3 Phylloquinone exchange in PS I.

A part of this thesis is devoted to the investigation of the quinone cofactor in its PS I specific protein site and its function in the electron transfer process. The function of the quinone as an acceptor and donor of electrons can be studied by two different but complementary methods. The first approach is based on the point mutagenesis of specific amino acids involved in the quinone binding site. The second method involves systematic variation of the structure of the quinone cofactor by means of incorporation of artificial quinones or quinone-likes compounds in PS I. The estimation of the binding constant of the artificial quinone, the location, the distance and the orientation relative to the P_{700} donor molecule yield useful information on the modified A_1 acceptor site and possibly about the relevant protein-cofactor interactions and their influence on protein function.

3.1 Biosynthetic pathway mutants

Several methods are available which allow the quinone in the PS I to be modified. For example, inactivation of the genes that code for enzymes involved in the pathway of phylloquinone biosynthesis Figure 3.1 [41]. The inactivation of those gene prevents the synthesis of corresponding protein in the organism and, thus, the PhQ biosynthesis can not be carried out during the cells growth. The first experiments in this direction gave unexpected results. For example, in the *menB* mutant the protein dihydroxynaphthoate synthase was inactivated. However, functional PS I protein complex could be isolated from *menB* mutants cells. It turned out that they contain another quinone acceptor in the A_1 site. By independent analytical chemical technique and EPR spectroscopy it was identified as plastoquinone-9 (PQ-9). That means that in the absence of PhQ PS I can accept the PQ-9 from the quinone pool normally used by PS II.

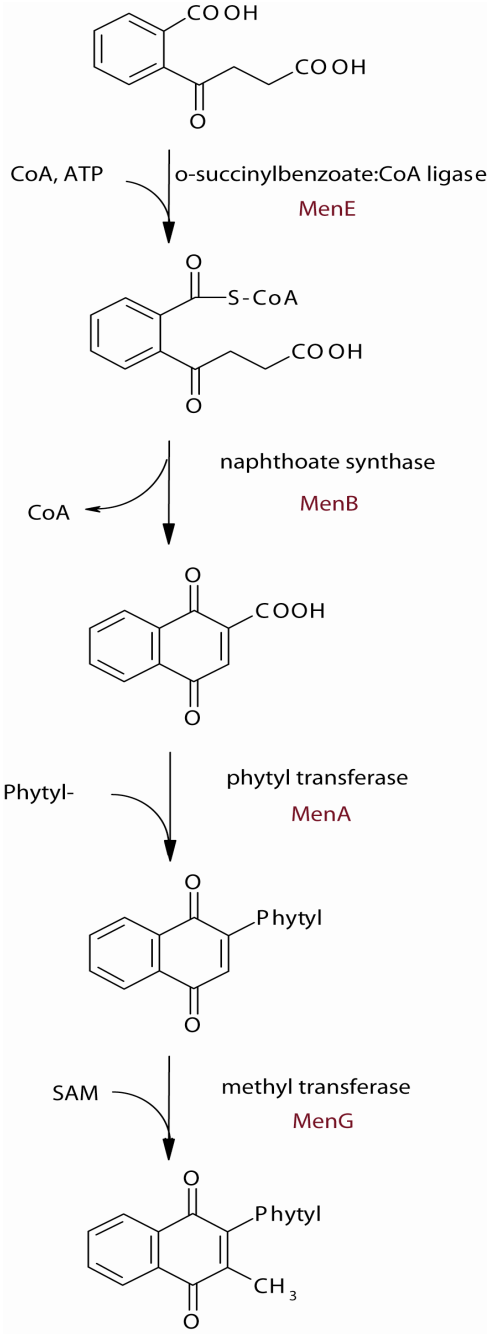

Figure 3.1. Biosynthetic pathway for phylloquinone synthesis in bacteria [41].

At room temperature, PQ-9 is able to function in accepting an electron from A_0^- and passing the electron forward to the iron-sulphur clusters. [42]. The rate of electron transfer from $(PQ\text{-}9)^-$ to iron-sulphur clusters is slower in comparison to the wild type. A lifetime of 15 μs (250 μs minor phase) was evaluated for $(PQ\text{-}9)^-$ from UV spectroscopy and electrometric measurements. The difference in the kinetics is consistent with a 130 mV more oxidizing redox potential of PQ-9 in the A_1 site. [43]. The TR EPR spectra measured at X-, Q- and W– band reveal the following prominent features of the $P_{700}^+Q^-$ radical pair: (i) $(PQ\text{-}9)^-$ has a larger *g*-anisotropy than native PhQ, as expected for one aromatic ring benzoquinone derivative, (ii) loss of the prominent methyl hyperfine couplings attributed to the 2-methyl group of PhQ, (iii) the orientation of the PQ-9 in the A_1 site as derived from the spin polarisation pattern remains similar to that of native PhQ in the wild type, (iv) out-of-phase electron spin echo modulation experiments on $P_{700}^+Q^-$ radical pair show that the dipolar coupling in the radical pair is the same as in the native PS I, i.e. the distance between P_{700}^+ and Q^- (25.3±0.3Å) is the same as between P_{700}^+ and A_1^- in the wild type [42]. Pulsed ENDOR spectroscopy was one of the critical methods for the characterisation of quinone molecular structure, it shows two sets of resolved spectral features with nearly axially symmetric hyperfine couplings. They were tentatively assigned to the two methyl groups of the plastoquinone-9, and their difference indicates a strong inequivalence among two methyl groups in A_1 site.

