± 0.47</td>
<td>28 ± 3</td>
<td>Mix</td>
</tr>
<tr>
<td>menB26' + 2-CO$_2$-3.5-NQ</td>
<td>3.3</td>
<td>78</td>
<td>PQ</td>
</tr>
<tr>
<td>menB26' + 2-CO$_2$-3.7-NQ</td>
<td>3.4</td>
<td>76</td>
<td>PQ</td>
</tr>
<tr>
<td>menB26' + 2-OH-NQ</td>
<td>4.2</td>
<td>56</td>
<td>PQ</td>
</tr>
<tr>
<td>menB26' + 2-OH-3-prenyl-1.4-NQ</td>
<td>2.4</td>
<td>31</td>
<td>PQ/Mix?</td>
</tr>
</tbody>
</table>

Table. P700$^-$ reduction kinetics in PS I complexes isolated from the menB mutant. Flash induced optical transient measured at 811 nm during a single flash. Sample conditions: 4-sided fluorescence cuvette containing sample at 10 µg/ml in Chl 25 mM Tris, pH 8.3, 0.04% β-DM, 10 mM ascorbate and 4 µM DPIP. Excitation wavelength, 532 nm. excitation energy, 1.4 mJ.
Figure Legends

Fig. 1. **Naphthoquinone supplements used in this study.** NQ and CO₂ are naphthoquinone and carboxy, respectively.

Fig. 2. **LC-MS analysis of the pigments extracted from PSI complexes of the menB26' cells that had been grown with 1,4-naphthoquinone supplement.** From top to bottom the chromatograms represent the total ion count at m/z 436, m/z 450, and m/z 748 which correspond to the mass of unmethylated PhQ (t = 17.6 min), PhQ (t = 19.9 min), and plastoquinone n = 9 (t = 29.1 min), respectively.

Fig. 3. **HPLC chromatogram of the pigment extracts of the menB26' cells that had been grown with 1,4-naphthoquinone supplement.** The peaks were detected with a UV-visible photodiode detector. Labeled inset spectra at 17.7 min, 20.1 min, 29.1 min represent PhQ minus methyl group (PhQ-Me), phyloquinone (PhQ), and plastoquinone (PQ), respectively. The chlorophyll and β-carotene peaks are at 19 min and 28.9 min, respectively.

Fig. 4. **P700⁺ Reduction Kinetics in PSI Complexes Isolated from the menB26' mutant.** Laser-flash induced optical transient measured at 811 nm. Time is plotted on a logarithmic scale, in which a deviation from the horizontal represents a kinetic phase. The experimental data is shown as dots and the computer-generated exponential fits are shown as solid lines, with the lifetimes of each phase shown. The relative contributions of each kinetic phase can be judged by the intersection of the fit line with the abscissa. The error between the experimental data and the fits are shown above each plot. The samples A-F are wild-type, menB26', menB26' + 2-Me-1,4-naphthoquinone, menB26' + 2-CO₂-1,4-naphthoquinone, menB26' + 2-CO₂-3,5-naphthoquinone, and menB26' + menaquinone (Vit. K₂), respectively.

Fig. 5. **Photoaccumulated Q-band CW EPR spectra of A₁⁺ and Q in PSI complexes isolated from wild type, menB' mutant, and menB' mutant with naphthoquinone supplements.** The photoaccumulation was carried out for 40 min at 205 K. Instrument settings: microwave power 1 mW, microwave frequency 34.056 GHz, modulation frequency 100 KHz, modulation amplitude 1 G, temperature 205 K, time constant 10 mS, conversion time 10 mS, 100 averaged scans.
Fig. 6. **Pulse Experiment to monitor in vivo incorporation of externally supplied phylloquinone into PSI.** Percentage of PhQ to total quinone (PhQ + PQ) extracted from PSI trimer pigments. At time equal zero 10 μM final concentration PhQ was added to the liquid media. Aliquots of cells were removed at intervals, pelleted and frozen. Frozen cells were later broken and PSI trimers extracted. Chlorophyll concentration and A730 were also recorded to determine if the cells were undergoing significant changes. Each value represents an average of two determinations. Data from one of two experiments is shown in the figure.
1.4-naphthoquinone

2-methyl-1.4-naphthoquinone (vitamin K₃)

2-hydroxy-1.4-naphthoquinone (Lacaphol)

2-carboxy-1.4-naphthoquinone

2-carboxy-3,5-naphthoquinone

2-carboxy-3,7-naphthoquinone

2,3-dichloro-1.4-naphthoquinone

5-hydroxy-1.4-naphthoquinone (2-methyl = plumbagin)

Fig. 1
Fig. 2
Fig. 3
Fig. 4
Fig. 5
Fig. 6
CHAPTER 6. RECRUITMENT OF A FOREIGN QUINONE INTO THE A₁ SITE OF PHOTOSYSTEM I

IV. IN VIVO REPLACEMENT OF THE NATIVE QUINONE IN THE PHYLLOQUINONE-LESS MUTANTS OF SYNECHOCYSTIS SP. PCC 6803 BY 1,2-NAPHTHOQUINONE

A paper to be submitted to the Journal of Biological Chemistry


Summary

The phylloquinone production has been short circuited by the interruption of the menA and menB genes, which are part of the phylloquinone biosynthetic pathway in Synechocystis sp. PCC 6803 (Johnson, T. W. et al. (2000) J. Biol. Chem. 275: 8523-8530; Zybailov, B. et al. (2000) J. Biol. Chem. 275: 8531-8538; Semenov et al. (2000) J. Biol. Chem. 275. 23429-23438). It has been determined that for the phylloquinone-less mutants that, plastoquinone-9 (PQ) occupies the A₁ site and functions as an electron transfer cofactor from A₀ to the FeS clusters in Photosystem I (PSI). This is the first example of a phytlylated naphthoquinone derivative, with the carbonyls in the 1,2 position instead of the 1,4 position, being incorporated into the A₁ site in vivo. The quinone also is utilized as an electron transfer intermediate from A₀ to Fₓ. This was achieved by supplementing the growth medium of menB⁻ mutant cells with 1,2-naphthoquinone. This result indicates that the specificity of the

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8 Faithful abbreviations: PQ, plastoquinone, plastoquinone-9; PSI, photosystem I; PSII, photosystem II; EPR, electron paramagnetic resonance; HPLC, high performance liquid chromatography; GC, gas chromatography; MS, mass spectroscopy; DPIP, 2,6-dichlorophenol-indophenol; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine;
phytyl transferase enzyme is relatively nonspecific in terms of the groups present at the 3- and 4-positions of the 1,2-naphthoquinone ring. There is a population of phytyl-1,2-naphthoquinone and methyl-phytyl-1,2-naphthoquinone. Acknowledging that phytyled naphthoquinone derivatives are present upon supplementing the growth medium of menB mutant cells with 1,2-naphthoquinone, the phytol tail is considered a necessary part of the molecule for displacement of plastoquinone-9 from the A1 site.

**Introduction**

Substituted benzo- and naphtho-quinones are utilized as electron and proton transfer cofactors in electron transport chains (1-3). These quinones have two distinct characteristics: a relatively polar aromatic ring which and a non-polar isoprenoid ‘tail’ of various chain lengths. Benzoquinones such as plastoquinone-9 (PQ) is comprised of a single substituted aromatic ring with a nine unit isoprenoid ‘tail’ and Ubiquinone (UQ) another substituted single aromatic ring with a long isoprenoid tail both function in membrane-bound protein complexes. Their purpose is to act as a electron/proton carrier during photosynthetic activity and/or as respiratory electron transport cofactor. PQ has multiple functions in photosystem II (PSII). It acts as a bound one-electron acceptor in the QA site and as an exchangeable 2-electron/2-proton acceptor in the QB site. The reduced PQH2 then dissociates from the QB site, diffusing into the membrane, which is then replaced by an oxidized PQ. Photosynthetic reaction centers (RCs) of purple bacteria use UQ or menaquinone in a similar manner. In photosystem I (PSI), phylloquinone (PhQ), a methyl-substituted NQ with a largely saturated phytol tail. It functions as bound one-electron redox intermediate in the A1 site. Each quinone displays redox properties that are different for each site of interaction in PSI and PSII (4) which is conferred largely by the protein environment.

