

119

Biological Incorporation of Alternative Quinones into Photosystem I

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119.1	Introduction.....	119-1
119.2	Phylloquinone Biosynthetic Pathway.....	119-2
119.3	Phylloquinone Biosynthetic Pathway Gene Disruption.....	119-6
119.4	Results of Phylloquinone Pathway Gene Disruption.....	119-6
119.5	High-Light-Tolerant Strains of <i>menB</i> and <i>menD</i> Mutant Strains.....	119-8
119.6	Modification of Phylloquinone through Gene Inactivation.....	119-9
119.7	<i>In Vivo</i> Recruitment of Media-Supplemented Naphthoquinones.....	119-10

119.1 Introduction

All well-characterized photosynthetic reaction centers contain quinone molecules. Type II reaction centers, such as Photosystem II (PS II), contain two quinones, one that functions as a bound one-electron acceptor, and the other that functions as a mobile two-electron (and two-proton) accumulator. In PS II, plastoquinone-9 (PQ-9) serves the role of the mobile quinone, which shuttles electrons to Photosystem I (PS I) via the cytochrome b_6f complex. Type I reaction centers, such as PS I of cyanobacteria and green plants, contain two bound phylloquinones (PhQ — vitamin K₁, 2-methyl-3-phytyl-1,4-naphthoquinone).¹⁻⁴ One or both PhQ molecules function as one-electron acceptor, shuttling electrons from the primary acceptor A_0 (a chlorophyll *a* monomer), to F_X (a [4Fe-4S] cluster).⁵ PhQ neither becomes protonated as part of the electron transfer process nor diffuses from the PS I complex as part of its normal function. The drop in Gibbs free energy from A_0 to F_X is estimated to be ca. 250 mV⁶ to 320 mV.^{7,8} Thus, PhQ plays an important role as an intermediate in the early stages of electron transfer in PS I.⁸

Phylloquinone has become a focus of a variety of structure function relationships due to its central role in electron transfer. The PhQ molecules can be extracted from PS I using dry or water-saturated diethyl ether^{9,10} or using hexane containing 0.3% methanol.^{11,12} Water-saturated ether extraction also removes a significant number of antenna chlorophylls, and all of the carotenoids (and probably lipids), while leaving the iron–sulfur clusters unaffected. As might be expected, both extraction procedures block room-temperature electron transfer from A_0 to the iron–sulfur clusters.^{4,9} Reconstitution of one PhQ fully restores electron transfer from A_0 to the iron–sulfur clusters.¹³ Iwaki and coworkers reported that PhQ could be replaced by a variety of quinones with appropriate redox potentials.¹⁴ Moreover, an assortment of non-native quinones can be inserted into the PhQ binding site *in vitro*.^{15,16} In particular,

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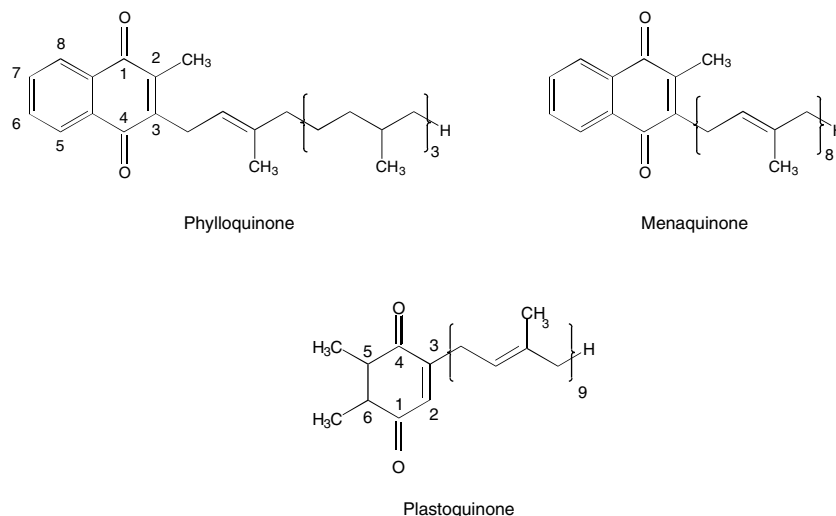


FIGURE 119.1 Structure of phylloquinone, menaquinone-8, and plastoquinone-9.

artificial quinones (benzo- naphtho-, anthraquinones) and quinoid compounds added *in vitro* to chemically extracted PS I bind to the PhQ site and are capable of forward electron transfer.^{13,17,18} Biggins suggested that in addition to energetic considerations, a necessary structural constraint was the presence of an alkyl tail.¹⁹ However, a recent review supported the assessment that a quinone head group of appropriate redox potential is alone capable of restoring electron transfer from A_0 to the iron-sulfur clusters.¹⁶

In this chapter, we focus on protocols that were developed for modifying or replacing PhQ *in vivo*. This method utilizes the biosynthetic machinery of the cell to replace PhQ with PQ-9, a molecule otherwise associated with PS II, and with a variety of naphthoquinone derivatives. We will first identify candidates for genes responsible for the biosynthetic pathway of PhQ. It will be shown that inactivation of these genes disallows PhQ production. We will then describe the physiological and bioenergetic changes that result from the insertion of PQ-9 and naphthoquinone derivatives into the PhQ binding site. These derivatives will be shown to result from a specific gene inactivation or from *in vivo* feeding of (naphtho- and anthra-) quinones to living cells.

