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PROTEIN–COFACTOR INTERACTIONS IN BIOENERGETIC COMPLEXES:
THE PHYLLOQUINONE COFACTORs IN PHOTOSYSTEM I

A Dissertation in
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by

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ABSTRACT

The electron transfer chain in Photosystem I contains two phylloquinone molecules, \(A_{1A}\) and \(A_{1B}\), that operate at highly reducing midpoint potentials. The phylloquinones exhibit significantly different redox properties even though they exist in near-equivalent protein environments. The work described in this dissertation explores the role of the single hydrogen bond between the backbone -NH of Leu722 and the \(C_4\) carbonyl group of \(A_{1A}\) in modulating the redox properties of the phylloquinone. The weakening of the hydrogen bond does not alter the structural integrity of the binding pocket but increases the rate of electron transfer from \(A_{1A}^-\) to \(F_X\), likely due to a change in the midpoint potential of the quinone and the reorganization energy of the surroundings. More importantly, the absence of the hydrogen bond renders the \(A_{1A}\) quinone susceptible to double reduction/protonation, thus generating a high potential thermodynamic well, which prevents electron transfer to the \(F_X\) cluster. The blockage of electron transfer along the PsaA-branch due to the formation of the doubly reduced \(A_{1A}\) phyllohydroquinone and the reduction of the iron-sulfur clusters \(F_A\) and \(F_B\) allows the direct observation of electron transfer along the PsaB-branch. This discovery led to the unambiguous assignment of an EPR spectroscopic signature to the previously elusive \(A_{1B}\) quinone. We propose that the primary purpose of the H-bond is to tie up the \(C_4\) carbonyl group of phylloquinone in a H-bond so as to prevent protonation and hence lower the probability of double reduction during periods of high intensity illumination.
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LIST OF ABBREVIATIONS

A\textsubscript{0A} primary electron acceptor of Type I photosynthetic reaction center on the A-branch

A\textsubscript{0B} primary electron acceptor of Type I photosynthetic reaction center on the B-branch

A\textsubscript{1A} quinone intermediate electron acceptor of Type I photosynthetic reaction center on the A-branch

A\textsubscript{1B} quinone intermediate electron acceptor of Type I photosynthetic reaction center on the B-branch

b\textsubscript{03}-QOX quinol oxidase enzyme

Chl chlorophyll

CW continuous wave

DFT density function theory

ENDOR electron nuclear double resonance

EPR electron paramagnetic resonance

ESEEM electron spin echo envelope modulation

F\textsubscript{A} terminal iron-sulfur cluster electron acceptor of Type I photosynthetic reaction center

F\textsubscript{B} terminal iron-sulfur cluster electron acceptor of Type I photosynthetic reaction center

F\textsubscript{X} interpolypeptide iron sulfur cluster in a Type I photosynthetic reaction center
<table>
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<tr>
<td>FDMR</td>
<td>fluorescence detected magnetic resonance</td>
</tr>
<tr>
<td>H-bond</td>
<td>hydrogen bond</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid</td>
</tr>
<tr>
<td>Hfc</td>
<td>hyperfine coupling</td>
</tr>
<tr>
<td>HYSCORE</td>
<td>hyperfine sublevel correlation</td>
</tr>
<tr>
<td>Nd-YAG</td>
<td>neodymium-doped yttrium aluminum garnet</td>
</tr>
<tr>
<td>NADP</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>P&lt;sub&gt;680&lt;/sub&gt;</td>
<td>primary donor of Photosystem II</td>
</tr>
<tr>
<td>P&lt;sub&gt;700&lt;/sub&gt;</td>
<td>primary donor of Photosystem I</td>
</tr>
<tr>
<td>P&lt;sub&gt;700&lt;/sub&gt;-F&lt;sub&gt;X&lt;/sub&gt; core</td>
<td>Photosystem I devoid of the PsaC, PsaD and PsaE subunits</td>
</tr>
<tr>
<td>pbRC</td>
<td>purple bacteria reaction center</td>
</tr>
<tr>
<td>PS I</td>
<td>Photosystem I</td>
</tr>
<tr>
<td>PS II</td>
<td>Photosystem II</td>
</tr>
<tr>
<td>PS I core</td>
<td>Photosystem I devoid of the PsaC, PsaD and PsaE subunits</td>
</tr>
<tr>
<td>Q&lt;sub&gt;A&lt;/sub&gt;</td>
<td>quinone intermediate electron acceptor of Type II photosynthetic reaction center</td>
</tr>
<tr>
<td>Q&lt;sub&gt;B&lt;/sub&gt;</td>
<td>terminal quinone electron acceptor of Type II photosynthetic reaction center</td>
</tr>
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To my mom and dad.
Thank you for teaching me to fight for what I believe in.
Chapter 1

General Introduction

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1.1 PHOTOSYNTHESIS

Life on planet Earth is sustained to a large extent by oxygenic photosynthesis. In this highly developed process, solar energy is used to convert CO\textsubscript{2} and H\textsubscript{2}O into carbohydrates, releasing O\textsubscript{2} to the atmosphere as a by-product. Photosynthetic organisms are able to perform this task so efficiently that the composition of the atmosphere underwent a complete transition ~2.3 billion years ago as a result of the arrival of cyanobacteria some ~400 million years earlier (1, 2). Indeed, it is the production of O\textsubscript{2} by photosynthetic prokaryotes that has made the emergence of highly advanced forms of eukaryotic life possible some 1.4 billion years ago (3-5). To this day, oxygenic photosynthesis remains the major source of O\textsubscript{2} in the atmosphere. Further, photosynthetic activity has generated enough biomass to provide all of the stored carbon-based fuel currently used by humankind. There is widespread belief that the economic deployment of solar biofuels will depend on the construction of artificial systems that closely mimic the process of natural photosynthesis. For this reason, how photosynthetic organisms convert light into chemical bond energy has become a compelling topic of study.

Of the six well-established phototrophic bacterial phyla, Firmicutes, Chloroflexi, Chlorobi, Proteobacteria, Acidobacteria and Cyanobacteria, only Cyanobacteria are capable of performing oxygenic photosynthesis. In addition, eukaryotes such as higher plants and algae also evolve oxygen during photosynthesis. Oxygenic photosynthesis can be divided into light and dark reactions. Dark reactions such as carbon fixation can occur even in the absence of light while light reactions such as transmembrane electron transport are light dependent. In cyanobacteria, photosynthesis occurs in a series of well-organized membrane structure in the cytoplasm called thylakoid membranes. These
highly folded membrane sacs allows the cell to pack a large surface area into a small space. Integral membrane protein complexes that are embedded in the thylakoid membrane carry out the light dependent reactions (Figure 1.1).

1.2 PHOTOSYNTHETIC REACTION CENTERS

The pigment-protein complexes that are responsible for light-induced electron transfer across the photosynthetic membrane are called photosynthetic reaction centers. They consist of antenna pigments that capture light energy, organic and inorganic molecules that function as electron transfer cofactors, and proteins that provide a scaffold for these components. The purpose of a photosynthetic reaction center is to generate and stabilize a charge-separated state over hundreds of milliseconds, which constitutes a biochemically relevant period of time (6). It carries out charge separation using the energy of a photon to generate a hole-electron pair against a highly unfavorable thermodynamic gradient, and stabilizes this state by transferring the electron and/or the hole through a series of cofactors to lengthen distance and hence the lifetime of the donor-acceptor pair. In general, two types of photosynthetic reaction centers exist and are classified depending on the nature of the terminal electron acceptor (7). Type I reaction centers use a bound iron-sulfur cluster and operate at the reducing end of the redox scale (8, 9). Type II reaction centers use a lipophilic quinone molecule and operate at the oxidizing end of the redox scale (10). During oxygenic photosynthesis, the Type II reaction center, Photosystem II (PS II), and the Type I reaction center, Photosystem I (PS I), function in series to oxidize water and reduce NADP\(^+\), respectively (11). Conceptually, the process is initiated at PS II, where light-induced charge separation generates a strong oxidant that
prompts a catalyst consisting of four Mn and one Ca to split H₂O into O₂, protons and electrons (12-20). Concomitantly, light-induced charge separation in PS I generates a strong reductant that ultimately reduces NADP⁺ to NADPH (21). Figure 1.2 depicts the Z-scheme showing the pathway of oxygenic electron transport. The energy of the proton gradient, generated as a result of H₂O oxidation and by passage of electrons through the cytochrome b₅f complex, is conserved in the generation of ATP from ADP and inorganic phosphate. The process culminates when CO₂ is converted into carbohydrate using the bond energy of ATP and the reducing power of NADPH (22).

1.2.1 Photosystem II, a Type II Reaction Center

Photosystem II (PS II) uses light energy to drive two chemical reactions; the oxidation of water and the reduction of plastoquinone (PQ), which can be summarized as:

$$2 \text{H}_2\text{O} + 2\text{PQ} + 4\text{H}^+ + (4h\nu) \rightarrow \text{O}_2 + 2\text{PQH}_2 + 4\text{H}^+$$

The absorption of four photons per cycle causes the splitting of two water molecules and the reduction of two plastoquinone molecule to plastoquinol with the evolution of one molecule of oxygen. The splitting of the water molecule is achieved in the catalytic redox center called the oxygen-evolving complex (OEC). A structural model for the OEC has been developed based on the 3.0 Å X-ray crystal structure of PS II (PDB ID: 2AXT) from *Thermosynechococcus elongatus* (23) and the X-ray absorption fine structure (EXAFS) studies on PS II crystals (24). A cluster comprising of four manganese ions and one calcium ion are involved in splitting two molecules of water, thereby generating dioxygen, four electrons and four protons. The electrons are subsequently transported via PS II and used in the reduction of plastoquinone. The protons released into the thylakoid
partially contribute to the generation of the proton gradient across the membrane that is used in ATP synthesis.

Excluding the peripheral antenna complexes, PS II consists of 20 different polypeptides, most of which are membrane bound. At the core of PS II reaction center are two proteins PsbA (39 kDa) and PsbB (56 kDa) that anchor all of the electron transport cofactors. PsbE (9.3 kDa) and PsbF (4.5 kDa) are involved in photoprotection and prevent radical formation under non-optimal conditions of electron flux (23). The electron transfer chain in PS II begins at the primary donor, P$_{680}$, which is a special pair of chlorophyll $a$ (Chl $a$) molecules named for its absorbance peak in the visible region. The exciton energy migrates among the antenna pigments and is funneled into P$_{680}$, whereupon it is excited to its singlet state. This triggers charge separation with the electron being transferred to the primary acceptor, a pheophytin molecule. A bridging Chl $a$ molecule acts as an intermediate acceptor between P$_{680}$ and the pheophytin. Although there are two branches of cofactors beyond the primary donor, electron transfer proceeds via the cofactors bound by the PsbA subunit. The charge separated state between P$_{680}^+$ and Pheo$^-$ is stabilized by forward electron transfer to the plastoquinone molecules Q$_A$ (bound by PsbA) and Q$_B$ (bound by PsbB) (7). Electron transfer from Q$_A$ to Q$_B$ is aided by the non-heme, Fe$^{2+}$ iron located between them.

The oxidized P$_{680}^+$ is reduced back to its ground state by the electron produced in the water-splitting reaction, creating a stable P$_{680}Q_AQ_B^-$ charge separated state. A second light-induced turnover of P$_{680}$ results in an unstable P$_{680}Q^-A^-Q_B^-$ charge separated state. Stabilization to the P$_{680}Q_A(Q_BH_2)$ state occurs with the uptake of two protons from the medium. Plastoquinol (Q$_BH_2$) is loosely bound to the Q$_B$ binding pocket and diffuses out
into the membrane to the cytochrome $b_{6f}$ complex (7). Here, plastoquinol is oxidized back to plastoquinone and the regenerated quinone diffuses back to the $Q_B$ binding pocket to take part in the next electron transport cycle. Concomitant to the quinone oxidation, by virtue of the protonmotive Q-cycle, the cytochrome $b_{6f}$ complex translocates up to two protons per electrons across the membrane. Cytochrome $b_{6f}$ passes the electron to a diffusible, soluble electron carrier, plastocyanin (a copper protein) or cytochrome $c_6$ (a heme protein), which eventually shuttles the electron to Photosystem I.

1.2.2 Photosystem I, a Type I Reaction Center

The overall reaction performed by Photosystem I (PS I) can be summarized as follows:

$$2\text{NADP}^+ + 2\text{H}^+ + 4e^- \rightarrow 2\text{NADPH}$$

The electron density map of trimeric PS I from the cyanobacterium *T. elongatus* has been solved to a resolution of 2.5 Å (25, 26), thereby allowing the architecture of pigments, cofactors and proteins to be accurately modeled. Each cyanobacterial PS I monomer, shown in Figure 1.3 (hereafter known as the PS I complex), is comprised of twelve protein subunits (PsaA to PsaF, PsaI to PsaM and PsaX), 96 chlorophyll $a$ (Chl $a$) molecules, 22 carotenoids, two phylloquinones (PhQ)$^1$, three [4Fe-4S] clusters, four lipids and a number of bound water molecules. The PsaA and PsaB polypeptides (amino acid sequence identity of 45%) assemble as a heterodimer and provide ligands for the majority of the electron transfer cofactors, which are located on both sides of a pseudo-C$_2$ axis of symmetry. Thus, a common overall binding frame exists for the two branches of electron transfer cofactors. The arrangement of the electron transfer cofactors in the PS I

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$^1$ Although *Synechococcus* sp. PCC 7002, *Gleobacter violaceus* and *Cyanidium caldarium* contain menaquinone-4 (2-methyl-3-all-trans-tetraisoprenyl-1,4-naphthalenedione) and *Anacystis nidulans* and *Euglena gracilis* contains 5-OH-phylloquinone (27) I will use the term ‘phylloquinone’ (2-methyl-3-(3,7,11,15-tetramethyl-2-hexadecenyl)-1,4-naphthalenedione) to denote the identity of the quinone in the $A_{1A}$ and $A_{1B}$ sites of PS I.
complex is depicted in Figure 1.4. The individual cofactors are labeled with their respective spectroscopic and structural names. The use of ‘A’ or ‘B’ in the structural name specifies the protein (PsaA or PsaB) that ligates the cofactor. The spectroscopic names therefore refer to the electron transfer pathway in which each cofactor participates. For instance, the cofactor Q_{K}A, also known as A_{1A}, is bound by the PsaA subunit and is on the A-branch of the electron transfer pathway.

Figure 1.5 shows the approximate redox potentials of each of the cofactors together with the kinetics of electron transport between them. Light-induced charge separation is initiated within the six excitonically coupled Chl molecules, which form the photoactive core of the PS I complex; Chl a, ligated by PsaB and Chl a’, the 13^2 epimer of Chl a ligated by PsaA that comprise P_{700} (28), and four Chl a molecules that comprise A_{A}, A_{B}, A_{0A}, and A_{0B}. When light is absorbed by any of the 90 antenna Chl molecules (29), the excited state migrates to the primary donor Chls, initiating charge separation and resulting in the formation of what has historically been considered the primary radical pair P_{700}^{+}A_{0A}^{-} (or P_{700}^{+}A_{0B}^{-}). The charge separation event occurs within ~3.7 ps (30, 31), but the details of the process remain obscure. Owing to the relatively large distance between P_{700} and A_{0A} (or P_{700} and A_{0B}), it is generally assumed that the accessory Chl A_{A} (or A_{B}) plays a role as a transient electron transfer intermediate. Recently, a new model (32) based on the analysis of ultrafast transient absorbance data has been proposed in which the initial charge separation occurs between A_{A} and A_{0A} (or A_{B} and A_{0B}), resulting in the primary radical pair A_{A}^{+}A_{0A}^{-} (or A_{B}^{+}A_{0B}^{-}). P_{700} quickly donates an electron to A_{A}^{+} (or A_{B}^{+}), thereby initiating the first step in the stabilization of the charge separated states and resulting in the first readily observable state, P_{700}^{+}A_{0A}^{-} (or P_{700}^{+}A_{0B}^{-}).
Following initial charge separation, the electron is transferred within ~30 ps to \( A_{1A} \) (or \( A_{1B} \)), resulting in the formation of the \( P_{700}^+ A_{1A}^- \) (or \( P_{700}^+ A_{1B}^- \)) radical pair that initiates the process of stabilizing the charge-separated state over longer periods of time (30, 33-37).

The primary acceptor and the phylloquinone molecules in the two branches are collectively referred to as \( A_0 \) and \( A_1 \). This nomenclature was used in the past due to considerable debate regarding the active involvement of the pseudo-\( C_2 \) symmetric \( A \)-and/or \( B \)-side branches in electron transfer. However, there is now a consensus that electron transfer in PS I follows a bidirectional scheme (38-50). When referring to unique spectroscopic characteristics or to electron transport via a particular branch, the cofactors are denoted with the subunit that anchors them. For instance, the quinone involved in electron transport through the \( A \)-branch is named \( A_{1A} \) and that in the \( B \)-branch is named \( A_{1B} \) while they are collectively referred to as \( A_1 \).

Although the involvement of both branches is an accepted paradigm, the protein factors that lead to the initial asymmetry that results in different amounts of \( A \)-branch and \( B \)-branch electron transfer in PS I from different organisms remains to be uncovered. Regardless of the actual pathway, it must be remembered that any consequence of bifurcated electron transfer is rendered moot by the convergence of the \( A \)- and \( B \)-branches at \( F_X \), an interpolypeptide \([4Fe-4S]^{1+,2+}\) cluster. \( F_X \) is ligated by two cysteine residues from PsaA and by two cysteine residues from PsaB. It is one of the most reducing iron-sulfur clusters known in biology (51, 52). The terminal \([4Fe-4S]^{1+,2+}\) clusters \( F_A \) and \( F_B \) are ligated by cysteine residues on PsaC, which is a bacterial-like dicluster ferredoxin bound on the stromal side of PS I (53, 54). The function of the three
Fe/S clusters is to serve as a molecular wire, lengthening the time of charge separation at the expense of a fraction of the transiently stored Gibbs free energy, and vectoring the electron out of the membrane into the soluble phase.

1.3 PARTICIPATION OF QUINONES IN ELECTRON TRANSFER

Quinones play an indispensable role in biology, wherein they function as bound electron transfer cofactors, as gates for controlling one-electron and two-electron chemistry, and as membrane diffusible carriers of electrons and protons between redox proteins. They are present in a wide variety of bioenergetic complexes including the respiratory complexes, *i.e.* the bc$_1$ complex and the b$_6$f complex; certain enzymes; *i.e.* bo$_3$ quinol oxidase, and nitrate reductase; photosystems, *i.e.* PS I and PS II in thylakoid membranes of Cyanobacteria; and photosynthetic reaction centers in membranes of the phyla Proteobacteria (purple non-sulfur bacteria) and Chloroflexi (green non-sulfur bacteria); (it is still not clear if the homodimeric type I reaction centers in members of the phyla Chlorobia (e.g. green sulfur bacteria) and Firmicutes (e.g. heliobacteria) contains a functional quinone). The roles played by the quinones in these diverse biological systems require them to possess a large range of midpoint potentials, which can be as reducing as in the quinone/semiquinone couple in PS I or as oxidizing as in the semiquinione/quinol couple in the Q$_o$ site of the cytochrome bc$_1$ complex. Yet, depending on the number of conjugated rings and the identity of substituent groups, quinones span only a limited range of redox potentials in aqueous and non-aqueous media. It is their interaction with the protein that is the key to extending the dynamic range of working potentials.
Two types of quinoid ring structures are present in photosynthetic reaction centers: those based on a 1,4-napthoquinone ring and those based on a 1,4-benzoquinone ring (Figure 1.6). Phylloquinone belongs to the subclass of quinones composed of a 1,4-naphthoquinone nucleus and a polyprenyl (phytyl) substituent. Plastoquinone-9 belongs to the subclass of quinones composed of a 1,4-benzoquinone nucleus and a polyprenyl (solanyl) substituent. Phylloquinone and plastoquinone-9 are synthesized only in oxygenic phototrophs and are bound to the $A_{1A}/A_{1B}$ and $Q_{A}/Q_{B}$ binding sites in PS I and PS II, respectively. Type II reaction centers are designed such that $Q_{A}$ serves as a bound, one-electron transfer cofactor and $Q_{B}$ serves as a mobile, one electron/two electron (plus two proton) gate. Here, the electron transfer occurs unidirectionally through the A-side cofactors; electron transfer through the B-side cofactors does not occur (55). The possible presence of dielectric asymmetry (56, 57) at the level of the initial cofactors and the requirement for a stable $Q_{B}$ semiquinone could be major factors in determining the directionality in the proteobacterial reaction center. Type I reaction centers are designed to promote (only) one-electron transfers from $P_{700}$ to the $F_{A}/F_{B}$ clusters. In the homodimeric Type I reaction centers of heliobacteria and green sulfur bacteria, the issue of uni- or bi-directional electron transfer is rendered moot by definition. In the heterodimeric Type I reaction centers of plants and cyanobacteria, the symmetry is broken. Any asymmetry in electron transfer through the two branches of chemically equivalent cofactors leading from the primary donor $P_{700}$ to the acceptor $F_{X}$ must be due to the surrounding protein. The larger unanswered question is how the relatively minor differences in local structure govern whether the electron favors the A- or B-branches of cofactors in PS I.
1.4 STRUCTURE OF THE A₁A AND A₁B PHYLLOQUINONE BINDING SITES

Detailed views of the phylloquinone-binding pockets in the A-branch and B-branch (rotated 180° about the C₂ axis of symmetry) of PS I are depicted in Figure 1.7. The three cofactors, A₀ (not shown), A₁, and Fₓ are linked by an intricate network of contacts and hydrogen bonds that bind them to the protein and promote electron transfer (58). M₆₈₈ₚₛₐₐ (M₆₆₈ₚₛₐₜ) is the axial ligand to A₀ₐ (A₀ₜ) and is H-bonded via its backbone oxygen to the side chain oxygen of S₆₉₂ₚₛₐₐ (S₆₇₂ₚₛₐₜ). The side chain oxygen of S₆₉₂ₚₛₐₐ (S₆₇₂ₚₛₐₜ) is also H-bonded to the indole ring nitrogen of W₆₉₇ₚₛₐₐ (W₆₇₇ₚₛₐₜ). This Trp is π-stacked with the phylloquinone A₁ₐ (A₁ₜ), which, in turn, is H-bonded to L₇₂₂ₚₛₐₐ (L₇₀₆ₚₛₐₜ). The backbone oxygen of L₇₂₂ₚₛₐₐ (L₇₀₆ₚₛₐₜ) is H-bonded to the Fₓ binding loop through R₆₉₄ₚₛₐₐ (R₆₇₄ₚₛₐₜ). Sequence alignment indicates that these residues are conserved in different species as well as between PsaA and PsaB (42).

Careful examination of the two binding sites reveals subtle differences despite the high degree of overall symmetry. The most apparent difference is the orientation of the phytanyl tails of the phylloquinones in the A- and B-branches (Figure 1.7). Other differences include the presence of carotenoids with different orientations and configurations in the vicinity of each of the two phylloquinone molecules, the arrangement of bound water molecules near the phylloquinones, the distribution of charged and neutral lipids, and the presence of a Trp contributed by PsaB just below the Fₓ cluster. The two most striking features of the A₁₁ₐ (A₁₁ₜ) quinone binding pocket are the π-stacked arrangement with W₆₉₇ₚₛₐₐ (W₆₇₇ₚₛₐₜ) and the presence of only one hydrogen bond to the protein backbone with L₇₂₂ₚₛₐₐ (L₇₀₆ₚₛₐₜ). The interaction with the Trp residue is assumed to destabilize the negative charge on the semiquinone anion radical, thereby lowering its
redox potential. In contrast, the H-bond withdraws electron density and stabilizes the negative charge on the semiquinone anion radical, thereby raising its redox potential. The question is how these and other factors conspire to confer the appropriate redox potential to the $A_{1A}$ and $A_{1B}$ quinones.

1.5 THERMODYNAMIC AND KINETIC PROPERTIES OF $A_{1A}$ AND $A_{1B}$

Despite the significant advances in the field of computational biochemistry, the exact details of how the protein matrix confers the necessary thermodynamic properties to the redox cofactors remain incompletely understood. The influence of protein is primarily electrostatic, consisting of discrete charges on metal centers, wholly or partially uncompensated charges on amino acid side chains, the summed polarities of the amide bonds in the protein backbone, the polarity of uncharged groups on amino acid side chains, and the presence of H-bonds (which are polar). Calculating the contributions of each of these factors to the net thermodynamic properties of any particular electron transfer cofactor is a difficult undertaking. However, due to a number of fortunate circumstances including the availability of high resolution crystal structures, the use of a brief flash of light to initiate electron transfer, the ability to alter the protein and cofactor composition, and the availability of powerful spectroscopic methods to probe structure-function relationships, PS I is rapidly becoming one of the most appropriate biological models for the study of protein-cofactor interactions.

1.5.1 Energetics of Electron Transfer through the $A_{1A}$ and $A_{1B}$ Phylloquinones

The rate of nonadiabatic electron transfer between two successive redox cofactors is described by the semi-classical Marcus equation, which is given by:
where, $k_{et}$ is the temperature dependent rate, $\Delta G$ is the free energy difference between the donor-acceptor pair, $\lambda$ is the reorganization energy, and $V$ is the electronic tunneling matrix element between the donor and acceptor pair. The exponential term, the Frank Condon factor, defines a parabola. As $-\Delta G$ increases, the rate of electron transfer increases; it attains a maximum value when $-\Delta G = \lambda$; and as $-\Delta G$ increases further, the rate of electron transfer decreases. The pre-exponential term, the electronic coupling element $V$, falls exponentially with edge-to-edge distance between the donor and the acceptor pair. Dutton and Moser (60-64) proposed a simplified empirical version of Eq. (1) for electron transfer in proteins:

$$\log_{10}k_{et} = 15 - 0.6R - 3.1(\Delta G + \lambda)^2 / \lambda \quad (1.2)$$

where, $R$ is the edge-to-edge distance between the donor and acceptor pair. A more complete treatment takes into account the different packing densities, $\rho$, of atoms within the matrix:

$$\log_{10}k_{et} = 13 - (1.2 - 0.8\rho)(R - 3.6) - 3.1(\Delta G + \lambda)^2 / \lambda \quad (1.3)$$

It should be noted that temperature dependence is incorporated into the numerical constants in Eqs. 2 and 3 (60, 62, 64). The edge-to-edge distance between cofactors is supplied by the X-ray crystal structure, and the reorganization energy is usually assumed to be 0.7 eV in proteins (however, see (65) for a discussion of alternative values). The Gibbs free energy change between donor and acceptor pairs, however, must either be calculated or determined experimentally.
1.5.2 Theoretical and Experimental Determination of Redox Potentials of $A_{1A}$ and $A_{1B}$

The midpoint potentials of $A_{1A}$ and $A_{1B}$ are difficult to assess, and therefore the range of estimated values are reported in literature. The direct titration of the phylloquinone/phyllosemiquinone couple in the $A_{1A}$ and $A_{1B}$ sites are complicated not only by the fact that the potentials are very highly reducing but also because the midpoint potential of the second electron reduction step (accompanied by protonation) to the hydroquinone is higher (i.e. more oxidizing) than that of the first electron reduction step to the semiquinone. As a consequence, measurement of the first electron reduction of isolated quinones must be carried out in a non-potic solvent, else protonation will open a channel for the two-electron phylloquinone/phyllohydroquinone redox couple. Most determinations of the midpoint potentials of $A_{1A}$ and $A_{1B}$ are carried out indirectly. Before it was known that two phylloquinones were redox-active in PS I, Vos and Van Gorkom (66) described the influence of an electric field on the individual electron transfer rate constants and compared the experimental curves of PS I electroluminescence with those from simulations. A midpoint potential of $-810$ mV was estimated for ‘$A_1$’.

Iwaki et.al. (67) incorporated the concept of solvent acceptor number (67, 68) to derive an empirical formula that relates the midpoint potential of a quinone in the ‘$A_1$’ site of PS I ($E_m$) to that in dimethyl formamide (DMF) ($E_{1/2}$):

$$ E_m + 700 \text{ mV} = 0.69 \left( E_{1/2} + 387 \text{ mV} \right) \quad (1.4) $$

By employing a value of $-497$ mV (vs. NHE) for the midpoint potential of 2,3-dimethylnaphthoquinone (a molecule structurally similar to phylloquinone) in DMF, a midpoint potential of $-778$ mV was estimated for phylloquinone in the ‘$A_1$’ site. The shift of $-281$ mV was attributed to the influence of the protein environment. The major insight
gained from this work was that to attain such low midpoint potentials, the ‘A1’ binding site must be in a low dielectric environment so as to destabilize the phylloquinone radical ion relative to the ground state. It has been pointed out (69, 70) that the measurement of the midpoint potentials of the cited reference quinone, 2,3-dimethylnaphthoquinone, did not incorporate a correction for the liquid junction potential between water and DMF. Liquid junction potential is a result of the difference in solvation energy between water and DMF that causes an unequal distribution of anions and cations between the two phases.