We hope to take advantage of protein induced altered redox properties upon replacement of the PhQ with alternative exogenously supplemented quinones. This will allow us understand the structural determinants that allow PhQ to function with high efficiency in the A1 site of PSI. The goal was the biological replacement of the native PhQ with a variety of benzylquinone and naphthoquinone derivatives. Itoh and coworkers, and Biggins and coworkers used organic solvents to extract phylloquinone from lyophilized PSI complexes, and reconstituted the empty A1 site with various benzoquinone, naphthoquinone,
and anthraquinone head groups (5-9). The extraction procedure removes up to 90% of the chlorophyll and most of the carotenoid molecules along with the two molecules of PhQ, leading to the significant possibility that structural perturbations might have occurred to the PSI complexes. To circumvent this problem, we developed a genetic technique to accomplish the \textit{in vivo} removal of the native PhQ. In a series of three papers (10-12), we reported the generation and characterization of PQ-less mutants of the cyanobacterium \textit{Synechocystis} sp. PCC 6803. We accomplished this by interruption mutagenesis of two genes, \textit{menA} which codes for a phytol transferase, and \textit{menB} which codes for 1,4-dihydroxy-2-naphthoate (DHNA) synthase. Our strategy was to preclude PhQ production with the intent of creating an empty A\textsubscript{1} site. However, we found that the mutant PSI complex is occupied by a PQ, supposedly recruited from the thylakoid membrane. By EPR and optical spectroscopic analyses PQ was show to occupy the A\textsubscript{1} site at the same distance from P700\textsuperscript{+} and with the same orientation as phylloquinone in the wild type. It also functions as an intermediate electron transfer carrier between A\textsubscript{0} and F\textsubscript{X}.

We have shown that it is possible to introduce various quinones in the A\textsubscript{1} site \textit{in vivo} by utilizing the unmodified phytol transferase and methyl transferase enzymes in the remaining biosynthetic pathway of the \textit{menB\textsuperscript{v}} mutant cells, chapter 5. However, this is the first example of a quinone with the carbonyls in the 1,2 positions being phytylated and incorporated into the A\textsubscript{1} site of the \textit{menB\textsuperscript{v}} mutant.

\textbf{Material and Methods}

\textit{Cyanobacterial Strains and Growth.}

A glucose-tolerant strain of \textit{Synechocystis} sp. PCC 6803 was used as the wild type strain. The \textit{menA} mutant strain lacks functional phytol transferase and has been previously described (10). The \textit{menB\textsuperscript{v}} strain used here (\textit{menB26\textsuperscript{v}}) was derived as a second site suppressor of the \textit{menB18\textsuperscript{v}} strain reported earlier (10). Description of the \textit{menB26\textsuperscript{v}} mutant is in chapter 5 of this dissertation. The wild type, \textit{menA} and \textit{menB26\textsuperscript{v}} mutant cells were grown in the BG-11 medium under condition described in Ref. (13) and in Chapter 5.

\textit{Growth Rates of the Wild type and Mutant Cells with 1,2-naphthoquinone Supplement.}

Monitoring growth rates and determining chlorophyll concentrations are described elsewhere in Chapter 5 and in Ref. (14), respectively.
Isolation of Thylakoid Membranes and PSI Particles

Thylakoid membranes were prepared from cells and stored as described by (15), with the subsequent preparation of the PSI complexes described in Chapter 5.

Measurement of Photosystem I Activity

Steady-state rates of electron transfer for isolated PSI complexes were measured using cytochrome c₅ as electron donor and flavodoxin as electron acceptor as described in (16). The measurement of P700 photooxidation in whole cells was based on the techniques developed by Harbison and adapted to cyanobacteria as described in (17).

Analysis of Phyloquinone using HPLC and Mass Spectrometry

PSI samples were prepared and quinones were analyzed on an equal chlorophyll basis, as previously described (10). However, the solvent gradient was modified to a 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 1.0 mL/min. A solution of PhQ (40 mM) was prepared in absolute ethanol and kept at -20°C as a standard for calibration.

Q-band EPR Spectroscopy of Photoaccumulated PSI Trimers

Photoaccumulation experiments were carried out as described in (10).

Optical Kinetic Spectroscopy in the nIR Region

Optical absorbance changes in the near-IR were measured using a laboratory-built spectrophotometer (12), following the same sample preparation procedures.

Results

Growth of Cells with Naphthoquinone Supplements

Growth rates of the wild type, menA', and menB26' mutants were measured in cells grown in BG-11 medium containing bicarbonate (Table 1). Photoautotrophic doubling times for the wild type (26 h) were nearly three times faster than the mutants (menA' 86 h; and menB26' 77 h). Photomixotrophic growth uses both respiration (5 mM glucose) and photosynthesis for energy metabolism. The wild type and menB26' mutant had similar doubling times of 14 h and 15 h, respectively, but the menA' strain was sluggish with a doubling time of 26 h. Photoheterotrophic growth included addition of glucose for respiration and of 10 μM DCMU to block PSII activity. This effectively prevented
photosynthesis. The wild type, \textit{menA} and \textit{menB26} mutant strains had similar doubling times indicating that the respiratory energy metabolism function normally.

When the cells attained a culture density of \( \sim 0.15 \text{ O.D.} \), 1,2-naphthoquinone was added to the growth medium of the wild type, \textit{menA} and \textit{menB26} mutant cells to a final concentration of 5 to 10 \( \mu \text{M} \). Wild type doubling times were not affected significantly by addition of 1,2-naphthoquinone to the growth medium.

The \textit{menA} mutant was stressed by the addition of the naphthoquinone derivative and subsequently died (Table 1). The \textit{menA} mutant can not attach the phytol tail to the naphthoquinone head group, these results would indicate that the 1,2-naphthoquinone does not displace PQ.

Adding 1,2-naphthoquinone to the \textit{menB26} mutant had only a marginal effect on growth rates. Compared with the decreasing doubling times of other supplemented naphthoquinones that are incorporated into PSI, the 1,2-naphthoquinone was unlikely to be phytolated and incorporated into PSI (Chapter 5). Vitamin K\(_{1}\) and 2-CO\(_2\)-1,4-naphthoquinone supplements have doubling times less than 50\% of that of basal growth rate. The 1,2-naphthoquinone supplement decreases the doubling time by only 21\%. This is more on the order of other naphthoquinones that are not phytolated (Chapter 5).

\textit{LC-MS Analysis of Pigment Extracts from PSI Complexes}

The presence of quinones in the extracts of purified PSI trimers was examined using HPLC coupled to a chemical ionization time-of-flight mass spectrometer. Chlorophyll \( a \) (m/z 892) was identified with a retention time of 19.0 minutes. Phylloquinone (m/z 450) and plastoquinone-9 (m/z 748) had characteristics retention times of 19.9 and 29.1 min, respectively (Fig. 2). Known standards were used to confirm each of these peaks. Nearly identical chromatograms were obtained for organic solvent extracts of the PSI trimer preparations of the wild type, \textit{menA} and \textit{menB26} mutants, except that the PhQ peak at 19.9 min was missing in the mutants (Table 2). Quantification by MS is difficult, given that each molecule has slightly varying ionization characteristics. We determined the number of counts at a particular molecular weight ion, thereby crudely calculating the area under the peak. The amount of plastoquinone-9 in the wild type trimers appears to be very small ( <
1% and has not been detected to be involved in electron transfer discussed further in Chapter 5.