119.2 Phylloquinone Biosynthetic Pathway

The only known function of PhQ in cyanobacteria and plants is to function as an electron transfer cofactor in PS I. In spite of its importance in cyanobacteria, the biosynthetic route of PhQ was not previously elucidated. Many prokaryotes contain the metabolic pathway for the biosynthesis of menaquinone (MQ), a PhQ-like molecule (Figure 119.1). In certain bacteria, MQ is used during fumarate reduction in anaerobic respiration.^{20,21} In green sulfur bacteria and in heliobacteria, MQ may function as a loosely bound secondary electron acceptor in the photosynthetic reaction center.²² The genes encoding enzymes involved in the conversion of chorismate to MQ were cloned in a variety of organisms. MQ differs from PhQ only in the tail portion of the molecule: an unsaturated C-40 side chain is present, rather than a mostly saturated C-20 phytol side chain. Therefore, the synthesis of the naphthalene rings in PhQ and MQ involves similar steps in both pathways.

Given that PhQ biosynthesis is comparable to MQ biosynthesis, the starting molecule for the naphthoquinone nucleus is isochorismate (Figure 119.2), which is ultimately derived from shikimic acid. The first committed step involves the loss of pyruvate and carbon dioxide with the attachment of 2-oxoglutarate to the benzene ring.²³ The next two steps involve stripping the aromatic ring of the hydroxyl group and subsequently attaching a CoA to the aromatic carboxylate group. A succinyl moiety provides the C-2 through C-4 carbons in the formation of the second aromatic ring, which is catalyzed by DHNA

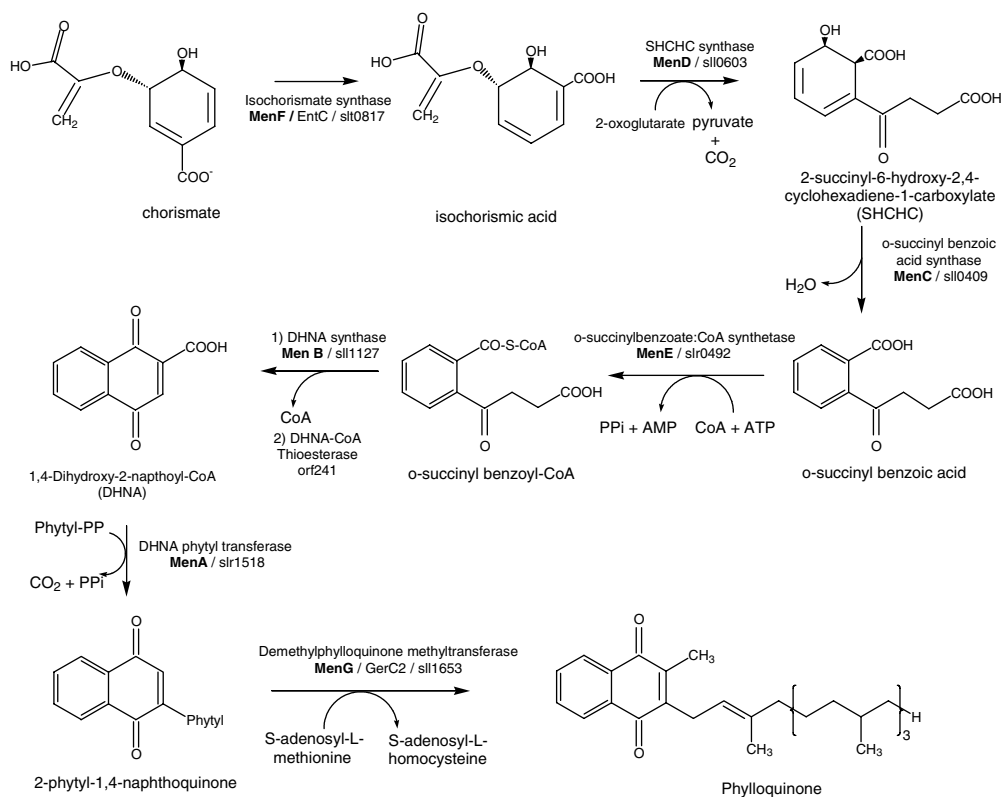


FIGURE 119.2 Proposed biosynthetic pathway of phyloquinone in *Synechocystis* sp. PCC 6803. The genes responsible for the biosynthesis of menaquinone were initially described in *E. coli*. The homologs of these genes were identified in the genome sequence of *Synechocystis* sp. PCC 6803, and *menA*, *menB*, *menD*, *menE*, and *menG* were confirmed by experiment.

synthase with subsequent removal of the CoA by a thioesterase. Both quinone oxygens are derived from water, not from molecular oxygen.²⁴ At this point, the naphthoquinone head group of PhQ is essentially formed. The biosynthesis of the phytol tail is thought to be generated from acetyl-CoA through the mevalonate pathway to form geranylgeranyl pyrophosphate.²⁵ This molecule is then hydrogenated to phytol pyrophosphate using NADPH as the contributing electron donor.²⁶ The synthesis of the PhQ phytol tail and the MQ isoprenoid tail is treated in greater detail in the literature.²⁷ The remaining steps involve attaching a phytol group in the C-3 position, with loss of pyrophosphate and carbon dioxide from the pyrophosphate-phytyl group and carboxylate, respectively. Finally, the demethyl-PhQ is methylated, with the methyl group derived from L-methionine, at the C-2 position.