An indirect approach to estimate the midpoint potential of ‘A1’ involves mathematically modelling the forward and backward electron transfer reactions in PS I complexes that contain a native and reduced set of electron acceptors. By using a quasi-equilibrium approach and rate constants derived from P700-F_A/F_B complexes, P700-F_X cores (stripped of PsaC) and P700-A1 cores (stripped of F_X), Shinkarev et al. (71) calculated a 205 mV free energy gap between ‘A1’ and F_A. If a consensus midpoint potential –530 mV is assigned to F_A, the midpoint potential of ‘A1’ would be –735 mV. It should be noted that the above studies were carried out before it was known that the phylloquinones on the two independent branches were active in electron transfer. With the knowledge of the 2.5 Å X-ray crystal structure of PS I, Santabarbara et al. (49) employed a similar quasi-equilibrium approach to calculate the electron transfer reactions in PS I, but with the added provisions of incorporating both a bidirectional scheme and Marcus theory in the model. The midpoint potential of F_X was calculated to be –680 mV, which is within 10 mV of the consensus midpoint potential of –688 mV determined experimentally. The midpoint potentials of A1B and A1A were calculated to be –696 mV and –671 mV,
respectively. In this formulation, electron transfer from A\textsubscript{1A} to F\textsubscript{X} would be endothermic by 9 mV and electron transfer from A\textsubscript{1B} to F\textsubscript{X} would be exothermic by 16 mV. Ishikita and Knapp (72) had earlier calculated the midpoint potentials of the A\textsubscript{1A} and A\textsubscript{1B} quinones by evaluating the electrostatic energies from the solution of the Poisson-Boltzmann equation. Usually the incorporation of water molecules leads to no improvement in the calculated values because their influence is considered implicitly in the high dielectric constant used for the modeled cavities (72). In the case of PS I, the A\textsubscript{1A} and A\textsubscript{1B} binding sites are both unusually close to a cluster of 5 and 6 H-bonded water molecules, respectively. When the waters were not included in the calculation, the midpoint potentials of A\textsubscript{1A} and A\textsubscript{1B} were calculated to be $-438$ mV and $-604$ mV, and when all of the waters are included, the values were $-639$ mV and $-776$ mV, respectively. When (only) water-37 was included in the calculation, the midpoint potentials of A\textsubscript{1A} and A\textsubscript{1B} were $-531$ mV and $-686$ mV, respectively. Ishikita et al. (72) preferred to use midpoint potentials calculated for A\textsubscript{1A} and A\textsubscript{1B} when (only) water-37 was included because these calculated values most closely matched the experimental values. Probably the most valuable result of this study is that the midpoint potential of A\textsubscript{1B} was found to be 137 mV to 166 mV more reducing than A\textsubscript{1A} (the value depends on the number of water molecules included in the calculation), a much larger difference than the 25 mV difference calculated in ref. (47). More recently, Ptushenko et al. (70) incorporated two novel elements in carrying out calculations to obtain the midpoint potentials of all of the electron transfer cofactors in PS I. The first was the use of the optical dielectric permittivity to calculate the pre-existing field induced by permanent charges. The second was the use of a heterogeneous distribution of static dielectric constants, which were
derived from prior electrometric measurements (73, 74). The PS I complex was divided into five planar layers, each with discrete dielectric constants, parallel to the membrane. This approach accounts for the difference in the intra-protein electric field induced by permanent charges. Taking into account the correction for the liquid junction potential, the midpoint potentials of A₁B and A₁A were calculated to be –844 mV and –671 mV, a difference of 173 mV. The operating midpoint potential of Fₓ was calculated to be –585 mV while the equilibrium midpoint potential of Fₓ was calculated to be –654 mV. Using these values, electron transfer from A₁B to Fₓ would be strongly favorable and electron transfer from A₁A to Fₓ would be (barely) favorable. It should be noted that the calculated values for the equilibrium midpoint potential of Fₓ are ~35 mV more oxidizing than the consensus value determined experimentally. The 173 mV difference in the calculated midpoint potentials of A₁A of A₁B is well in line with the 137 mV to 166 mV difference calculated by Ishikita et al. (72). What is particularly noteworthy about this work is that the 173 mV energy gap between A₁A and A₁B corresponds to a 28-fold faster rate of electron transfer from A₁B to Fₓ than from A₁A to Fₓ. This agrees quite well with the experimentally-determined ratio of the rates of fast to slow electron transfer in Synechocystis sp. PCC 6803 attributed to the A₁B to Fₓ and A₁A to Fₓ electron transfer steps, respectively (75).

In summary, all of the theoretical and experimental methodologies are in agreement that the A₁B to Fₓ electron transfer step is thermodynamically favorable. There is more uncertainty about the A₁A to Fₓ electron transfer step, which in most determinations is mildly unfavorable. The consequence of an uphill electron transfer step from A₁A to Fₓ is that as the temperature is lowered, the amount of thermal energy available ultimately
becomes insufficient to span the Gibbs free energy gap. At this critical temperature, $A_{1A}^-$ to $F_X$ electron transfer would be expected to cease while $A_{1B}^-$ to $F_X$ electron transfer would remain unaffected. Schlodder et al. (65) showed that below the glass transition temperature of a water/glycerol mixture, illumination of PS I complexes from *T. elongatus* results in irreversible charge separation between $P_{700}$ and $F_A/F_B$ in ~35% of PS I complexes, whereas in ~45% of the complexes, the electron cycles between $P_{700}$ and $A_1$, and in the remaining ~20%, it cycles between $P_{700}$ and $F_X$. Heathcote and colleagues (47) proposed that the irreversible fraction represents the thermodynamically favorable electron transfer step from $A_{1B}^-$ to $F_X$ and that the reversible fraction is a consequence of the thermodynamically unfavorable step from $A_{1A}^-$ to $F_X$. This correlates quite well with experimental measurements, which show a thermally activated slow kinetic phase of electron transfer from $A_{1A}$ to $F_X$ with a lifetime of ~200 ns and an activation energy larger than a (nearly) non-activated fast kinetic phase of electron transfer from $A_{1B}^-$ to $F_X$ with a lifetime of ~10 ns (75).

1.6 SUMMARY

In this chapter, a concise introduction to the protein complexes that participate in the light-dependent reactions of photosynthesis was presented, with emphasis on PS I. The phylloquinone molecule is an integral part of PS I and functions as intermediate electron acceptor. Of the many interactions that exist in the binding pocket, the H-bond formed between the phylloquinone and the protein backbone is thought to play a key role in determining its functional properties. The following chapters deal with the characterization of the H-bond. In Chapter 2, the number of H-bonds formed by the
quinone in its functionally active state is probed by transient electron paramagnetic resonance spectroscopy. Determination of spin density distribution around the quinone ring allows us to assign a single H-bond to the phylloquinone. In Chapter 3, the functional role of the H-bond is sought. It is determined that the H-bond prevents the double reduction/protonation of the phylloquinone under periods of high illumination. It is shown that in the absence of the H-bond, double reduction renders the phylloquinone incapable of participating in electron transfer. Chapter 4 deals with the detailed kinetic characterization of a PS I variant with a significantly weak H-bond. The weakening of H-bond is shown to have a profound effect on the redox potential of the quinone as well as the reorganization energy of the binding pocket. In Chapter 5, I probe the difference in the H-bond strength between the quinone in the two branches of PS I as a possible source of their different redox properties. Based on coupling constants derived by high-resolution hyperfine sublevel correlation spectroscopy, I conclude that the variation in the H-bond strengths between the two binding pockets is not significant enough to account for the estimated differences in midpoint potential. This dissertation presents a comprehensive study of the H-bond in the phylloquinone binding pocket of PS I and describes the number and strength of H-bonds formed by the quinone and its functional significance.
1.7 REFERENCES


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1.8 FIGURE LEGENDS

**Figure 1.1** A schematic representation of the arrangement of protein complexes in the thylakoid membrane of cyanobacteria. PS II, Photosystem II (PDB ID 1S5L); PS I, Photosystem I (PDB ID 1JB0); Cyt b₆f, the cytochrome complex (PDB ID 2ZT9); PC, plastocyanin (PDB ID 1BXV); FD, ferredoxin (PDB ID 2CJO); FNR, ferredoxin–NADP⁺ oxidoreductase (PDB ID 1QUE).

**Figure 1.2** The Z scheme showing the pathway of oxygenic photosynthesis. The vertical axis indicates approximate equilibrium midpoint potentials (Eₘ) of the electron transport cofactors. The abbreviations used are as follows; Mn, tetranuclear manganese cluster; Tyr tyrosine-161 on PsbA protein; P₆₈₀, primary donor of Photosystem II; P₆₈₀*, excited electronic state of P₆₈₀; Pheo, pheophytin; QA, the bound plastoquinone; QB, the mobile plastoquinone; PQ, a pool of mobile plastoquinone molecules; the brown box is a protein complex containing cytochrome b₆ (Cyt b₆), iron–sulfur protein (FeS) and cytochrome f (Cyt f); PC, plastocyanin; P₇₀₀, primary donor of Photosystem I; P₇₀₀*, excited electronic state of P₇₀₀; A₀, chlorophyll a molecule; A₁, phylloquinone; Fₓ, Fₐ, Fₜ, iron sulfur clusters; FD, ferredoxin; FNR, ferredoxin–NADP⁺ oxidoreductase; NADP⁺, nicotinamide–adenine dinucleotide phosphate.

**Figure 1.3** Structural model of monomeric PS I at 2.5 Å resolution. The twelve proteins (PsaA-PsaF, Psal-PsaM and PsaX) that comprise the PS I complex are shown in different colors. The three major subunits (PsaA-PsaC) that anchor the electron transfer cofactors are indicated. The two terminal electron transport cofactors anchored by PsaC on the
outer side of the membrane are depicted.

**Figure 1.4** Arrangement of electron transport cofactors in PS I. The two branches are denoted as the A- and the B- branch. The spectroscopic names and the structural names (in parantheses) are indicated near each cofactor. The use of ‘A’ or ‘B’ in the structural names refers to the protein subunit (PsaA or PsaB) that ligates the cofactor. The use of ‘A’ or ‘B’ in the spectroscopic names refers to the electron transfer pathway in which the cofactor participates.

**Figure 1.5** The components of the PS I reaction center depicted with redox potential on the y-axis together with rate of electron transfer.

**Figure 1.6** Structure of isoprenoid quinone rings. Phylloquinone is an analog of napthoquinone with a phytol substituent while plastoquinone-9 is an analog of benzoquinone with a solanyl substituent.

**Figure 1.7** The phylloquinone binding pocket from the 2.5 Å X-ray crystal structure (3) (PDB ID 1JB0) in the A- (top) and the B-branch rotated 180° about the C_2 axis of symmetry (bottom). The intricate network of H-bonding along with the π–stacked W697_{PsaA}/W677_{PsaB} and the H-bonded L722_{PsaA}/L706_{PsaB} residues is shown.
Figure 1.4
Figure 1.5
Figure 1.6

Phylloquinone
C_{31}H_{46}O_{2}
M: 450.35 g/mol

Plastoquinone 9
C_{53}H_{80}O_{2}
M: 748.64 g/mol
Figure 1.7
Chapter 2

Determination of the Number of H-bonds Between the Protein and the $A_{1A}$ Phyllosemiquinone

[Published in part as an article titled “single-sided hydrogen bonding to the quinone cofactor in Photosystem I probed by selective $^{13}$C-labeled naphthoquinones and transient EPR” by Irina Karyagina, John Golbeck, Nithya Srinivasan, Dietmar Stehlik and Herbert Zimermann in *Applied Magnetic Resonance*, 2006, 30, 287 – 310]
2.1 ABSTRACT

The hydrogen (H-) bond between the carbonyl group of the phylloquinone and the protein backbone is one of the many significant protein-cofactor interactions that exist within the phylloquinone binding pocket in Photosystem I (PS I). Although the X-ray crystal structure indicates that in its ground state, the quinone forms one H-bond with the protein backbone, it has been suggested that in its semiquinone anionic form, the quinone may form a second H-bond. In this chapter, the spin density distribution at the nuclei of the carbonyl groups of the quinone molecule is estimated to gain insight into the H-bonding scheme. The spin density at the ring carbon positions can be accessed experimentally via EPR-determined hyperfine coupling tensor elements of quinones with selective $^{13}$C isotope labels in one of the two carbonyl groups. Complete hyperfine coupling tensor data are presented here for each of the quinone with $^{13}$C label in its functional charge-separated state in cyanobacterial PS I complexes. A highly asymmetric H-bonding scheme with a single dominant H-bond to one of the carbonyl groups of the $A_{1A}$ quinone is confirmed. A comparison with the quinone binding sites of other protein-cofactor systems containing more complex H-bonding schemes reveals the uniqueness of the PS I site. The experimental agrees well with calculations based on density function theory (DFT), with a minor discrepancy for the high spin density $^{13}$C position associated with the H-bonded carbonyl group. The dominant hyperfine coupling component (and spin density) is underestimated in the DFT calculations, not only for the high asymmetry case in PS I, but also for other quinone binding sites. The consequences and relevance for biological functions are discussed.
2.2 INTRODUCTION

2.2.1 Strength and Number of H-Bonds to the A1A Phylloquinone

The single hydrogen (H-) bond between the protein and the phylloquinone depicted in the 2.5 Å X-ray crystal structure of cyanobacterial PS I spans a distance of 2.69 Å from the backbone nitrogen of L722PsaA to the carbonyl O at C4 of the phylloquinone ring (Figure 1.7). Similarly, a H-bond spans 2.75 Å from the backbone nitrogen of L706PsaB to the carbonyl O at C4 of the phylloquinone ring in the A1B site. The O atoms of S692PsaA and S672PsaB are too distant at 3.28 Å and 3.36 Å and at too acute an angle to provide H-bonds to the carbonyl O at C1 of the phylloquinone rings in the A1A and A1B sites, respectively. However, the X-ray crystal structure depicts only the ground state. Electron nuclear double resonance (ENDOR) studies of photoaccumulated PS I have been interpreted to suggest the formation of a second H-bond to the functionally active phyllosemiquinone anions in the A1A and A1B sites (1-3). Could there be movement of the phylloquinone so that a second H-bond is formed after reduction? To answer this question, the electron spin density around the phyllosemiquinone ring was mapped.

The average spin density for a symmetry-related ring position in a semiquinone is higher in a protic (i.e. isopropanol) than in an aprotic (i.e. 2-methylytetrahydrofuran) solvent. This is because in protic solvents, partial electron withdrawal by the two identical H-bonds formed by the quinone alters the electron spin density at the C1 and C4 carbonyl O atoms equally. In the A1A and A1B sites of PS I, the presence of a single H-bond to the phylloquinone would result in a distinctively different pattern of electron spin densities than in protic solution. A straightforward valence bond model (Figure 2.1) can be
invoked to correlate asymmetric H-bonding to the two carbonyl groups with the observed asymmetric and alternating spin density distribution around the naphthosemiquinone ring. The highest spin and charge density in a phylloquinone radical anion is expected at the two carbonyl groups because of the high electronegativity of the oxygen atoms. Formally, the charge is placed on the oxygen, while the unpaired electron is placed on the carbon. When a single H-bond is present at O₄ its electron withdrawing effect is expected to increase the charge on O₄ and the spin density at C₄ compared to O₁ and C₁, respectively. In agreement with the possible resonance structures, the spin density will shift to higher values at the position C₄, and at the alternating positions C₂, C₉, and O₁. At the odd ring positions, C₁, C₃, and C₁₀, the spin density will shift to lower values. The presence of the methyl group at C₂, a high spin center, results in pronounced electron-nuclear hyperfine couplings and gives rise to a quartet with relative intensities 1:3:3:1 centered near the g yy component of the electronic g-tensor of the quinone. As a result, the axial hyperfine coupling from the methyl group is partially resolved and can be seen in X-, Q- and W-band spectra of photoaccumulated Α₁⁻ (4). The dominant hyperfine axis points in the direction of the g yy tensor axis; hence, the splittings are observed largely in the midfield region. Evidence for a relatively large hyperfine coupling can be observed by transient EPR at X-band as the notable feature at 345.2 mT and the minor features at 344.7 mT and 346.0 mT (Figure 2.2). The principal values of this hyperfine coupling, measured in a static photoaccumulated phyllosemiquinone radical by CW ENDOR spectroscopy (1) were found to be a ll = 12.8 MHz and a ⊥ = 9.1 MHz, and in a flash induced spin-correlated radical pair by transient ENDOR were found to be a ll = 12.3 MHz and a ⊥ = 8.8 MHz (5). The two values are similar, but not identical, and the difference may be due to the
electrostatic field generated by \( F_A^- \), \( F_B^- \) and \( F_X^- \) in photoaccumulated PS I (all of which are in the ground state in the transient EPR measurement). These values are considerably larger than the \( a_{\parallel} = 10.0 \) MHz and \( a_{\perp} = 6.8 \) MHz measured for the phylloquinone radical \textit{in vitro} in alkaline ethanol (1). They are also at least twice as large as the values for the corresponding ring position of \( Q_A \) in the pbRC consistent with a more asymmetric H-bonding pattern in PS I. From this data, the H-bonding pattern of the phyllosemiquinone radical would agree best with a single dominant backbone H-bond to the oxidized phylloquinone found in the X-ray structure. However, to confirm the occurrence and significance of a single H-bond, the spin density distribution in other relevant ring positions needed to be measured by introducing isotopically-labeled quinones into the \( A_{1A} \) site for the purpose of mapping the electron spin distribution around the naphthoquinone ring.

2.2.2 Incorporation of Artificial Quinones in the \( A_1 \) Binding Pocket

The phylloquinones are tightly bound in PS I, yet perdeuterated phylloquinone can exchange with native phylloquinone, residing in the \( A_{1A} \) (and likely \( A_{1B} \)) site(s) with an orientation identical to wild-type PS I (6). The exchange is efficient at elevated temperatures with an excess of isotopically labeled phylloquinone; although, exchange is possible at 4°C over extended time periods (7). There is no indication that phylloquinone self-exchange occurs \textit{in vivo} or that phylloquinone exchange would even have physiological significance. No non-native quinone has yet been identified that will exchange naturally with phylloquinone, but this is likely the consequence of natural selection, which has provided for a very high binding constant for phylloquinone (relative to plastoquinone-9, which is the only other abundant quinone in photosynthetic
membranes). Two methods have been developed for introducing foreign quinones into PS I. The native phylloquinone can be removed using organic solvents (8-13), and native (14, 15) or non-native quinones (15-20) can be introduced in vitro. Alternately, the genetic interruption of the phylloquinone biosynthetic pathway allows a loosely bound plastoquinone-9 to be incorporated into the A₁A and A₁B sites, which can then be displaced either in vivo or in vitro with native or non-native naphthoquinones (21-24). The latter method is preferred since it is less harsh and does not affect the structure of the binding pocket or the pigment composition of the PS I complex.

Biosynthesis of phylloquinone in cyanobacteria and higher plants proceeds by a pathway similar to the menaquinone biosynthesis pathway of Escherichia coli, a gram-negative bacterium (25). Menaquinone synthesis occurs via eight enzymatic steps as shown in Figure 2.3. Ring cyclization, which converts the one ring benzoquinone derivative to a two ring naphthoquinone derivative, occurs through the activation of 1,4-hydroxynapthoyl-CoA synthase (MenB) (26).

Targeted inactivation of the menB gene in Synechocystis sp. PCC 6803 (21, 27) and in Synechococcus sp. PCC 7002 (28) have been carried out by introducing an antibiotic cartridge into the coding region of these genes. In both cases, phylloquinone synthesis was prevented, demonstrating that these genes are essential for its biosynthesis in cyanobacteria. Quite unexpectedly, the cyanobacterial menB null mutants grew photoautotrophically, albeit at a slower rate, in the absence of phylloquinone (21, 27). It was thought highly unlikely that an electron could span the distance between A₀ and Fₓ in a realistic period of time at room temperature, so a substitute for phylloquinone was sought, and found, as a UV-absorbing cofactor (21, 29). This moiety, which was present
only in the null mutant, was identified as plastoquinone-9 based on its UV-visible absorption spectrum and mass. Based on this study, it can be stated for certain that in cyanobacteria, i) the menB gene is essential for phylloquinone biosynthesis, ii) plastoquinone-9 occupies the A_{1A} (and likely the A_{1B}) binding site(s) when phylloquinone biosynthesis is interrupted, iii) plastoquinone-9 is oriented similar to phylloquinone at the same distance from P_{700} and iv) plastoquinone-9 is capable of supporting forward electron transfer at room and low temperatures in spite of its considerably higher midpoint potential.

Plastoquinone-9 can be displaced from the A_{1A} and A_{1B} sites of PS I by supplementing the growth medium of the menB null mutant or by incubating isolated PS I complexes with various naphthoquinones (24). The displacement of plastoquinone-9 is so efficient that the reconstitution of exogenous naphthoquinones is quantitative. 2-methyl-1,4-naphthoquinone is an ideal candidate for incorporation into the A_{1A} (and likely A_{1B}) sites of PS I isolated from the menB null mutant. The transient EPR polarization pattern shows that 2-methyl-1,4-naphthoquinone head group is present in the A_{1A} site with the same orientation as the native phylloquinone head group. The 1:3:3:1 hyperfine splittings show that the methyl group is located meta to the H-bond, which is the same high spin position occupied by the methyl group of phylloquinone in the wild-type. The only significant difference is that the –CH_{3} splittings are better resolved due to the reduced inhomogeneous linewidth resulting from the absence of the unresolved hyperfine couplings from the methylene group of the phytol tail (Figure 2.2).
2.2.3 Spin Density Distribution Around the Quinone Ring

2-methyl-1,4-naphthoquinone is the perfect choice for the purpose of mapping the electron spin distribution around the naphthoquinone ring because the lack of the phytanyl tail reduces the inhomogeneous linewidth broadening, making the hyperfine coupling tensor more pronounced than in phylloquinone (Figure 2.2). The primary motivation for this work is that it allowed for an accurate test for the number of H-bonds to the phylloquinone in the A_{1A} site. A secondary motivation was that predictions of electron spin density distribution in the phyllosemiquinone radical based on DFT have become available, and the experimental verification of the theoretical calculations is therefore timely. Even though the highest spin density would be expected on the carbonyl C and O atoms, the hyperfine tensor values of isotope-labeled ^{17}O nuclei are, unfortunately, relatively insensitive to H-bond induced changes in the \( \pi \) electron spin density of aromatic rings \((30)\). Hence, when ^{17}O-substituted 2-methyl-1,4-naphthoquinone was exchanged into PS I from the menB variant, the small asymmetry simply confirmed that the ^{17}O hyperfine coupling tensor is an unreliable marker for the presence of the H-bond. The next best choices are the carbonyl C_4 to measure a proposed high spin density position and carbonyl C_1 to measure a proposed low spin density position. By incorporating 2-methyl-1,4-naphthoquinone labeled with ^{13}C at C_1 and C_4, ^{13}C hyperfine coupling parameters and spin densities at these carbon ring positions could be determined.

Here, a previous study \((31)\) with only one selective ^{13}C label in the high spin density ring position 4 of 2-methyl-1,4-naphthoquinone is extended to the quasi-symmetric low spin density ring position 1. While the expected strong asymmetry for the ^{13}C hyperfine
coupling tensors could be established by comparison of the $^{13}\text{C}_4$ hyperfine coupling tensor in the A$_{1A}$ site and in protic isopropanol solution, quantitative comparison with a set of independent DFT calculations requires data for the selectively $^{13}\text{C}$ labeled ring position 1 as well. Two additional arguments motivate the extra effort: the asymmetry in the $^{13}\text{C}$ hyperfine coupling tensors concerns the most sensitive positions in the molecule, and the quinone binding site in PS I represent thus far a unique case of a quinone cofactor with a single H-bond. The model of a predominant H-bond to the carbonyl group at ring position 4 is confirmed, in particular, for the functional, transient charge-separated $P_{700}^{\cdot+}$ naphthoquinone$^{\cdot-}$ radical-ion pair state in PS I. The overall comparison to the results of the DFT calculations was found to be satisfactory.

2.3 MATERIAL AND METHODS

2.3.1 Synthesis of Isotope Labeled 2-methyl-1-$^{13}\text{C}$-1,4-naphthoquinone

The synthesis follows, to some extent, the general reaction sequence described earlier for 2-methyl-4-$^{13}\text{C}$-1,4-naphthoquinone (31). Major modifications were introduced in the steps from tetralone to 2-methyl-naphthoquinone (see Figure 2.4).

4-phenyl-2-butanole (I) was converted to 2-bromo-4-phenyl-butane (II) with PBr$_3$. The corresponding Grignard reagent (III) was carboxylated with $^{13}\text{CO}_2$ (from BaCO$_3$, -30° C, ether) to 1-$^{13}\text{C}$-2-methyl-4-phenyl-butanoic acid (IV). The cyclization to 1-$^{13}\text{C}$-2-methyl-1-tetralone (V) was accomplished with methane-sulphonic acid instead of using the polyphosphoric acid cyclization reaction of the acid chloride (32). The usual Clemmensen reduction to the tetraline, followed by dehydrogenation and oxidation was
avoided by introducing the one step dehydrogenation and oxidation of the 1-$^{13}$C-2-methyl-1-tetralone to 1-$^{13}$C-2-methyl-1.4-naphthoquinone with CrO$_3$/CH$_3$COOH/ H$_2$SO$_4$:

**Oxidation solution:** 14g CrO$_3$ dissolved in 9 ml conc. CH$_3$COOH was diluted with 14ml H$_2$O and acidified with 1.4 ml conc. H$_2$SO$_4$.

**Oxidation:** 3g (V) was mixed with 14ml conc. CH$_3$COOH. To the stirred solution the chromium reagent was dropwise added at 5°C within 30 min. After the addition the mixture was stirred at 50°C for two hours, warmed up to room temperature and diluted with water in an ice bath until crystals appeared. Yield = 1.5g (VI). TLC, Silica, hexane/ether 8:2, one spot. Fp=103.5-104.5°C.

The precise labeling of 1-$^{13}$C-2-methyl-1.4-naphthoquinone, the percentage of $^{13}$C, as well as the chemical purity were checked by $^{13}$C and $^1$H-NMR and mass-spectrometry, and were found to be greater than 99%.

2.3.2 Preparation of PS I with $^{13}$C Labeled 2-methyl-1,4-naphthoquinone

A hundred fold molar excess of quinone (10 µl of 0.034 M solution of quinone in ethanol) was added to 150 µl of PS I sample in buffer (Tris buffer pH 8.3 containing 0.2 % Triton X-100) isolated from menB mutant cells of Synechocystis sp. PCC 6803 (21). Incubation was carried out at room temperature (2-4 h) with intensive stirring. The PS I complexes were washed twice with 150 µl buffer to remove the excess quinone. Prior to transient EPR measurements, 10 µl of 1 M sodium ascorbate solution was added as an external electron donor. The sample was dark-adapted and stored frozen darkness.

2.3.3 Transient EPR Spectroscopy

Transient EPR spectroscopy can be performed with various microwave frequencies. For X-band transient EPR experiments, a Bruker ER046 XK-T microwave bridge was
equipped with a Flexline dielectric resonator and an Oxford liquid helium gas-flow cryostat. The loaded $Q$-value for this dielectric ring resonator was about 3000, equivalent to a rise time of $\tau_r = Q/(2\pi\nu_{mw}) \approx 50$ ns. At X-band, the hyperfine coupling tensors dominate the spectrum and such experiments give useful insight into the strength of the H-bond. Q-band (35 GHz) transient EPR spectra of the samples were measured with the same set-up except that a Bruker ER 056 QMV microwave bridge equipped with a home built cylindrical resonator with light access was used. At Q-band, the $g$-tensor of the radical dominate the spectrum and hence, information about the relative orientation of the species involved in the radical pair formation can be obtained. The samples were illuminated using a Spectra Physics Nd-YAG/MOPO laser system with a pulse width of ~10 ns, operating at 10 Hz repetition rate, at the 2$\text{nd}$ harmonic (532 nm). All experiments were performed either at a temperature of 80 K. The available microwave power of about 200 mW was reduced by 30 dB in order to the obtain transients free from nutation distortion. See (33) for a detailed description of transient EPR spectroscopy as applied to Photosystem I.

2.4 RESULTS

2.4.1 Orientation of the Naphthoquinones in the $A_1\alpha$ Binding Site

Figure 2.5 presents Q-band spin polarized transient spectra of the radical pair state $P_{700}^\cdot + A_1^- \cdot$ in PS I complexes with (a) native phylloquinone (wild-type), (b) 2-methyl-1,4-naphthoquinone, and labeled 2-methyl-1,4-naphthoquinone with the $^{13}$C label in either ring position 1 (c) or 4 (d) in the $A_1$ site. Neglecting for the moment the differences due to the methyl proton and the $^{13}$C spin related hyperfine splittings, the transient spectra
exhibit the same overall polarization pattern (E/A/A/E/A), showing emissive (E) and absorptive (A) polarization extremes from the low to high field side (Figure 2.5). This pattern is most sensitive to the orientation of the quinone molecular axes with respect to the C_2 axis of symmetry (see (33) for review) and can be treated as a signature of the quinone orientation in the A_{1A} site for the charge separated state. The geometric parameters obtained from simulation of the spectra agree well with the orientation determined by the X-ray structure for the ground state (PDB ID: 1JB0). The polarization pattern in Figure 2.5 confirms that within experimental accuracy, all three substituted 2-methyl-1,4-naphthoquinones occupy the A_{1A} site with the same orientation as does native phylloquinone in wild-type PS I. In all the above cases, conclusions can be made only about the A_{1A} binding pocket since transient EPR can detect only the A-branch electron transfer.