When *menB26* cells were grown with 1,2-naphthoquinone, the LC-MS analysis of pigments extracted from isolated PSI complexes showed a peak at the same retention time and with the identical mass (m/z 450) as phylloquinone (Figure 2). It is likely that this peak represents the analog of phylloquinone, the methyl derivative of a methyl-phytyl-1,2-naphthoquinone (1,2-phyloquinone). Accordingly, the positions of the carboxyls apparently do not appear to affect the retention times of the 1,2- and 1,4- substituted naphthoquinones. Spiking the 1,2-phyloquinone derivative with authentic phylloquinone showed one peak at 19.9 minutes with some line broadening; however, the peak was not shifted nor did it create a doublet, data not shown. These samples also contained a species with a mass of 436 m/z and retention time of 17.6 min. This species likely represents unmethylated phytyl-1,2-naphthoquinone. The ratio of phytyl-1,2-naphthoquinone to the 1,2-phyloquinone derivative is 2 in the *menB26* mutant with supplemented 1,2-naphthoquinone. This suggests that the rate of methylation is slower than the rate of phytolation, and that the pool of unmethylated derivative is available for assimilation into the A₄ site. The presence of demethyl 1,2-phyloquinone in the PSI complex also shows that the methyl group is not required for the assembly into the A₄ site. The total 1,2-phyloquinone derivative (methylated and unmethylated species) to plastoquinone ratio is 3:1 for 1,2-naphthoquinone. The location of the phytol tail and methyl group on 1,2-naphthoquinone are not known, but were likely to be on the 3 and 4 positions. However, the location of substitutions are not known. Investigation by a collaborator, Dr. Gobleck, is underway.

**HPLC Analysis of Pigment Extracts**

To complement the LC-MS analysis of the pigment extracts from PSI complexes, we examined the absorption spectra of the HPLC peaks with a UV-visible range diode array detector. Quantitative data can be estimated from the UV-visible, since the extinction coefficients of the molecules are known. The retention times in the HPLC analysis were consistent to those in the LC-MS studies, with slight variations that are attributed to different lengths of the connecting tubes between instruments.
Coelution with other pigments, namely chlorophyll $a$ at 19 minutes and $\beta$-carotene at 28.5 minutes, allowed comparison of the wild type chromatograms to the phyloquinone-less mutants. Elution and absorption properties of phyloquinone and plastoquinone have been described previously in Chapter 5 and Ref. (10). Plastoquinone is only observed in the PSI trimers of phyloquinone-less mutants.

Identification of the phytated 1,2-naphthoquinone compounds was complicated by coelution with chlorophyll and other molecules. 1,2-phytyloquinone has been identified at 20 min. However, phytyl-1,2-naphthoquinone identified at 17 minutes by LC-MS but not confirmed by absorption spectroscopy. The phytolated-1,2-naphthoquinone was not identified as it was masked under the chlorophyll peak and that absorption detection is three orders of magnitude less sensitive (18).

The 1,2-phyloquinone to plastoquinone ratio is 1.5:1 with the chlorophyll to total quinone ratio at 63 based on absorption spectroscopy. Supposedly, the remainder of the $A_1$ sites are either empty or occupied with the phytyl-1,2-naphthoquinone.

$P^{700^+}$ Optical Recombination Kinetics

To determine if the quinone is within the $A_1$ site, we probed its activity by observing the kinetic backreaction charge recombination of $P700^+$. In PSI trimers isolated from the wild type, after a saturating flash the reduction of the $P700^+$ RC is multiphasic (12,19). When measured by 810 nm near-IR optical spectroscopy and in the absence of external electron acceptors, the typical wild type spectrum shows a majority of the $P700^+$ being reduced with a lifetime of $60 - 80$ ms (12,19). There is also a long-lived (>2000 ms) kinetic phase of $P700^+$ reduction from direct reduction of the $P700^+$ by reduced DCPIP that contributes to 20% of the total absorbance change. In PSI trimers isolated from the $menB26$ mutant, the reduction of $P700^+$ is multiphasic (Figure 3). When $menB26$ is measured in the absence of external electron acceptor, $P700^+$ is reduced with lifetimes of approximately $3.0$ ms and $19$ ms in a $7:1$ ratio. The $3$ ms phase has been attributed to plastoquinone in the $A_1$ site with the electrons originating from the iron sulfur clusters (12). The mutants also exhibit a minor long-lived kinetic phase attributed to electron donation of DCPIP to the oxidized $P700^+$ that contribute the remainder of the absorbance change. The
kinetic back reaction phases for both the \textit{menB26'} mutant are consistent with plastoquinone being in the \( A_1 \) site of PSI and acting as an electron intermediate from \( A_0 \) to \( F_X \) (12).

Monitoring the kinetic back reaction of the quinone supplemented cells indicates whether plastoquinone still remains active in the \( A_1 \) site or is replaced by a naphthoquinone. Table 4. The 1,4-naphthoquinone head group in the \( A_1 \) site causes a loss or decrease in contribution from the 3 ms phase as seen in chapter 5. The 1,2-naphthoquinone supplement was fit to four exponentials instead of the usual three. In fitting the curve, three exponentials was not adequate in defining all the phases. With four exponentials there is a clearly defined minor 3 ms phase (8\%) attributed to plastoquinone. The 34 ms (27\%) and 10 ms (25\%) phases may be the active phytylated 1,2-naphthoquinone within the \( A_1 \) site. Two phases may be the result of PSI being in different confirmations (12). An alternative is that both pathways through PSI (and \( A_1 \)) are accepting electrons. The latter option has not been shown in cyanobacteria. The long lived decay of 2060 ms (40\%) is attributed the direct reduction of \( P700^* \) by DPCIP (12). Given that the plastoquinone phase is present in reduced amounts indicates that there is another quinone also in the active site. The longer phases (2060 ms) are in higher contributions than normal, indicating more damaged reaction centers or that 1,2-naphthoquinone is not allowing reduction of \( P700^* \).

This result confirms that not only are the naphthoquinone is contained in PSI, but also active within the \( A_1 \) site as well. This data coupled with the pigment extract analysis suggests that the phytylated quinone has displaced plastoquinone.

\textit{CW EPR Spectroscopy at Q-Band of Photoaccumulated \( A_1 \)}

To further probe the contents of the \( A_1 \) site, we photoaccumulated the \( A_1 \) signal for Q-band EPR. Plastoquinone has been previously identified in the \( A_1 \) site of the \textit{menB} mutants (12). Figure 4 shows the Q-band EPR spectra of photoaccumulated \( A_1^* \) in PSI complexes isolated from wild type, \textit{menB26'}, and \textit{menB26'} with 1,2-naphthoquinone supplemented. The \textit{menB26'} mutant strain shows the previously identified plastoquinone photoaccumulated in the \( A_1 \) site with wild type shown for comparison. The \textit{menB26'} + 1,2-naphthoquinone spectrum contains a naphthoquinone group that is being photoaccumulated. Some hyperfine splitting is evident indicating the 1,2-naphthoquinone ring is also at least
partially methylated. This spectrum complements the P700* back reaction rates indicating that there is an active naphthoquinone within the A₁ site.

Discussion

We generated cyanobacterial mutant strains that are unable to synthesize phylloquinone, to understand function of phylloquinone in photosynthetic organisms (10). The PSI complexes of these mutants contain plastoquinone in their A₁ site (11). The redox potential of phylloquinone is significantly different in the PSI environment as compared to being free in solution ((12), and references therein). To further probe the structure-function relations of quinones in the A₁ site of PSI, we added 1,2-naphthoquinone as a supplement during growth of phylloquinone-less mutants. Once inside the cell, the supplemented quinone could undergo the remaining natural steps in phylloquinone synthesis: attachment of the phytol tail by phytyl transferase (the menA product) and possible methylation by methylase (the product of menG). The newly synthesized phylloquinone or derivative competed with and replaced the plastoquinone in the A₁ site of the PSI complexes in the mutants.

Much effort has been spent in extracting the phylloquinone from the A₁ site to insert alternative quinones in vitro (20,21) (22,23). These benzo-, naphtha-, and anthraquinones have been shown to bind in A₁ and to accept electrons from A₀ (20,24). But only 1,4-naphthoquinones with a hydrocarbon tail can transfer the electron forward to the [4Fe-4S] cluster F₅ (25). Two hypotheses have been proposed: the electron transfer is dependent on an appropriate redox potential or that a specific orientation of the quinone is necessary, which is provided by the isoprene tail.