The search for the proteins involved in the PhQ biosynthetic pathway in *Synechocystis* sp. PCC 6803 begins with a search for the genes involved in MQ biosynthesis in other organisms. The genes encoding enzymes involved in the conversion of chorismate to MQ were cloned in *Escherichia coli*^{28–34} and *Bacillus subtilis*.^{35–38} Specific genes involved in the pathway were identified in spinach³⁹ and *Bacillus stearothermophilus*.⁴⁰ MQ biosynthetic genes were also identified by homology in a variety of other genomes (Table 119.1), including *Haemophilus influenzae* and *Arabidopsis thaliana*.⁴¹ The genome database for *Synechocystis* sp. PCC 6803⁴² contains homologs for several genes that encode enzymes for MQ biosynthesis: *menF* (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 MQ biosynthesis protein). Possible homologs of *menC* and ORF241 (the DHNA thioesterase) were also identified in database searches. The proposed pathway is illustrated in Figure 119.2. We will focus here

TABLE 119.1 Percent Identity and Percent Homology of Enzymes of Proposed Homologous Genes in the PhQ Biosynthetic Pathway of *Synechocystis* sp. PCC 6803 to Menaquinone Genes in Four other Organisms: *Escherichia coli*, *Bacillus subtilis*, *Haemophilus influenzae*, and *Arabidopsis thaliana*

Gene	Percentage Identity and Homology Compared to <i>Synechocystis</i> sp. PCC 6803 (%H/%I)			
	<i>Escherichia coli</i>	<i>Bacillus subtilis</i>	<i>Haemophilus influenzae</i>	<i>Arabidopsis thaliana</i>
<i>menD</i>	49/32	52/33	42/29	42/28
<i>menC</i>	37/22	40/23	42/25	46/26
<i>menE</i>	42/25	42/24	40/27	46/29
<i>menB</i>	78/64	79/67	82/70	67/54
<i>orf241</i>	42/28	45/25	^a	43/25
<i>menA</i>	44/26	42/20	41/24	46/30
<i>menG</i>	50/30	51/33	^a	44/32

^a No homologous proteins were identified.

on the dedicated steps to PhQ synthesis from isochorismate, involving products of the *menD*, *menC*, *menE*, *menB*, ORF241, *menA*, and *menG* genes. The product of the *menF* gene is involved as a dedicated step, even considering that isochorismate also participates in the synthesis of serine and enterochelin.⁶⁰ Each protein in the PhQ biosynthetic pathway was examined individually for domain/active site homologies between *Synechocystis* sp. PCC 6803 and organisms ranging from bacteria to higher plants. The phylogenetic tree was determined for each gene product, suggesting overall evolutionary ties among organisms.

In general, the phylogenetic tree comparisons for *menD*, *menC*, *menE*, *menB*, and *menA* indicate that *Synechocystis* sp. PCC 6803 (*Syn*) and *B. subtilis* (*BSU*) tend to be paired first. *E. coli* (*Eco*) and *H. influenzae* (*HIN*) also tend to form an initial pair throughout the biosynthetic pathway. Those two first pairs (*Syn/BSU* and *Eco/HIN*) are usually joined before *A. thaliana* (*Art*) intersects. The *Syn* enzymes tend to be more closely conserved with other bacteria, with *Art* showing the greatest discrepancy in protein alignment.

The *menD* (SHCHC synthase) and *menC* (o-succinyl benzoic acid synthase) genes are the first two genes coding for enzymes that are likely to be involved exclusively in PhQ biosynthesis. Starting with isochorismic acid, SHCHC synthase produces 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate (SHCHC), with the subsequent enzyme o-succinyl benzoic acid synthase producing o-succinyl benzoic acid. Both *menD* genes from *E. coli*²⁸ and *B. subtilis*³⁷ were cloned. The *menC* gene was also cloned in *E. coli*³⁰ and *B. subtilis*.^{36,43} The protein alignment of SHCHC synthase from both organisms shows a 42 to 52% similarity. The similarity is 37 to 46% for o-succinyl benzoic acid synthase (Table 119.1). Protein alignments for SHCHC synthase show a highly conserved N-terminal domain. This domain may be part of the active site or a possible substrate-binding site for 2-oxoglutarate, which is consumed in the reaction with pyruvate and carbon dioxide released as side products. O-succinyl benzoic acid synthase (*menC*) has no well-defined domains that can be identified as active sites. However, there are several sets of residues that have complete identity in the direction of the C-terminal end. The reaction involves loss of an hydroxyl from the substrate without cofactor involvement, and this may suggest that a smaller less-conserved active site may be present.

The largest enzyme in the pathway is o-succinyl benzoate:CoA synthetase, which has ca. 450 residues and is encoded by the *menE* gene. The homology comparison of the sequence of this protein in *Synechocystis* sp. PCC 6803 to other organisms is lower compared to the other protein similarities probed in this pathway. There is a C-terminal region of identity that could represent the active site domain. This generally basic domain has conserved hydrophobic, basic, and hydroxyl amino acids from residues 350 to 400 and also contains two conserved proline residues. The enzyme attaches 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 *menB* gene product (DHNA synthase) is the most conserved protein within the PhQ biosynthetic pathway among different organisms (Table 119.1). The *menB* gene in *A. thaliana* has the lowest identity