4.4.2 Hyperfine Induced Spectral Differences

In spite of the similar polarization pattern, the spectra in Figure 2.5 differ significantly with respect to their partially resolved hyperfine structure. The methyl proton hyperfine splitting (1:3:3:1 multiplet) is better resolved for 2-methyl-1,4-naphthoquinone than for native phylloquinone because the former lacks broadening resulting from the interaction with the phytol tail protons (34). Most significantly, the additional ^13C hyperfine splitting over the whole spectral range is obvious in spectrum (c) and (d) of Figure 2.5.

For a more detailed comparison of the hyperfine-induced differences, the samples containing the three 2-methyl-1,4-naphthoquinones (unlabeled, 1-^13C and 4-^13C labeled) in the A_{1} site are analyzed at X-band frequencies. The transient spin polarized P_{700}{^+}A_{1}{^-} spectra recorded at X-band are shown in Figure 2.6. The following qualitative features
are apparent; (a) the spectra of both $^{13}$C labeled naphthoquinones extend over a wider field range than does unlabeled naphthoquinone. The spectrum of the 4-$^{13}$C sample (broken line) extends further than that of the 1-$^{13}$C sample (dotted line), especially in the high-field region of the spectrum. This is mostly determined by the out-of-plane $A_{zz}$ component of the $^{13}$C hyperfine coupling tensor in the high-field region and the in-plane components in the central and low-field region. (b) The spectral patterns differ for the two $^{13}$C labeled naphthoquinones, reflecting the expected asymmetric spin density distribution that arises from the asymmetric H-bond, and (c) the field positions of the absorptive and emissive polarization maxima vary considerably over the whole spectrum between the three samples. This indicates that all components of the $^{13}$C hyperfine coupling tensor are involved in the observed spectral differences.

The spectral differences introduced by the selectively $^{13}$C labeled quinone carbonyl groups are more pronounced in PS I than in the other protein-cofactor systems with well characterized quinone binding sites, in particular, the purple bacterial reaction center (pbRC) (35) and quinol oxidase (bo$_3$-QOX) (36). Note that the signs of the hyperfine coupling tensor elements cannot be determined from our experimental spectra. However, they can be assigned by analogy to cases where $^{13}$C hyperfine coupling tensors have been determined (e.g. Triple ENDOR, see (35)). Moreover, the signs follow from basic molecular model considerations as implemented for the calculated $^{13}$C hyperfine coupling tensor elements.

2.4.3 $^{13}$C Hyperfine Tensor Parameters from Spectral Simulations

In order to evaluate the $^{13}$C hyperfine coupling tensor elements, the transient spectra were simulated using the well established (33) correlated radical pair (CRP) concept. A large
number of magnetic interaction and structural parameters are required for the spectral simulation. Fortunately, nearly all parameters are known from independent experimental data and are listed in Table 2.1. Convincing spectral simulations have been achieved for the P_{700}^{++}A_{1}^{-} state of wild-type PS I as well as with 2-methyl-1,4-naphthoquinone substituted into the A_{1A} site (34). It is convenient to choose the latter spectrum as a reference.

The influence of each individual $^{13}$C hyperfine tensor component can be judged best by varying each component separately. Based on previous studies, it is expected that the hyperfine coupling tensor of a labeled $^{13}$C in the aromatic ring positions of high spin density is (nearly) axially symmetric. The largest (positive) parallel component, $A_{zz}$ is associated with the out-of-naphthoquinone-plane axis z.

Figure 2.7 depicts the simulations in which the $A_{zz}$ component is systematically varied. Equal values are used for the $A_{xx}$ and $A_{yy}$ (negative) components, however, different values (see Figure legend for details) are used for the spectra corresponding to the two labeled quinones to account for the different hyperfine splittings observed in the mid- and low-field region. Experimental spectra of 4-$^{13}$C labeled 2-methyl-1,4-naphthoquinone in and 1-$^{13}$C labeled 2-methyl-1,4-naphthoquinone are included for comparison. Large $A_{zz}$ values are required for a good agreement between experimental and simulated spectra for 2-methyl-4-$^{13}$C-1,4-naphthoquinone while small $A_{zz}$ values are appropriate for 2-methyl-1-$^{13}$C-1,4-naphthoquinone. Variations in $A_{zz}$ produce the most prominent changes in the peak/edge/shoulder appearance of the broad up-field emissive feature. However, smaller peak shifts occur also over the entire spectrum. For 2-methyl-1-$^{13}$C-1,4-naphthoquinone, the fairly large perpendicular components ($A_{xx} = A_{yy}$ about 15 MHz, required to simulate
the low-field region) causes negligible inward shift of the up-field edge below $A_{zz} = 8$ MHz.

Figure 2.8 presents simulation results when the in-plane hyperfine coupling tensor components, $A_{xx}$ and $A_{yy}$ are varied in the range of 15 MHz. When one component is varied, the other is maintained at a fixed value of 15 MHz. The $A_{zz}$ value is fixed at 6 MHz. The variation of the in-plane components produce a small but systematic peak shifts. As expected for the characteristic quinone g-anisotropy ($g_{xx} > g_{yy}$), variation in $A_{xx}$ yields changes predominantly in the low-field region while variation in $A_{yy}$ affects the mid-field region. Note that the features in the high-field edge are influenced to a minor extent (provided $A_{zz}$ is kept small).

The simulations show that while the spectral positions of the partly resolved hyperfine lines are reproduced quite well, the intensities are not. The spin polarization patterns of the simulated spectra can be further refined by introducing anisotropic linewidth parameters, which introduces two adjustable parameters per radical ion to the simulation procedure. Usually, the anisotropic linewidth parameters are not based on a physical model (e.g. anisotropic cofactor motion, see discussion) but are free-floating in a fitting approach. It remains doubtful whether this adds to the reliability of the evaluated hyperfine coupling tensor parameters. The simulations shown in Figures 2.7 and 2.8 use a single residual Gaussian linewidth parameter per radical ion: $P_{700}^{++}$ ($\Delta B=0.50$ mT) and naphthosemiquinone radical ($\Delta B=0.30$ mT). By choosing the same linewidths for all simulations, the focus is on spectral shifts resulting from variation of the hyperfine coupling tensor parameters. The linewidth of $P_{700}^{++}$ has essentially no influence on the spectral line positions. The comparison of experimental and calculated patterns shows the
obvious need to broaden the up-field A/E features induced by $^{13}$C hyperfine coupling. This can be achieved by introducing anisotropic broadening of the residual $A_1^-$ linewidth. This aspect will be addressed in section 2.5.2.

The $^{13}$C-hyperfine coupling tensor components obtained from simulations together with tensors determined from previous studies (31, 37, 38) are shown in Table 2.2. Analogous results for the $Q_A$ site in pbRC (35, 38-40) and for the $Q_{\text{H}}$ site in bo$_3$-QOX (36) are also listed for comparison. The most prominent observations are; (a) the $^{13}$C hyperfine tensor parameters of the quinone in the $A_{1A}$ site of PS I exhibit a large asymmetry with respect to the two carbonyl groups. This is consistent with a single-sided H-bond as rationalized by the valence-bond model in Figure 2.1. The reduced asymmetries observed in other membrane-bound protein-cofactor systems are consistent with additional H-bonds to the respective quinone cofactor. (b) The asymmetry is most pronounced for the $A_{zz}$ component, with a value of 40(2) MHz for the H-bonded C$_4$-O$_4$ group versus 8(2) MHz for the C$_1$-O$_1$ group without the H-bond (the value in parenthesis is the approximate error). The in-plane components are $(-)10.5(2.0)$ and $(-)15(2)$ MHz respectively. Note that the in-plane component is larger for the C$_1$–O$_1$ group. Consequently, protic hyperfine coupling constant $a_{iso}=1/3\text{Tr}(A)$ is larger, which indicates the presence of increased spin density at the nuclei neighboring the C$_1$ position.

2.5 DISCUSSION

One immediate outcome of this work is the confirmation of the presence of only one H-bond to the quinone in its functional, charge-separated state, which in agreement with the ground state X-ray crystal structure (1JB0). Hyperfine couplings of $^1$H, $^{13}$C and $^{17}$O in
positions 2, 4, and O1, respectively, of the quinone head group (31, 41) and direct observation of a 1H-hyperfine coupling in the H-bond (42) provide further support to the assignment of one H-bond to the quinone. This work substantiates the valence bond model shown in Figure 2.1 using quinones with 13C in ring positions 1 and 4, which has asymmetric hyperfine coupling tensors that are very sensitive to changes in electron spin density distribution. The orientation of the 13C hyperfine coupling tensors, with one out-of-plane component $A_{zz}$ and nearly equal in-plane components $A_{xx}$ and $A_{yy}$, do not deviate substantially from the naphthoquinone molecular symmetry. Corresponding 13C hyperfine coupling parameters available for the QA site in pbRC (35) and the QH site in bo3-QOX (36) are listed in Table 2.2 for comparison.

2.5.1 Comparison with Related Quinone Binding Sites and DFT Calculations

Independent sets of DFT calculations on quinone binding sites have been published in the last decade (37-39, 43) (see Table 2.2). Most of the calculations have been performed prior to the experimental evaluation of the 13C hyperfine coupling tensors, in particular for the A1 site in PS I. The A1 site in PS I is unique since, of the many protein-quinone complexes studied, it is the only one where the quinone forms one H-bond. Since the other carbonyl oxygen atom is not involved in H-bond formation, the quinone exhibits highest asymmetry in the most significant hyperfine coupling tensors. Hence, the A1 site in PS I should be a sensitive indicator of the predictive power of DFT calculations.

Qualitatively, the general features of the measured 13C hyperfine coupling tensors are comparable to values obtained from DFT calculations. The in-plane $A_{xx}$ and $A_{yy}$ components are reproduced quite well. The in-plane components are larger for the C1-position and lower for the C4 position. On the other hand, the out-of-plane $A_{zz}$ component
is larger for the C\textsubscript{4}-position and lower for the C\textsubscript{1} position. A\textsubscript{zz}, the most prominent and experimentally accessible hyperfine tensor component, is dramatically underestimated in DFT calculations. Note that this specific discrepancy is not restricted to the A\textsubscript{1} site in PS I. The observed discrepancy is less pronounced in other systems due to the presence of multiple H-bonds that reduce the asymmetry. However, in protic solvent where two symmetric H-bonds are formed, the A\textsubscript{zz}(C\textsubscript{4}) values calculated theoretically (30 MHz) are identical to experimentally determined values (30.3 and 29.0 MHz). However, comparison of theoretically calculated A\textsubscript{zz} values for the quinone in isopropanol (30.3 MHz) and the quinone in A\textsubscript{1} site (32.7 MHz) indicate that the asymmetry-induced increase in the tensor is underestimated. In contrast, the experimental value for A\textsubscript{zz}(C\textsubscript{4}) in the A\textsubscript{1} site (40(2) MHz) is much higher than the experimentally determined value in isopropanol (30(2) MHz). The same trend, albeit to a less pronounced extent, is observed when comparing the respective calculated and measured A\textsubscript{zz} values in solution and in the other quinone binding sites (Table 2.2).

It is important to realize that the discrepancy between experimental and calculated A\textsubscript{zz}(C\textsubscript{4}) values manifests as a discrepancy in the respective spin density distribution, \( \rho_\pi(C_4) \). This is especially true for large A\textsubscript{zz} values where, a direct proportionality exists between the two quantities. This proportionality A\textsubscript{zz}(C\textsubscript{4}) \( \sim \rho_\pi(C_4) \) may appear as similar to the famous McConnell relation, \( a_\text{iso} = Q \rho \) where, \( a_\text{iso} \) is the protic hyperfine coupling constant. However, the origin of the former proportionality is very different. It is applicable to a particular hyperfine coupling tensor component and holds good only for very restricted cases.
According to the valence bond model (Figure 2.1) for a quinone involved in one H-bond similar to the phylloquinone in the A1 site, the spin density at the C4-position is higher and the spin densities at the neighbor ring carbon positions (C3 and C10) are negligible. In addition, the unpaired π-spin density in semiquinone radicals is concentrated on the carbonyl groups (~70%). In other words, the only significant contributions to $A_{zz}(C_4)$ originate from the π spin densities (in the $p_z$ orbitals) at the C4- and O4-position. Based on previous studies, (44) the following conclusions can be drawn; (a) the dipolar hyperfine coupling interaction between the unpaired electron spin density in the $p_z(C_4)$ orbital and the $^{13}$C nuclear spin located in the orbital center results in a large positive $A_{zz}(C_4) \sim \rho_{\pi}(C_4)$ contribution, (b) due to the planar geometry, the out-of-plane dipolar $A_{zz}(C_4)$ interaction component, integrated over the spin density carrying $p_z(O_4)$ orbital, is close to zero (analogous to the McConnell/Strathdee consideration for the aromatic CH fragment). In addition, the contributions to the isotropic scalar hyperfine coupling $a_{iso}(C_4)$ have opposite signs, which argues for a largely dipolar character. This is indeed observed for the $^{13}$C hyperfine coupling tensor in the high spin density C4-position.

The $A_{zz}(C_4) \sim \rho_{\pi}(C_4)$ proportionality as well as the dipolar nature of the hyperfine coupling tensor is indeed confirmed by the DFT calculations. In solution, the spin density is calculated as 0.14 for both the 1- and 4- positions (43, 45) while in the A1-site it increases to 0.16 in the 4-position and reduces to 0.05 in the 1-position (46) (Figure 2.9). The increase in $\rho_{\pi}(C_4)$ from 0.14 to 0.16 corresponds well to the calculated increase in $A_{zz}(C_4)$ from 30.3 to 32.7 MHz and is consistent with the $A_{zz}(C_4) \sim \rho_{\pi}(C_4)$ proportionality. Assuming $\rho_{\pi}(C_4) \sim 0.14$ and $A_{zz}(C_4) = 30.3$ MHz in solution, the experimentally observed increase of $A_{zz}(^{13}C_4)$ to 40(2) MHz in the A1-site corresponds to
\( \rho_{\pi}(C_4) \sim 0.19(1) \). As a means of verification, one may compare these values to the spin density at \( C_2 \), another high spin density ring position \((31)\). The spin density increases from \( \rho_{\pi}(C_2) \sim 0.11(1) \) in isopropanol to 0.15(1) in the \( A_1 \)-site. The ratios of the spin density in the \( A_1 \) site to spin density in isopropanol for the two positions (0.19/0.14 for \( C_4 \) and 0.15/0.11 for \( C_2 \)) are nearly identical.

The increase in spin density at \( C_4 \) obtained from calculation (0.02) when phylloquinone is removed from isopropanol to the \( A_1 \) site is smaller than that calculated based on experimentally determined tensors (0.05). A possible reason for both these values being insubstantial could be the relocation of spin density in the quinone ring, away from the \( C_1/C_4 \) positions. According to the calculated spin density distribution (see Figure 2.9) the relocation occurs mainly from \( C_1/C_4 \) to \( O_1/O_4 \) and to \( C_2/C_3 \). As a consequence, the long established good agreement between measured and calculated \(^1\text{H} \) hyperfine coupling tensor of the methyl group in \( C_2 \) position may be accidental because it depends on relocation of spin density from \( C_4 \) to \( C_2 \).

2.5.2 Physical Models for Orientation Dependent Line Broadening Effects

The comparison of experimental and simulated spectra in Figure 2.7 and Figure 2.8 resulted in satisfactory agreement only with respect to the spectral line positions. However, the intensities of the spectral features deviate substantially between experiment and simulation, in particular at the low- and high- field region. Freely adjustable, orientation-dependent linewidth parameters can fix this but are not deemed an acceptable procedure, as they do not provide physical insight. On the other hand, the experimental polarization patterns indicate quite obvious orientation-dependent broadening effects, most clearly at the high-field end of the spectra. This spectral region is dominated by the
\[ g_{zz} \] tensor components, which happen to be nearly equal for the \( P_{700}^{•+} \) and naphthoquinone\(^{•−} \) contributors to the observed radical pair. Variation of just the linewidth \( \Delta B(P_{700}^{•+}) \) (Figure 2.10) was found to leave the up-field emissive feature of spectra corresponding to 2-methyl-4-\(^{13}\)C-1,4-naphthoquinone unaffected. This also applies to the high-field edge of spectra corresponding to 2-methyl-1-\(^{13}\)C-1,4-naphthoquinone. Both results nicely confirm the expectation that these high-field features are exclusively due to the \(^{13}\)C hyperfine coupling-induced effects in the field region associated with \( g_{zz} \) of naphthoquinone\(^{•−} \) only. There are several possible mechanisms for selective broadening in this region (and to a lesser extent at the low-field end of the spectra) associated exclusively with the \( g_{zz} \) component of naphthoquinone.

Orientation dependent \( T_2 \) spin-spin relaxation effects have been studied extensively for the \( Q_A^{−} \) and \( Q_B^{−} \) site of pbRC using high-field pulsed EPR \((47, 48)\). Two motional models have been distinguished: (a) anisotropic slow librational motion; the axis, defined by the H-bonds from the protein environment to the two colinear carbonyl groups, was identified to be the main quinone anchor axis in the protein around which preferential motion occurred, and (b) essentially isotropic motion due to collective vibrational motion embedded in the environment. The latter process is observed to dominate at temperatures above 160 K. The two types of motion are associated with distinct signatures in the orientation dependence of the relaxation times, \( T_{2,\text{eff}}(B) \). In all cases, the longest time constants are observed when the field is oriented along the quinone molecular axes identical with the g-tensor axes. While convincing evidence for meaningful motional models has been gathered, it is important in the present context to note that all characteristic powder pattern features, in particular the edges, were observed to remain
unchanged over the full time regime that the orientation dependent $T_2$ relaxation occurred. Note also, that $T_{2,\text{eff}}(B)$ induced linewidth contributions would necessarily cause narrowing at the edges of the powder pattern. Therefore, these intriguing and highly informative motional models, currently also investigated in PS I, can be excluded as the origin of the broadening mechanisms that are likely to affect the $^{13}$C hyperfine coupling induced features of the transient EPR spectra in Figure 2.6.

An anisotropic distribution of quinone orientations or H-bond constellations may be another option for the observed broadening effects. The well-resolved hyperfine coupling features associated with the in-plane $A_{xx}(^{13}$C) and $A_{yy}(^{13}$C) components (also applicable to the partially resolved methyl $^1$H hyperfine coupling) would require a selective distribution of (only) the $A_{zz}(^{13}$C) values. This cannot be rationalized within a simple molecular model because the distribution induced broadening effects would have to be averaged out for the in-plane components. Hence, due to lack of a reasonable physical model, simulations using freely variable anisotropic linewidth parameters were not performed.

### 2.5.3 Biological Significance of the Hyperfine Coupling Tensors

The prime purpose behind all efforts to obtain more refined spectroscopic and local structural data of cofactor binding sites is a better understanding of how structural and magnetic interaction properties relate to functional properties of the relevant biological process. In the present case, the latter would consist of the sequence of forward electron transfer processes that proceed via the quinone during light-induced charge separation. The most important question relates to the fact that PS I is designed for high reducing
strength at the terminal acceptors. As a consequence, the quinone in the A₁ binding site possesses one of the most negative redox potentials known for any quinone cofactors. The backbone H-bond is an important structural element of the A₁ binding site and is able to stabilize the anionic state after charge separation. But first the putative role for a redox potential shift is considered. As a general rule it is often assumed that every H-bond to a quinone carbonyl group will shift the redox potential by an average positive amount. Little reliable information is available, however, on the actual amount of the shift. Recent estimates based on electrostatic calculations (49) suggest a rather small redox potential contribution (on the order of 50 meV). On the other hand, values up to 250 meV per H-bond are used in the literature, e.g. (37) and should be rated as strongly exaggerated. If each H-bond is accompanied with an even small positive shift of the redox potential, the confirmation of a single H-bond to the quinone in the A₁ site of PS I in this study, in comparison with two H-bonds (one per carbonyl group) for the QA-site in pbRC points in the proper direction. In this context, it should be recalled that the redox potential of semiquinones is known to shift to more negative values by (~400meV) when the solvent is varied from high polarity (protic solvents, e.g. methanol, water) to low polarity (aprotic solvents, e.g. ether). Similarly, reliable correlations have been established between quinone redox potentials and a variety of polarity indicators for a wide range of solvents as well as between magnetic interaction parameters (g-anisotropy, hyperfine coupling tensor components, H-bond strength) (43, 45). The striking similarity between the magnetic resonance parameters of the semiquinone radical anion in the A₁ site and in aprotic solvents suggests the assignment of the same effective polarity to both environments. The suggested rationale for the highly negative redox potential of the
quinone radical anion in the A₁ site of PS I is a highly hydrophobic (non-polar) binding environment as compared to a very hydrophilic (polar) environment in the QA site in pbRC. This rationale is certainly appealing with respect to an effective local polarity parameter model. But it also raises the question of how this polarity parameter can be related to or even derived from the respective atomic structure model and electrostatic considerations such as distribution of local (partial) charges, dipoles, protonatable groups. The apparent opposing extremes in the local polarity parameter for the two types of binding site can be contrasted against several similarities in the structural details of the respective immediate binding site environments.

The structural comparison of the quinone sites in type I (A₁A in PS I, PDB ID: 1JB0) and type II RCs (QA in pbRC, PDB ID: 1PCR), reveals several structural similarities: (i) In both binding sites the quinone is in π-stacking configuration with a tryptophan residue (Trp697PsaA for A₁A in PS I and TrpM252 for QA in pbRC) with the most favourable overlap in the A₁ site, (ii) the quinone in each site is H-bonded to the NH-group in the protein backbone (Leu722PsaA in PS I and AlaM260 in pbRC), (iii) the backbone H-bonds are in close vicinity to bound water networks identified in the respective X-ray structure models, (iv) both backbone H-bonds have comparable deviation of the O…H-N bond direction from the quinone ring plane and, (v) in both RCs the backbone H-bonds involves the carbonyl group ortho to the extended hydrocarbon tail. Note that the common naphthoquinone notation used here assigns the tail to ring position 3; this differs from the standard numbering of the tail position used for the benzoquinone derivatives in the QA site of pbRC. The main differences between the two binding pockets are (i) the overall orientation of the quinone with respect to the common C₂-symmetry axis and, (ii)
the Qₐ-site includes one more H-bond to the other carbonyl group from the His M219 residue. This histidine also ligates the non-heme Fe. A comparison of the $^{13}$C hyperfine coupling tensors of the individually labeled C₁ and C₄ ring positions listed in Table 2.2 (35), shows C₁/C₄ asymmetry in the Qₐ-site. As expected, due to multiple H-bonding, the asymmetry is smaller than that for the A₁ site and has an opposite sign. This inverted asymmetry in the Qₐ site is indeed observed and calculated (see Table 2.2). It reflects a stronger H-bond from HisM219 to one carbonyl group compared to a weaker backbone H-bond to the other carbonyl group.

Direct access to the H-bond properties has been achieved with the application of orientation selective proton and deuteron ENDOR spectroscopy to the nuclear spin within the H-bond. The main challenges of such an experiment include selective nuclear isotope labeling and accounting for possible interfering resonances from the protein-cofactor background. Recently, a long and persistent experimental effort (35, 46, 50, 51) has succeeded in the accurate determination and full assignment of the hyperfine coupling (and quadrupole) tensors of the $^1$H (and $^2$H) spins in the two H-bonds of the Qₐ-site in pbRC. Identification of the H-bond ENDOR lines turns out to be tricky mainly because of spectral congestion from background ENDOR lines due to either other cofactor radical ions or the binding site protein environment (52, 53). The issue of assignment of H-bonds will be addressed in Chapter 5 with hyperfine coupling constants obtained using higher resolution techniques such as hyperfine sublevel correlation spectroscopy.

2.6 SUMMARY

The work presented in this chapter conclusively shows that the phylloquinone, A₁ₐ (and by inference A₁ₕ) forms a single H-bond with the protein backbone. The spin density
distribution over the naphthoquinone headgroup is highly asymmetric and alternating, consistent with a single H-bond. Since each H-bond is accompanied by small positive shift in the redox potential, the confirmation of the presence of a single H-bond to the quinone in the A₁ site of PS I in this study is consistent with a protein environment designed to drive the midpoint potential of the quinone more negative in comparison to the bacterial reaction centers. In the next chapter, I attempt to understand the role of the H-bond in maintaining the structural and functional integrity of the binding pocket.
2.7 REFERENCES


### 2.8 TABLES

**Table 2.1** Structural and magnetic interaction parameters used for the simulation of transient EPR spectra of the P\textsuperscript{700}••Q•• radical pair state

<table>
<thead>
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<th>$g_{xx}$</th>
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<tr>
<td>$\alpha$</td>
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<td>$T_2=1.3\mu s$</td>
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<td>$KT=(1ms)^{-1}$</td>
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<th>Residual Inhomogeneous Linewidth</th>
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<td>$\Delta B(P_{700}^{•+})=0.50mT$</td>
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<tr>
<td>$\Delta B(A_1^{•−})=0.30mT$</td>
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Table 2.2 $^{13}$C hfc tensor data for selectively isotope labeled quinones. Comparison between the A$_1$ site of PS I and quinones in other binding sites and results from DFT calculations are shown. (Experimental values have approximate errors in parentheses).

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<tr>
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<th>$^{13}$C in C$_1$-Position</th>
<th>$^{13}$C in C$_4$-Position</th>
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<tbody>
<tr>
<td></td>
<td>ref.</td>
<td>$A_{xx}$</td>
<td>$A_{yy}$</td>
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<tr>
<td>A$_1$ in PS I</td>
<td>2-CH$_3$ (13C)-1,4-NQ</td>
<td>a, this work</td>
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</tr>
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<td>g</td>
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<td>C$_1$ and C$_4$ equivalent in cw-EPR</td>
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<td>13C-VK$_3$ in IP</td>
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<td>13C UQ-2</td>
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$^a$(31), $^b$(37), $^c$(39), $^d$(35), $^e$(40), $^f$(36), $^g$(38), $^S$Sinnecker, S. “DFT calculations on a A$_1$ radical anion model in PS I” (2006); summary of computational details and results is discussed in ref. (43). Results for naphthoquinone (NQ) derivatives in frozen isopropanol solution shown in (45). VK$_3$, vitamin K$_3$; IP, isopropanol; UQ, ubiquinone. [signs] with experimental values are added by analog, see text.
2.9 FIGURE LEGENDS

**Figure 2.1** A valence bond scheme for the semiquinone π-radical anion with a single H-bond. The electron withdrawing ability of the single H-bond increases the charge density on O₄ and the spin density on C₄ and creates an alternating electron spin density distribution in the ring. Three resonance structures are shown with the unpaired π-electron spin located at phylloquinone ring positions: 4, 2, and O₁ (right to left).

**Figure 2.2** A closer view of the A₁A binding pocket occupied by phylloquinone. 2-methyl-1,4-napthoquinone is shown as an inset (top). The transient EPR spectrum PS I with 2-methyl-1,4-napthoquinone (inset bottom) show highly pronounced hyperfine splitting when compared to the wild-type.

**Figure 2.3** The menaquinone biosynthetic pathway. SAM – S-adenosyl-L-methionine; TPP-SS – thiamine-pyrophosphate-succinic semialdehyde.

**Figure 2.4** Scheme describing the synthesis of 2-methyl-1-¹³C-1,4-naphthoquinone

**Figure 2.5** Spin-polarized transient EPR spectra of the light-induced P₇₀₀⁺⁺Q⁻⁻ radical anion pair state of PS I complexes at Q-band (35 GHz) and 80K. Comparison of spectra with (a) native phylloquinone (wild-type), (b) 2-methyl-1,4-napthoquinone, (c) 2-methyl-1-¹³C-1,4-napthoquinone, (d) 2-methyl-4-¹³C-1,4-napthoquinone in the A₁ site of the manB deletion mutant. The spectra have been extracted from the full time/field data set by integrating the signal intensity in a time window from 500 to 2000 ns
following the laser flash. Identical overall polarization patterns indicate the same orientation of the substituted naphthoquinone in the A1A site.

**Figure 2.6** Comparison of spin-polarized transient EPR spectra of the P700\(^{+}\)Q\(^{-}\) radical ion pair state of PS I complexes from the menB mutant, at X-band and 80K. The PQ-9 recruited into the A1 site of menB PS I is exchanged with 2-methyl-1,4-naphthoquinone (solid line), 2-methyl-1-\(^{13}\)C-1,4-naphthoquinone (dotted line) and 2-methyl-4-\(^{13}\)C-1,4-naphtoquinone (broken line). The spectra have been extracted from the full time/field data sets by integrating the signal intensity in a time window from 500 to 1500 ns following the laser flash.