Initially, we studied the growth characteristics of the menB26' mutant strain and wild type with naphthoquinone supplement (Table 1). In chapter 5, several of the phytylated naphthoquinone supplements resulted in decreased the doubling times of the cells. It was further shown that those phytylated-naphthoquinones were utilized in PSI. The remaining unutilized naphthoquinones did not significantly change the growth rates. Wild type cell growth rate slowed upon addition of supplemented quinones, except for 1,2-naphthoquinone. It appears at first glance that 1,2-naphthoquinone is not being utilized by
the cells given that the growth rate does not significantly increase for \textit{menB26}'. But the compound was apparently toxic to the \textit{menA'} strain.

The pigment extracts separated by HPLC and analyzed by both mass spectrometry (Table 2) and ultraviolet absorbance (Table 3) detect molecules identified as phytlyl-1,2-naphthoquione and methyl-phytyl-1,2-naphthoquinone. The locations of the phytlyl and methyl groups are not known but likely arranged on the 3 and 4 carbon positions. By mass spectroscopy the area under the curve shows that there is about twice as much unmethylated derivative as the 1,2-phyloquinone. It shows that both molecules are capable of inserting into the A\textsubscript{i} site. However, we can not speculate as to the cause of the ratio. It is possible that the order of the enzymatic reactions becomes important given that open sites for substitution are now different than with the 1,4-naphthoquinone carboxyls. The distribution of substitutions are not resolvable by this type of spectroscopy. The amounts of the phytlylated compounds are too small to perform NMR or other structural determining techniques. The ratios of phytlylated naphthoquinone to plastoquinone is the low, indicating that either 1,2-naphthoquinone is not efficiently phytlylated by phytlyl transferase or that the head group does not fit well into the A\textsubscript{i} site decreasing the binding affinity.

The activity PSI, as monitored by the flash induced P700 back reaction measures the content of the A\textsubscript{i} site. Table 4. The 1,2-naphthoquinone supplemented cells still have a distinct 3 ms phase attributed to plastoquinone (Figure 3). However, the contribution of the phase has decreased from approximately 65\% in \textit{menB26'} to 8\%. The contribution of the longer phases of 10 ms and 2060 ms are greater also in this sample than in the controls. The 10 ms phase may be attributed to damage reaction centers (12). The 2060 ms phase is the direct reduction of P700\textsuperscript{+} by the external donor. The high amount of contribution from this phase may be from the electron being very slow unable to overcome the thermodynamic barrier or that the electron is essentially trapped in A\textsubscript{i}\textsuperscript{-} with the external electron donor reducing P700\textsuperscript{-}. Measuring the forward electron transfer from A\textsubscript{i} to the iron sulfur clusters (\textit{F\textsubscript{x}/F\textsubscript{A}/F\textsubscript{B}}, monitored at 430/480 nm) has yielded inconclusive results as to the electron transfer from A\textsubscript{i}\textsuperscript{-} to FX, data not shown. The identity of the 34 ms phase has not been determined, but could be the back reaction from the iron sulfur clusters through the 1,2-naphthoquinone. This is plausible given that phytloquinone has a kinetic back reaction time
of about 60 - 85 ms (observed and Ref. (12)) and the demethylphyloquinone (generated from the menG methyl transferase gene disruption) has a time of about 50 ms (26). The 34 ms phase is comparable to a properly aligned naphthoquinone head group occupying A1. We have not yet determined if it is from the methylated or unmethylated 1,2-naphthoquinone head group.

Photoaccumulated EPR reveals that menB26 with 1,2-naphthoquinone supplement does not contain plastoquinone (Figure 4). Wild type and menB26 differ in both g-tensor principle values and linewidths (11). The 1,2-naphthoquinone supplement generates a photoaccumulated spectrum closer aligned with a naphthoquinone ring than benzoquinone. Some hyperfine details are also visible, albeit at low resolution. Thus some form of phytylated 1,2-naphthoquinone is being incorporated into the A1 site.

As reported earlier, plastoquinone occupies the A1 site of PSI in the phyloquinone-less mutants for menB and menA of Synechocystis sp. PCC 6803 (10,11). We show that the addition 1,2-naphthoquinone, the cells complete the synthesis by attaching a phytol tail producing phyloquinone derivatives. These phytylated compounds out compete and replace the resident plastoquinone in the existing PSI complexes. We used HPLC and LC-MS to detect the presence of phytylated-1,2-naphthoquinone molecules in PSI complexes. Inside the PSI complex, the phytylated naphthoquinone derivatives did apparently function as an intermediate in the electron transfer from A0 to Fx. Optical and EPR spectroscopy was used to study activity of the phytylated-1,2-naphthoquinone molecules in the A1 site of the PSI complexes. When the foreign quinones replaced plastoquinone partially, proportion of kinetic phases in the P700 reduction kinetics was used to indicate relative contributions of the resident plastoquinone and foreign quinone. This is the first example of a utilized quinone with the carbonyls in the 1,2- position. This confirms that a variety of quinones that contain a phytol tail can be incorporated in vivo into the A1 site of PSI and perform electron transfer.

References


Table 1

Growth of wild type and mutant strains of *Synechocystis* sp. PCC 6803 with supplemented naphthoquinones

<table>
<thead>
<tr>
<th>Supplement</th>
<th>Doubling Time (h)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-type</td>
<td><em>menA</em>&lt;sup&gt;-&lt;/sup&gt;</td>
<td><em>menB</em>&lt;sup&gt;26-&lt;/sup&gt;</td>
</tr>
<tr>
<td>None</td>
<td>26 ± 3.0</td>
<td>86 ± 5.0</td>
<td>77 ± 4.0</td>
</tr>
<tr>
<td>Glucose</td>
<td>14 ± 2.5</td>
<td>26 ± 8.7</td>
<td>15 ± 3.4</td>
</tr>
<tr>
<td>DCMU + Glu</td>
<td>17 ± 2.0</td>
<td>19 ± 4.3</td>
<td>19 ± 2.3</td>
</tr>
<tr>
<td>1,2-NQ</td>
<td>25 ± 2.2</td>
<td>died</td>
<td>61 ± 5.5</td>
</tr>
</tbody>
</table>

Light intensity was 50 μE, normal light. Error is in the fit linear slope and was between 5-15%, with an n = 3 or more. Concentrations of naphthoquinones, DCMU, and glucose were 5 μM, 10 μM, and 5 mM, respectively. Initial cell density was 0.10 O.D. at 730 nm. The “none” column is basal growth rate in BG11 medium. * Indicates that the cells doubled once and then died. N.D. = Not done. Numbers without a statistical error were derived from less than three observations.
Table 2

Quinone analysis of the PSI pigment extracts by LC-MS of the wild type and menB26\textsuperscript{'} mutant with naphthoquinone supplements

<table>
<thead>
<tr>
<th>Sample</th>
<th>DeMePhQ/PhQ</th>
<th>PhQ/PQ</th>
<th>NQ\textsubscript{T}/PQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>nd</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>menB26\textsuperscript{'}</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>menB26\textsuperscript{'} + PhQ</td>
<td>nd</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>menB26\textsuperscript{'} + 1.2-NQ</td>
<td>2</td>
<td>1</td>
<td>3</td>
</tr>
</tbody>
</table>

Table: Chromatograms were run on 0.026 mg chlorophyll content of the PSI trimers. Sample injections (35 \mu L) were made on a 4.6 mm x 25 cm Ultrasphere C\textsubscript{18} column (4.6 mm x 250 mm) with 5 \mu m packing (Beckman) 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 1.0 mL/min. Mass 436, 450, and 748 represents PhQ minus a methyl group (or isomer). PhQ, and PQ n = 9, respectively. Mass ratios are based on the intensities of the selected peaks. The peak areas used for the ratios are the m/z ± 1.0 mass units. For a given standard deviation there are 4 to 6 independent results (n = 4 to 6). nd = not detected.
Table 3

Quinone analysis of the PSI pigment extracts by HPLC-UV/vis of the wild type and \( \text{menB}^\prime \) mutant with naphthoquinone supplements