menB_Art.t	MADSNELGSA	SRRLSVUINH	LIPIGFSPAR	ADSVELCSAS	SMDDRFHKUH	50
menB_BSU.t	M-----	-----	-----	---AE---	-----	3
menB_Syn.t	M-----	-----	-----	---D---	-----	2
menB_HIN.t	MDNPKD---	-----	--DULYAP--	---VE---	-----	14
TmenB_Eco.t	MIYPDE---	-----	--AMLVAP--	---VE---	-----	14
Consensus	M.....	-----P--	---VE---	-----	50
menB_Art.t	GEUPTHEVVA	KKTDFFGEGD	NKEFVDIIVE	KALDESTAKI	TINRPEVANA	100
menB_BSU.t	-----	---KT-----	KRTVDEIIVE	T-VN-GIAKI	TINRPEVANA	34
menB_Syn.t	-----	---HI-----	AKHYDEIIVE	K-AG-GIAKI	TINRPHKANA	33
menB_HIN.t	-----	---IDH-----	SEGYSDIIVE	KSTD-GIAKI	TINRPEVANA	47
TmenB_Eco.t	-----	---HDC-----	SEGFEDIIVE	KSTD-GIAKI	TINRPEVANA	47
Consensus	-----Y.DIIVE	K...D-GIAKI	TINRPEVANA	100
menB_Art.t	FPPPTUKEIM	RAFNDARRDD	SUGUIILTGR	GTKAFCSGGD	QALRTQDGYA	150
menB_BSU.t	FIPKTVHEI1	DAFADARRDD	NUGUIILTGR	GDK-----	AFCSGGDQK	76
menB_Syn.t	FPPPTUFEIV	DAFCNARRDN	RIGUIILLTGR	GPH---SDGK	YAFCSGGDQS	80
menB_HIN.t	FPPPTUKEIM	RAFNDARRDE	NIGUIILLTGE	GDK-----	AFCSGGDQK	89
TmenB_Eco.t	FPPPTUKEI1	DAFADARRDD	NIGUIILLTGR	GDK-----	AFCSGGDQK	89
Consensus	FPPPTUKEI1	.AF.DARR.D	NIGUIILLTGR	G.K-----	AFCSGGDQK	150
menB_Art.t	DPNDUGALNV	LDLQUQIRAL	PKPUIAHLUP	EVDLDFDGLS	QHSVRIISGG	200
menB_BSU.t	URGHG---V	UG-DDQIPRL	NULDQLRLIR	UIP--KPUVA	MUGSVRIISGG	120
menB_Syn.t	URGEG---V	ID-DQSTPRL	NULDQLRLIR	SMP--KUUIA	LUGSVRIISGG	124
menB_HIN.t	URGDYB---GV	KD-DSGVHLL	NULDFQRDIR	SCP--KPUVA	MUGSVRIISGG	134
TmenB_Eco.t	URGDYB---GV	KD-DSGVHLL	NULDFQRDIR	TCP--KPUVA	MUGSVRIISGG	134
Consensus	URGD.G---.V	.D-D.G..R	NULD..QLRLR	..P--KPUVA	MUGSVRIISGG	200
menB_Art.t	HALHMLCDLT	IARDNAIFGQ	TGPKUGSFDQ	GMBSSIMSR	UIELTITAMQ	250
menB_BSU.t	HALHMLCDLT	IARDNAIFGQ	TGPKUGSFDQ	GMBGVLARI	UGH-----	163
menB_Syn.t	HALHMLCDLT	IARDNAIFGQ	TGPKUGSFDQ	GMBGVLARI	UGQ-----	167
menB_HIN.t	HALHMLCDLT	IARDNAIFGQ	TGPKUGSFDQ	GMBGVMARI	UGQ-----	177
TmenB_Eco.t	HALHMLCDLT	IARDNAIFGQ	TGPKUGSFDQ	GMBGVMARI	UGQ-----	177
Consensus	HALHMLCDLT	IARDNAIFGQ	TGPKUGSFDQ	G.MBSSVMARI	UGQ-----	250
menB_Art.t	YPKSNKHLI	KUYECIQGVP	KKAREIIVLC	RDYADERLD	MGLUNTUUP	300
menB_BSU.t	-----	-----	KKAREIIVLC	RDYADERLD	MGLUNTUUP	193
menB_Syn.t	-----	-----	KKAREIIVLC	RDYADERER	MGLUNTUUP	197
menB_HIN.t	-----	-----	KKAREIIVLC	RDYADERLD	MGLUNTUUP	207
TmenB_Eco.t	-----	-----	KKAREIIVLC	RDYADERLD	MGLUNTUUP	207
Consensus	-----	-----	KKAREIIVLC	RDYADERLD	MGLUNTUUP	300
menB_Art.t	EDLEKETVKA	CREMLNSPT	ALADLKAAFN	ADCGDAGLQ	ELAGNATLIF	350
menB_BSU.t	EDLESEIKN	CEEMLEKSP	ALADLKAAFN	ADTDGLAGLQ	DFAGDATALY	243
menB_Syn.t	DALESEIKN	AKEMLEKSP	ALADLKAAFN	ADCGDAGLQ	ELAGNATLIF	247
menB_HIN.t	ADLEKETVKA	CREMLNSPT	ALADLKAAFN	ADCGDAGLQ	ELAGNATLIF	257
TmenB_Eco.t	ADLEKETVKA	CREMLNSPT	ALADLKAAFN	ADCGDAGLQ	ELAGNATLIF	257
Consensus	.DLEKETV..K	CREMLNSPT	ALADLKAAFN	ADCGDAGLQ	ELAGNATLIF	350
menB_Art.t	MYTDERTEGR	TAYMHRFPD	FSKFRFP			378
menB_BSU.t	MYTDERKEGR	DSFKEKRPD	FSQFRFP			271
menB_Syn.t	MYTDESSEGR	QRFLEKRPD	FSQYPLP			275
menB_HIN.t	MYTDESSEGR	NRFNEKRPD	FSKFRFP			285
TmenB_Eco.t	MYTDESSEGR	NRFNQKRPD	FSKFRFP			285
Consensus	MYTEES.EGR	.RF.EKRPD	FSKFRFP			378

FIGURE 119.3 Homology comparison of the *menB* gene. Abbreviations for each organism: Art is *Arabidopsis thaliana*, BSU is *Bacillus subtilis*, Eco is *E. coli*, HIN is *Haemophilus influenza*, and Syn is *Synechocystis* sp. PCC 6803.

at 54%, which may be due to the presence of an intron in the N-terminal region (Figure 119.3). The overall homology of the bacterial proteins is about 80%. The C-terminal half is highly conserved. Given the high degree of similarity, the second ring closure can be considered a critical step, converting a substituted benzene ring into naphthoquinone. DHNA synthase is the only step that does not produce by-products or consume high-energy cofactors. This suggests that the enzyme mechanism is refined, hence imparting a high degree of functional and structural similarity between species.