**Figure 2.7** The X-band experimental polarization pattern (solid line) of the P700\(^{+}\)Q\(^{-}\) radical pair state of PS I complexes with 2-methyl-4-\(^{13}\)C-1,4-naphthoquinone in the A1A-binding site is compared with simulations. Top: The A\(_{zz}\) value of the \(^{13}\)C hfs tensor is varied in 8 MHz steps as indicated while the perpendicular components are fixed to \(|A_{xx}|=|A_{yy}|=10.5\) MHz. Position of the spectral features is best reproduced for \(A_{zz} = 40\) (2) MHz. Bottom: 2-methyl-1-\(^{13}\)C-1,4-naphthoquinone in the A1A-binding site. The A\(_{zz}\) value of \(^{13}\)C hfs tensor is varied as indicated and the perpendicular components are fixed to \(|A_{xx}|=|A_{yy}|=15\) MHz. Position of the spectral features is best reproduced for \(A_{zz} = 8\) (2) MHz. The values in paranthesis represent approximate errors.
Figure 2.8 The X-band experimental pattern of the $P_{700}^{+}Q^{-}$ radical pair state of PS I complexes with 2-methyl-1-$^{13}$C-1,4-naphthoquinone in the $A_{1A}$-binding site is compared with simulations using separate variation of each of the in-plane principal $^{13}$C hfs tensor parameters. Top: Variation of the $A_{xx}$ value of $^{13}$C hfs tensor as indicated. The other hyperfine coupling tensor components are fixed to $|A_{yy}|=15$ MHz and $A_{zz}=6$ MHz. Bottom: Variation of the $A_{yy}$ value of $^{13}$C hfs tensor as indicated. The other hyperfine coupling tensor components are fixed to $|A_{xx}|=15$ MHz and $A_{zz}=6$ MHz.

Figure 2.9 Spin density distribution according to DFT calculations. (a) Phylloquinone in protic solvent with one H-bond to each carbonyl group, (b) Phylloquinone in the $A_{1}$ binding site with one H-bond to the protein backbone and (c) 2-methyl-1,4-napthoquinone in protic solvent. Maximum asymmetry is expected in (b).

Figure 2.10 The X-band experimental polarization pattern (black) of the $P_{700}^{+}Q^{-}$ radical pair state of PS I complexes with 2-methyl-4-$^{13}$C-1,4-naphthoquinone (top) and 2-methyl-1-$^{13}$C-1,4-naphthoquinone (bottom) in the $A_{1A}$-binding site is compared with simulations. The hyperfine tensor parameters are taken from the best simulations in Figure 2.7 and 2.8, respectively. Only the isotropic Gaussian linewidth parameter $\Delta B$ ($P_{700}^{+}$) is varied as 0.35 mT, 0.50 mT and 0.65 mT.
Figure 2.1
Figure 2.2
Figure 2.3

Chorismate $\xrightarrow{\text{MenF}}$ Isochorismate $\xrightarrow{\text{MenD}}$ 2-Succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate

Dihydroxynaphthoate-CoA $\text{MenB} \xrightarrow{\text{ATP,CoASH}}$ o-Succinylbenzoate-CoA $\text{MenE} \xrightarrow{\text{AMP,PPI}}$ o-Succinylbenzoate

CoASH $\xrightarrow{\text{MenH}}$ Dihydroxynaphthoate

Demethylmenaquinone/demethylphyloquinone $\text{MenA} \xrightarrow{\text{Prenyl-PPI}}$ Menaquinone/phyloquinone

Menaquinone/phyloquinone $\text{MenG/UbiE} \xrightarrow{\text{SAM}}$ Menaquinone/phyloquinone
Figure 2.4

I \xrightarrow{\text{PBr}_3} \text{II}

\text{III} \xrightarrow{\text{CO}_2, \text{H}^\oplus} \text{IV}

\text{V} \xrightarrow{\text{CH}_3\text{SO}_3\text{H}, \text{Cycl}} \text{V}

\text{V} \xrightarrow{\text{CrO}_3, \text{CH}_3\text{COOH, H}_2\text{SO}_4} \text{VI}
Figure 2.5

EPR Intensity (arb. unit) vs. $B_0$/mT
Figure 2.7

![EPR Spectra](image-url)
Figure 2.9
Figure 2.10

(a) EPR intensity (arb. unit) vs. $B_0$ (mT)

(b) EPR intensity (arb. unit) vs. $B_0$ (mT)
Chapter 3

Functional Significance of the Hydrogen Bond Between the Protein and the Phylloquinone in Photosystem I probed by Transient EPR Spectroscopy

[Published in part as an article titled “Role of the Hydrogen Bond from Leu722 to the A1A Phylloquinone in Photosystem I” by Nithya Srinivasan, Irina Karyagina, Robert Bittl, Art van der Est, and John H. Golbeck in Biochemistry, 2009, 48 (15), 3315-3324]
3.1 ABSTRACT

In the previous chapter, it was established that the phylloquinone molecule forms one hydrogen (H-) bond with the protein backbone in its functionally active state based on the asymmetric spin density distribution around the quinone ring. Here, I describe a series of experiments that probe the functional significance of the H-bond. The two molecules of phylloquinone in Photosystem I (PS I) function as electron transfer cofactors at highly reducing midpoint potentials. It is therefore surprising that each phylloquinone is hydrogen bonded at the C₄ position to the backbone –NH of a Leu residue given that a H-bond stabilizes the semiquinone relative to the ground state, which serves to drive the midpoint potential more oxidizing. To better understand the precise role of the H-bond, a PS I variant was generated in which L722_PsaA was replaced with a bulky Trp residue. This change was designed to alter the conformation of the A-jk(1) loop and therefore change the strength of the H-bond to the PsaA-branch phylloquinone. Transient EPR studies at 80 K show that the A₁A site in the PS I variant is fully occupied with phylloquinone but the absence of methyl hyperfine couplings in the quinone contribution to the P₇₀₀⁺⁺A₁⁻⁻ radical pair spectrum indicates that the H-bond has been severely weakened. In wild-type PS I, reduction of Fₐ and Fₐ with sodium dithionite causes a ~30% increase in the amplitude of the P₇₀₀⁺⁺A₁⁻⁻ transient EPR signal due to the added contribution of the PsbB-branch cofactors to low temperature reversible electron transfer between P₇₀₀ and A₁A. In contrast, the same treatment to the L722W_PsaA variant leads to a reduction in the amplitude of the P₇₀₀⁺⁺A₁⁻⁻ transient EPR signal. This behavior suggests that A₁A has undergone double reduction to phyllohydroquinone, thereby preventing electron transfer past A₀A. The P₇₀₀⁺⁺A₁⁻⁻ radical pair spectrum shows an altered spin polarization pattern
and pronounced methyl hyperfine couplings characteristic of a highly asymmetric H-bonding to the phylloquinone. Numerical simulations of the polarization pattern indicate that this radical pair spectrum arises from electron transfer between P_{700} and A_{1B}. The altered reduction behavior in the L722W_{PsaA} variant suggests that the primary purpose of the H-bond is to tie up the C_{4} carbonyl group of phylloquinone in a H-bond so as to prevent protonation and hence lower the probability of double reduction during periods of high light intensity.
3.2 INTRODUCTION

While it is convenient to consider the pigments and electron transfer cofactors as discrete entities, the protein melds the two together by tuning electron transfer. In one role, the protein serves as a scaffold to position the electron transfer cofactors at fixed distances and geometries so as to promote efficient and rapid electron transfer among the bound cofactors. The principles that govern electron transfer are described by Marcus theory (1). Briefly, the protein matrix serves as a medium to tune the thermodynamic properties of the cofactors so as to balance the need for a strong driving force to promote forward electron transfer against the need to conserve the maximum amount of energy in the final charge-separated state. This compromise is ultimately the key to understanding the design principles of PS I.

3.3 INFLUENCE OF THE PROTEIN ENVIRONMENT ON THE REDOX POTENTIALS OF A_{1A} AND A_{1B}

Just what are the protein determinants that cause these seemingly large differences in the midpoint potentials of the phylloquinones in the A- and B-branches? According to the electrostatic calculations carried out by Ishikita and Knapp (2), the most significant factor in conferring the extremely low midpoint potential of the A_{1A} and A_{1B} quinones is the influence of the formal –2 charge on the F_X cluster, which lowers A_{1B} by –256 mV and A_{1A} by –237 mV. The formal –2 charges on the F_A and F_B clusters also lower the midpoint potentials of A_{1B} and A_{1A} but by smaller amounts of –59 mV and –50 mV, respectively. W697_{PsaA} and W677_{PsaB}, which are π-stacked with A_{1A} and A_{1B} are calculated to contribute –27 mV to the midpoint potential of the respective quinone. The
latter value is significantly less than the contribution of \(-50\) to \(-150\) mV determined by quantum chemical calculations (3). This discrepancy is a possible result of computational constraints that limit the calculation in the latter case to the phylloquinone, the \(\pi\)-stacked Trp residue and the backbone NH from the Leu residue that contributes the H-bond to the quinone. Although the above factors influence the redox potential of \(A_{1A}\) and \(A_{1B}\), they do not account for the asymmetry in the calculated values. The large calculated difference between the redox potentials of \(A_{1A}\) and \(A_{1B}\) has been attributed mainly to two factors: the protein backbone, particularly \(S692_{PsaA}\) and \(S572_{PsaB}\), and the side chain of \(D575_{PsaB}\). \(D575_{PsaB}\) is much closer to \(A_{1A}\) than to \(A_{1B}\), and it was proposed that its protonation state is strongly coupled to the reduction of \(A_{1A}\) (its symmetry counterpart \(Q588_{PsaA}\) is neutral and non-titratable). This is understandable since reduction of \(A_1\) has been shown to induce long range electrostatic interactions that perturbs cofactors such as \(A_0\) (4). Under fully ionizing conditions, \(D575_{PsaB}\) and \(Q588_{PsaA}\) drive the redox potential more negative (i.e. more reducing) by identical amounts: \(-154\) mV for \(A_{1B}\) and \(-192\) mV for \(A_{1A}\). However, when the \(A_{1A}\) phylloquinone is 50% reduced, the protonation state of (only) \(D575_{PsaB}\) changes, thereby driving the midpoint potential of \(A_{1A}\) +82 mV more positive (i.e. more oxidizing) than \(A_{1B}\). The net result is that the \(A_{1B}\) phylloquinone is judged to be much more reducing than the \(A_{1A}\) phylloquinone, a conclusion supported most recently by the calculations of Ptushenko et al. (5). Karyagina et al (6) undertook the task of measuring the rate of oxidation of \(A_{1A}^-\) by transient EPR and by time-resolved optical spectroscopy after \(D575_{PsaB}\) was changed from a negatively charged residue to a neutral Ala and to a positively charged Lys. Unexpectedly, the rate of electron transfer from \(A_{1A}^-\) to \(F_X\) was found to decrease only slightly according to the sequence Asp < Ala < Lys (6).
Although the direction of the change was in agreement with theory, the magnitude of the change was very much smaller than expected indicating that the mutation influences the redox potential of the FX cluster. Thus, the difference in Gibbs free energy between the A\textsubscript{1A}/A\textsubscript{1B} and FX redox pairs is not significantly different than in wild-type PS I.

3.3.1 The π–Stacked Trp Residue

The Trp residues in van der Waals contact with the phylloquinones in the A\textsubscript{1A} and A\textsubscript{1B} sites were among the first targeted for study. The inter-planar distances between the quinone ring and the indole ring is between 3 Å and 3.5 Å from the X-ray crystal structure (7, 8) and 3.7 Å from ESEEM studies (9). According to quantum chemical calculations, the presence of the π -stacked Trp residues should result in a −50 to −150 mV shift of the midpoint potential of phylloquinone (3). Partial or total removal of the π overlap by changing the Trp to a Phe or Ala residue should result in a lower the Gibbs free energy difference between A\textsubscript{1} and FX. Assuming that electron transfer between A\textsubscript{1} and FX is in the normal region of the Marcus curve, this should result in a slower rate of oxidation of the phyllosemiquinone anion. The idea is that if the ~200 ns kinetic phase were due to A-side electron transfer, then alteration of the W693\textsubscript{PsaA} to Phe should affect the slow, and not the fast, kinetic phase; similarly, if the ~15 ns kinetic phase were due to B-side electron transfer, then alteration of W673\textsubscript{PsaB} should only affect the fast, and not the slow, kinetic phase. Redding and colleagues (10) generated the single and double mutants W693F\textsubscript{PsaA}, W673F\textsubscript{PsaB}, and W693F\textsubscript{PsaA}/W673F\textsubscript{PsaB} in C. reinhardtii and measured the rate of forward electron transfer from A\textsubscript{1}− to FX in whole cells using pump-probe optical spectroscopy. In the W693F\textsubscript{PsaA} variant, the 143 ns lifetime of the slow component was slowed to 490 ns, while the fast component remained relatively
unaffected. In the W673F_{PsaB} variant, the 13 ns lifetime of the fast component was slowed to 73 ns, while the slow component remained relatively unaffected. In a W693F_{PsaA}/W673F_{PsaB} double variant, the fast and slow kinetic phases were both affected. Even though the lifetimes of the two components varied in a complementary fashion, the ratio of the fraction of PS I associated with each component did not change. This would be the expected result if there were no redirection of electrons through the alternate branch. The straightforward explanation is that the two kinetic phases represent the electron transfer via the two branches of cofactors and that an alteration in a given branch only affects the electron transfer through that branch. This conclusion is eminently reasonable given that the ratio of A-branch to B-branch electron transfer has most certainly been decided by the time the electron reaches the A_{1A} and A_{1B} phylloquinones. These results strongly support the proposal of Joliot and Joliot (11) that the two kinetic phases represent the electron transport through the two bifurcating pathways of electron transfer cofactors in PS I.

Theoretical studies have shown that a neutral quinone and an indole molecule prefer the π-stacked arrangement while a semiquinone radical anion prefers a T-stacked configuration with significant N–H−π hydrogen bonding interaction (3). Phylloquinone however maintains its π-stacked orientation even upon reduction (12). It had been widely thought the orientation of phylloquinone is fixed by its extended tail; however, quinone replacement studies with 2-methyl-1,4-naphthoquinone showed that this is not the case (13). For a fixed arrangement, the extent of intermolecular π-π interaction is smaller upon one-electron reduction and could therefore lower the redox potential of the \( P_{700}^{+}A_{1}^{-} \) radical pair. A recent theoretical analysis of the protein environment surrounding the
phyloquinone suggests that the contribution may not be as profound as the −50 to −150 mV originally suggested (3). In fact, the contribution could be as little as −27 mV (2).

3.3.2 The H-bond Leucine Residue

Continuing the theme of how protein-cofactor interactions relate to and control the functional properties of the phyloquinone in PS I, I now focus on the role of the H-bond. At first sight, the H-bond to the A₁A and A₁B phyloquinones appears at odds with the requirement that PS I produce an extremely low redox potential on the acceptor side. In a H-bond, charge redistribution occurs between the H-bonded partners such that electron density is withdrawn from the quinone ring. This stabilizes the reduced form of the (semi)quinone relative to the non-reduced form, thereby raising the redox potential to a higher (i.e. more positive) value. Because every H-bond to a quinone carbonyl group will shift the redox potential to a more positive value (14), it is appropriate that Qₐ and Qₜₜ in PS II are bound by two H-bonds, rather than just one, to the protein. In PS I, however, the protein environment must drive the midpoint potential of each phyloquinone even lower (i.e. more reducing) than would otherwise be necessary to compensate for the effect of the H-bond. This raises the question of why there even exists a H-bond to the A₁A and A₁B phyloquinones.

Here, I focus on L722ₚₐₐ, the residue involved in the formation of the single H-bond with the C₄ carbonyl group of the transient EPR-detectable A₁A phyloquinone. Because it is formed with the backbone nitrogen of L722ₚₐₐ, the H-bond will not necessarily be affected by a simple change in the side chain of the amino acid. However, the introduction of a residue such as Trp, with a bulky side chain, might be expected to cause a reorientation of the A-jk(1) loop of the PsA protein thereby weakening the H-bond to
the phylloquinone. In this chapter, I describe the initial characterization of the L722W<sub>PsaA</sub> variant using low temperature transient EPR spectroscopy. The spin-polarized spectra of P<sup>700</sup><sup>•+</sup>A<sub>1A</sub><sup>•−</sup> indicate that the spin density distribution on the phyllosemiquinone is altered in a manner that is consistent with a weakening of the hydrogen bond. The data suggest that the A<sub>1A</sub> phylloquinone in the variant is highly susceptible to double reduction in the presence of dithionite in comparison to the wild-type. I propose that this difference arises because protonation of the phylloquinone, which is required for double reduction, occurs more easily when the H-bond is weakened. The selective alteration of the properties of the A<sub>1A</sub> phylloquinone also allows the Q-band spectrum of the spin-polarized P<sup>700</sup><sup>•+</sup>A<sub>1B</sub><sup>•−</sup> radical pair to be accurately recorded for the first time.

### 3.4 MATERIALS AND METHODS

#### 3.4.1 Generation of the Point Mutants

The point mutant L722W<sub>PsaA</sub> was generated as described in ref. (15). To generate mutations in the A<sub>1A</sub> binding site, the pIBC plasmid was constructed through cloning of a DNA fragment that contained most of the psaA gene, the psaB gene and a 760-bp region downstream of the psaB gene into a pBluescript II KS vector. A chloramphenicol-resistant gene was inserted after the 3′ terminator of the psaB gene. PCR mutagenesis was carried out using the QuikChange site directed mutagenesis kit (Stratagene Inc). The construct with the specific mutation in the psaA gene was generated through PCR mutagenesis using the pIBC plasmid DNA as the template and appropriate primers for L722W<sub>PsaA</sub>. The plasmid with the desired psaA mutations derived from pIBC was used to transform the *Synechocystis* sp. PCC 6803 recipient strain pWX3. The pWX3 recipient
strain was constructed by replacing the 1130-bp 3’ end of the psaA gene and the entire psaB gene with a spectinomycin resistance gene. Transformants with chloramphenicol resistance were selected under low light intensities. To verify the full segregation of the transformants, DNA fragments containing the mutation sites were amplified through PCR from the genomic DNA of the mutant strains and sequenced to confirm the desired nucleotide change.

3.4.2 Cell Culture and PS I Isolation

The *Synechocystis* sp. PCC 6803 wild-type and mutant strains were cultured in β–HEPES medium with 5 mM glucose under low light intensities. Preparation of thylakoid membranes and isolation of trimeric PS I complexes were carried out using the non-ionic detergent n-dodecyl-β-D-maltoside according to previously published procedures (15).

3.4.3 Multi-Frequency Spin-Polarized Transient EPR

The X-band (9 GHz) transient EPR experiments were carried out on a laboratory built spectrometer using a Bruker ER046 XK-T microwave bridge equipped with an ER-4118XMD-5W1 dielectric ring resonator and an Oxford CF935 helium gas flow cryostat. The loaded *Q*-value for this dielectric ring resonator was about 3000, equivalent to a rise time of $\tau_r = Q/(2\pi \times \nu_{mw}) \approx 50$ ns. Q-band (35 GHz) transient EPR spectra of the samples were also measured with the same set-up except that a Bruker ER 056 QMV microwave bridge equipped with a home-built cylindrical resonator was used. All samples contained 1 mM sodium ascorbate as the external electron donor and were frozen in the dark. For the chemical reduction experiments, PS I complexes were incubated in the dark with 10 mM sodium dithionite at pH 10.0 under anaerobic conditions for 30 minutes and frozen in complete darkness. The samples were illuminated using a Spectra Physics Nd-
YAG/MOPO laser system operating at 10 Hz.

3.5 RESULTS

3.5.1 Low Temperature Spin-Polarized Transient EPR Spectra of \( P_{700}^{+}A_{1A}^- \) at X- and Q-Bands

Figure 3.1 shows the low temperature spin-polarized transient EPR spectrum of PS I from the \( L722W_{PsaA} \) variant at X-band and Q-band together with the corresponding wild-type spectrum. The radical pair spectrum of the \( L722W_{PsaA} \) variant shows an E/A/E (where E is emission and A is absorption; see (16) for a detailed discussion on the origin of the emissive and absorptive features in the spin-polarized transient EPR spectra of the \( P_{700}^{+}A_{1A}^- \) radical pair) polarization pattern at X-band and an E/A/A/E/A polarization pattern at Q-band that is similar to the wild-type but with some noticeable differences. The most significant of these differences are the loss of the pronounced shoulder in the mid-field region of the X-band spectrum and an increase in the amplitude of the low field E/A pattern relative to the high field emission in the Q-band spectrum. (With the chosen normalization, this appears as a decrease in the intensity of the up-field region.) As described in the previous chapter, this shoulder in the mid-field region of the X-band spectrum arises from the hyperfine coupling of the methyl group in the \( C_2 \) position on the phylloquinone head group. The loss of this shoulder in the \( L722W_{PsaA} \) variant implies that the methyl hyperfine coupling has been weakened. Figure 3.2 shows numerical simulations in which the expected effect of a weakening of the methyl hyperfine coupling is explored. The dashed curves are calculations using the known parameters for the radical pair \( P_{700}^{+}A_{1A}^- \) (17, 18). The solid curves are simulations using the same
parameters except that the principal values of the methyl hyperfine coupling tensor have been reduced by a factor of two. The spectra have been normalized to give the same maximum amplitude. The reduction of the methyl hyperfine coupling leads to the absence of the shoulder and an increase in the relative intensity of the low field region of the Q-band spectrum as observed experimentally. Thus, the spectra in Figure 3.1 confirm that the methyl hyperfine coupling is weaker in the L722W$_{PsaA}$ variant. The magnitude of the coupling is difficult to estimate accurately from the spectra, thus the data only show qualitatively that the coupling has been reduced and is not a quantitative measure of the reduction. Nevertheless, the presence of methyl hyperfine couplings has proven to be a sensitive indicator of the environment of the phylloquinone from which the asymmetry and strength of the H-bond can be deduced (13, 19). In PS I from the wild-type, the H-bond withdraws electron density, producing an alternating pattern of electron spin density around the quinone ring as described by the valence bond model (Figure 2.1) such that the spin density is highest at C$_2$ and C$_4$, and lowest at C$_1$ and C$_3$. The interaction of the three methyl protons with the electron spin density on C$_2$ gives rise to a quartet with relative intensities 1:3:3:1 centered near the $g_{yy}$ component of the electronic $g$-tensor of the quinone. In PS I from the L722W$_{PsaA}$ variant, the loss of the methyl hyperfine coupling indicates lower spin density on C$_2$, which can arise from a more symmetrical spin distribution either due to the absence of the single H-bond or the presence of two H-bonds of similar strength. The simplest explanation is that the bulky Trp has altered the configuration of the A-jk(1) loop containing residue L722$_{PsaA}$, thereby severely weakening the single H-bond to the phylloquinone. In principle, the absence of the H-bond could lead to a lower binding constant for
phyloquinone, resulting in a significant fraction of unfilled A₁ₐ sites and/or an altered orientation of phyloquinone in the A₁ₐ site. Both can be assessed by transient EPR spectroscopy. In the unfilled sites, the recombination between P₇₀₀⁺⁺ and A₀ₐ⁻⁻ would generate a P₇₀₀ triplet with a characteristic spin-polarized EPR spectrum. However, no such spectrum was detected in the L722Wₚₕₐₐ variant, implying that the A₁ₐ sites are fully occupied (data not shown). The orientation of phyloquinone in the A₁ₐ site is difficult to assess at X-band because hyperfine couplings dominate the spectrum. However, at Q-band and higher frequencies, the electronic g-tensor is better resolved; the spin polarization pattern of the P₇₀₀⁺⁺A₁ₐ⁻⁻ radical pair is particularly sensitive to the relative orientation of the g-tensors g(P₇₀₀⁺⁺) and g(A₁ₐ⁻⁻) with respect to the vector that connects the spin density centers involved in the radical pair (16). The fact that both the wild-type and the L722Wₚₕₐₐ variant have an E/A polarization pattern on the low-field side of their spectra (see Figure 3.1, bottom) indicates that the orientation of the A₁ₐ phyloquinone in the variant is similar to the wild-type.

3.5.2 Low Temperature Spin-Polarized Transient EPR Spectra of Chemically Reduced PS I Complexes

Figure 3.3 shows the low temperature X-band transient EPR spectrum of PS I from the wild-type and the L722Wₚₕₐₐ variant before and after chemical reduction of the terminal Fₐ/Fₕ clusters with sodium dithionite. In wild-type PS I, the most significant difference between the non-reduced and the reduced samples is the increase in signal intensity by 32%. This increase in the signal amplitude represents the irreversible fraction of PS I that would have passed electrons forward irreversibly to the Fₐ/Fₕ clusters at low temperature, but are now forced to undergo reversible electron transfer between P₇₀₀ and
A1. Contrary to expectation, chemical reduction resulted in an opposite trend in L722W\textsubscript{PsaA}; here, the signal of the reduced sample decreased to 27% of the amplitude of the non-reduced sample.

The weaker P\textsubscript{700}\textsuperscript{+}A\textsubscript{1}\textsuperscript{-} spectrum in the pre-reduced sample of the L722W\textsubscript{PsaA} variant suggests that the A\textsubscript{1A} phylloquinone is more easily reduced to phyllohydroquinone than in the wild-type. It is also possible that the A\textsubscript{1A} phyllohydroquinone is lost from the binding site in the L722W\textsubscript{PsaA} variant under reducing conditions since the phyllosemiquinone form is known to be somewhat labile in native PS I (20, 21).

Consistent with double reduction and/or loss of the quinone, a strong spectrum of the triplet state of P\textsubscript{700} is generated due to the recombination of P\textsubscript{700}\textsuperscript{+} and A\textsubscript{0}\textsuperscript{-} (data not shown). Closer inspection of Figure 3.3 (middle) shows that shape of the P\textsubscript{700}\textsuperscript{+}A\textsubscript{1}\textsuperscript{-} spectrum from the L722W\textsubscript{PsaA} variant also changes when the sample is reduced. The most noticeable differences are the reappearance of the shoulder on the central absorptive peak of the reduced L722W\textsubscript{PsaA} sample, indicative of partially resolved methyl hyperfine splitting and a change in the relative intensity of the emissive feature on the high field end of the spectrum. Since the simplest explanation for the EPR spectrum of the untreated samples in Figures 3.1 and 3.3 is that the H-bond between the protein and the A\textsubscript{1A} phylloquinone is disrupted in the L722W\textsubscript{PsaA} variant, the reappearance of the hyperfine coupling upon dithionite treatment must be either due to a small population of A\textsubscript{1A} that has re-established the H-bond or due to electron transfer involving the A\textsubscript{1B} phylloquinone in which the H-bond from L706\textsubscript{PsaB} remains unaltered.

Figure 3.4 compares the X- and Q-band spectra of the reduced L722W\textsubscript{PsaA} variant with a non-reduced wild-type sample. The spectra have been normalized to the same intensity in
the low-field region, which is dominated by the contribution from the phylloquinone to the spin polarized radical pair. As can be seen in the Q-band spectrum, the $P_{700}^{+}A_{1}^{-}$ polarization pattern changes from E/A/A/E/A in the wild-type to E/A/E/A/E in the chemically-reduced $L722W_{PsaA}$ variant, with the maximum change occurring in the high field region of the spectrum. The fact that the two spectra are different clearly suggests that the spectrum of the variant is not due to a fraction of the complexes in which the H-bond to $A_{1A}$ is re-established. Instead, the spectrum of the reduced variant appears to represent the spin-polarized EPR spectrum of the $P_{700}^{+}A_{1B}^{-}$ radical pair that becomes observable after i) blocking forward electron transfer from $A_{1B}^{-}$ via $F_{x}$ to $F_{A}$ and $F_{B}$, and ii) blocking electron transfer from $A_{0A}^{-}$ to $A_{1A}$.