<table>
<thead>
<tr>
<th>Sample</th>
<th>Chl/Q(_T)</th>
<th>NQ(_T)/PQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>42</td>
<td>71</td>
</tr>
<tr>
<td>( \text{menB26}' )</td>
<td>55</td>
<td>---</td>
</tr>
<tr>
<td>( \text{menB26}' + \text{PhQ} )</td>
<td>44</td>
<td>59</td>
</tr>
<tr>
<td>( \text{menB26}' + 1.2\text{-NQ} )</td>
<td>63</td>
<td>2</td>
</tr>
</tbody>
</table>

Table: Chromatograms were run on 0.026 mg chlorophyll content of the PSI trimers. Sample injections (35 \( \mu \text{L} \)) were made on a 4.6 mm x 25 cm Ultrasphere C\(_{18}\) column (4.6 mm x 250 mm) with 5 \( \mu \text{m} \) packing (Beckman) 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 1.0 mL/min. PhQ-CH\(_3\), PhQ, and PQ \( n = 9 \), represents PhQ minus a methyl group. PhQ, and plastoquinone \( n = 9 \), respectively. Differences in retention times relative to LC-MS are attributed to slightly different lengths of the connecting tubes. Retention times for the components are PhQ-OH = 11.0 min, demethylPhQ = 17.7 min, PhQ = 19.9 min, PQ = 29.1 min. For a given standard deviation there are 4 to 6 independent results (\( n = 4 \) to 6).
### Table 4

<table>
<thead>
<tr>
<th>Sample</th>
<th>ms back reaction</th>
<th>% of contribution</th>
<th>A&lt;sub&gt;i&lt;/sub&gt; content</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>62</td>
<td>40</td>
<td>PhQ</td>
</tr>
<tr>
<td>menB&lt;sub&gt;26&lt;/sub&gt;</td>
<td>2.8</td>
<td>65</td>
<td>PQ</td>
</tr>
<tr>
<td>menB&lt;sub&gt;26&lt;/sub&gt; + PhQ</td>
<td>10</td>
<td>20</td>
<td>PhQ</td>
</tr>
<tr>
<td>menB&lt;sub&gt;26&lt;/sub&gt; + 1,2-NQ</td>
<td>2.9</td>
<td>9</td>
<td>Mix</td>
</tr>
</tbody>
</table>

**Table.** P<sub>700</sub><sup>+</sup> reduction kinetics in PS I complexes isolated from the *menB* mutant. Flash induced optical transient measured at 811 nm during a single flash. Sample conditions: 4-sided fluorescence cuvette containing sample at 10 µg/ml in Chl 25 mM Tris, pH 8.3. 0.04% β-DM, 10 mM ascorbate and 4 µM DPIP. Excitation wavelength, 532 nm. excitation energy, 1.4 mJ.
Figure Legends

Fig. 1. Figure of phylloquinone, plastoquinone and 1,2-naphthoquinone.

Fig. 2. LC-MS analysis of the pigments extracted from PSI complexes of the menB26\(^{-}\) cells that had been grown with 1,2-naphthoquinone supplement. From top to bottom the chromatograms represent the total ion count at m/z 436, m/z 450, and m/z 748 which correspond to the mass of unmethylated 1,2-PhQ (t = 17.6 min), 1,2-PhQ (t = 19.9 min), and plastoquinone n = 9 (t = 29.1 min), respectively.

Fig. 3. \(P700^+\) Reduction Kinetics in PSI Complexes Isolated from the menB26\(^{-}\) mutant. Laser-flash induced optical transient measured at 811 nm. Time is plotted on a logarithmic scale, in which a deviation from the horizontal represents a kinetic phase. The experimental data is shown as dots and the computer-generated exponential fits are shown as solid lines, with the lifetimes of each phase shown. The relative contributions of each kinetic phase can be judged by the intersection of the fit line with the abscissa. The error between the experimental data and the fits are shown above each plot. The samples A-C are wild type, menB26\(^{-}\), and menB26\(^{-}\) + 1,2-naphthoquinone, respectively.

Fig. 4. Photoaccumulated Q-band CW EPR spectra of \(A_{i}^+\) and Q in PSI complexes isolated from wild type, menB26\(^{-}\) mutant, and menB26\(^{-}\) mutant with naphthoquinone supplements. The photoaccumulation was carried out for 40 min at 205 K. Instrument settings: microwave power 1 mW, microwave frequency 34.056 GHz, modulation frequency 100 KHz, modulation amplitude 1 G, temperature 205 K, time constant 10 mS, conversion time 10 mS, 100 averaged scans.
1,2-naphthoquinone

Phyloquinone

Plastoquinone

Fig. 1
Fig. 3
Fig. 4
CHAPTER 7. GENERAL CONCLUSIONS

Both plants and animals depend on phylloquinone to function in distinctly different roles. In animals, phylloquinone is essential for initiating blood clotting and covalently modifying specific glutamic acid residues. There also has been much effort in understanding the physiological effects of a lack of phylloquinone in the diets of animals and molecules that inhibit phylloquinone's actions. In plants phylloquinone is only utilized as an electron transport intermediate in photosystem I. Each of these uses for phylloquinone is necessary for the optimum survival of the organism. But how critical phylloquinone is to the function of photosystem I has not been probed. Therefore we have undertaken a project to understand how phylloquinone is biosynthetically produced. We also strove to understand how photosystem I operated, if at all, when phylloquinone can not be synthesized.

Initially we genetically disallowed phylloquinone biosynthesis by disrupting four individual genes in the pathway (*menA, menB, menD,* and *menE*). The *menD* and *menE* genes are relatively early in the pathway and code for enzymes that modifies chorismate into a naphthoquinone precursor. The *menB* gene codes for an enzyme that produces the naphthoquinone head group in phylloquinone. The *menA* gene codes for an enzyme that attaches the phytyl tail onto the ring. These genes were chosen disruption for different reasons. The *menD* and *menE* genes were disrupted to understand where in the biosynthetic pathway is the production of phylloquinone dedicated by these enzymes. The *menB* gene product was disabled because it prevented phytylation of the ring in the next step. The *menA* gene product was disabled because it prevented the phytylation of the intact naphthoquinone head, which was thought to be a possible candidate for A1 site, given the lack of phylloquinone. In all cases, phylloquinone was not produced. The implications here are that four of the seven genes have no alternative pathway for the production of the specific molecules. The *menD* gene codes for an enzyme in the first dedicated step in the pathway. The *menA* disruption shows that the head group DHNA is not the most suitable replacement of phylloquinone in the mutants.

However, instead of finding an empty A1 site, plastoquinone was determined to be present *in vivo* in the phylloquinone-less mutants. Plastoquinone is a fair substitute for
phylloquinone activity in PSI. The orientation of plastoquinone is similar to phylloquinone, however there are distinctly different energetic properties. Plastoquinone is less reducing than phylloquinone and that change in redox potential has significant effects on the electron transfer rates through $A_1$. Efforts in a variety of collaborating laboratories are continuing to probe these differences between plastoquinone and phylloquinone.

We also determined that phylloquinone and derivatives can be reinserted into the $menB$ mutant \textit{in vivo} by the addition of phylloquinone or the product of the $menB$ reaction (DHNA). Addition of naphthoquinone analogs of DHNA (vitamin K$_3$, 1,4-naphthoquinone) also produces phylloquinone and, possibly, derivatives. The remaining machinery for production of phylloquinone is not affected, allowing us to supplement the cells with alternative quinones which the cells, in turn phytlylate and insert into PSI. The phytlylated quinones displace plastoquinone. We also show that a 1,2-naphthoquinone is also phytlylated and methylated and inserted into the $A_1$ site. This is the first example of a 1,2 substituted quinone in PSI \textit{in vivo}. The $menB$ mutant that has been 'fed' these quinones show distinctly different physiological characteristics and changes in the activity of PSI. In the cases where phylloquinone like molecules are in PSI, wild type characteristics emerge.