Given the small amount of information on *orf241*, we will only acknowledge that it is a likely component of the MQ/PhQ biosynthetic pathway. The homology is relatively low among the organisms compared (Table 119.1). It is thought to be a thioesterase that releases CoA from DHNA-CoA.

The DHNA phytyl transferase encoded in the *menA* gene is clearly an important step, and it differentiates the production of PhQ from MQ in *Synechocystis* sp. PCC 6803. The homology comparisons among organisms are surprisingly similar, given that DHNA phytyl transferase in *Synechocystis* sp. PCC 6803 transfers to the naphthoquinone a C-20 mostly saturated phytyl tail, whereas in other bacterial organisms, it transfers an unsaturated C-40 group.

The *menG* gene codes for a demethyl-PhQ methyl transferase. The gene involved in the PhQ biosynthetic pathway has proven difficult to identify because methylation is a common cellular reaction and is performed by many enzymes on a variety of substrates.⁴⁴ Although the homology comparison among organisms is comparable to other enzymes in this pathway, the protein alignment fails to yield a consistent domain for the active site.

Genes involved in PhQ biosynthesis of *Synechocystis* sp. PCC 6803 were thus identified. The percent homology and identity among organisms vary significantly, leading to tentative identification. To prove that these proposed genes code for enzymes involved in the biosynthetic pathway of PhQ, several were selected for gene disruption studies.

119.3 Phyloquinone Biosynthetic Pathway Gene Disruption

Five of the seven identified genes (*menA*, *menB*, *menD*, *menE*, and *menG*) in the PhQ biosynthetic pathway were disrupted. With the pathway interrupted at specific points, our goals were to characterize the physiology of the mutant cells and to study the effect on PS I electron transfer.

The *menB* and *menA* genes were chosen for the first set of inactivation studies. The *menB* gene encodes DHNA synthetase, which is responsible for the biosynthesis of the naphthoquinone head group. By disrupting the *menB* gene, we hoped to prevent the synthesis of a molecule that would be capable of binding in the PhQ site. We expected that *menB* gene inactivation would lead to an empty PhQ binding site. The *menA* gene was chosen for disruption as a complement to the *menB* gene. The *menA* gene encodes phytyl transferase, which is responsible for displacing the carboxy group with a phytyl group. According to Itoh and coworkers, tail-less naphthoquinones are capable of binding to the PhQ site.¹⁶ By disrupting the *menA* gene, synthesis halts at the production of DHNA, which we thought might be incorporated into the PhQ binding site. This could allow us to determine if phytylation is a necessary precondition for binding, and whether the naphthoquinone head group, with an appropriate redox potential, is the only requirement for function. The results of these mutations are discussed in the next section.

The *menD* and *menE* genes were chosen for the next set of inactivation studies. The *menD* gene was chosen because its product was thought to be the first dedicated step in the pathway. The *menE* gene was chosen to confirm the gene's assignment, given the relatively low sequence homology. Isochrosmate, the product from the previous step (coded by *menF*), is an intermediate found in many other pathways and yet has been recently determined necessary for PhQ biosynthesis.⁶⁰

The identification of the *menG* gene was difficult, and after careful consideration of several potential methylases in a variety of genomes, disruption as the open reading frame *sll1653* in *Synechocystis* sp. PCC 6803 confirmed its involvement in the PhQ biosynthetic pathway.^{45,55} This disruption is important, not only in identifying the proper methylase, but also for generating demethyl-PhQ, a potentially interesting substitute for PhQ in PS I.

119.4 Results of Phyloquinone Pathway Gene Disruption

The *menA* and *menB* genes were inactivated by disrupting and partially removing the targeted gene with an antibiotic resistance cassette, thereby preventing a fully functional enzyme from being translated.⁴⁶ High-performance liquid chromatography coupled with detection by UV-Vis (HPLC/UV-Vis) and mass spectroscopy (LC-MS) showed that pigments extracted from membranes and PS I trimers of the *menA* and *menB* deletion mutant strains lacked detectable levels of PhQ. Therefore, one immediate conclusion was that the *menA* and *menB* genes in the *Synechocystis* sp. PCC 6803 genome code for phytyl transferase and DHNA synthase, respectively, in the PhQ biosynthetic pathway. However, interesting observations were made regarding function. The *menA* and *menB* deletion mutant strains continued to grow photoautotrophically, albeit with increased doubling times, indicating that photosynthesis was still functional. Plastoquinone-9 (PQ-9) was found by HPLC/UV-Vis and LC-MS analysis in extracted pigments of PS I

trimers from the *menA* and *menB* deletion mutant strains but not from the wild type. Using low-temperature electron paramagnetic resonance (EPR) spectroscopy, it could be shown that an electron derived from P700 reduced the terminal iron–sulfur clusters, F_A/F_B . Additionally, steady state electron transfer rates of cytochrome c_6 to flavodoxin in PS I complexes from the *menA* and *menB* deletion mutant strains were about 80% that of the wild type. This led us to two possible conclusions: (1) that the PhQ-binding site is bypassed as the electron is transferred from A_0 to the iron–sulfur clusters or (2) that a foreign molecule is recruited into the PhQ binding site, and that it participates as a cofactor in forwarding the electron transfer.