A similar hypothesis is invoked to explain the observations on the reduced wild-type sample, in which the formation of a trapped fraction via electron transfer through the $PsaB$-branch leads to the increase in signal intensity (22, 23). If correct, the increase in the intensity of the wild-type $P_{700}^{+}A_{1}^{-}$ spectrum upon reduction of $F_{A}$ and $F_{B}$ would be due to an additional contribution from the $PsaB$-branch radical pair $P_{700}^{+}A_{1B}^{-}$. Hence the difference between the spectra of the reduced and non-reduced wild-type samples should produce the spectrum of the $PsaB$-branch radical pair. The bottom trace in Figure 3.3 shows a comparison of this difference spectrum and the spectrum of the reduced $L722W_{PsaA}$ variant. As can be seen, their shapes are indeed the same, which strongly suggests they are both due to $P_{700}^{+}A_{1B}^{-}$. 
3.5.3 Simulation of the Low Temperature X- and Q-Band Spin-Polarized Transient EPR Spectra

The spectra of P$_{700}^{+}$A$_{1A}^{-}$ and P$_{700}^{+}$A$_{1B}^{-}$ differ because the spin density on P$_{700}^{+}$ is localized on the Chl $a$ molecule associated with PsaB (24, 25) which leads to a different orientation of the dipolar coupling vector relative to the g-tensor axes of P$_{700}^{+}$ in the radical pairs P$_{700}^{+}$A$_{1A}^{-}$ and P$_{700}^{+}$A$_{1B}^{-}$. To confirm that the spin-polarized spectrum of the reduced L722W$_{\text{PsaA}}$ sample represents the P$_{700}^{+}$A$_{1B}^{-}$ radical pair, numerical simulations were carried out (Figure 3.5) using geometric parameters calculated from the X-ray structural model and known orientation of the g-tensor axes of P$_{700}^{+}$ and the phyllosemiquinones in their respective molecular frames (17). The angles describing the relative orientations of the dipolar coupling vector and the two g-tensors are given in the caption to Figure 3.5 (26). All other parameters used in the simulation are the same as given in refs. (17) and (18). As can be seen from the comparison of Figure 3.4 and Figure 3.5, the numerical simulations of P$_{700}^{+}$A$_{1A}^{-}$ and P$_{700}^{+}$A$_{1B}^{-}$ closely resemble the spectra of the wild-type and the L722W$_{\text{PsaA}}$ variant following reduction by dithionite respectively, which supports the idea that the spectrum of the reduced L722W$_{\text{PsaA}}$ variant is due to electron transfer through the PsaB-side cofactors.

3.6 DISCUSSION

3.6.1 Effect of the Mutation on the Strength of the H-Bond

The relative strength of the H-bond from the backbone nitrogen of Leu722$_{\text{PsaA}}$ to the A$_{1A}$ phylloquinone can be assessed with the use of transient EPR spectroscopy. The distinct absence of the methyl hyperfine coupling observed in the L722W$_{\text{PsaA}}$ variant can come
about as a result of a more symmetrical H-bond arrangement, either from a second H-bond, in this instance to the C$_1$ carbonyl group of the phylloquinone, or a weakened H-bond, in this instance from L722$_{PsaA}$ to the C$_4$ carbonyl group. Under both conditions, the electron spin density distribution at C$_2$ would be decreased, leading to a weaker hyperfine coupling with the methyl protons. This is because the H-bond to the phylloquinone in the A$_{1A}$ site has a large effect on the spin density distribution around the quinone ring. The highest spin and charge density in a phylloquinone radical anion is expected at the two carbonyl groups because of the high electronegativity of the oxygen atoms. Formally, the charge is placed on the oxygen, while the unpaired electron is placed on the carbon. In vacuum or a homogeneous protic solvent, in which H-bonding to the two oxygen atoms is the same, the spin density on C$_1$ and C$_4$ is identical. In contrast, when a single H-bond is present at O$_4$ its electron withdrawing effect is expected to increase the charge on O$_4$ and the spin density at C$_4$ compared to O$_1$ and C$_1$, respectively. In accordance with the resonance structures of a conjugated system, the single H-bond leads to an alternating electron spin density distribution in the ring such that it is highest at C$_4$ and C$_2$ and lowest at C$_3$ and C$_1$ (Figure 2.1). It is the high spin density at C$_2$ that is responsible for the pronounced hyperfine coupling from the –CH$_3$ group of phylloquinone in wild-type PS I.

Turning the argument around, the magnitude of the methyl hyperfine coupling can serve as a sensitive probe of the strength and number of H-bonds to the A$_{1A}$ phylloquinone.

The crystal structure of PS I at 2.5 Å resolution shows that the side chain of L722$_{PsaA}$ points away from the A$_{1A}$ site suggesting that upon substitution of a Trp residue, the bulky imidazole side chain would not result in steric hindrance and therefore not interfere with side chains that make up the phylloquinone binding pocket. Consistent with this
expectation, the transient EPR data do not show any significant alteration in the orientation of the semiquinone ring with respect to $P_{700}^+$, indicating that the change in the H-bond strength is probably a result of alterations of the conformation of the protein backbone near $L722_{PsaA}$. One possibility is that the A-jk(1) switchback loop, which contains the $L722_{PsaA}$ residue, moves from its normal equilibrium position, thereby weakening the single H-bond to the $A_{1A}$ quinone. A second possibility is that more distant shifts in the protein backbone would allow for the formation of a second H-bond with the $C_1$ carbonyl group. These two possibilities can be distinguished because the rate of electron transfer from $A_{1A}^-$ to $F_X$ in the $L722W_{PsaA}$ variant is sensitive to the number and strength of the H-bonds. Assuming electron transfer occurs in the normal region of the Marcus curve, a weaker H-bond would be expected to shift the midpoint potential of $A_{1A}$ to a more negative value, and thereby increase the driving force for electron transfer from $A_{1A}^-$ to $F_X$. A second H-bond would be expected to shift the midpoint potential of $A_{1A}$ to a more positive value, and thereby decrease the driving force for electron transfer from $A_{1A}^-$ to $F_X$. Measurements carried out at 240 K show that the rate of electron transfer from $A_{1A}^-$ to $F_X$ increases by about an order of magnitude compared to the wild-type, thereby favoring the simpler hypothesis that a single H-bond is weakened rather than the more complicated hypothesis that a second H-bond is formed (27). The temperature study of forward electron transfer will be discussed in Chapter 4.

3.6.2 Reversible and Irreversible Electron Transfer to the $A_{1A}$ and $A_{1B}$ Phylloquinones

Chemical reduction with sodium dithionite leads to very different effects in PS I from the wild-type and the $L722W_{PsaA}$ variant. Below the glass transition temperature, two distinct fractions are formed such that $\sim 75\%$ of wild-type PS I undergoes reversible electron
transport between $P_{700}$ and the $A_{1A}$ phylloquinone, generating the transient EPR-observable $P_{700}^{+}A_{1A}^{-}$ radical pair (28) and the other $\sim 25\%$ of the reaction centers do not contribute to the transient EPR spectrum because on the first several flashes, irreversible $P_{700}^{+}[F_{A}/F_{B}]^{-}$ charge separated pair is formed. In the currently accepted model, irreversible electron transfer to $F_{A}$ and $F_{B}$ proceeds via the $A_{1B}$ phylloquinone. These observations suggest that electron transfer from $A_{1A}^{-}$ to $F_{X}$ is thermodynamically unfavorable while electron transfer from $A_{1B}^{-}$ to $F_{X}$ is thermodynamically favorable (2, 29, 30). This would be consistent with the observation that the slow phase of $A_{1}^{-}$ oxidation exhibits a strong Arrhenius type temperature dependence with a higher activation energy than the fast phase of $A_{1}^{-}$ oxidation, which is nearly temperature independent and has a lower activation energy (28, 29). However, the factors that result in the two fractions are not fully understood. When $F_{A}$ and $F_{B}$ are chemically reduced, this otherwise transient EPR silent fraction begins to undergo reversible electron transfer and its contribution adds to the amplitude of the $P_{700}^{+}A_{1A}^{-}$ radical pair spectrum, increasing its intensity by $\sim 25\%$. The results presented here also suggest that this contribution arises from $PsaB$-side electron transfer.

The chemically reduced $L722W_{PsaA}$ sample behaves anomalously. Three significant features require further elaboration: (i) the decrease in the amplitude of the radical pair spectrum after reduction of $F_{A}$ and $F_{B}$; (ii) the reappearance of the prominent methyl hyperfine couplings and (iii) the formation of spin-polarized $P_{700}$ triplet. If the H-bond were weakened, as I suspect is the case in the $L722W_{PsaA}$ variant, the $A_{1A}$ phylloquinone might become susceptible to protonation and undergo facile double reduction. The alteration in the equilibrium position of the $A$-jk(1) switchback loop could further result
in the opening of a water channel, allowing for easier protonation. Were this to occur, forward electron transfer to F_X (as well as backward electron transfer to A_0) would be blocked because the phyllosemiquinone/phyllohydroquinone couple has a higher (more positive) redox potential than the phylloquinone/phyllosemiquinone couple (31). The 3/4 fraction of PS I complexes that had originally given rise to the P_{700}^{+}A_{1A}^{-} radical pair would now become transient EPR silent, leading to the decrease in the spectral amplitude. I propose that the spin-polarized signal in the chemically reduced L722W_{PsaA} variant represents the population of PS I that was transient EPR silent in the non-reduced sample. This fraction would correspond to the population of PS I that irreversibly charge separates at low temperature to P_{700}^{+}[F_A/F_B]^{-}. The reappearance of the methyl hyperfine coupling in the X-band spectrum shows the involvement of a quinone with an intact H-bond, supporting the proposal that the spectra arise from the spin-polarized P_{700}^{+}A_{1B}^{-} radical pair. The observation that upon chemical reduction spin polarization pattern of the L722W_{PsaA} variant become equivalent to that of the difference spectrum (reduced minus non-reduced) of the wild-type provides excellent support to the above argument. In the fraction of PS I complexes in which electron transport past A_{0A} is blocked, charge recombination would occur between P_{700}^{+} and A_{0A}^{-}. During such recombination two products are formed: the singlet P_{700} species (yield ~ 70%) and the triplet P_{700} species (yield ~30%) that decay by intersystem crossing to the singlet ground state in a few microseconds (see (32)). Consistent with this picture, a strong triplet P_{700} is observed in the reduced L722W_{PsaA} variant. However, quantification of the amplitude and comparison with the wild-type is complicated by the possibility of stable reduction of A_{0A} in the variant and A_{1A} in the wild-type. Hence, the intensity of the triplet spectrum is
difficult to use as a quantitative measure of the amount of electron transfer beyond \( A_{0A} \). Nonetheless, it is reasonable to conclude that the observed \( P_{700} \) triplet signal together with the \( \sim 75\% \) decrease in the radical pair signal strength indicates an essentially complete block in PsaA-side electron transfer past \( A_{0A} \) in the \( L722W_{PsaA} \) variant under reducing conditions.

3.6.3 Spectrum of the Spin-Polarized \( P_{700}^{++} A_{1B}^- \) Radical Pair

The differences in the spectra of the two radical pairs \( P_{700}^{++} A_{1A}^- \) and \( P_{700}^{++} A_{1B}^- \) arise from the difference in their geometrical arrangement. The 2.5 Å resolution X-ray crystal structure of PS I shows that the two branches of cofactors are arranged symmetrically about an axis that passes through \( P_{700} \) and \( F_X \) (8). This structural symmetry does not, however, translate to electronic symmetry (Figure 3.6). The spin density distribution on \( P_{700}^{++} \) is asymmetric with the spin mainly localized on the Chl \( a \) molecule ligated by the PsaB subunit (as opposed to the Chl \( a' \) molecule ligated by the PsaA subunit) (24, 25). As a result of this asymmetry the vector \( z_d \), which connects the centers of \( P_{700}^{++} \) and \( A_1^- \), is oriented differently relative to the \( g \)-tensor axes of \( P_{700}^+ \) in the two radical pairs. This difference has a considerable effect on the \( P_{700}^{++} \) region of the spin polarization pattern of the transient EPR spectrum as can be seen in the simulated spectra shown in Figure 3.5 and in ref. (33). Consistent with this effect, the transient EPR spectra of the photoaccumulated sample (33) and the \( L722W_{PsaA} \) mutant (this work), show the largest change in the high field region of the spectrum, where the main contribution is derived from \( P_{700}^{++} \). Based on the similarity between the observed Q-band spectrum and the simulated \( P_{700}^{++} A_{1B}^- \) spectrum, the presence of resolved hyperfine coupling in the X-band spectrum and the large decrease in intensity upon reduction, I conclude that the spectrum
of the reduced L722W<sub>PsaA</sub> sample arises primarily from P<sub>700</sub><sup>++</sup>A<sub>1B</sub><sup>−</sup>. In an alternate scenario, a shift of the asymmetric spin distribution of P<sub>700</sub><sup>++</sup> from the B-branch Chl <i>a</i> to the A-branch Chl <i>a</i> such that the interspin vector connects the Chl <i>a</i> and A<sub>1A</sub><sup>−</sup> could result in an identical spectrum. This possibility is less likely for two reasons: (i) such redistribution would require a perturbation around P<sub>700</sub>, which is not expected at a distance so far from L722<sub>PsaA</sub>, and (ii) the appearance of the hyperfine coupling in the X-band spectrum of the reduced L722W<sub>PsaA</sub> sample indicates the involvement of the unmodified A<sub>1B</sub> site rather than the modified A<sub>1A</sub> site. The involvement of A<sub>1B</sub> is further supported by the similarity between the difference spectrum of the reduced and non-reduced wild-type and the reduced L722W<sub>PsaA</sub> variant shown in Figure 3.3. Thus, the results for the L722W<sub>PsaA</sub> variant are in agreement with the findings of Poluektov et al. (33), who showed that when A<sub>1A</sub> is completely reduced, a spin-polarized spectrum consistent with the P<sub>700</sub><sup>++</sup>A<sub>1B</sub><sup>−</sup> radical pair is obtained.

### 3.6.4 Phylloquinone-Protein Interactions Necessary for Binding to the A<sub>1A</sub> Site

To understand the influence of the protein environment on the functional properties of any given cofactor, it is necessary to restrict changes to the near vicinity of the cofactor. This is especially true in the case of phylloquinone, as the route of electron transfer along the PsaA- and PsaB-branches is likely to be completely decided prior to the A<sub>0A</sub> to A<sub>1A</sub> (and A<sub>0B</sub> to A<sub>1B</sub>) electron transfer steps. Phylloquinone is tightly bound in the A<sub>1A</sub> and A<sub>1B</sub> binding sites, but the interactions that anchor it to the protein appear to be primarily hydrophobic (<i>i.e.</i> entropic). Nevertheless, a wide range of structurally different compounds are able to bind to the A<sub>1A</sub> and A<sub>1B</sub> sites. If the 2-methyl group is removed, the 2-phytyl-1,4-naphthoquinone tightly occupies the A<sub>1A</sub> site in an orientation similar to
phyloquinone in the wild-type (34) (see (35) for a review of quinone substitution studies). The only effect is a predictable slowing of electron transfer due to a positive shift in the midpoint potential of the quinone. If the phytol tail of phylloquinone is removed, the 2-methyl-1,4-naphthoquinone occupies the A\textsubscript{1A} site also in an orientation similar to phylloquinone in the wild-type (13, 36) and again the forward electron transfer kinetics from A\textsubscript{1A} are altered in agreement with a positive shift in the midpoint potential of the 2-methyl-1,4-naphthoquinone. Plastoquinone-9, which is a single ring quinone with a much longer tail, can be recruited into the A\textsubscript{1A} site and is also oriented similar to that of phylloquinone in the wild-type (37-41). However, in contrast to the modified phylloquinones, it is loosely bound and can be easily displaced by a variety of substituted 1,4-naphthoquinones. Studies using solvent extracted PS I have shown that anthraquinones also bind while benzoquinones often bind poorly (42-45). In all of the studies involving naphthoquinone derivatives, the EPR parameters indicate that the H-bond from L722\textsubscript{PsaA} to the C\textsubscript{4} carbonyl group of the phylloquinone in the A\textsubscript{1A} site (and from L706\textsubscript{PsaB} to the C\textsubscript{4} carbonyl group of an equivalent phylloquinone in the A\textsubscript{1B} site) is retained. However, as shown in this work, the H-bond is structurally dispensable: phylloquinone occupies the A\textsubscript{1A} site with an orientation similar to that in the wild-type. Thus, any one of the three most prominent features of phylloquinone - the C\textsubscript{2} methyl group, the C\textsubscript{3} phytol tail, or the C\textsubscript{4} H-bond - can be altered (or eliminated) and yet the phylloquinone binds in the A\textsubscript{1A} site and functions in electron transfer. Given that the presence of the H-bond requires the protein environment to drive the redox potential more reducing than would otherwise be necessary and given that the H-bond does not serve a structural role in binding or orienting phylloquinone in the A\textsubscript{1A} and by inference
in the A1B site, its role must be sought elsewhere. I propose that because the A1A phylloquinone in the L722W<sub>PsaA</sub> variant is inactivated under reducing conditions, the primary role of the H-bond between the A1A phylloquinone and the protein backbone is to ensure function of the phylloquinone between its oxidized quinone and singly-reduced semiquinone states. The presence of the H-bond would tie up the C4 carbonyl group, preventing protonation and rendering the phylloquinone less susceptible to double reduction and possible dissociation from the binding site under periods of high illumination. The phylloquinone in cyanobacterial PS I complexes has been shown to undergo double reduction when illuminated in the presence of sodium dithionite (31, 46). The transient accumulation of reduced electron acceptors, F<sub>B</sub><sup>−</sup>, F<sub>A</sub><sup>−</sup>, F<sub>X</sub><sup>−</sup>, and A<sub>1</sub><sup>−</sup>, upon illuminating dark-adapted cyanobacterial cells (40, 47) indicate that double reduction of phylloquinone may be a realistic occurrence under physiologically-relevant conditions.

3.7 SUMMARY

The studies presented in this chapter indicate that the H-bond is extremely important in maintaining the functional integrity of the binding pocket. If the H-bond is significantly weakened, the quinone can still occupy the binding pocket in an orientation similar to that in the wild-type. Although the H-bond is structurally expendable, it plays a key role in ensuring that the quinone can take part in electron transfer. In the absence of the H-bond, protonation-coupled-double reduction of the quinone becomes energetically feasible resulting in the formation of phyllohydroquinol, which is incapable of participating in electron transfer. By tying up the carbonyl group, the H-bond prevents double reduction/protonation and allows the quinone to function as a one electron gate.
The presence of the H-bond likely drives the midpoint potential of the quinone more oxidizing making the protein environment overcompensate for its presence. In the next chapter I present a detailed room-temperature kinetic characterization of the PS I variant that has a significantly weakened H-bond to understand its effect on the rate of electron transfer through the phylloquinone.
3.8 REFERENCES

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3.9 FIGURE LEGENDS

**Figure 3.1** Top: X-band (top) and Q-band (bottom) spin-polarized transient EPR spectra of PS I complexes isolated from L722WPsaA (solid lines) compared with wild-type (dashed lines) at 80 K. The spectra are normalized to give the same maximum amplitude. Positive signals correspond to absorption (A) and negative signals correspond to emission (E). The spectra represent the P$_{700}^{+}$$A_{1A}^{-}$ radical pair and they have been extracted from full time/field data sets by averaging the signal intensity in a time window from 1.0 ms to 1.75 ms (X-band) and ~0.4 ms to 1.1 ms (Q-band) following the laser flash.

**Figure 3.2** Simulated spectra of P$_{700}^{+}$$A_{1A}^{-}$ at X-band (top) and Q-band (bottom). The parameters are the same as those used in ref. (17, 18) except that in the solid spectra the principal values of the phylloquinone methyl hyperfine coupling has been reduced by a factor of two.

**Figure 3.3** The effect of reduction by dithionite on the X-band spin-polarized transient EPR spectra of the wild-type (top) and L722WPsaA (middle). The top and middle parts of the figure show spectra measured at 80 K before (solid lines) and after (dashed lines) reduction. Note the opposite trend in the signal intensity between the wild-type and the mutant and the reappearance of the hfc structure upon reduction of L722WPsaA. The bottom part of the figure shows a comparison of the spectrum of the L722WPsaA variant following reduction (solid lines) and the reduced minus non-reduced difference spectrum of the wild-type (dashed lines). The two spectra have been normalized to the same maximum absorption intensity. The E/A/E spectra of the non-reduced samples (solid
traces top and middle) are attributed primarily to $P_{700}^{\cdot+}A_{1A}^{\cdot-}$ while the two spectra in the bottom trace are assigned to $P_{700}^{\cdot+}A_{1B}^{\cdot-}$. The contribution from $^3P_{700}$, which gives a sloping background to the spectra of the reduced wild-type and L722W$_{PsaA}$ samples has been removed by a linear baseline correction.

**Figure 3.4** X-band (top) and Q-band (bottom) spin-polarized transient EPR spectra of wild-type (dashed lines) and reduced L722W$_{PsaA}$ (solid lines) recorded at 80 K. The difference is more prominent at Q-band. The E/A/A/E/A polarization pattern in the wild-type changes to E/A/E/A/E in reduced L722W$_{PsaA}$. The contribution from $^3P_{700}$, which gives a sloping background to the spectra of the reduced wild-type and L722W$_{PsaA}$ samples has been removed by a linear baseline correction. The spectra are normalized to give the same amplitude for the low-field emissive feature.

**Figure 3.5** Simulated X-band (top) and Q-band (bottom) spin-polarized transient EPR spectra of $P_{700}^{\cdot+}A_{1A}^{\cdot-}$ (dashed lines) and $P_{700}^{\cdot+}A_{1B}^{\cdot-}$ (solid lines). Euler angles in degrees as defined in (26) describing g($A_1$) in g($P_{700}^{\cdot+}$): $P_{700}^{\cdot+}A_{1A}^{\cdot-}$ (26, 126, 81); $P_{700}^{\cdot+}A_{1B}^{\cdot-}$ (101, 80, -220). Angles $q$ and $f$ describing the dipolar vector $z_d$ in g($P_{700}^{\cdot+}$) in degrees: $P_{700}^{\cdot+}A_{1A}^{\cdot-}$ (36, 78); $P_{700}^{\cdot+}A_{1B}^{\cdot-}$ (87, 75). All other parameters are the same as those used in ref. (17, 18).

**Figure 3.6** The arrangement of cofactors in PS I from 2.5 Å X-ray crystal structure seen parallel to the membrane plane (8). A vertical axis in grey passes through $F_X$ representing the $C_2$ axis of symmetry. The axis in black passes through the Chl $a$ molecule of $P_{700}$
ligated by PsaB, on which the majority of the charge on P_{700}^{•+} is localized. The vector connecting the centers of P_{700}^{•+} and A_{1}^{•−} is oriented differently relative to the g-tensor axis of P_{700}^{•+} in the two radical pairs, P_{700}^{•+}A_{1A}^{•−} and P_{700}^{•+}A_{1B}^{•−}. 
Figure 3.1

X-band

--- wild type
--- $L722W_{PsaA}$

Q-band

--- wild type
--- $L722W_{PsaA}$

$B_0$/mT

343 344 345 346 347 348 349

1204 1206 1208 1210 1212
Figure 3.2

X-band

--- dashed: wild type hfs
--- solid: reduced hfs

--- A
--- E

343 344 345 346 347 348 349
B₀ / mT

Q-band

--- dashed: wild type hfs
--- solid: reduced hfs

--- A
--- E

1204 1206 1208 1210 1212
B₀ / mT
Figure 3.3
Figure 3.4

X-band

- $L722W_{PsaA}$ (10 mM dithionite)
- wild type

$B_0 / \text{mT}$

343 345 347 349

Q-band

- $L722W_{PsaA}$ (10 mM dithionite)
- wild type

$B_0 / \text{mT}$

1204 1206 1208 1210 1212
Figure 3.5
Figure 3.6
Chapter 4

Kinetic Characterization of the L722W_{PsaA} Variant with a Significantly Weakened H-bond to the Phylloquinone in Photosystem I
4.1 ABSTRACT

In the last chapter, I showed that the H-bond between the carbonyl oxygen of the phylloquinone and the backbone NH of the L722PsaA residue could be disrupted by substitution with a bulky Trp residue. Here, I present a detailed room temperature kinetic characterization of the variant. Time-resolved optical and EPR studies show that at 293 K, (i) the rate of forward electron transfer from $A_{1A}^-$ to $F_x$ is increased by a factor of approximately three in the L722W$_{PsaA}$ variant compared to the wild-type; (ii) a minor fraction of $A_{1A}^-$ is involved in charge recombination from $A_0^-$, and (iii) the rate of charge recombination between $[F_A/F_B]^-$ and $P_{700}^+$ is slower in the L722W$_{PsaA}$ variant than in the wild-type. Pulsed EPR measurements at 80 K show that the distance between $P_{700}^+$ and $A_{1A}^-$ is the same in the L722W$_{PsaA}$ variant and in the wild-type. An activation energy of $180 \pm 10$ meV is calculated from the Arrhenius plot for the L722W$_{PsaA}$ variant, a value lower than the $220 \pm 10$ meV of the wild type. The Arrhenius plot also shows that the temperature dependence of electron transfer is altered and becomes almost independent of temperature below ~220 K. The increase in rate and decrease in the activation energy of forward electron transfer and the decrease in rate of charge recombination are consistent with an increase in the driving force ($-\Delta G$) due to a more reducing redox potential of $A_{1A}$, which is expected in the absence of the H-bond. The weakened H-bond may also change the reorganization energy of the phylloquinone. The change in the properties and environment of $A_{1A}$, however, have only a minor affect on electron throughput to $F_A/F_B$, as indicated by the accumulation of $F_A^-$ and $F_B^-$ in continuous light and by steady-state measurements of flavodoxin reduction.
4.2 INTRODUCTION

All photosynthetic reaction centers (RCs) share a common motif that consists of a protein dimer, which anchors the majority of the electron transfer cofactors in a $C_2$ symmetric fashion. Although the symmetry of the heterodimeric RC core allows for two independent electron transfer pathways, variations in the functional properties of the cofactors due to subtle differences in the protein environment dictate the flux of electrons through each branch. The dissimilarity between the two pathways is already evident at the level of $P_{700}$, which in its cationic state exhibits an asymmetric charge distribution on the Chl $a'/a$ special pair ($I$). Probing the initial charge separation that involves the six chl $a$ molecules is fraught with complications due to the trapping of the excited state by the chemically equivalent antenna pigments. In contrast, the $A_{1A}^-/A_{1B}^-$ to $F_X$ electron transfer steps can be readily studied by a variety of time-resolved optical and magnetic resonance techniques, thereby providing a convenient way to follow the kinetics as well as the pathway of electron transfer.

Early time-resolved optical studies indicated that the oxidation of $A_1^-$ occurred with a half-time of 15 ns in spinach chloroplasts ($2$) and 200 ns in cyanobacterial PS I complexes ($3$), while transient electron paramagnetic resonance (EPR) studies on both species reported a half-time of 180 ns ($4$, $5$). This discrepancy was resolved when the kinetics of $A_1^-$ oxidation was found to be biphasic in both species, with half-times of 25 ns and 150 ns in spinach PS I ($6$) and 7 ns and 190 ns in *Synechocystis* sp. PCC 6803 PS I ($7$). The ratio of the amplitudes of the slow and the fast phases however, is not fixed. Analysis of the spin polarized pattern of transient EPR spectra indicated that the contribution of the fast phase to the total amplitude was $\sim$30% in cyanobacterial PS I, but
the contribution increased to ~50% in spinach and Chlamydomonas reinhardtii PS I. The use of a weak non-ionic detergent to isolate PS I complexes resulted in a relatively high ratio of slow to fast kinetic phases whereas the use of strong detergents such as Triton X-100 resulted in a relatively low ratio of slow to fast kinetic phases. Studies on whole cells of a PS II-deficient mutant of Chlorella Sorokiniana (8) showed the oxidation of A$_1^-$ to be inherently biphasic, with half-times of 18 ns and 160 ns, thereby showing conclusively that the fast phase is not entirely a detergent induced artifact. The change in ratio was recently explained as a redirection of electron transfer through A$_{1B}$ at the expense of A$_{1A}$ due to the loss of the H-bond from Tyr696$_{PsaA}$ to A$_{0A}$ (9). Based on the lack of influence of the membrane potential on the relative amplitude of A$_1^-$ oxidation and the presence of the slow component upon the removal of F$_A$/F$_B$, it was initially proposed that the presence of two kinetic phases was due to electron transfer through a single branch that exists in two conformations or to electron transfer via the two branches of cofactors (8). There is now a consensus that the electron transfer in cyanobacterial PS I is bidirectional, albeit with the majority of the electrons being transferred via the PsaA branch of cofactors (see (10) for a detailed review).

The phylloquinones in the A$_{1A}$ and A$_{1B}$ binding sites possess some of the most negative redox potentials in biology (~ – 600 to – 800 mV vs NHE) (11). In contrast, plastoquinone-9 (PQ-9) in the Q$_B$ and Q$_A$ sites of Photosystem II (PS II) operates at a more oxidizing potential of 0 to – 150 mV (12). In the menB and menA mutants of Synechocystis sp. PCC 6803, PQ-9 is recruited into the A$_{1A}$ and A$_{1B}$ sites of PS I (13-15), where it assumes a redox potential of ~ – 500 to – 700 mV (16), a value 400 to 650 mV more reducing than the Q$_A$/Q$_B$ sites in PS II. The large variation in the midpoint potential
of PQ-9 between PS I and PS II clearly illustrates the influence of protein in modulating the cofactor’s thermodynamic properties. The redox potentials of $A_{1A}$ and $A_{1B}$ have been evaluated computationally by calculating the electrostatic energies from the solution of the Poisson-Boltzmann equation based on the 2.5 Å X-ray crystal structure of cyanobacterial PS I (17). The values depend on how many of the crystal water molecules near the $A_{1A}$ and $A_{1B}$ quinones are included in the calculation. Of the several values reported, midpoint potentials of $-531$ mV for $A_{1A}$ and $-688$ mV for $A_{1B}$, calculated when only water-37 was included, most closely matched the experimental results. More refined calculations that incorporate optical dielectric permittivity and a distribution of static dielectric constants, both of which account for protein-induced permanent charges, estimate the midpoint potentials of $A_{1A}$ and $A_{1B}$ to be $-671$ mV and $-844$ mV respectively (18). Because the equilibrium midpoint potential of $F_X$ is estimated to be $-654$ mV (18), the electron transfer from $A_{1B}$ to $F_X$ is strongly favorable, and the electron transfer from $A_{1A}$ to $F_X$ either barely favorable or unfavorable, depending on the values selected. These potentials are in reasonable agreement with the experimental observation of a thermally activated slow phase, which is attributed to the $A_{1A}^-$ to $F_X$ electron transfer step (19) and a non-activated fast phase attributed to the $A_{1B}^-$ to $F_X$ electron transfer (20).