Another example of specific gene inactivation is the inactivation of *menG* which is responsible for synthesis of methyltransferase enzyme participating in the PhQ biosynthesis Figure 3.1. This enzyme is responsible for transferring the methyl group to 2-phytyl-1,4-naphthoquinone Figure 3.1. Mass spectrometric measurements show that targeted inactivation of the *menG* gene prevents the completion of the final step in the biosynthesis of PhQ and leads to the accumulation of 2-phytyl-1,4-naphthoquinone in PS I.

Correspondingly, the characterisation of this mutant by EPR spectroscopy shows the loss of the specific methyl hfs couplings. In addition, the spin polarisation pattern indicates that 2-phytyl-1,4-naphthoquinone is in the same orientation as native PhQ, and out-of-phase spin echo modulation spectroscopy shows the same P_{700}^{+} to Q^{-} center-to-center distance as in wild type PS I. TR-EPR studies indicate that forward electron transfer from Q- to F_X is slowed from 290 ns in the wild type to 600 ns in the *menG* mutant. The estimation of redox potential for 2-phytyl-1,4-naphthoquinone was 50-60 mV more oxidizing than PhQ in the A_1 site [44].

3.2 *In vivo* and *in vitro* quinone substitution in the biosynthetic pathway mutants

If the *menB* mutant growth medium is supplemented with naphthoquinone (NQ) derivatives they might be incorporated (*in vivo*) into the A_1 site. For example, the native supplement 2-CO_2H-1,4NQ and mono-substituted 2-CH_3-1,4NQ quinones can both be recognised as substrates by phytyltransferase enzyme in the cells and addition of a phytyl tail accompanies their incorporation in to A_1 site. Other quinones, which have no substituent in position 2 of the NQ ring, can also be incorporated into A_1 site but only partially and without either the phytyl tail or the methyl group. An example of this is 1,4NQ [45].

If the PS I protein complex is isolated from the cells of the *menB* mutant quinone substitution should be possible *in vitro* as well. The *in vitro* replacement has a certain advantage because it avoids additional enzymatic modification of the quinone substrate. *In vitro* quinone replacement will be used extensively in this thesis for the preparation of PS I complexes with the artificial quinones.

3.3 Quinone substitution after organic solvent extraction of native phylloquinone

The PS I can be modified through the selective extraction of cofactors and their subsequent reconstitution with artificial substituents. Extraction of lyophilised PS I complexes by organic solvents (diethyl ether or hexane/methanol mixture) depletes more than 90% of chlorophyll molecules, all carotenoids, and two secondary acceptor phylloquinones. The photochemical electron transfer activity from the primary donor P_{700} to the acceptor chlorophyll A_0 is preserved. A variety of artificial quinones (benzo-, naphtho-, anthraquinones) have been incorporated into the A_1 site of PS I after such extraction [6, 46, 47]. Some artificial quinones are able to mediate electron transfer between A_0 and F_x if their redox potential (E_m) values of Q-/Q in A_1 site are appropriate. The quinone/quinonoids with E_m more positive than that of F_x-/F_x (-720 mV) are unable to reduce F_x. The quinones that exhibit redox potential in organic solvent ($E_{1/2}$ measured in the DMF) equal to –400 mV were shown to reduce equal amounts of P_{700}^+ and iron-sulphur clusters. The example is 2-methylnaphthoquinone (vitamin K_3). Quinones with more negative potentials reduce higher amounts of F_x. On the other hand, quinones with extremely low $E_{1/2}$ could not be reduced by A_0^-, suggesting their E_m to be comparable to or more negatives then that of A_0.

The abilities of quinones/quinonoids to accept electrons from A_0^- or to reduce F_x did not depend on their molecular structures such as number of aromatic rings, alkyl tail length, addition of halogens, amino, or hydroxyl side groups, but significantly dependent on their redox properties in situ at the A_1 site [48].

The PhQ in native PS I has been shown to be exchangeable with deuterated PhQ by the incubation of PS I at elevated temperature [49]. Although each method of quinone

exchange differs to some extent, nearly all gave almost consistent results with respect to unchanged location and orientation of the quinone in binding site.