A series of careful magnetic resonance experiments identified a foreign quinone as this intermediate cofactor in PS I.⁴⁷ Initially, a semiquinone anion radical (Q^-) was observed transiently by EPR spectroscopy at X-band (9 GHz) when living whole cells of the *menA* and *menB* deletion mutant strains were illuminated with white light. The molecule responsible for this radical was not only capable of accepting an electron from P700, but also, it was able to discharge electrons forward to the iron–sulfur clusters. When the radical was photoaccumulated at low temperatures and studied at higher microwave frequencies (34 GHz), the larger g -anisotropy hinted that the quinone contained a single benzoquinone ring rather than a naphthoquinone ring. Also, the prominent hyperfine splittings due to the 2-methyl group of PhQ⁴⁸ were missing. ENDOR spectroscopy showed that Q^- remained the same in the *menA* and *menB* deletion mutant strains, and there were two asymmetric methyl groups on the molecule. These structural features are consistent with the identity of the intermediate cofactor as PQ-9. Spin-polarized transient EPR at three microwave frequencies (X, Q, and W-bands) indicated that the vector connecting the quinone carbonyls is pointed toward P700, which is the same orientation as in native PhQ.⁴⁹ Pulsed EPR techniques indicated that the distance from P700 and Q^- is 25.3 Å, which is close to the P700⁺ and A_1^- distance.^{50,51} Hence, Q^- is the same orientation and distance from P700 as PhQ. The accumulated magnetic resonance data, therefore, prove that Q^- is PQ-9, and that it is likely bound to the same site as PhQ.

We next focused on the kinetics of electron transfer in PS I complexes from the *menA* and *menB* deletion mutant strains. Electron transfer rates are sensitive to distance, Gibbs free energy, and reorganization energies among donor and acceptor pairs. The replacement of PhQ with PQ-9 translated into changes in forward and backward electron transfer rates through the quinone. In particular, the forward electron donation from the PhQ⁺ to F_X slowed by about a factor of 10 relative to the wild type. In wild-type PS I, the charge recombination between P700⁺ and $[F_A/F_B]^-$ is multiphasic after a saturating flash.⁶ When measured in the absence of an external electron acceptor, the reduction of P700⁺ is biphasic, with typical lifetimes of 10 to 30 ms and 80 to 100 ms. The reduction of P700⁺ in *menA* and *menB* deletion mutant strains reduction are also biphasic, with lifetimes of ca. 3 and ca. 10 ms. The 30-fold increase in the P700⁺ reduction kinetics in the *menA* and *menB* deletion mutant strains was confirmed using CW EPR spectroscopy. The 3 ms phase has proven to be a highly useful characteristic that allows one to determine whether PQ-9 or PhQ is in the binding site. The changes in the electron transfer kinetics are explained by a change in the redox potential of PQ-9. It is estimated to be ca. +95 mV more oxidizing than PhQ, resulting in a thermodynamically “uphill” electron transfer step from Q^- to F_X .⁶

The *menD* and *menE* genes code for enzymes that function and reside earlier in the PhQ biosynthetic pathway. These genes were targeted for gene inactivation due to the possibility that there may be an alternative pathway that circumvents the *menD* and *menE* gene inactivations. The *menD* and *menE* deletion mutant strains were similarly engineered by inserting an antibiotic-resistant cassette into the gene.⁵² The physiological characteristics of the *menD* and *menE* deletion mutant strains were found to be similar to the *menA* and *menB* deletion mutant strains. The cells grew at a fraction of the wild-type rate, and all showed high light sensitivity, a lower chlorophyll content a per cell, and a lower ratio of PS I:PS II. All of the mutant strains had comparable rates of whole-chain and PS II oxygen evolution. Using LC-MS and HPLC-UV/Vis detection of extracted pigments we showed that the mutant strains did not contain PhQ in the thylakoid membrane or the purified PS I complexes. As with the *menB* deletion mutant strain, PQ-9 was identified in PS I complexes by Q-band EPR spectroscopy. A 3 ms lifetime was also observed for the kinetic back-reaction. Therefore, similar to the *menA* and *menB* deletion mutant strains, PQ-9 is recruited into the PhQ binding site of PS I in the *menD* and *menE* deletion mutant

strains. We therefore find the same general physiological and phenotypic responses when PQ-9 is in the PhQ binding site of PS I, regardless of which gene in the PhQ biosynthetic pathway is inactivated. A corollary of this conclusion is that no alternative pathways exist for the biosynthesis of PhQ in *Synechocystis* sp. PCC 6803.

119.5 High-Light-Tolerant Strains of *menB* and *menD* Mutant Strains

All of the original PhQ biosynthetic pathway mutant strains (including *menA*, *menB*, *menD* and *menE*) have a low content of PS I and a low chlorophyll content per cell, and they are sensitive to high-light intensities.^{46, 52–54} Previously known as the *menB* mutant strain, the original *menB18* deletion mutant strain is high-light sensitive and was used for the experiments described here. Two high-light-tolerant strains were isolated from the *menD* and *menB* deletion mutant strains. Colonies of the mutant strains were streaked onto BG11 agar plates containing antibiotics and were placed under high-light intensities ($160 \mu\text{M m}^{-2} \text{s}^{-2}$). The *menD*-R1 and *menB26* mutant strains were two of several isolates that could grow under high-light intensities. The latter mutation also occurred spontaneously in liquid cultures grown under normal-light intensities (40 to $60 \mu\text{M m}^{-2} \text{s}^{-2}$), in which the cells were recultured continually for many generations. Presumably, these high-light-tolerant, suppressor mutations can be generated in any of the PhQ biosynthetic pathway gene disruption mutations. To prevent the development of high-light tolerance, liquid cultures should only be grown for two to five generations under low-light intensities ($5 \mu\text{M m}^{-2} \text{s}^{-2}$). Cells stored on BG11 plates in low light have not been found to become high-light tolerant.

Approximately 20 *menB* deletion mutant strains were characterized for growth rate, PS I to PS II ratio, PhQ/PQ-9 content, and oxygen evolution.⁵⁴ Of the 20, 10 were high-light tolerant. The other strain, *menB26*, was selected for robustness, the ability to tolerate high-light intensities, and the ability to grow in the presence of supplemented (benzo-, naphtho-, and anthra-) quinones. This trait was necessary for the naphthoquinone feeding experiments described later. The other strains did not have the ability to thrive in liquid cultures containing low levels ($10 \mu\text{M}$) of supplemented quinone.