In addition to electrostatic interactions, the H-bond between the quinone carbonyl oxygen and the backbone NH of a Leu residue, and the $\pi$–stacking interactions with a Trp residue are also involved in modulating the redox potential of $A_{1A}$ and $A_{1B}$. Electrostatic calculations indicate that $Trp_{697}^{PsaA}$ and $Trp_{677}^{PsaB}$ contribute an equivalent $-27$ mV to the midpoint potentials of $A_{1A}$ and $A_{1B}$ (17). Pump-probe optical studies of the $Trp_{697}^{PsaA}/Trp_{677}^{PsaB}$ variants in *C. reinhardtii* (21) and *Synechocystis sp.* PCC 6803
(22, 23) showed a slowing of the slow phase when the mutation was on the A-branch and a slowing of the fast phase when the mutation was on the B-branch. Transient EPR spectra of the P\textsubscript{700}\textsuperscript{+} A\textsubscript{1A}\textsuperscript{−} radical pair and Q-band CW EPR spectra of the photoaccumulated A\textsubscript{1A}\textsuperscript{−} radical indicated a stabilization of the semiquinone anion radical, consistent with an increase in redox potential. The charge recombination kinetics in the Trp to Phe mutants in C. reinhardtii indicated a 60 mV increase in redox potential, a value somewhat greater than that predicted from electrostatic calculations.

It is difficult to design an experimental strategy to determine the contribution of the H-bond to the redox potential of the quinone because the bond is formed between the backbone NH of Leu722\textsubscript{PsaA}/Leu706\textsubscript{PsaB} and the C\textsubscript{4} carbonyl oxygen of the naphthoquinone headgroup. Electrostatic calculations suggest that the contribution from the H-bond could be as little as -50 mV (17) while DFT calculations suggest that it could be as much as -250 mV (24). In the previous chapter, I presented the initial characterization of a variant in which the H-bonded Leu722\textsubscript{PsaA} was replaced with a bulky Trp (25). Briefly, transient EPR studies showed the phylloquinone remained tightly bound in the pocket with an orientation similar to the wild-type. The partially resolved hyperfine feature in the spin polarized transient EPR spectrum of the P\textsubscript{700}\textsuperscript{+} A\textsubscript{1A}\textsuperscript{−} radical pair typically seen in the wild-type, was nearly absent in the L722W\textsubscript{PsaA} variant, indicating that the spin density distribution on the A\textsubscript{1A}\textsuperscript{−} phyllosemiquinone was altered in a manner consistent with a significant weakening of the hydrogen bond. It was suspected that the bulky Trp residue displaces the equilibrium position of the A-\textsubscript{jk}(1) loop that contains the L722\textsubscript{PsaA} residue, thereby altering the H-bond distance. In the absence of the H-bond, the A\textsubscript{1A} quinone was susceptible to double reduction by sodium dithionite,
which resulted in the interruption of A-branch electron transfer. Under these conditions, the electron transfer through the B-branch was clearly observed by transient EPR spectroscopy. I concluded that the purpose of the H-bond is to tie up the C₄ carbonyl group thereby preventing its double reduction under periods of high illumination. A doubly reduced hydroquinone is in a thermodynamic well because it has a higher redox potential than either A₀ or Fₓ, thereby rendering the reaction center in operative in ferredoxin reduction.

In this chapter, a detailed kinetic characterization of the L722W_PsaA variant at room temperature is presented. The absence of the H-bond leads to an increased rate of electron transfer from A₁A⁻ to Fₓ as observed by room temperature transient EPR and time-resolved optical spectroscopy due to alteration of the midpoint potential of the A₁A to a more negative value. The change in the midpoint potential of the phylloquinone is reflected in an altered rate of charge recombination between [Fₓ/Fₐ]⁻ and P₇₀₀⁺. The substitution of Leu with Trp results in a distribution of H-bond strengths between the protein backbone and the quinone as indicated by the decay-associated optical spectra. I provide evidence that the absence of the H-bond also influences the reorganization energy in the environment of the quinone, which manifests as an alteration of the temperature dependence of forward electron transfer.

4.3 MATERIALS AND METHODS

4.3.1 Cell Culture and PS I Isolation – Synechocystis sp. PCC 6803 wild-type and mutant strains were cultured in β-HEPES medium with 5 mM glucose under low light intensity. The preparation of thylakoid membranes and the isolation of PS I trimers were carried
out according to a previously published procedure (23).

4.3.2 Steady State Electron Transfer Rates of Flavodoxin Photoreduction – Steady state electron transfer rates of flavodoxin photoreduction were measured using wild-type and mutant PS I complexes at 5 µg of Chl/ml in 50 mM Tris/HCl, pH 8.3. A typical sample contained 50 mM MgCl₂, 6 µM phenazine methosulfate, 20 µM cytochrome c₆, 15 µM flavodoxin, 6 mM sodium ascorbate, and 0.05% n-dodecyl-β-D maltoside. Measurements were made by monitoring the rate of change in the absorption at 580 nm using a Cary 50 Bio UV-Visible spectrophotometer with appropriate blocking filters for the actinic and measuring beams. The actinic illumination was provided by high-intensity red light-emitting diodes (Hansatech Instruments).

4.3.3 Low Temperature Continuous Wave EPR – Low-temperature EPR spectroscopy was conducted using a Bruker-ECS 106 X-band spectrometer equipped with an Oxford liquid helium cryostat and temperature controller. The spectrometer conditions were: microwave power, 100 mW; microwave frequency, 9.47 GHz; receiver gain, 20000; modulation amplitude, 20 G at 100 kHz. The signal-to-noise ratio was improved by averaging eight scans. PS I samples assayed for light-induced electron transfer contained 10 mM sodium ascorbate and 300 µM DCPIP as the external electron donor and mediator, respectively. In-cavity actinic illumination was provided by an argon ion laser, which was operated at 2.0 W in all-lines mode. In all cases, the dark spectrum (sample frozen in darkness) was subtracted from the light-induced spectrum to generate a light-induced difference spectrum of the reduced iron-sulfur clusters.

4.3.4 Out-of-Phase Echo Modulation – Electron spin-echo (ESE) amplitude-modulation curves were obtained on an Bruker Elexsys E580 spectrometer by collecting the
amplitude of the echo in the out-of-phase channel. The echo height was measured at time \( T \approx \tau_2 \) after the second microwave pulse of a laser flash-t1 -90-t2 -180 pulse sequence as function of \( \tau_2 \) (see (26) for details).

4.3.5 Time-Resolved Optical Spectroscopy at 480 nm – The kinetics of \( A_{1A}^- \) and \( A_{1B}^- \) oxidation were measured optically on the ns time scale by monitoring flash-induced transient absorption changes at 480 nm using a time-resolved spectrophotometer similar to that described in ref. (27). The excitation beam was provided by a frequency-doubled (\( l = 532 \) nm), Q-switched Nd:YAG laser (DCR-11, Spectra Physics, Mountain View, CA) operated in the short pulse mode (ca. 3 ns). The measuring light was provided by a xenon flash that was tailored with a bank of inductors and capacitors to produce a relatively flat top for 5 \( \mu s \). The 480 nm measuring beam was filtered using a combination of narrow-band (8 nm) interference and colored glass filters (to block the scattered laser flash) prior to the sample and in front of the Si photodiode detector (FND 100Q from EG&G). The photocurrent was changed to a voltage across a 50 \( \Omega \) resistor, and the signal was amplified using a laboratory-built gain block (30 dB, 500 Hz-1.7 GHz) and recorded using a digitizing oscilloscope (DSA 602A with amplifier plug-in 11A52 from Tektronix, Beaverton, OR). A photodiode connected to a 11A72 plug-in detected the laser flash and initiated the data acquisition. The rise-time of the detection system was measured using Ru(Bipy)\(_3\)Cl\(_2\) luminescence to be 3 ns. The baseline was recorded after every flash by mechanically blocking the excitation flash, and the no-flash transient was subtracted from that recorded with flash excitation on every cycle. Typically, 1024 or 2048 pairs of flash minus no-flash transients were averaged at a repetition rate of 1 Hz. Software written in LabView controlled the timing sequence and the acquisition and manipulation of the
data. The sample was contained in a 10×10mm standard quartz cuvette. All spectroscopic measurements were performed at room temperature. Kinetic traces were analyzed by fitting with a multiexponential function using Marquardt least-squares algorithm that was programmed in IGOR Pro v. 5.2 (Wavemetrics, Lake Oswego, OR).

4.3.6 Pump-Probe Optical Spectroscopy in the Near-UV and Visible Region – Measurements of the kinetics of semiquinone oxidation in the ns to µs time scale were performed on isolated PS I trimers in a pump-probe spectrophotometer as described previously (28). The PS I complexes were suspended at a concentration of 40 µg/ml in 50mM Tris buffer with sodium ascorbate and 2,6-dichlorophenol-indophenol (DCPIP) concentrations of 10 mM and 40 µM, respectively. Decay-associated spectra of the kinetic phases were derived from a global multi-exponential fit of the kinetic components obtained at each wavelength, using the program MEXFIT adapted from (29). Charge separation was induced by a 5-ns (full width at half-maximal) light pulse at 700 nm using a Nd:YAG pumped LDS 698 dye exciting ~70% of the PS I complexes. Absorbance changes were followed from 5 ns to 20 µs using detecting flashes provided by an OPO Continuum (OPO Panther, type II) from 300 to 540 nm, frequency doubled for wavelengths less than 410 nm. For detection in the UV, a fluorescent glass (Sumita Optical Glass, Lumilass-B) was used to convert the UV photons to blue photons to minimize noise.

4.3.7 Optically Detected Magnetic Resonance – The set-up used to record fluorescence detected magnetic resonance (FDMR) has been previously described in detail (30). All samples were diluted to a concentration equivalent to 100 µg Chl/ml in a buffer containing 0.1 M sorbitol, 10 mM NaCl, 5 mM MgCl₂ and 60% w/v glycerol
immediately before measurement. Prior reduction of PS I electron acceptors was accomplished by a photo-accumulation procedure as described by Carbonera et al., (31) with minor modifications. Sodium dithionite was added to the sample to a final concentration of 10 mM, incubated under anaerobic conditions in the dark for 5 minutes, diluted into glycerol-containing buffer to a final concentration of 60% w/v, and illuminated for 5 minutes with a focused white light from a 1000-W halogen lamp. Deconvolution of the FDMR spectra in terms of Gaussian bands was carried out as described (30).

4.3.8 Transient EPR Spectroscopy – X-band (9 GHz) transient EPR experiments were carried out on a laboratory built spectrometer using a modified Bruker 200D-SRC spectrometer equipped with an ER-4118XMD-5W1 dielectric ring resonator and an Oxford CF935 helium gas flow cryostat. The loaded $Q$-value for this dielectric ring resonator was about $Q = 3000$, equivalent to a rise time of $\tau_r = Q/(2\pi \times \nu_{mw}) \approx 50$ ns. All samples contained 1 mM sodium ascorbate and 50 µM phenazine methosulfate as the external electron donor in 50 mM Tris buffer at pH 8.3, and were frozen in the dark. The samples were illuminated at 532 nm using the second harmonic of a Continuum Surelite Nd YAG laser operating at 10 Hz.

4.3.9 Time Resolved Optical Spectroscopy in the Near-infrared Region – The kinetic of the $P_{700}^+ [F_A/F_B]^{-}$ backreaction were measured in the near-IR were using a laboratory-built, time-resolved spectrophotometer. The samples were placed in a quartz cuvette with a path length of 10 mm. The sample contained PS I trimers at 50 µg/mL Chl a in 50 mM Tris-HCl (pH 8.2), 10 mM sodium ascorbate, 4 µM 2,6-dichlorophenolindophenol (DCPIP), and 0.04% Triton X-100. The samples were prepared in an anaerobic chamber
with an atmosphere of 10% hydrogen and 90% nitrogen (Coy Laboratories, Grass Lake, MI). The kinetic traces were analyzed by fitting a multiexponential decay using the Marquardt least-squares algorithm (Igor Pro, Lake Oswego, OR).

4.4 RESULTS

Before presenting the detailed studies of the kinetics and energetics of the $A_{1A}^-$ to $F_x$ electron transfer, it is important to first characterize the overall function and electron transport properties of PS I complexes from the L722W$_{PsaA}$ variant.

4.4.1 Electron Transfer Throughput at 298 K – The effect of the mutation on the ability of the complexes to catalyze light driven electron transfer from a high potential donor to a low potential acceptor at room temperature is best studied by measuring steady-rate rates of flavodoxin reduction. The rates of flavodoxin reduction supported by the wild-type and L722W$_{PsaA}$ variant under saturating light conditions were 1300 $\mu$mol mg Chl$^{-1}$ hr$^{-1}$ and 900 $\mu$mol/mg Chl$^{-1}$ hr$^{-1}$ respectively. Assuming 100 Chl/P$_{700}$, these rates correspond to 36 e$^-$ PS I$^{-1}$ s$^{-1}$ for the wild-type and 25 e$^-$ PS I$^{-1}$ s$^{-1}$ for the L722W$_{PsaA}$ variant. It is noteworthy that the light dependence of the flavodoxin reduction in L722W$_{PsaA}$ is comparable to the wild-type (Figure 1), indicating that the relative quantum efficiency is not affected by the mutation. This is not surprising given that the mutation is not expected to affect light harvesting or charge separation. These results show that although H-bond between residue L722$_{PsaA}$ and the A-branch phylloquinone is significantly weakened (25), the variant PS I complexes are capable of supporting a rate of electron transfer to flavodoxin, which is only slightly lower than in wild type PS I.
4.4.2 Yield of $F_A/F_B$ Reduction at 15 K – At low temperature in PS I complexes from the wild type, several fractions with different electron transfer properties exist \((19, 32, 33)\). In one fraction, electron transfer to the terminal iron-sulfur clusters $F_A/F_B$ leads to irreversible charge separation. In another fraction, reversible electron transfer between $P_{700}$ and $A_{1A}$ occurs. Some reversible electron transfer between $P_{700}$ and $F_X$ has also been reported \((19)\). The origin of these various fractions is not well understood but it is known that the reversible electron transfer occurs between $P_{700}$ and $A_{1A}$ \((22, 23, 34)\). Hence it is of interest to investigate whether alteration of H-bond to the A-branch phylloquinone has any effect on the ability of the complexes to transfer electrons to $F_A/F_B$ at low temperature. Stable reduction of the iron-sulfur clusters is most easily observed by EPR spectroscopy. Because re-reduction of $P_{700}^+$ does not occur at low temperature, illuminating a dark-frozen sample at 15 K leads to either reversible electron transfer or trapping of a single electron on either $F_A$ or $F_B$ in a given PS I complex. The resulting spectrum is the sum of the individual spectra of $F_A^-$, with $g$ values of 2.07, 1.95, and 1.85 and $F_B^-$ with $g$ values of 2.05, 1.93, 1.88. Figure 2 (top) shows that relative intensities of the resonances of reduced $F_A^-$ and $F_B^-$ are similar in the wild-type and the L722W$_{PsaA}$ variant, indicating that the mutation does not interfere with the ability to promote electrons to the terminal iron sulfur clusters at low temperature. When the samples are illuminated while slowly freezing, re-reduction of $P_{700}^+$ can occur before charge recombination and it becomes possible to trap more than one electron on the iron sulfur clusters and hence to reduce both $F_A$ and $F_B$ within the same PS I complex. Under these conditions, a so-called magnetic interaction spectrum is observed due to the close proximity of the two spins, with apparent $g$-values of 2.05, 1.94, 1.92, and 1.88 in both
the wild-type and L722W_{PsaA} variant (Figure 2, bottom). The similar intensities of the resonances in the wild-type and L722W_{PsaA} variant indicate that the entire population of PS I complexes are capable of carrying out electron transfer to the terminal iron-sulfur clusters.

4.4.3 Distance Measurement between P^{700+} and A_{1A}^- – In principle, the weakening of the H-bond seen in the L722W_{PsaA} variant could be accompanied by a change in the position of the quinone, which would be expected to have an important impact on the electron transfer rates. Hence, it is important to determine whether the position of the quinone is altered in the variant. Out-of-phase electron spin echo envelope modulation (OOP-ESEEM) experiments allow the distance between the P^{700+} and A_{1A}^- radicals to be probed (see (35) for a review) and give an indication of whether the position of the quinone is affected by the mutation. The electron spin echo of a singlet-born, weakly coupled, spin-correlated radical pair exhibits deep amplitude modulations as a function of the pulse spacing and is phase-shifted by 90° when compared with that of a radical. The modulation frequency is determined by spin–spin coupling, the dipolar part of which is a sensitive indicator of the distance between the spin density centers of the respective radicals. A comparison of the OOP-ESEEM signal of the radical pair P^{700+}A_{1A}^- in the wild type and the L722W_{PsaA} variant is shown in Figure 3. As is apparent, the two modulation curves are virtually indistinguishable indicating that the dipolar coupling and hence the distance between state P^{700+} and A_{1A}^- is unaltered by the mutation. Disorder in the binding site would be expected to cause a distribution of distances and modulation frequencies, which would lead damping of the modulation curve ((26),34). Since no difference in the decay of the modulation is observed for the L722W_{PsaA} variant and the
wild type, I conclude that the phylloquinone does not experience greater disorder as a result of the mutation. Thus, the position of the phylloquinone and the degree of disorder in its binding site appear to be unaltered in spite of the significant weakening of the H-bond.

Together, the preceding results show that the electron transport efficiency at room temperature and low temperature and the position of the quinone are not affected to any great extent by the L722Wₚₛₐₐ mutation.

4.4.4 Time-Resolved Optical Measurements – Figure 4 depicts flash induced absorption changes in PS I trimers isolated from the wild-type and the L722Wₚₛₐₐ variant using time-resolved optical spectroscopy. Forward electron transfer from $A_{1A}^-$ and from $A_{1B}^-$ to $F_X$ are typically monitored at 380 nm, wherein a laser flash results in an increase in absorbance due to the formation of a semiquinone anion, which then decays bi-exponentially as the electron is transferred from the phylloquinones to the iron-sulfur cluster $F_X$. In the L722Wₚₛₐₐ variant, the absorbance changes at 380 nm were weak in comparison to the wild-type. I therefore carried out the measurement at 480 nm, a wavelength at which the phyllosemiquinone anion causes an electrochromic bandshift in a nearby carotenoid molecule (36). The kinetic traces obtained at this wavelength are similar to those at 380 nm except that an additional, positive contribution due to long-lived $P_{700}^+$ is also observed. Bi-exponential fits for the wild-type sample measured at 480 nm yield lifetimes of 17 ns and 208 ns for the two kinetic phases, with extrapolated relative amplitudes of 0.21 and 0.79 respectively. Similar fits for the L722Wₚₛₐₐ sample yield lifetimes of 15 ns and 86 ns for the two kinetic phases, with extrapolated relative amplitudes of 0.47 and 0.53 respectively. According to current thinking, the fast kinetic
decay represents forward electron transfer from $A_{1B}^-$ to $F_X$ and the slow kinetic decay represents forward electron transfer from $A_{1A}^-$ to $F_X$. In both samples, the relative amplitude of the slow kinetic phase is larger than that of the fast kinetic phase, a finding that agrees with previously published data at 380 nm for cyanobacterial PS I. These results show that the slow kinetic phase becomes faster in the $L722W_{PsaA}$ variant, consistent with a more reducing midpoint potential for $A_{1A}$ as a result of the weakened H-bond (see discussion). The fast kinetic phase remains unchanged, but the ratio of fast to slow kinetic phases appears to have increased in the $L722W_{PsaA}$ variant relative to the wild-type.

To obtain further information about the changes in the kinetics induced by the mutation, transient absorption changes were examined in the ns to µs scale at discrete wavelengths in the near-UV and blue region by pump-probe optical spectroscopy in PS I particles. Global analysis of the transients obtained yielded three kinetics components: two that decay in the ns time range, and one that is long-lived and does not decay within the detection time of the experiment. The decay-associated spectra (DAS) of these components are shown in Figure 5 along with the initial absorbance change at time zero. The spectrum of the non-decaying component is similar to that of the $P_{700}^{+} [F_{A}/F_{B}]^-$ minus $P_{700} [F_{A}/F_{B}]$ difference spectrum (37). The two ns time components display positive absorption between 370 nm and 400 nm and are characteristic of the oxidation of the $A_{1A}^-$ and $A_{1B}^-$ phylloquinones. These spectra also show positive changes in the phylloquinone absorption between 370 nm and 400 nm and a bathochromic band shift of a carotenoid centred around ~450 nm. The spectra of the exponential decays normalized to the non-decaying component are shown for both wild-type and the $L722W_{PsaA}$ variant
(Figure 5). Panel A depicts the fast phase due to $A_{1A}^-$ to $F_X$ electron transfer; panel B depicts the slow phase due to $A_{1B}^-$ to $F_X$ electron transfer; panel C shows the normalized spectra of the non-decaying phase together with the additional 4 µs phase obtained for $L722W_{PsaA}$ and panel D shows the initial spectra extrapolated to time zero. Considerable variation in the lifetime of the decay, as well as subtle changes in the shape of the decay-associated spectra (DAS) are observed for the $L722W_{PsaA}$ variant. The major effect of replacing the H-bonded Leu residue in the A-branch with Trp is a change in the lifetime of the slow kinetic phase, which decreases from 285 ns in the wild type to 105 ns in the $L722W_{PsaA}$ variant. The fast kinetic phase remains relatively unchanged. In agreement with the *in-vitro* study at 480 nm, ratio of the fast to the slow component is higher in the $L722W_{PsaA}$ variant (ratio at 370 nm; 50:50) than in the wild type sample (ratio at 380 nm; 20:80), and the spectrum shows a hypsochromic shift in the blue region, with the dominant peak at 380 nm being shifted to ~ 370 nm. This is likely the reason that the absorption change of the $L722W_{PsaA}$ variant using the time-resolved instrument at 380 nm was weak. The DAS of the nanosecond component also display a marked trough in the 435 to 445 nm region. Furthermore, an additional component, a 4 µs phase, is observed in the $L722W_{PsaA}$ variant. I attribute this component to the decay of the $^3P_{700}$ species formed during the recombination event between $P_{700}^+$ and $A_0^-$. In wild-type PS I trimers, the amplitude of this component is so small that a spectrum could not be extracted.

In a previous study (25), I have shown that the $A_{1A}$ quinone in the $L722W_{PsaA}$ variant is susceptible to double reduction/protonation, which might explain the observed recombination. However, in order to rule out the possibility that the phase is an artifact
produced by the presence of redox mediators such as ascorbate, I performed experiments on whole cells. The spectra of the exponential decays normalized to the non-decaying component are shown for both wild-type and the L722W<sub>PsaA</sub> variant (Figure 6). Panel A depicts the fast phase due to A<sub>1A</sub>←F<sub>X</sub> electron transfer; panel B depicts the slow phase due to A<sub>1B</sub>←F<sub>X</sub> electron transfer; panel C shows the reduction of P<sub>700</sub><sup>+</sup> by cytochrome<sub>c<sub>6</sub></sub> together with the normalized spectra of the non-decaying component; and panel D shows the initial spectra extrapolated to time zero. The DAS obtained from whole cells are similar to those obtained from PS I particles. The 6 µs component depicted in panel C is attributed to the reduction of P<sub>700</sub><sup>+</sup> based on its typical bleaching at 430 nm. The presence of ‘chl-like’ bleaching in the ns component together with the decrease in amplitude of the t<sub>0</sub> component indicates contribution from the P<sub>700</sub><sup>+</sup> A<sub>0</sub><sup>−</sup> recombination event. It should be noted that quantifying the fraction of PS I that generates triplets or that is involved in recombination between A<sub>0</sub><sup>−</sup> and P<sub>700</sub><sup>+</sup> based on the DAS is a relatively difficult undertaking.

4.4.5 Quantification of <sup>3</sup>P<sub>700</sub> Yield – The yield of <sup>3</sup>P<sub>700</sub> formed by charge recombination from P<sub>700</sub><sup>+</sup> A<sub>0</sub><sup>−</sup> provides another measure of the efficiency of forward electron transfer and is most accurately measured by fluorescence-detected magnetic resonance (FDMR). An FDMR spectrum arises from changes in the steady-state fluorescence when a microwave field is applied in resonance with transitions between the spin levels of the triplet state of the emitting species. The fluorescence intensity is measured as a function of the microwave frequency and typically, three transitions are observed at frequencies 2|E|, |D| + |E| and |D| − |E|, where |D| and |E| are the two zero-field splitting parameters that define the difference in the energy levels of the triplet state. For Chl molecules, these transitions
are in the region of 100 to 1000 MHz. FDMR spectra of thylakoid membranes isolated from the wild type and the L722W\textsubscript{PsA} variant are shown in Figure 7. The |D| + |E| and |D| – |E| transitions, which induce a change in the fluorescence intensity at 720 nm, are shown for both dithionite-treated as well as non-treated samples. Both samples display resonance transitions with maxima at ~715 MHz (|D| - |E|) and ~940 MHz (|D| + |E|). To accurately quantify the relative \(^3\text{P}_{700}\) yields in the variant with respect to the control, the FDMR spectra were globally deconvoluted as Gaussian bands. To estimate the maximal amount of \(^3\text{P}_{700}\) that can be generated in the PS I complexes for a given Chl \(a\) concentration, the samples were incubated with dithionite and illuminated to reduce the iron-sulfur clusters and A\(_1\). Under these conditions, electron transfer past A\(_0\) is blocked and recombination between P\(_{700}\)^+ and A\(_0^–\) generates \(^3\text{P}_{700}\). FDMR measurements with the terminal acceptors reduced and oxidized are compared in Figure 7. If I assume that the FDMR intensity found under reducing conditions represents triplet formation in all of the PS I complexes, the ratio of the intensities for the two sets of conditions gives the percentage of complexes in which \(^3\text{P}_{700}\) is formed under ambient redox conditions. In the L722W\textsubscript{PsA} variant, I find that 9 to 12 % of the PS I complexes generate triplets, which is somewhat greater than the 2 to 4% of PS I complexes found for the wild-type sample. The formation triplets could be caused either by a population of PS I complexes in which the phylloquinone binding site is empty or the phylloquinone is unable to act as an acceptor. Although this population does appear to increase in the L722W\textsubscript{PsA} variant it remains a very small fraction of the PS I complexes. Hence, I can conclude that the yield of electron transfer past A\(_0\) is not significantly reduced by the mutation.
4.4.6 Room Temperature Spin Polarized Transient EPR Measurements of Forward Electron Transfer—The transient absorbance data presented in Figures 4.6 indicate that the rate of electron transfer from $A_{1A}^-$ to $F_x$ is increased in the L722W$_{PsaA}$ variant. Spin polarized transient EPR (TREPR) measurements provide a convenient way to confirm this finding. For wild type PS I, two consecutive polarization patterns are observed in the TREPR data at temperatures above 220 K (38). As the electron is transferred from $A_{1A}^-$ to $F_x$, the E/A/E polarization pattern (early spectrum) due to the $P_{700}^{+}A_{1A}^{-}$ radical pair changes to one with net emission (late spectrum) that arises from the $P_{700}^{+}$ contribution of the $P_{700}^{+}(Fe/S)^-$ radical pair. Although it is known that the electron transfer proceeds via $F_X$ (4), the radical pair is referred to as $P_{700}^{+}(Fe/S)^-$ to allow for the possibility that the $F_X^-$ to $F_A$ step is faster than the $A_{1A}^-$ to $F_X$ step (39). Only the slow phase of electron transfer from $A_{1A}^-$ to $F_X$ that occurs via the A-branch is kinetically resolved in the transient EPR. The kinetic phase from $A_{1B}^-$ to $F_X$ through the B-branch occurs faster than the risetime of the spectrometer and therefore a signal due to $P_{700}^{+}(Fe/S)^-$ that rises with the spectrometer risetime is observed from complexes in which B-branch electron transfer occurs. Figure 8 (top) shows the spin polarization pattern of PS I complexes from the wild-type and the L722W$_{PsaA}$ variant at 293 K. The spectra are taken at time windows centered at 100 ns, 300 ns and 1240 ns after the laser flash. The solid curves are simulations (see (40) for parameters used in simulations) and the dashed curves are experimentally observed spectra. In the wild-type sample (Figure 8, top right), at 100 ns, the spectrum is predominantly due to the radical pair $P_{700}^{+}A_{1A}^-$. With increasing time, the polarization pattern evolves into the mainly emissive spectrum due to $P_{700}^{+}(Fe/S)^-$. The decay of the $P_{700}^{+}A_{1A}^-$ radical pair is seen most clearly by the disappearance of the
emissive feature associated with $A_{1\Delta}^-$ at field position (a). In the L722W$_{PsaA}$ variant (Figure 8, top left), the early radical pair spectrum representing $P_{700}^{\cdot+}A_{1\Delta}^-$ has almost completely decayed within ~100 ns following the laser flash and is only visible as an extremely weak emissive feature at field position (a). The majority of the contribution in all three time windows shown in Figure 8 arises from $P_{700}^{\cdot+}$ in the $P_{700}^{\cdot+}(Fe/S)^-$ radical pair indicating that the electron transfer to $F_\Delta$ is much faster in the L722W$_{PsaA}$ variant. A comparison of the late spectrum (1240 ns) of the wild-type and variant indicates that its polarization pattern is unaltered, showing that the spin dynamics in the preceding radical pairs remain unaffected in the L722W$_{PsaA}$ variant. The faster rate of electron transfer in the L722W$_{PsaA}$ variant is also apparent in the comparison of the transients shown in Figure 8 (bottom). At position (a) only $P_{700}^{\cdot+}A_{1}^-$ contributes to the signal and it is evident that its lifetime is close to the risetime of the spectrometer (50 ns), which results in a very weak response.