The use of the phylloquinone-less mutants is continuing. We are now able to, at will, substitute quinones into the $A_1$ site. We also know what the requirements are for displacing plastoquinone. This leads us in new directions for synthesizing tailor made quinones that meet a particular structure or redox potential. Alternative uses include probing the electron transport from $A_0$ through the desired quinone in $A_1$ to $F_X$. This will not only allow us a better understanding of the $A_1$ site, but its interactions with its electron transport partners.
APPENDIX A. DEVELOPED PROCEDURES AND PROTOCOLS SPECIFIC TO DISSERTATION EXPERIMENTS


A glucose-tolerant strain of *Synechocystis* sp. PCC 6803 was used as the wild type strain. The cells were grown in the BG-11 medium, with 5 mM glucose and 1 µg/ml antibiotic for the mutants (Rippka et al., 1979). Agar plates for the growth of the stock cells were kept at low light intensity (2 to 10 µE m\(^{-2}\) s\(^{-1}\)) on a 5 mM glucose and 2 µg/ml antibiotic. Liquid cultures of the wild type and mutant strains were grown heterotrophically under normal light conditions (40 to 60 µE m\(^{-2}\) s\(^{-1}\)) and were bubbled with sterile filtered air. Usually two sets of cells were kept growing. The first set was at normal light levels and the second set at low light levels to keep the growth rate reduced. Less glucose or no glucose can also be used to keep a liquid stock of cells growing slowly without having to reculture and potentially contaminate the cells as frequently as cells grown with glucose or at normal light levels. Cell growth was monitored by measuring the optical density at 730 nm (A\(_{730}\)) with a Shimadzu spectrophotometer. It is important to be consistent with taking the reading to prevent artificial error. Cells from liquid cultures in the late exponential phase of growth (A\(_{730}\) = 0.8-1.2) were harvested by centrifugation at 5000 x g for 15 min.

Growth on a large scale was achieved by starting a 3 L culture at 0.050 O.D.\(_{730}\) and adding 2.5 mM glucose final concentration in addition to antibiotics for the mutants. The mutants contain antibiotic cassettes that impart resistance to specific antibiotics. Addition of quinones was previously done at around 0.20 – 0.30 O.D.\(_{730}\). Presently quinones were added in the late stage log phase of the growing cells and incubated for 1-3 hours before harvesting.

A2. Growth rates of the wild-type and mutant cells.

Fresh cyanobacterial cultures were grown twice with glucose and once or twice, depending on the growth rates of the strain, without glucose to deplete any exogenous glucose stored in the cells. In the late exponential phase of the third liquid culture, the cells were pelleted by centrifugation, washed twice with BG11 medium, and resuspended in BG11 and the appropriate antibiotics for the mutants at approximately 10 O.D.\(_{730}\) concentration. For estimating growth rates, the cultures were grown in 6 well culture plates.
with 8 ml total liquid medium in each well. All cultures were adjusted to the same initial cell density (O.D_{730} = 0.08 - 0.1). A test well was done to determine the amount of concentrated cells and BG11 that were needed to dilute in the 8 ml liquid culture. From this 1 ml of liquid is drawn and O.D_{730} was determined. This reading is the initial O.D_{730} for the cells in the growth experiment. Final concentration of glucose and DCMU was 5 mM and 10 μM, respectively. The order of addition to the well was: 1) cells; 2) glucose; 3) DCMU; 4) quinone or other additives; 5) balance of BG11. Make sure that the cells do not come into contact with the other added ingredients before adding the BG11. Adding the BG11 liquid last will ensure thorough mixing so as not to spike the cells with overly concentrated solutions. The 6-well plates were sealed with parafilm and put on an orbital shaker at 110 rpm under a bank of fluorescent lights at normal light conditions (40 to 60 μE m^{-2} s^{-1}). Plates were deposited in only one layer on the shaker. Given the differences in shakers, find a speed that the liquid approaches the top of the well but does not go over or up to the lip. Tape the wells down if on the edge of the shaker.

Each mutant strain or wild type must be in their own 6-well plate. If you mix strains in an individual plate you will see a growth rate cross over effect. Duplicates are highly advised for each run. The experiments were completed 3-7 times in duplicate.

Cell density was measured by removing 200 μl of culture add 800μl of water (total 1 ml) and measuring the O.D_{730} and multiplying by 5. When the cells are thicker (~0.50 O.D_{730}), 100 μl of culture was drawn with the balance water.

To determine the doubling time, record the O.D_{730}, time and date of each reading starting when the cells were mixed. Convert the time into minutes and plot time verses the natural log of the O.D_{730} of each reading. Determine the slope only for the linear part of the graph. The slope of the line is in units of 1/minutes which should be on the order of 10^{-1} to 10^{-5}. Dividing 115 by the first two digits in the slope (y.zx 10^{-4}) will give the doubling time of the cells in hours. The number 115 is from

\[
t = \left( \frac{\ln 2}{(K \text{ (1/min)})(60 \text{ (min/hr))}) \right) \times 10,000
\]
The advantage of this method is that you can do two or three times as many samples at once and be assured of nearly homogeneous conditions. It is reasonable to do 12 6-well plates at once, given enough shaker space.

**A3. Chlorophyll Analysis and Oxygen Evolution Measurements.**

Chlorophyll was extracted from whole cells and thylakoids with 100% methanol. Chlorophyll concentrations were determined according to Ref. (MacKinney, 1941).

Oxygen evolution measurements were performed using a Clark-type electrode as described in (Shen et al., 1993). Cells were prepared by starting cultures at the same time and grown photomixotrophically with 5mM glucose for two subculturing. The final liquid culture was made without addition of glucose and grown until mid log growth phase (0.4 – 0.6 OD) and then analyzed. From these cells the initial O.D.\(_{730}\) was recorded and the chlorophyll concentration by methanol extraction of chlorophyll. The cells were pelleted and washed with small amounts of 40mM HEPES/NaOH buffer pH 7.0. The cells were resuspended at about 6 – 12 O.D.\(_{730}\) ml\(^{-1}\) in 40mM HEPES/NaOH buffer pH 7.0. Increasing the concentration past 12 O.D.\(_{730}\) will cause the cells to pellet out. The cells were kept in the dark during the experiments and are only good for 4 – 8 hours after the initial prep. Preparation of the cells were staggered during the experimental runs. The temperature of the electrode chamber was maintained at 25°C by a circulating water bath. Measurements were done on 5 O.D. of cells. Whole-chain electron transport (H\(_2\)O to CO\(_2\)) measurements were determined after the addition of 5 mM NaHCO\(_3\); oxygen evolution mediated by PS II only was determined after addition of 4 mM \(p\)-benzoquinone (BQ). More BQ was used if the stock solution was not fresh or has significant amounts of black oxidized quinone. Note that an excess of BQ is not going to affect the rate significantly, since there must be an excess of electron acceptor for PSII to operate maximally. The light intensity was 1840 \(\mu\)E m\(^{-2}\) s\(^{-1}\) as created by neutral density filters. Experiments were repeated in triplicate and averaged. Also one of the initial runs was repeated at the end to determine that the instrument readings have not drifted over time. The final experiment was adding sodium dithionite to distilled air saturated with bubbled water. Add the powder until the oxygen readings do not drop. The difference between the starting point and bottom is your delta (\(\Delta\)), which will be used later to determine the rate of oxygen evolution.
The units are properly expressed as \( \mu \text{mol O}_2/\text{A}_{730} \text{ h ml} \). The number 0.253 mmol/ml is the concentration of oxygen in air saturated water at 25°C. There is a conversion of 3600 s/h to convert from readings in seconds to hours. Delta (\( \Delta \)) is from above.