The doubling time of the *menD*-R1 mutant strain (30 h) was comparable to the wild type (26 h), which grows nearly three times faster than the *menD* mutant strain (80 h).^{52,54} Both the *menB18* and *menB26* mutant strains had comparable doubling times (over 70 h). Additionally, *menB26*, unlike *menB18*, was more tolerant of quinones added to the liquid growth media. The *menB18* strain tended to die or enter an early stationary phase upon addition of quinones.

The high-light tolerant *menD*-R1 and *menB26* strains are otherwise similar to the original high-light sensitive *menD* and *menB18* strains.⁵⁴ All mutant strains contain active PQ-9 in the PhQ binding site, and the chlorophyll content per cell is reduced to 67 to 85% that of the wild type. To determine if the quinone-binding pocket contained a secondary site mutation, the appropriate regions of the *psaA* and *psaB* genes were sequenced and found to be identical to the wild type, *menD*, and *menB18* strains. The quinone-to-chlorophyll ratio for all mutant strains is similar to that of the wild type, 1:50.

The differences that arise between the original mutant strains (*menD* and *menB18*) and the high-light-tolerant strains (*menD*-R1 and *menB26*) relate to the PS I to PS II ratio.⁵⁴ Phenotypic differences are noted in several ways. Shown in Figure 119.4 is the 77 K fluorescence emission of whole cells on an equal cell number basis; the 720 nm emission is characteristic of PS I, while the 685/695 nm emission is characteristic of PS II. When compared to the wild type, the original *menD* strain and the original *menB18* strains reduced ratios of PS I:PS II. This is also true of the *menA* and *menE* mutant strains (not shown). The high-light-tolerant *menD*-R1 and *menB26* strains have PS I:PS II ratios comparable or greater than those of the wild type. Oxygen evolution rates provide a striking example in the differences in the high-light-tolerant strains relative to the wild-type strains and the *menD* and *menB18* mutant strains. Under the growth conditions of these experiments, the wild type, and the *menD*, *menB18*, and *menE* mutant strains have approximately a 1:3 ratio of water-to-bicarbonate whole-chain oxygen evolution versus PS II oxygen evolution alone. In the high-light-tolerant strains, this ratio drops to ca. 1:1. Whole-chain

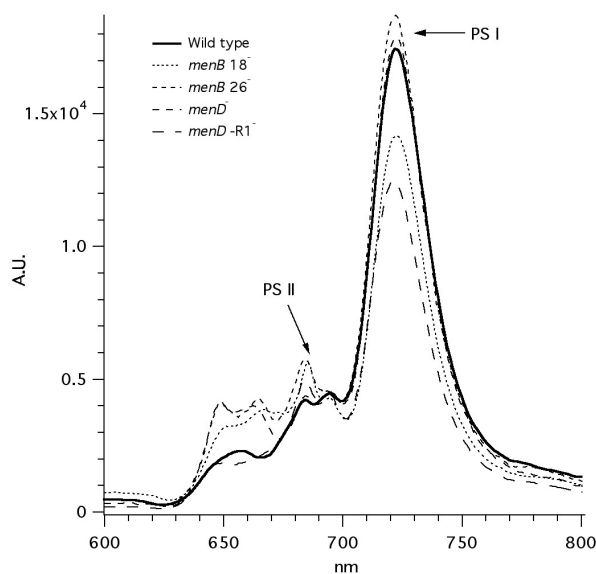


FIGURE 119.4 Fluorescence emission spectra at 77 K of whole cells from *Synechocystis* sp. PCC 6803 wild type, *menB* mutant strains, and *menD* mutant strains. Spectra were recorded at the same cell density and normalized relative to PS II. Each spectrum was the average of the four measurements performed in duplicate. 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 721 nm.

electron transfer rates decrease 86% and 58% in the *menD*-R1 and *menB*26 strains, compared to the original strains (*menD* and *menB*18), respectively. The PS II oxygen evolution levels of *menD*-R1 and *menB*26 decreased to 30% and 49% of the levels in the original *menD* and *menB*18 mutant strains, respectively. The change in oxygen evolution for *menD*-R1 and *menB*26, coupled with the 77 K fluorescent measurements, indicate that the cell has downregulated the amount of PS II. The lower level of PS II per cell generates less reductant under high-light conditions, an amount that can be adequately processed by the lower amount of PS I. This leads to a lower level of stress on the cells under high-light conditions, which, in turn, allows the cells to grow.

119.6 Modification of Phylloquinone through Gene Inactivation

A novel PhQ derivative was introduced biologically into the PhQ binding site by inactivating a specific methyltransferase coded by the *menG* gene, one that methylates the naphthoquinone ring ortho to the phytyl tail.⁵⁵ Mass spectrometric measurements show that pigments extracted from PS I complexes of the *menG* mutant strain lack the $m/z = 450$ peak characteristic of PhQ and instead reveal an $m/z = 436$ peak characteristic of 2-phytyl-1,4-naphthoquinone. EPR spectroscopy of the photoaccumulated semiquinone $Q^{\cdot-}$ signal in PS I complexes showed the presence of a hyperfine doublet that indicated the presence of an H in place of the CH_3 group ortho to the phytyl tail.