The rates of electron transfer in the two samples can be obtained from a global simulation of the time/field dataset as described in (40). In these simulations, only the rate of electron transfer from $A_{1\Delta}^-$ to $F_\Delta$, the spin-lattice relaxation time and the percentage of electron transfer in the two branches are allowed to vary and their values are determined from a fit to the dataset. The results of the simulation are shown as the solid spectra (Figure 8, top) and solid time traces (Figure 8, bottom).

The lifetime of the $A_{1\Delta}^-$ to $F_\Delta$ electron transfer obtained from the fit is $\tau = 270 \pm 30$ ns for the wild-type and $\tau = 50 \pm 30$ ns for the L722W$_{PsaA}$ variant. These values are in reasonable agreement with the lifetimes obtained from the transient absorbance measurements and support the conclusion that replacement of the Leu722$_{PsaA}$ residue...
with Trp causes a significant increase in the rate of forward electron transfer from $A_{1A}^-$ to $F_X$. In the fits of the TREPR datasets, the lifetime of the $A_{1B}^-$ to $F_X$ electron transfer was held constant at 20 ns and values of less than 10% B-branch transfer gave best agreement with the experimental data for both the wild type and the $L722W_{PsaA}$ variant. There is a large uncertainty in the amount of B-branch transfer obtained from the fit but qualitatively, the TREPR data are in agreement with the optical results which indicate that only a minor fraction of electrons are transferred via the B-branch.

4.4.7 Temperature Dependence of the Rate of Electron Transfer from $A_{1A}^-$ to $F_X$ – In the wild type, the slow component of the $A_{1A}^-$ to $F_X$ step is known to be strongly activated \([19, 20]\) and a change in this activation energy is the most likely reason for the faster electron transfer rate in the $L722W_{PsaA}$ variant. The observed increase in the rate of the forward electron transfer in the $L722W_{PsaA}$ variant implies a decrease in the activation energy and therefore a weaker temperature dependence. I have tested this idea by measuring the temperature dependence of the electron transfer by TREPR. In wild-type PS I, the slow phase of electron transfer is directly observable in the spin polarized TREPR signals at temperatures above 220 K. Below this temperature, the rate of forward electron transfer becomes slower than the spin relaxation processes and the late signal falls below the detection limit of the instrument. In contrast, the forward electron transfer kinetics from $A_{1A}^-$ to $F_X$ in the $L722W_{PsaA}$ variant is sufficiently fast that it can be clearly seen at temperatures down to 100 K. Figure 9 shows the Arrhenius plot of the natural logarithm of the electron transfer rate \(versus\) the inverse temperature. For the wild type, data taken from \([19]\) are plotted along with the rates from the optical and TREPR measurements reported here. The straight line through these data specifies an activation
energy of 220 ± 10 meV and a maximum rate of (2.6 ± 0.1) × 10^{10} \text{s}^{-1}. For the L722W_{PsaA} mutant, the points do not lie on a single straight line and the slope is considerably different above and below ~220 K. Above this temperature the slope and intercept give an activation energy of 180 ± 10 meV and a maximum rate of (2.6 ± 0.1) × 10^{10} \text{s}^{-1} respectively. Below ~200 K, the rate is almost independent of temperature. Possible reasons for this behavior will be discussed in more detail below. However, it appears that above ~200 K the activation energy is lower than in the wild type.

4.4.7 Charge Recombination Kinetics between $P_{700}^+$ and $[F_A/F_B]^-$ – In wild-type PS I complexes, the recombination between $P_{700}^+$ and $[F_A/F_B]^-$ at room temperature occurs with a lifetime of 80 ms after a saturating flash (Figure 10, Top). The lifetime of the charge recombination between $P_{700}^+$ and $[F_A/F_B]^-$ in the L722W_{PsaA} variant is slowed to 120 ms (Figure 10 Bottom). In both samples, a slow kinetic phase that accounts for a minor fraction of the total decay amplitude is observed. This phase represents donation of an external electron donor to $P_{700}^+$ in those reaction centers in which $[F_A/F_B]^-$ has donated an electron to an oxidant, most probably molecular oxygen. A weaker H-bond between phylloquinone and the protein is expected to push the midpoint potential of the quinone more negative leading to a change in $\Delta G$ for both forward and reverse electron transfer between $A_{1A}$ and $F_X$. This change in $\Delta G$ (and/or a change in the reorganization energy) results in a smaller activation energy and faster forward electron transfer in the L722W_{PsaA} variant than in the wild type. This implies that the activation energy and lifetime for the reverse reaction from $F_X^-$ to $A_{1A}$ increases. If charge recombination from $F_A/F_B$ occurs by thermal repopulation of the electron acceptors, and it proceeds via the A-branch quinone it should therefore be slowed as observed here.
4.5 DISCUSSION

Our results show that the weakened H-bond between the carbonyl oxygen of the phylloquinone and the backbone NH of Leu722PsaA results in: i) no significant change in the overall throughput of electrons though PS I ii) no change in the position of A<sub>1A</sub> iii) an approximate 3- to 4-fold increase in the rate of forward electron transfer from A<sub>1A</sub><sup>-</sup> to F<sub>X</sub> at 293 K, iv) a 1.5 fold slowing of the backreaction between P<sub>700</sub><sup>+</sup> and [F<sub>A/F_B</sub>]<sup>-</sup> and v) two distinct activation energies and maximum rates for the A<sub>1A</sub><sup>-</sup> to F<sub>X</sub> electron transfer in different temperature regimes.

4.5.1 Possible Structural Role of the H-bond – Since the main function of H-bonds in biological systems is in forming ordered structures it is natural to expect that the H-bond to phylloquinone might play a structural role and be important for binding the quinone to the protein. If this were true disruption of the H-bond in the L722W<sub>PsaA</sub> variant might be expected to result in partial loss of phylloquinone from the A<sub>1A</sub> binding site and possibly a change in the position of the quinone. Time-resolved optical spectroscopic studies on the L722W<sub>PsaA</sub> variant suggest some recombination at the level of A<sub>0</sub> does occur. Normalizing the spectra based on the non-decaying phase indicates that the amplitude of the 6 ms component and the amplitude of the spectrum extrapolated to time zero is larger for L722W<sub>PsaA</sub> when compared to wild-type. This together with the prominent trough in the 435 to 445 nm region of the two ns components indicates a contribution of an additional kinetic event. This can be accounted for by singlet recombination of P<sub>700</sub><sup>+</sup>A<sub>0</sub><sup>-</sup> based on the (A<sub>0</sub><sup>-</sup> minus A<sub>0</sub>) difference spectrum, which has a characteristic bleaching minimum at 438 nm (39). However, accurate estimation of this contribution is difficult due to spectral overlap with other components. Moreover, solvent extraction studies (41)
show that when phylloquinone is lost from the binding site, triplet recombination between P$_{700}^+$ and A$_0^-$ gives rise to $^3$P$_{700}$ that also decays in ~ 6 µs. In whole cells, it is not possible to distinguish between this component and the reduction of P$_{700}^+$ by plastocyanin, which also occurs in ~ 6 µs. Using isolated PS I trimers, and ascorbate as the exogenous electron donor donation to P$_{700}^+$ is much slower and the two component can be distinguished. Under these conditions, only a minor contribution from a 6 µs component that exhibits bleaching at ~ 435 nm indicative of $^3$P$_{700}$ is observed for the L722W$_{PsaA}$ variant. The FDMR data shown in Figure 7 provide the most accurate estimation of the yield of $^3$P$_{700}$. As discussed above, these data show that the triplet yield in both the L722W$_{PsaA}$ variant and the wild type is small and that it is only slightly increased in the variant. The low yield of $^3$P$_{700}$ was also confirmed by low temperature transient EPR spectroscopy, which showed no evidence of the characteristic spin polarization pattern of a triplet state formed by charge recombination (data not shown). I envision two scenarios to explain the minor non-functional A$_{1A}$ sites; they are either empty or contain functionally inactive phyllohydroquinone. In vitro incorporation of added 2-methyl-1,4-naphthoquinone to isolated PS I complexes of the L722W$_{PsaA}$ variant did not produce a detectable change in the polarization pattern of the transient EPR spectrum (data not shown) indicating the absence of empty sites.

It is interesting to consider the possibility of double reduction that results in the formation of phylohydroquinone. In a recent study (25), I showed that addition of dithionite led to double reduction of A$_{1A}$ in the L722W$_{PsaA}$ variant. I postulated that this was because in the absence of the H-bond the reduced quinone could become protonated. Because the midpoint potential of the semiquinone/hydroquinone couple is higher (i.e. more positive)
than that of the quinone/semiquinone couple, the electron would reside in a thermodynamic trap, thereby preventing forward (as well as backward) electron transfer. It was proposed that the function of the H-bond is to tie up the carbonyl oxygen group so that protonation, which is a prerequisite for double reduction, cannot occur during periods of high intensity illumination.

4.5.2 Energetic Role of the H-bond – At first glance, the H-bond between the protein backbone and phylloquinone appears to be at odds with the requirement that the quinone function at a very low redox potential. The H-bond stabilizes the semiquinone anion radical relative to the ground state, thereby raising the redox potential to a higher (i.e. more positive) value. This means that other factors in the environment must drive the midpoint potential of the phylloquinones even lower (i.e. more reducing) than would otherwise be necessary to compensate for the effect of the H-bond. The magnitude of this effect is difficult to estimate accurately. However, according to electrostatic calculations (17) the H-bond could contribute as little as +50 mV and according to DFT calculations as much as +250 mV (24) to the midpoint potential of the quinone. In a recent study (42), amine-substituted naphthoquinones, which contained one or two internal H-bonds, were synthesized in an attempt to relate H-bond strength to midpoint potential. A series of 11 phylloquinone derivatives, each lacking the extended phytol side chain but featuring H-bond donor amides at selected peri positions on the quinone head group were evaluated. Depending on the electron withdrawing capability of the substituent, the phylloquinone reduction potential was driven more oxidizing by as little as +76 mV or by as much as +207 mV. Clearly, the H-bond has the ability to alter the redox potential of the A_{1A} phylloquinone by a significant amount.
The effect weakening of the H-bond on the driving force (−ΔG) for electron transfer from A_{1A}⁻ to F_X depends on whether the reaction is exergonic or endergonic. Estimates in the literature indicate that A_{1A} and F_X are nearly isoenergetic so that the electron transfer is energetically slightly uphill or slightly downhill (see (10) for a review). In the case of exergonic electron transfer pushing the quinone potential more negative increases the driving force, whereas if ΔG is positive the energy difference is decreased. However, in either case the change is expected to result in a lower activation barrier and faster electron transfer.

For downhill electron transfer, the non-adiabatic rate can be described by Marcus theory (43) according to Equation 1.

\[ k_{et} = \frac{2\pi |V|^2}{\hbar \sqrt{4\pi k_B T}} \exp \left\{ -\frac{(\Delta G + \lambda)^2}{4\lambda k_B T} \right\} \]  

(4.1)

where, \( V \) represents the electronic coupling between the initial and final state electronic wavefunctions, which is expected to decrease exponentially with the edge-to-edge distance, \( R \), between the redox pairs, according to the relation \( V = \exp (-\beta R) \). In the case of uphill electron transfer the rate is related to the corresponding downhill reaction in the reverse direction by the Boltzmann factor (44).

\[ k^{\text{uphill}}_{et} = k^{\text{downhill}}_{et} e^{\frac{\Delta G}{kT}} \]  

(4.2)

The term \((\Delta G + \lambda)^2/4\lambda \) in Eq. (1) represents the activation energy, E_a. \( \lambda \) and \( \Delta G \) are the reorganization energy and the Gibbs free energy change respectively. \( \lambda \) is difficult to measure experimentally however, a wide range of reactions in purple bacterial reaction centers have been shown to be consistent with a common value of ~ 0.7 eV (45). For the
A1A- to Fx step the activation energy (~0.2 eV) is close to λ/4 (~0.18 eV) indicating that the reorganization energy dominates E_a.

The two factors under experimental control that influence the rate of electron transfer between two cofactors are the inter-cofactor distance, which influences the electronic coupling, and ΔG, which influences the activation energy. Based on out-of-phase echo modulation studies, the distance between P_{700}^+ and the A1A- phylloquinone in the L722W_PsaA variant is identical to the wild-type and previously published transient EPR studies show that the orientation of the phylloquinone in the L722W_PsaA variant is essentially the same as that of the wild-type (25). Because the Leu to Trp substitution is distant from the Fx cluster, significant structural changes in the Fx binding pocket are not expected. Thus, the distance between A1A and Fx in the L722W_PsaA variant is probably not significantly altered compared to the wild-type. Accordingly, no change in the electronic coupling is expected and the same maximum rate of A1A- to Fx should be found. The change in the electron transfer rate would then be due to a change in E_a resulting from a change in the midpoint potential of the quinone.

The Arrhenius plots (Figure 9) is consistent with expectations, the value of E_a in the L722W_PsaA variant (180 ± 10 meV) is slightly smaller than in the wild type (220 ± 10 meV), while the maximum rate is unchanged. If l is assumed to be the same in the wild type and the variant, the change in E_a is due to a change in the midpoint potential of the quinone. For l ~0.7 eV, the values of E_a derived from Figure 9 yield DG > 0 with DG for the wild type ~75 meV greater than that for the variant. With l ~1.0 eV, the E_a values correspond to DG < 0 and DG for the L722W_PsaA variant ~ 90 meV more negative than that for the wild type. This suggests that the weakening of the H-bond caused by the
mutation pushes the midpoint potential of the phylloquinone more negative by approximately 75-90 mV provided that it does not alter the reorganization energy. If this estimate of the change in DG is correct, the effect of the mutation on the electron transfer kinetics is very similar to replacing phylloquinone by antthraquinone, which has a midpoint potential that is ~ 100 mV more reducing than phylloquinone. Indeed, the change in the kinetics observed when anthraquinone is incorporated into solvent extracted PS I (46) is quite similar to that found for the L722W_PsaA variant.

Although the changes in the kinetics and activation energy are consistent with a lowering of the midpoint potential of the quinone, it is also possible that the contribution of λ to the activation energy changes. It has been suggested that λ is typically higher for one electron reduction of quinones in solvent mixtures that can form H-bonds in comparison to solvent mixtures that cannot form H-bonds (47). The weakening of the H-bond in the L722W_PsaA variant can therefore be expected to reduce λ, thereby lowering E_a.

A striking feature of Figure 9 is the fact that in the L722W_PsaA variant, the electron transfer rate becomes nearly independent of temperature below ~200 K, while it is strongly activated above this temperature. There is also some indication of a possible change in the slope of the Arrhenius plot of the wild type data from (19) at ~200 K. A similar change in slope has also been reported for anthraquinone substituted PS I (46).

Such behavior is expected for activated electron transfer as a result of the zero-point vibrational energy. At temperatures at which kT is small compared to the zero-point energy the thermal energy becomes independent of temperature. Hence, the electron transfer rate is also temperature independent. The data in Figure 9 can be fit very well by a model suggested by Hopfield that takes the zero-point energy into account (48).
However, the activation energy obtained from the fit is unreasonably large, presumably because assumptions contained in the model are not valid for $A_1$ to $F_X$ electron transfer. For example, the model assumes that no entropy change occurs during the electron transfer, while it has been shown from photoacoustic spectroscopy data that for the $A_{1A}^-$ to $F_X$ electron transfer step, entropy is the main contribution to the free energy and therefore drives the reaction (49). Nonetheless, the model provides an adequate qualitative explanation for the change in slope. The temperature at which the change occurs corresponds to $kT = \frac{1}{2} \hbar \omega$ where $\omega$ is the characteristic frequency of the vibrational mode that is coupled to the electron transfer. From this rough approximation we obtain a frequency of $\sim 140 \text{ cm}^{-1}$ for the mode(s). The slope change appears to occur at slightly higher temperature for the L722W$_{PsaA}$ variant, compared to the wild type, which suggests that the frequency may increase when the H-bond is weakened. In this context it is interesting to compare the kinetics of the $A_{1A}^-$ to $F_X$ transfer in the L722W$_{PsaA}$ with the two branches in the wild type. The $A_{1B}^-$ to $F_X$ step has a faster rate and is nearly independent of temperature (20) while $A_{1A}^-$ to $F_X$ electron transfer is slower and is more strongly activated. Thus, the behavior of the L722W$_{PsaA}$ variant kinetics is roughly between that of the wild type A-branch and B-branch kinetics. This suggests that in addition to a difference in midpoint potential there may also be a difference in the frequency of the modes that couple to the electron transfer in the two branches. However, it is not clear what role, if any, the H-bonds in the two phylloquinone binding sites would play.

4.5.3 Possible Heterogeneity in the H-bonding – Since it is possible that a distribution of H-bond strengths exists, it is important to consider what its impact would be. The
observation that the orientation of the quinone was identical to that in the wild type and that no change was observed in the distance between P$_{700}$ and A$_{1A}$ suggests that the mobility of the protein backbone in the loop containing L722$_{_{PsaA}}$ may play an important role in determining the H-bond strength. Structural heterogeneity within the A$_{1A}$ binding pocket could also explain why an apparent increase in the amplitude of the fast phase is seen by time-resolved optical experiments. A distribution of H-bond strengths would result in a distribution of electron transfer rates and could lead to a fraction of PS I complexes in which the rates in the two branches are nearly equal. The presence of such a fraction would lead to an apparent increase the relative amplitude of the fast phase. The fit of the transients would yield the two dominant components in the distribution of electron transfer lifetimes. A similar effect has been proposed in a variant of PS I in which, the Met residue that ligates the Mg$^{2+}$ of A$_{0A}$, the Chl $a$ molecule that functions as the primary acceptor in the A-branch, was replaced with a Leu residue (50). It was suggested that the resulting disorder in the binding site produced a distribution of lifetimes for A$_{0A}$. 

4.5.4 Charge Recombination Kinetics - The observed slowing of the charge recombination in the L722W$_{_{PsaA}}$ variant agrees well with the finding that the activation energy for forward electron transfer from A$_{1A}$ to F$_x$ is smaller and the proposal that the midpoint potential of the quinone is pushed more negative. Although charge recombination in PS I is an incompletely understood process, it is known that it occurs via thermal repopulation and involves A$_1$. The lowering of the activation energy for the forward reaction from A$_{1A}$ to F$_x$ means that activation energy for the reverse reaction should increase. Similarly, a more negative midpoint potential for A$_1$ increases the
driving force for recombination of $P_{700}^+ A_{1}^-$, which would slow the reaction because it lies in the inverted region. However, care must be taken in interpreting the charge recombination rates in terms of the $A_{1A}$ midpoint potential and the activation energy of $A_{1A}^-$ to $F_x$ electron transfer because the directionality of the recombination is not known. Hence, there are several possible interpretations. The most straightforward explanation for the change in the backreaction kinetics is that the reaction occurs via the A-branch quinone and either the $F_x^-$ to $A_{1A}$ or the $A_{1A}^-$ to $P_{700}$ step is rate limiting. However, the possibility that the change in the lifetime occurs because the mutation alters the recombination pathway or has a remote effect on the iron-sulfur clusters cannot be excluded.

4.6 SUMMARY

The experimental studies described in this chapter clearly indicate that the H-bond plays a significant role in modulating the redox properties of the phylloquinone, affecting both the kinetics and energetics of electron transfer in PS I. I further confirm that the weakening of the H-bond does not cause a large change in the distance of the semiquinone from $P_{700}^+$. Forward electron transfer through the quinone is accelerated in the variant and can be rationalized in terms of classical Marcus theory with the shift in the redox potential of the quinone to a more negative value. The H-bond possibly plays a role in determining the reorganization energy and frequency of the mode coupled to the electron transfer as evidenced from the temperature dependence of the forward electron transfer. Furthermore, a distribution of H-bond strengths could exists in the binding pocket. The change in the electron transfer kinetics, however, do not affect electron
throughput to $F_A/F_B$, as indicated by the accumulation of $F_A^-$ and $F_B^-$ accumulation in continuous light and by steady-state measurements of flavodoxin reduction. This study shows that, in addition to preventing the double reduction/protonation of $A_{1A}$, the H-bond influences the midpoint potential of the $A_{1A}$ (and by inference $A_{1B}$) phylloquinone(s) in PS I. In the next chapter, I probe the relative differences in H-bond strengths between the two quinones as a possible source of the differences in their redox properties using hyperfine sublevel correlation spectroscopy (HYSCORE), a high-resolution EPR technique. This study will corroborate that each quinone forms one H-bond and provide information about the differences in strength, length and geometry of the H-bonds between the $A_{1A}$ and $A_{1B}$ binding pockets.
4.7 REFERENCES


4.8 FIGURE LEGENDS

Figure 4.1 Steady state rates of flavodoxin photoreduction in PS I complexes isolated from wild-type and L722W_{PsaA} (µmol flavodoxin mg Chl\(^{-1}\) hr\(^{-1}\)) plotted against relative light intensity.

Figure 4.2 EPR spectra of F\(_{A}\) and F\(_{B}\) in PS I complexes isolated from wild-type and L722W_{PsaA}. All samples contained 0.2 mg/ml chl, 10 mM sodium ascorbate and 300 µM DCPIP. For one electron transfer reaction (top), the dark-frozen samples were illuminated in-cavity at 15 K. For two electron transfer reaction (bottom), the sample was illuminated at ~200 K and frozen immediately. All spectra represent the illumination minus the dark spectrum. Spectrometer settings: microwave power, 100 mW; microwave frequency, 9.478 GHz; receiver gain, 2 X 10\(^4\); modulation amplitude, 10 G at 100 kHz.

Figure 4.3 Comparison of the out-of-phase echo modulation of P\(_{700}^{+}\)A\(_{1}^{-}\) in PS I from wild-type (dashed) and L722W_{PsaA} (solid) at 80 K.

Figure 4.4 Time-resolved optical kinetics at 480 nm from wild type (top) and L722W_{PsaA} (bottom). The data is presented with a linear time axis so that the lifetime and contributions of the two phases can be distinguished easily. The residuals for each fit are shown above the main trace.

Figure 4.5 Spectra of the kinetic phases obtained by global decomposition of the pump-probe optical spectroscopy experiment on PS I particles isolated from wild type and
L722W_{PsaA} variant in the near-UV and blue region. Panel A: comparison of the fast kinetic component; Panel B: comparison of the slow kinetic component; Panel C: normalized non-decaying component together with the kinetic component representing the recombination event in L722W_{PsaA}; Panel D: initial spectra extrapolated to time zero ($t_0$).

**Figure 4.6** Spectra of the fast and slow kinetic phases obtained by global decomposition of the pump-probe optical spectroscopy experiment on whole cells of wild type and L722W_{PsaA} variant in the near-UV and blue region. Panel A: comparison of the fast kinetic component; Panel B: comparison of the slow kinetic component; Panel C: normalized non-decaying component together with the kinetic component representing $P_{700}^{+}$ reduction; Panel D: initial spectra extrapolated to time zero ($t_0$).

**Figure 4.7** FDMR spectra recorded in isolated thylakoids from the wild type and the L722W_{PsaA} variant either under ambient redox conditions or following illumination in the presence dithionite. Experimental conditions: emission wavelength, 720 nm; Temperature, 1.8 K; modulation amplitude, 33 Hz.

**Figure 4.8** Top: Transient EPR spectrum of L722W_{PsaA} at X-band observed at 293 K compared to the wild-type. Positive signals correspond to absorption (A) and negative signals correspond to emission (E). The E/A/E pattern is assigned to the $P_{700}^{++}A_1^{--}$ radical pair and the (E/A) pattern is assigned to $P_{700}^{++}(Fe/S)^{--}$. Bottom: The transients and the fits
used for determining the lifetime of the decay is shown for the wild-type (right) and L722W$_{\text{PsaA}}$ (left).

**Figure 4.9** Arrhenius plot of the electron transfer rate from $A_{1A}^-$ to $F_X$ in the L722W$_{\text{PsaA}}$ variant and the wild type. Filled circles: L722W$_{\text{PsaA}}$ EPR data, diamonds: L722W$_{\text{PsaA}}$ optical data, open circles: wild type EPR data, squares: wild type optical data (this work); triangles: wild type data taken from (19). The straight lines through the data give the following activation energies and maximum rates: wild type, $k_{\text{max}} = (2.6 \pm 0.1) \times 10^{10} \text{ s}^{-1}$, $D_G^* = 220 \pm 10 \text{ meV}$; L722W$_{\text{PsaA}}$ variant, $k_{\text{max}} = (2.6 \pm 0.1) \times 10^{10} \text{ s}^{-1}$, $D_G^* = 180 \pm 10 \text{ meV}$.

For the L722W$_{\text{PsaA}}$ variant, the lifetimes determined from the EPR data are as follows; 100 K, 2.8 µs; 120 K, 2.0 µs; 140 K, 1.5 µs; 160 K, 0.7 µs; 180 K, 0.6 µs; 200 K, 0.8 µs; 220 K, 0.5 µs; 260 K, 170 ns; 293 K, 50 ns.

**Figure 4.10** $P_{700}^+$ reduction kinetics in PS I complexes isolated from wild-type (top) and L722W$_{\text{PsaA}}$ (bottom). Laser induced optical transients measured at 820 nm is shown. The time is plotted on a logarithmic scale with the computer generated fits shown in solid lines. The lifetimes of each phase is indicated. PS I concentration was 50 µg/ml. All samples contained 10 mM sodium ascorbate and 4 µM DCPIP as electron donor and mediator respectively.
Figure 4.1
Figure 4.2

Wild Type
L722W<sub>PsaA</sub>

300 320 340 360 380 400

B<sub>IP</sub> mT

Wild Type
L722W<sub>PsaA</sub>
Figure 4.3
Figure 4.4

Wild-type

17 ns

208 ns

L722W

15 ns

86 ns
Figure 4.5

A

\( A_i \) (a.u.)

-0.3

-0.2

-0.1

0.0

0.1

0.2

350 400 450 500

Wavelength (nm)

B

\( A_i \) (a.u.)

-0.3

-0.2

-0.1

0.0

0.1

0.2

350 400 450 500

Wavelength (nm)

C

\( A_i \) (a.u.)

-1.5

-1.0

0.0

0.5

350 400 450 500

Wavelength (nm)

D

\( A_i \) (a.u.)

-1.5

-1.0

0.0

0.5

350 400 450 500

Wavelength (nm)

- 8.6 ns
- 22 ns

- 285 ns
- 100 ns

- n.d.
- 4.5 \( \mu \)s
- n.d.