The data was converted to ASCII and imported into a math spreadsheet, Igor Pro 3.14 (Wavemetrics, Inc., Lake Oswego, OR). By linear regression the slope of linear part of the oxygen evolution line was fitted. It was entered into the following calculation.

\[
\left( \frac{0.253 \mu \text{mol/ml}}{\Delta} \right) \left( \frac{3600 \text{ s}}{1 \text{ hr}} \right) \text{(Slope)} =
\]

**A4. 77 K Fluorescence Emission Spectra.**

The low temperature fluorescence emission spectra were measured using a SLM 8000C spectrofluorometer as described (Shen and Bryant, 1995). Cells from the exponential phase of growth were harvested by pelleting and resuspended in 25 mM HEPES/NaOH, pH 7.0 buffer or water. Cells (5 \( \mu \text{g Chl.} \)) were diluted in 25 mM HEPES/NaOH, pH 7.0 to a final volume of 30 \( \mu \text{l} \) and dark adapted for 30 minutes on ice. To the solution, 70 \( \mu \text{l} \) of neat glycerol was added, mixed, prior to quickly freezing in liquid nitrogen. The excitation wavelength was 435 nm. On the monochromator, the excitation slit width was set at 4 nm and the emission slit width was set at 2 nm. The emission was scanned twice from 600 nm to 800 nm and averaged.

Sodium fluorescein is a recommended internal standard for maize chloroplasts. It is difficult to use with cyanobacteria, since it intercalates into the cell walls and produce two peaks, a bound molecule spectrum and a free molecule spectrum.

Sample cuvettes are long stemmed (10 in.) disposable borosilicate glass pipettes from Fisher. Flame tip closed by a Bunsen burner adjusted to a hot flame by sticking 2 – 3 mm of the glass tip into the flame until orange hot. Take care to use pipettes that are relatively straight. A curved pipette will not align properly in the beam path of the fluorometer.

To set up the fluorometer properly, the following items are needed: fluorometer low temperature kit (face plate, sample holder bracket, top plate with dewer holder and cover, quartz dewer, sample holder which fits into dewer, screws, hex wrench), two 2 L liquid
nitrogen dewers. 200 µl pippetmen, 20 µl pippetmen, tips, neat glycerol, distilled water, 100% ethanol. 12 in small gauge needle with syringe adaptor. 0.5 ml syringe, flashlight, small mirror, ring stand with clamp for quartz dewer, long thin wooden (not metal) stick, sample cuvettes, kimwipes, tube filled with dryrite and connecting hoses, and icebucket for samples. First assemble the low temperature kit onto fluorometer and attach the air hose with dryrite tube to the face plate. The air blown through the dryrite tube prevent condensation from forming on the liquid nitrogen filled quartz dewer during a scan. Add to the quartz dewer enough liquid nitrogen to fill the clear glass section. Remove bubbles coming from the lower section of the dewer by touching the wooden rod to the bubble at the point of origin. Fill the dewer the rest of the way up with liquid nitrogen. Keep all liquid nitrogen dewers covered at all times to minimize condensation build up. Snow introduced into the dewers will collect onto the tip of the samples cuvette and block the beam path. Third, prepare sample and inject about 2 in. of sample into bottom of cuvette. Shake liquid to bottom. Air bubbles will shatter the cuvette when frozen. Put the sample in the sample holder. Freeze the sample quickly in one of the two 2 L dewers of liquid nitrogen. The spectrum of the glycerol/cell mixture will change if the mixture is left unfrozen. Put the sample in the quartz dewer and align it so that the sample is in the middle of the round flask. Place the dewer in the fluorometer. It may be necessary to align the sample in the beam paths to achieve a reasonable signal. The sample cuvette is a single use piece. The cuvette is non-recoverable and usually breaks upon melting.

A5. Analysis of phylloquinone using HPLC and mass spectrometry.

On an equal chlorophyll basis, the samples were prepared and quinones were analyzed as previously described (Johnson et al., 2000), with the following changes. Membranes containing 0.025 mg chlorophyll were centrifuged at 10,000 x g for 60 min and the supernatant was removed. PSI trimers were concentrated by a Centracon and then lyophilized to dryness. Care must be taken to remove all sucrose from the sucrose gradient first. The pigments were sequentially extracted with 0.5 ml methanol, 0.5 ml 1:1 (v/v) methanol:acetone, and 0.4 ml acetone, and the three extracts were combined. The resulting solution was concentrated by vacuum at 4°C in the dark to dryness. Dry pigment samples were stored at -80 °C until used. If the samples remain dry, dark, and in a -20 °C or colder.
the samples will remain stable for several weeks. Solutions will decompose within hours. faster with light. The pigments were resuspended in a 60 μl mixture of 1:1 methanol:isopropanol at ca. 0.8 mg Chl ml⁻¹. HPLC separations were monitored with photodiode array UV-visible detection using a Hewlett Packard (Agilent Technologies, Palo Alto, CA) model 1100 quaternary pump and model G1316A photodiode array detector. Sample injections (35 μl) were made on a reverse phase 4.6 mm × 25 cm Ultrasphere C₁₈ column (4.6 mm × 250 mm) with 5 μm packing (Beckman Instruments, Palo Alto, CA. using a gradient elution at (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 1.0 ml/min. The column was washed after each run (from 100% B to 100% A at 10 min. 0.5 ml/min, 100% A for 5 min at 1.0 ml/min.). A solution of phylloquinone (40 mM) was prepared in absolute ethanol and kept at −20 °C as a standard for calibration and peak identification. 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 post-column flow splitter delivered column eluent to the electrospray ion source at 10 μL min⁻¹.

For the UV/Vis detector, the peak spectrum was analyzed and compared to known standards or analogs. Phylloquinone has a nicely resolvable doublet at 248 nm and 270 nm by the diode array. Plastoquinone has a singlet at 256 nm. Other compounds like chlorophyll and β-carotene are readily identifiable. The spectra are readily quantifiable. Morton’s book on Biochemical Spectroscopy (John Wiley and Sons, 1975) contains useful quantitative data. Extinction coefficients used are, PQ-9, 256 nm, 15.200 cm•l/mol; PhQ. 248 nm, 18.600 cm•l/mol; Chl., 615 nm, 15.800 cm•l/mol.

Mass detection is much more sensitive than UV/Vis but less quantifiable. The MS spectrum was scanned for a particular mass peak to help determine its position on the absorption spectrum. It is also used to determine if there is any amount of a particular molecule that is not concentrated enough for UV/Vis detection. The following masses were searched for, PhQ. m/z = 450; PQ-9, m/z = 748. Chlorophyll appears at different masses depending on what kind of adduct it forms (Na⁻ or Cl⁻).
A6. Optical Kinetic Spectroscopy in the Visible Region.

Optical absorbance changes in the visible were measured using a laboratory-built spectrometer consisting of a 400-watt tungsten-halogen source (Oriel), a spectrometer consisting of a 400-watt tungsten-halogen source (Oriel), a 400-mm focal length monochromator (Jarrell Ash Model 82-410) prior to the sample cuvette (to select a measuring wavelength), a sample compartment for a 1 cm x 1 cm fluorescence (4-sided clear) cuvette, a second spectrometer consisting of a 400-watt tungsten-halogen source (Oriel) after the sample cuvette (to reject the flash and fluorescence artifacts), and a PIN-10 photodiode detector (UDT). Suitable lenses were placed to focus the light on the monochromator slits and to provide a collimated beam through the sample cuvette. The photocurrent was converted to a voltage with a 30 kOhm resistor, the voltage was amplified using a Model 100 amplifier (EG&G) set to a DC bandwidth of 100 kHz, and a signal was digitized using a Model 100 digital oscilloscope (Nicolet Instruments, Austin, TX) in a Power Macintosh 7100/80 computer. The data were transferred to the computer and stored as binary files using LabView 4.1 (National Instruments, Austin, TX) and further processed in Igor Pro 3.14 (Wavemetrics, Inc., Lake Oswego, OR). Actinic flashes were supplied using a frequency doubled ND-YAG laser (Spectra Physics) operating as 532 nm at a flash energy of 1.4 mJ. Each kinetic trace represents 8 to 32 averages within the digital oscilloscope or on the computer, depending on the setup. The sample cuvette (10 mm x 10 mm) contained 1.0 ml of PSI complex at 50 μg/ml Chl suspended in 25 mM Tris, pH 8.3 with 0.04% β-DM, 10 μM sodium ascorbate and 4 μM DCPIP. The samples were prepared anaerobically from stock solution mixtures.