Unlike PQ-9 containing mutant strains, the phenotype of the demethyl-PhQ mutant strain is similar to the wild type.⁵⁵ The doubling times of the *menG* deletion mutant strain under photoautotrophic and mixotrophic conditions are the same as those of the wild type. The chlorophyll content is also comparable. The steady state rates of electron transport for PS I complexes of the *menG* deletion mutant strain were virtually identical to those of the wild type. Transient EPR shows that the lifetime for the forward electron transfer from $Q^{\cdot-}$ to the iron-sulfur clusters slowed from a lifetime of 290 ns in the wild type to 600 ns

in the *menG* deletion strain. This would be expected if the redox potential of 2-phytyl-1,4-naphthoquinone were +50 to +60 mV more oxidizing than PhQ. The lifetime of the $P700^+[F_A/F_B]$ backreaction also decreased from 80 ms in the wild type to 20 ms in the *menG* deletion mutant strain.

119.7 *In Vivo* Recruitment of Media-Supplemented Naphthoquinones

Success at genetically disrupting the PhQ biosynthesis pathway allowed us to embark on a project aimed at the biological replacement of the native PhQ with alternative quinones. The initial gene disruptions resulted in a PQ-9 molecule occupying the PhQ site of PS I. These mutant strains allowed us to introduce a variety of phytylated benzyl- and naphthoquinone in order to probe the structural requirements for quinones to function in PS I. This method has proven to be a useful adjunct to PhQ removal by organic solvents^{16,19,56,57} and has additional advantages because it allows physiological observations of living cells. We found that it is possible to introduce phytylated quinones into the PhQ site *in vivo* by utilizing the phytyltransferase in the *menB* mutant strain and the methyltransferase in the *menA* mutant strain and by selecting tolerant strains (Figure 119.2).⁵³ For example, the *menB26* mutant strain prevents the naphthoquinone head group from forming, yet it allows phytylation of a quinone supplemented in the growth medium. Because the *menA* mutant strain is incapable of phytylation, it serves as a control to determine if the supplemented quinone head group alone is capable of displacing PQ-9 and functioning in forward electron transfer.⁵³

To assess the stability of PhQ over time and to gauge the relative binding of PQ-9, an *in vivo* PhQ pulse experiment was conducted on the living *menB26* cells. Within 15 min of the addition of PhQ to the growth media, PQ-9 was completely displaced by PhQ in PS I. Hence, PhQ must enter the PhQ binding site by diffusion, and it must displace the resident PQ-9. This rapid incorporation of PhQ suggests a significant difference in the affinity of the binding site for PhQ and PQ-9. This displacement of the loosely bound PQ-9 is the key for incorporating alternative phytylated quinones.

Reincorporation of PhQ was also achieved by supplementing the *menB26* growth media with the enzymatic product of DHNA synthetase, 2-CO₂-1,4-naphthoquinone, which resulted in a 94% replacement of PQ-9 with PhQ.⁵³ The Chl:Q_{total} in the supplemented *menB26* deletion mutant strain is comparable to that of the wild type. This proves that phytyltransferase (the *menA* gene product) and methylase (*menG* gene product) remain functional in the mutant strains. Furthermore, PQ-9 is retained when the *menA* deletion mutant strain is supplemented with 2-CO₂-1,4-naphthoquinone. Because the *menA* gene codes for phytyltransferase, we concluded that the phytyl group is important for binding the quinone into the site.

Reincorporation of PhQ also occurs with compounds such as 2-CH₃-1,4-naphthoquinone.⁵³ Supplementation with the deuterated derivative of 2-CD₃-1,4-naphthoquinone yields a d-PhQ product that still contains the CD₃ moiety. This shows that the deuterated methyl group is unaffected by the phytyl- and methyltransferase enzymes. The addition of 1,4-naphthoquinone led to the detection by LC-MS of a mixture of phytylated and phytylated/methylated molecules.⁵³ However, there was clear EPR evidence for *in vivo* incorporation of 1,4-naphthoquinone into the PhQ site with an orientation similar to that of PhQ in the wild type. This was an unexpected result, considering that previous *in vitro* incorporation of 1,4-naphthoquinone in solvent-extracted PS I complexes led to the incorporation of 1,4-naphthoquinone with an altered orientation.^{49,58}

It may even be possible to incorporate 1,2-naphthoquinone into the PhQ site.⁵⁴ Growth media supplementation with unsubstituted 1,2-naphthoquinone led to an admixture of phytylated and methylated/phytylated naphthoquinone molecules as well as to the retention of significant amounts of PQ-9 when assayed by LC-MS. The replacement of PQ-9 with phytylated 1,2-naphthoquinone was therefore low (<50%) in the *menB26* mutant strain. The chlorophyll-to-quinone ratio was 63, which is higher than that for the wild-type Chl:Q_T of 42.⁵⁹ This suggests, although it does not prove, the presence of a subpopulation of PS I that lacks a bound quinone molecule. The contribution of the 3 ms P700⁺

backreaction drops to <10% in the 1,2-naphthoquinone supplemented cells. Two new phases were observed, with lifetimes of 10 ms and 34 ms in a 1:1 ratio, totaling 50% of the total decay. Photoaccumulation of Q^- at 200 K revealed a complex EPR spectrum, suggesting that PQ-9 and a 1,2-naphthoquinone derivative are reduced. More work is obviously required, but this experiment hints at the promise of biological incorporation of novel quinones into the PhQ binding site of PS I.

Finally, a double gene knockout of the *menB* and *menG* genes has been generated (*menBG*). This mutant strain has the potential to phytolate naphthoquinones without additional methylation. The *menBG* mutant strain should allow for comparisons of novel, phytolated quinones in the PhQ binding site, with or without an accompanying methyl group. The *menBG* mutant strain, therefore, adds an additional option for probing the structure and function of molecules in the PhQ binding site of PS I.

Acknowledgments

T.W.J. gratefully acknowledges support from Professor Parag Chitnis. This work was supported by grants from the National Science Foundation (MCB-0117079) and the United States Department of Energy (DE-FG-02-98-ER20314).

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