A7. Q-band EPR Spectroscopy of Photoaccumulated PS I Trimers.

Photoaccumulation experiments were carried out using a Bruker ER300E spectrometer outfitted with an ER 5106 QT-W1 resonator, which is equipped with a port for in-cavity illumination. Cryogenic temperatures were maintained with an ER4118CV liquid nitrogen cryostat, controlled with an ER4121 temperature control unit. The microwave frequency was measured with a Hewlett-Packard 5352B frequency counter and the magnetic field was measured with a Bruker ER035M NMR Gaussmeter. The magnetic field was calibrated using γ,γ bisdiphenylene-β-phenylallyl (BDPA) complexed 1:1 with benzene.
Prior to the photoaccumulation, the pH of the sample (60 μl final) was adjusted with a glycine buffer pH 10.0, final concentration 233 mM, and sodium dithionite was added to a final concentration of 50 mM. This is done in an anaerobic glove box. After incubation in the dark for at least 20 min, the sample was frozen in the dark and placed into the resonator and the temperature was adjusted to 205 K. The sample was illuminated with a 20 mW He-Ne laser at 630 nm for a variable amount of time, usually around 40 minutes. The purpose of a variable illumination time is to remove the A₀ peak while fully reducing the A₁ site. The dark background was subtracted from the photoaccumulated spectra. EPR spectral simulations were carried out on a Power Macintosh 7300/200 computer using a Windows 3.1 emulator (SoftWindows 3.0, Insignia Solutions, UK) and SimFonia software (Bruker Analytik GMBH).

**A8. References**


APPENDIX B. RECRUITMENT OF A FOREIGN QUINONE INTO THE A₁ SITE OF PHOTOSYSTEM I

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

A paper published in the Journal of Biological Chemistry


Summary

Electron paramagnetic resonance (EPR) and electron-nuclear double resonance studies of the photosystem(PS) I quinone acceptor, A₁, in phylloquinone biosynthetic pathway mutants are described. Room temperature continuous wave EPR measurements at X-band of whole cells of menA and menB interruption mutants show a transient reduction and oxidation of an organic radical with a g-value and anisotropy characteristic of a quinone. In PS I complexes, the continuous wave EPR spectrum of the photoaccumulated Q'r radical, measured at Q-band, and the electron spin-polarized transient EPR spectra of the radical pair P7001Q", measured at X-, Q-, and W-bands, show three prominent features: (i) Q' has a larger g-anisotropy than native phylloquinone, (ii) Q' does not display the prominent methyl hyperfine couplings attributed to the 2-methyl group of phylloquinone, and (iii) the orientation of Q' in the A₁ site as derived from the spin polarization is that of native phylloquinone in the wild type. Electron spin echo modulation experiments on P700¹Q' show that the dipolar coupling in the radical pair is the same as in native PS I, i.e. the distance between P700¹ and Q' (25.3 ± 0.3 Å) is the same as between P700⁻ and A₁ in the wild type. Pulsed electron-nuclear double resonance studies show two sets of resolved spectral features with nearly axially symmetric hyperfine couplings. They are tentatively assigned to the two methyl groups of the recruited plastoquinone-9, and their difference indicates strong inequivalence among the two groups when in the A₁ site. These results show that Q (i) functioning accepting an electron from A₀⁻.
and in passing the electron forward to the iron-sulfur clusters. (ii) occupies the A1 site with an orientation similar to that of phylloquinone in the wild type, and (iii) has spectroscopic properties consistent with its identity as plastoquinone-9.
APPENDIX C. THE RED-ABSORBING CHLOROPHYLL a ANTENNA STATES OF PHOTOSYSTEM 1: A HOLE-BURNING STUDY OF SYNECHOCYSTIS SP PCC 6803 AND ITS MUTANTS

A paper published in the Journal Physical Chemistry B

M. Ratsep, T. W. Johnson, P. R. Chitnis, G. J. Small

Summary

Low temperature (4.2 K) absorption and hole-burned spectra are presented for the trimeric (wild-type, WT) photosystem I complex of the cyanobacterium Synechocystis sp. PCC 6803, its monomeric form, and mutants deficient in the PsaF, K, L, and M protein subunits. High-pressure- and Stark-hole-burning data for the WT trimer are presented as well as its temperature dependent Qy-absorption and -fluorescence spectra. Taken as a whole, the data lead to assignment of a new and lowest energy antenna Qy-state located at 714 nm at low temperatures. It is this state that is responsible for the fluorescence in the low-temperature limit and not the previously identified antenna Qy-state near 708 nm. The data indicate that the 714 nm state is associated with strongly coupled chlorophyll a molecules (perhaps a dimer) and possesses significant charge transfer character. The red chlorophylls absorbing at 708 and 714 nm do not appear to be directly bound to any of the above protein subunits. The results are consistent with a location close to the interfacial regions between PsaL and M and the PsaA/B heterodimeric core. It is likely that the red chlorophylls are bound to PsaA and/or PsaB.
APPENDIX D. ULTRAFAST PRIMARY PROCESSES IN PHOTO SYSTEM I REACTION CENTERS WITH A FOREIGN QUINONE IN THE A₁ SITE

A paper to be submitted to Journal of Physical Chemistry B

Sergei Savikhin, T. Wade Johnson, Peter Martinsson, Parag R. Chitnis, and Walter S. Struve

Summary

Ultrafast primary processes have been studied in photosystem I core antenna - reaction center complexes from a menB mutant of the cyanobacterium Synechocystis sp. PCC 6803, in which the native phylloquinone secondary electron acceptor A₁ has been replaced by a foreign quinone (plastoquinone-9). The kinetics of antenna excitation equilibration and trapping at the P700 reaction center are essentially the same as in native PS I. The steady-state (P700⁺ - P700⁻) absorption difference spectrum in the menB mutant virtually coincides with the wild type, indicating that replacement of the phylloquinone does not strongly perturb the electronic structure of the special pair chlorophylls. However, the difference between time-resolved transient absorption profiles for menB PS I with open and closed reaction centers (excited at 660 nm and probed at 690 nm) is strikingly different from the wild type. The latter (open - closed) absorption difference profile, which isolates the kinetics of reaction center processes, indicates that the A₀⁻→Q electron transfer in the menB mutant requires 1.4 ± 0.4 ns. This is ~45 times slower than the upper limit of ~30 ps that has been inferred for the time scale of the corresponding A₀⁻→A₁ transfer in native PS I.
APPENDIX E. ULTRAFAST PRIMARY PROCESSES IN A CYANOBACTERIAL PHOTOSYSTEM I REACTION CENTER

A paper to be submitted to Journal of Physical Chemistry B

Sergei Savikhin, T. Wade Johnson, Peter Martinsson, Parag R. Chitnis, and Walter S. Struve

Summary
The kinetics of primary events in photosynthetic reaction centers have previously been well characterized only in purple photosynthetic bacteria; these have provided a basis for widely used theories of electron transfer in proteins. The primary charge separation in photosystem I of the cyanobacterium Synechocystis sp. 6803 is found to be slower by a factor of 2-3, and the subsequent electron transfer from the primary to secondary electron acceptor is faster by two orders of magnitude, than the corresponding processes in purple bacteria. These differences are intriguing, because they are not in accord with current predictions based on the known reaction center geometries.
APPENDIX F. LIST OF UNREFEREED PUBLISHED PAPERS

F.1 MUTATIONAL ANALYSIS OF PHOTOSYSTEM I
Function of Phylloquinones


F.2 MUTATIONS IN THE PHYLLOQUINONE BIOSYNTHETIC PATHWAY:
A Foreign Quinone is Recruited into the A1 Binding Site after Inactivation of the menA and menB Genes in Synechocystis sp. PCC 6803

Boris Zybaiov, Gaozhong Shen, Ilya R. Vassiliev, Donald A. Bryant, Ricardo Reategui.
Wade Johnson, Wu Xu, Parag R. Chitnis, and John H. Golbeck
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The road towards a doctorate degree has been a long one filled with forks and roundabouts. I ended up in a traveling a different path than I originally set out on, but am much better for it.

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