- \( t_0 \)
- \( t_0 \)
Figure 4.6
Figure 4.7
Figure 4.8
Figure 4.9
Figure 4.10

Wild Type

L722W_{RamA}

AA (mOD)

10^1

2 3 4 5 6 7 8 9 10

Time, ms

10^2

2 3 4 5 6 7 8 9 10

Time, ms

10^3

2 3 4 5 6 7 8 9 10
Chapter 5

High Resolution Imaging of the Phylloquinone Binding Pocket in Photosystem I Using Hyperfine Sublevel Correlation Spectroscopy
5.1 ABSTRACT

In the preceding chapters, I attempted to elucidate the structural and the functional role of the H-bond to the A\textsubscript{1A} quinone. In this chapter, I confirm that each quinone forms a single H-bond with the protein backbone and probe the relative differences in H-bond strengths between the two quinones as a possible source of the differences in their redox properties using two-dimensional hyper sublevel correlation spectroscopy. The phylloquinones, A\textsubscript{1A} and A\textsubscript{1B}, exist in near-equivalent protein environments but possess distinct electron transfer properties. A\textsubscript{1A} is involved in thermodynamically uphill electron transfer to F\textsubscript{X} with a lifetime of ~200 ns while the A\textsubscript{1B} is involved in thermodynamically downhill electron transfer to F\textsubscript{X} with a lifetime of ~20 ns. According to recent computational studies, A\textsubscript{1B} is ~173 mV more reducing than A\textsubscript{1A}. The protein determinants responsible for these different redox properties are incompletely understood. Photoaccumulation of PS I complexes at pH 8.0 results in A\textsubscript{1A} being trapped. The experiments described in this chapter indicate that A\textsubscript{1A} forms one H-bond of ~1.59 Å in length. Studies on PS I complexes photoaccumulated at pH 10.0 show two H-bonds of unequal strengths corresponding to bond lengths of 1.62 Å and 1.71 Å. The second H-bond observed at pH 10.0 is assigned to A\textsubscript{1B}. Thus, I assign the stronger H-bond to the A\textsubscript{1A} quinone and the weaker H-bond to the A\textsubscript{1B} quinone and I propose that the difference in the H-bond strength between the two quinones is one of the factors that contribute to their differences in midpoint potential.
5.2 INTRODUCTION

The primary purpose behind efforts to obtain more refined spectroscopic and local structural data of cofactor binding sites is to better understand how structural and magnetic properties relate to the biological function of the cofactor. The phylloquinones in the A\textsubscript{1A} and A\textsubscript{1B} sites of Photosystem I (PS I) are difficult to distinguish spectroscopically because they exist in near-equivalent protein environments. Nevertheless, advances in time-resolved optical spectroscopy and EPR spectroscopy, combined with site-directed mutagenesis techniques have led to the distinction of the A\textsubscript{1A} and A\textsubscript{1B} phylloquinone binding pocket. As discussed in detail in section 1.5, the electron transfer properties of the two phylloquinone molecules are distinct; the A\textsubscript{1A} phylloquinone transfers the electron to F\textsubscript{X} in \(\sim 200\) ns, the A\textsubscript{1B}\textsuperscript{−} to F\textsubscript{X} electron transfer step occurs in \(\sim 20\) ns \((1)\). While the former is strongly activated and slows down at lower temperatures, while the latter is temperature independent \((2)\). The current model that explains these observations suggests that electron transfer on the A-branch is barely favorable while electron transfer via the B-branch is thermodynamically downhill, implying that A\textsubscript{1A} is more oxidizing than A\textsubscript{1B} \((3)\). This conclusion is backed up by computational studies, which indicate that A\textsubscript{1B} is \(\sim 173\) mV more reducing than A\textsubscript{1A} \((4, 5)\). These observations lead to the obvious question of why two chemically identical species possess such diverse electron transfer properties. Although it is expected that the protein environment plays a major role in modulating the redox properties of the quinones, the protein determinants that cause this seemingly large difference remain unclear. The 2.5 Å X-ray crystal structure points to a few subtle differences in the binding pocket \((4, 5)\). One possible explanation for the observed differences in redox
potentials and hence electron transfer rates could be the variation in the lipid environment of the two quinones. On the A-branch, a negatively charged phospholipid is present ~11 Å from the quinone while on the B-branch, a neutral galactolipid is located also at ~11 Å from the quinone. The negative charge on phospholipid is expected to increase the $A_{1A}$ redox potential relative to the uncharged galactolipid that is close to the $A_{1B}$ side, thereby slowing down electron transport via $A_{1A}$. However, electrostatic calculations, which take into account the four lipids in PS I, show that the contribution of the lipid to the redox potential of $A_1$ is negligible (an 8 mV increase for $A_{1A}$ and a 5 mV decrease for $A_{1B}$) (6). This result is corroborated by studies on the $pgsa$ deletion mutant that lacks the ability to synthesize the phospholipid. The decrease in the overall photosynthetic activity was attributed to a decrease in PS II activity rather than PS I (7). Another factor that could lead to a difference in reorganization energy between the two branches and therefore the electron transfer rate via the two branches is the difference in the arrangement of water molecules near the $A_{1A}$ and $A_{1B}$ binding sites. The six water molecules located near the $A_{1B}$ form a hexagon, which is a well-established low energy configuration for water molecules. On the other hand, the water molecules located in the vicinity of $A_{1A}$ do not have a well-defined arrangement. This could result in lower reorganization energy for B-branch, resulting in faster electron transfer via $A_{1B}$. Another important difference is the presence of a Trp residue ($W673_{PsaB}$) on the B-branch whose counterpart on the A-branch is a Gly residue. The direct contribution of the Trp residue, determined by electrostatic calculations, was negligible (a 4mV decrease for $A_{1A}$) (6). However, it is possible that the indole side chain may support electron transfer between $A_{1B}$ and $F_X$ by function as bridging molecule and therefore speed up electron transfer via the B-branch. In a PS I
variant from *Chlamydomonas reinhardtii* in which W677$_{\text{PsaB}}$ was replaced with Phe (8),
electron transfer via the B-branch slowed down but was still faster than the A-branch.
This study indicated that W677$_{\text{PsaB}}$ does not play a significant role in determining the rate
of electron transfer via the B-branch. Other minor differences include the difference in
the orientation of the phytol tail of the quinone and the presence/absence of certain
carotenoids.
Based on the 2.5 Å X-ray crystal structure of PS I, it is thought that the two important
interactions between the quinone and the protein environment (the π–stacking with the
Trp and the H-bonding to the Leu) are conserved between the two binding pockets. In the
previous chapters, the significance of the H-bond to the phylloquinone molecule was
elucidated and the number of H-bonds to the A$_{1A}$ was estimated. The single H-bond
formed between one of the carbonyl groups of the phylloquinone molecule and the
backbone NH of the protein plays a key role in maintaining the functional integrity of the
binding pocket. Although its primary purpose is to prevent protonation/double reduction
of the phylloquinone under periods of high illumination, the H-bond plays a key role in
modulating the redox potential of the phylloquinone. In this chapter, I confirm that the
A$_{1A}$ as well as A$_{1B}$ quinone form a single H-bond with the protein backbone. I also probe
the relative differences in H-bond strengths between the two quinones as a possible
source of the differences in their redox properties.

*5.2.1 H-bond Strength Probed by ENDOR*
Electron nuclear double resonance (ENDOR) spectroscopy combines the high sensitivity
of EPR spectroscopy with the high selectivity and high resolution of an NMR
experiment. In an EPR experiment, the unpaired electron in a radical is placed in a
magnetic field and swept with microwave radiation. The unpaired electron absorbs the energy from the microwave radiation to change its orientation with respect to the applied magnetic field. It changes from a low energy state where it is aligned with the applied field, to a high-energy state where it is aligned against the applied field. When the microwave radiation is removed, the unpaired electron relaxes back to the low energy state. If too much microwave power is applied continuously, the energy levels equilibrate leading to loss of signal. However, when such a state interacts with a nuclear spin in its vicinity, the transitions of the nuclei will allow the electron to relax. When a radiofrequency drives the nuclear transition (analogous to NMR transitions), the saturated EPR signal desaturates and increases in intensity, resulting in an ENDOR signal. A typical ENDOR spectrum depicts the changes in the intensity of the saturated EPR line as a function of the radiofrequency causing the nuclear transitions. ENDOR allows the measurement of the NMR spectra of nuclei in a particular species in the sample i.e. the radical containing the unpaired electron. This is especially useful when dealing with PS I, since it is too large for the successful application of conventional NMR techniques.

The phylloquinone radical probed by an ENDOR experiment can be generated by a photoaccumulation protocol that produces $A_{1A^-}$ and $A_{1B^-}$ radicals. In this protocol, the $F_A$ and $F_B$ clusters are first chemically reduced and light is used to successively generate the $F_{x^-}$, $A_{1A^-}$ and $A_{1B^-}$ states at pre-selected temperatures and pH values. A high yield of a given photoaccumulated state depends on contributions from multiple light-induced turnovers, rapid electron donation to $P_{700^+}$, and the different statistics of charge recombination versus forward electron donation to slowly accumulate the desired reduced acceptor set. Chemical reduction of PS I with sodium hydrosulfite at pH 8.0, followed by
brief illumination at 205 K has been shown to fully reduce $F_A/F_B$, partly reduce $F_X$, and somewhat reduce $A_1$ (9). Longer periods of illumination result in the complete reduction of $F_X$, ultimately generating one spin from ‘$A_1^-$’ per $P_{700}$ (9). Under these conditions ENDOR studies revealed hyperfine couplings with $a_{iso} = 10.3$ and $10.2$ MHz in PS I complexes from *Anabena variabilis* and spinach, respectively, due to the –CH$_3$ fragment at C$_2$ of the phyllosemiquinone radical (see Section 2.2.1) (10). These values are significantly different from the $a_{iso} = 7.9$ MHz of the phylloquinone anion radical in alkaline methanol (10), and are attributed to the unusually high spin density on C$_2$ of the PS I-bound phyllosemiquinone. Additional hyperfine couplings were reported, which were assigned to two H-bonds formed by the two carbonyl oxygen of the phylloquinone, However, the relative strengths of the two H-bonds would need to be significantly different to account for the highest electron spin density on C$_2$ (10-12). It should be noted that the presence of a second H-bond is not supported by transient EPR studies, which suggest only a single H-bond to (what was later identified as) the $A_{1A}$ quinone (see Chapter 2). Studies on the W693$_{PsaA}$ mutant of *Chlamydomonas reinhardtii* showed an alteration in the electronic structure of the photoaccumulated ‘$A_1^-$’ radical, suggesting that the signal is derived from $A_{1A}^-$, i.e., from electron transfer along the A-branch of cofactors (12). A second electron spin identified as ‘$A_0^-$’ was generated by photoaccumulating PS I at slightly higher temperatures of 220 K to 240 K (13-15). It was reported that it is possible to photoaccumulate a total of four spins by chemically reducing PS I with sodium hydrosulfite at pH 10.0 with subsequent illumination at 220 K (14, 15) (although this result has been recently challenged on theoretical grounds (16)). Additional hyperfine couplings were generated under these conditions, and a difference
ENDOR spectrum was extracted by subtracting the two-spin ENDOR spectrum from the three-to-four spin ENDOR spectrum. The anisotropy of the methyl group hyperfine couplings suggested that the resonances from the difference ENDOR spectrum could be assigned to a phyllosemiquinone radical. Because these additional spins were, by default, due to electron transfer along the B-branch of cofactors, the phylloquinone photoaccumulated under these conditions was assigned to $A_{1B}$. Further, the resonances were unperturbed by changes made to A-branch cofactors such as replacing $W_{693PsaA}$ with a Phe, further supporting its assignment to $A_{1B}$. Two hyperfine couplings were additionally reported for $A_{1A}^{-}$ and for $A_{1B}^{-}$, and interpreted as evidence for the presence of two H-bonds, one to each oxygen in both phylloquinones (14). However, it should be pointed out that these experiments were carried out at X-band, where it can be difficult to distinguish between a semiquinone radical and a Chl radical. This note of caution is warranted from work carried out at Q-band, where the $g$-tensor resolution of the phyllosemiquinone radical is markedly better. Using deuterated phylloquinones in a background of protonated PS I, the hyperfine coupling tensor associated with only a single H-bond could be identified in the pulsed Q-band ENDOR spectrum of $A_{1A}^{-}$ (17). The magnitude of the measured dipolar tensor corresponded to an unusually short H-bond, estimated to be $1.5 \pm 0.1$ Å from point-dipole approximations. The implication is that the resonance attributed to the second H-bond to $A_{1A}$ may have been derived instead from a proton associated with $A_{0}^{-}$, the formation of which cannot be avoided to some degree in the photoaccumulation procedure. Nevertheless, this study was the first to probe the possible differences in the H-bond strength between the two quinones.
5.2.2 HYSCORE- A Novel Method to Detect H-bonds in PS I

Hyperfine sublevel correlation (HYSCORE) spectroscopy is a sophisticated, pulsed EPR technique. In a traditional pulsed EPR experiment, the sample is subjected to a continuous magnetic field with the application of short and intense pulses of microwaves. The microwave signal generated due to the magnetization of the sample is recorded as a function of time between the pulses and then Fourier transformed to obtain the frequency spectrum of the sample. The most common pulsed EPR technique is electron spin echo envelope modulation (ESEEM), which yields information about the interaction of the unpaired electron with the surrounding nuclei. In an ESEEM experiment, the sample is subjected to two microwave pulses; a $\pi/2$ pulse and a $\pi$ pulse separated by a time, $\tau$ (see Figure 5.1). The first pulse lays down the unpaired spin along the Y-axis, which then begins to ‘fan out’ and dephase in the XY-plane. A second $180^\circ$ pulse will flip the magnetization of the spins about the X-axis allowing the decaying spins to come together. The spin packets that have now come together will begin to dephase again, which gives rise to an echo as a function of $\tau$. A Fourier transform of the echo gives the typical ESEEM spectrum. The peaks observed are essentially an NMR spectrum of nuclei that are coupled to the electron. ESEEM experiments can also be performed with a three-pulse sequence of $\pi/2-\tau-\pi/2-\pi\tau-\text{echo}$ in which time $T$ is swept (Figure 5.1). A two pulse ESEEM experiment is sensitive to spin-spin relaxation times while a three pulse experiment is more sensitive to spin-lattice relaxation times.

When there is a large number of nuclei that contribute to the ESEEM spectrum, as is the case in PS I, it is advantageous to apply 2D techniques such as HYSCORE that enables the separation of overlapping peaks observed by ESEEM. HYSCORE is a four-pulse
ESEEM technique wherein microwave pulses are applied in the following series: two $\pi/2$ pulses, a $\pi$ pulse followed by a $\pi/2$ pulse. The times $T_1$ and $T_2$ between the pulses are scanned (see Figure 5.1). After double Fourier transformation, off-diagonal peaks are observed like in two-dimensional NMR spectroscopy, in which nuclear frequencies that arise from the same nucleus can be correlated and grouped together. A cross peak correlates a nuclear transition frequency of one sublevel to a nuclear transition frequency of the same nucleus, but of the other sublevel. The cross peaks spread out the nuclear frequencies thereby allowing an easy analysis of complicated spectra. The signal to noise ratio is greatly enhanced due to the second Fourier transform that separates overlapping peaks along a second dimension. Furthermore, weak interactions, which are not observed in one-dimensional ESEEM experiments, are easily seen as low intensity hyperfine peaks in a HYSCORE experiment.

Peaks appearing in the upper right and lower left quadrants usually arise from nuclei in which the hyperfine coupling is less than the Larmor frequency. They appear at the Larmor frequency, separated by the hyperfine coupling. Peaks from nuclei in which the hyperfine interaction is greater than the Larmor frequency appear in the upper left and lower right quadrants. Information about the full hyperfine tensors of the various magnetic nuclei can be elucidated from simulations of the spectra, even for samples with molecules in different orientations. Information such as isotropic hyperfine coupling constant, spin density distribution and anisotropic hyperfine coupling constants can also be extracted for the HYSCORE data set.

In a recent study, the H-bonding pattern of a series of model benzoquinones in protic and aprotic solvents was probed (18). The isotropic coupling constants and spin density
distribution estimated by HYSCORE correlated well with those calculated from other EPR experiments. Based on the tensor components, a relationship between H-bond, spin density distribution and reduction potential was established. The effect of substituents such as methyl and phenyl groups at various quinone ring position was estimated. In dimethyl sulfoxide, an aprotic solvent, the quinones are incapable of forming H-bonds. Nevertheless, an increase in spin density distribution is observed when substituents such as a methyl group are introduced in the quinone ring. This observed increase is a direct manifestation of the electron donating nature of the methyl group. In a protic solvent such as isopropanol, benzoquinone is capable of forming four H-bonds, two for each carbonyl group. For 2,5-dimethyl benzoquinone in isopropanol, the four H-bonds are conserved, while a similar increase in the spin density distribution over the quinone ring is observed. The protic solvent however, amplifies the electron donating ability of the methyl group; the spin density increases by 8% in isopropanol when compared to 5% in dimethyl sulfoxide. Given that the spin density distribution is key in determining the midpoint potential of the quinone, it is possible to relate the microenvironment of the cofactor with its redox properties based on HYSCORE experiments. This proves to be a valuable resource when probing the strength of the H-bond formed between the A1A/A1B quinones and the protein environment in PS I.

5.3 MATERIALS AND METHODS

5.3.1 Preparation of PS I Complexes – Growth of Synechocystis sp. PCC 6803 wild-type cells and isolation of PS I complexes were performed as described in section 3.2.1.
5.3.2 Generation of $A_{1A}^-/A_{1B}^-$ Semiquinone – The sample was incubated with 50 mM sodium dithionite under anaerobic conditions for 30 minutes. Illumination at low light intensities of ~2.5 mW at 205 K for 40 minutes resulted in the photoaccumulation of PS I to generate the $A_{1A}^-/A_{1B}^-$ semiquinone.

5.3.3 Generation of $P_{700}^+$ – The PS I sample was incubated with 5 mM potassium ferricyanide to oxidize the primary donor and generate the $P_{700}^+$ cation.

5.3.4 2D HYSCORE – For obtaining the 2D HYSCORE spectra, the pulsed echo amplitude was measured using the sequence $\pi/2$-$\tau$-$\pi/2$-$t_1$-$\pi$-$t_2$-$\pi/2$-echo with a $\tau$ of 132 ns and a 12 ns detector gate (centered at the maximum of the echo signal); the delays are defined as the differences in the pulse starting points. The echo intensity was measured as a function of $t_1$ and $t_2$, where $t_1$ and $t_2$ were incremented in steps of 8 or 16 ns from their initial values of 24 and 40 ns, respectively. Equal amplitude pulses of 16 ns for $\pi/2$ and 32 ns for $\pi$ were used to record a 256 X 256 matrix. The 16 ns time difference between the initial values of $t_1$ and $t_2$ and $\pi/2$ and $\pi$ were set equal to obtain more symmetric spectra. The application of a 16-step phase cycling procedure eliminated the unwanted echoes and antiechoes. The echo decay was eliminated by a low order polynomial baseline correction and tapered with a Hamming function. Prior to 2D Fourier transformation, the data was zero filled to a 1024 X1024 matrix and the magnitude spectra were calculated using the Bruker X-EPR software (Bruker BioSpin, Billerica, MA). The spectra are presented as contour plots prepared in Matlab R2008a.

5.3.5 Simulations – The “saffron” function of the EasySpin software package was used for the numerical simulation of the experimental $^{14}$N and $^1$H HYSCORE spectrum.
5.4 RESULTS AND DISCUSSION

Figure 5.2 depicts the (+, +) quadrant of the 2D $^1$H HYSCORE spectrum of PS I complexes photoaccumulated at pH 8. Under these conditions, only the A$_{1A}^-$ semiquinone is generated (14). In a 2D HYSCORE spectrum, the electron–nuclear hyperfine interactions appear as cross-peaks in different quadrants depending on the strength of the coupling between the electron and the nucleus. In the sample studied here, the cross peaks are seen in the (+, +) quadrant and correspond to weakly interacting nuclei. They are centered about the diagonal located at the Zeeman frequency of the interacting nuclei. In this case, $|T + 2a_{iso}| >> 4\nu_1$, where $T$ is the anisotropic hyperfine tensor, $a_{iso}$ is the isotropic hyperfine coupling, and $\nu_1$ is the nuclear Larmor frequency. The cross peaks (ridges) are located close to the anti-diagonal, which intersects with the diagonal at the $^1$H Zeeman frequency of 14.7 MHz. Two distinct ridges exhibiting a minor shift from the anti-diagonal are observed. The shift is largely due to the anisotropic components of the hyperfine coupling tensor. The ridge that is closest to the anti-diagonal is assigned to overlapping cross peaks that arise from hyperfine interactions from intrinsic ring protons and β–methylene protons of the phytol tail. The pronounced ridge labeled ‘H–bond I’ shows a larger shift from the anti-diagonal, indicating the presence of a strong anisotropic contribution to the electron–nuclear hyperfine interaction. This allows us to assign the cross peaks to hyperfine interaction of the semiquinone anion with a H-bonded proton. More specific conclusion about the strength and geometry of the H-bond can be drawn after evaluation of the hyperfine coupling tensors from their line shapes.

In general, the three principal components of an electron-nuclei hyperfine tensor can be
presented as 
\[(a_{iso} - T(1 - \delta), a_{iso} - T(1 + \delta) \text{ and } a_{iso} + 2T),\]
where \(a_{iso}, T, \) and \(\delta\) are the isotropic, dipolar, and rhombic components of the tensor, respectively. In the case of axial symmetry \((\delta = 0)\) in powder samples, the proton ridges represent straight line segments when plotted in squared frequency coordinates of \(\nu_{\alpha}^2\) versus \(\nu_{\beta}^2\) (19). The contour line shape of the powder 2D spectrum due to a proton nuclei with nuclear spin \(I = 1/2\) for axial hyperfine interactions is described by equation 5.1.

\[v_{\alpha(\beta)} = (Q_{\alpha(\beta)}v_{\beta(\alpha)}^2 + G_{\alpha(\beta)})^{1/2} \]  

where

\[Q_{\alpha(\beta)} = \frac{2a_{iso} + T \mp 4v_1}{2a_{iso} + T \pm 4v_1} \]  

and

\[G_{\alpha(\beta)} = \pm 2v_1 \frac{4v_1^2 - a_{iso}^2 + 2T^2 - a_{iso}T}{2a_{iso} + T \pm 4v_1} \]  

\(v_1\) is the proton Zeeman frequency (14.71 MHz). The slope, \(Q_{\alpha(\beta)}\), and intercept, \(G_{\alpha(\beta)}\), can then be used to calculate possible solutions of \(a_{iso}\) and \(T\) with the same value of \(|2a+T|\) and interchanged \(A_\perp = |a-T|\) and \(A_\parallel = |a+2T|\)

\[T = \pm \sqrt{\frac{16}{9(1-Q_{\alpha(\beta)})^2} \left\{ G_{\alpha(\beta)} + \frac{4v_1^2Q_{\alpha(\beta)}}{1-Q_{\alpha(\beta)}} \right\}} \]  

and

\[a_{iso} = \pm 2v_1 \frac{1+Q_{\alpha(\beta)}}{1-Q_{\alpha(\beta)}} - \frac{T}{2} \]  

The squared frequency plot obtained for the phyllosemiquinone anion photoaccumulated at pH 8.0 is shown in Figure 5.3a. In this coordinate system, the ridges straighten out and
the points taken from the middle of these ridges can be fit well with a straight line (Figure 5.3b). The slope and intercept are shown in Table 5.1. The hyperfine coupling parameters calculated from these values are shown in Table 5.2. The H-bonded proton has an $a_{iso}$ value of $-0.27$ MHz and a T value of $3.93$ MHz.

The hyperfine parameters obtained from the linear analysis carry some degree of uncertainty. First, 2D HYSCORE is only sensitive to the relative sign of the hyperfine components. Second, the slope and the intercept are identical if the values for the parallel and perpendicular components of the anisotropic hyperfine coupling are interchanged. The uncertainty was minimal for the methyl protons when the values that are shown in Table 5.2 are compared to previous published data (20). For the H-bonding interactions, 2D $^1$H HYSCORE simulations were performed using the “saffron” function of the EasySpin software package (21). Figure 5.4 compares the spectrum obtained from simulations (bottom) with experimental spectrum (top). The simulations showed that in both cases, the absolute values of the perpendicular components are smaller than the absolute values of the parallel components (Figure 5.4b). Thus, the sign of the components can be deduced from the point-dipole approximation. Assuming a spin density of 0.2 on the carbonyl oxygen (22) and using point-dipole approximation, the H-bond distance is estimated to be 1.59 Å.

Figure 5.5 depicts the (+, +) quadrant of the 2D $^1$H HYSCORE spectrum of PS I complexes photoaccumulated at pH 10.0. In contrast to pH 8.0, three distinct ridges are present in the spectrum. Under these conditions, both the $A_{1A}^-$ and $A_{1B}^-$ semiquinones are generated (14, 23). The ridge that is closest to the anti-diagonal displays the strongest intensity and is assigned to overlapping cross peaks that arise from hyperfine interactions
of the semiquinone with the ring protons and β–methylene protons of the phytyl tail. The spectrum also displays an additional ridge that is located farther on the antidiagonal. This is assigned to the hyperfine interaction of the semiquinone with the three protons of the methyl group on the quinone head group. An almost identical feature was observed for PS I photoaccumulated at pH 8.0 (data not shown). The most important feature of the spectrum shown in Figure 5.5 is the presence of two resolved ridges labeled H–bond I and H–bond II. Both ridges are significantly shifted from the anti-diagonal, indicating the presence of strong anisotropic dipolar interactions characteristic of hyperfine interaction of the semiquinone with H-bonded protons.

The squared frequency plot obtained for the phyllosemiquinone anion photoaccumulated at pH 10.0 is shown in Figure 5.6a and the points taken from the middle of these ridges can be fit well with a straight line (Figure 5.6b). The slope and intercept are shown in Table 5.1. Figure 5.7 shows a comparison of the experimental (top) and simulated (bottom) spectra. Hyperfine coupling parameters obtained from spectral simulations (Figure 5.7b) are shown in Table 5.2. The H-bonded protons, H–bond I and H–bond II, exhibit T values of 3.71 MHz and 3.14 MHz and $a_{iso}$ values of –0.22 MHz and 0.20 MHz, respectively. Pronounced differences in the magnitude of the anisotropic hyperfine couplings of the two H-bonds are observed. The greater the shift of a ridge from the anti-diagonal, the larger is the anisotropic hyperfine coupling constant and stronger is the corresponding H-bond. A larger T value for H–bond I indicates that it is stronger than H–bond II. Since the value of the anisotropic hyperfine tensor is determined by the distance between the H-bonding partners, the two H-bonds are expected to have different lengths. This difference could arise from a change in the orientation of the H-bond donor and/or
the substituent groups on the phylloquinone. Assuming a spin density of 0.2 on the carbonyl oxygen (22) and using point-dipole approximation, the H-bond distances for H–bond I and H–bond II were estimated to be 1.62 Å and 1.71 Å, respectively.

The hyperfine coupling constants obtained for H–bond I at pH 8 closely match those obtained for H–bond I at pH 10.0 indicating that the corresponding cross peaks arise from hyperfine interaction between the H-bonded proton and semiquinone $A_{1A}^-$. The $^1H$ cross peak, H–bond II, observed at pH 10.0 could, in principle, originate from (i) a second H-bond to $A_{1A}^-$ or (ii) a H-bond to $A_{1B}^-$. The former possibility can be ruled out because no indication of a second H-bond was observed at pH 8.0 where $A_{1A}^-$ is the only contributing species. The assignment of a single (stronger) H-bond to $A_{1A}^-$ permits the assignment of the second (weaker) H-bond observed at pH 10.0 to $A_{1B}^-$. Therefore, I confirm that in cyanobacterial PS I, the $A_{1A}$ as well as $A_{1B}$ quinone is involved in one H-bond in its semiquinone anionic form, in accordance with the X-ray crystal structure. This assignment agrees well with transient EPR studies on PS I with $^{13}C$ labeled reconstituted naphthoquinones that show the presence of only one H-bond between phylloquinone $A_{1A}$ and the protein (22, 24). Whereas by transient EPR studies, it was possible to obtain information about the H-bond to the $A_{1A}^-$ semiquinone, in the present study, H-bonds to both $A_{1A}^-$ and $A_{1B}^-$ could be clearly distinguished.

The assignment of the stronger H-bond to $A_{1A}^-$ and the weaker H-bond to $A_{1B}^-$ agrees qualitatively with experimental and theoretical calculations. Electrostatic calculations and kinetic characterization of PS I indicate that $A_{1A}$ is more oxidizing than $A_{1B}$ (25) (see Chapter 1 for detailed discussion). Because a H-bond is expected to drive the redox potential of a quinone cofactor more positive, the stronger H-bond would be expected to
be associated with $A_{1A}$. This is in line with experimental evidence, which shows that weakening the H-bond to $A_{1A}$ causes the electron transfer rate from $A_{1A}$ to $F_X$ to become similar to that of $A_{1B}$ to $F_X$ (26). However, it is difficult to attribute the large calculated difference in the redox potential ($A_{1A}$ is $\sim173$ mV more oxidizing than $A_{1B}$) (3, 6) entirely to the difference in the strengths of the H-bonds measured in this study. The difference in the midpoint potential between $A_{1A}$ and $A_{1B}$ appears to be the sum of many small factors that add up, the most significant of them being the electrostatic interactions that are present in the vicinity of the $A_{1A}$ and $A_{1B}$ binding pockets. Nevertheless, it is appropriate to infer that the difference in the H-bonding pattern in the two pockets could contribute, albeit to a minor extent, to the differences in the redox properties of $A_{1A}$ and $A_{1B}$.

Based on the calculated $a_{iso}$ values, inferences can be drawn about differences in the geometry of H-bonds to $A_{1A}$ and $A_{1B}$. The hyperfine interaction of semiquinone anion radicals in protic and aprotic solvents have previously been characterized by ENDOR and 2D HYSCORE spectroscopy (27-29). In a protic solvent, the H-bond formed $in\ vitro$ between the carbonyl oxygen atom of an unsubstituted semiquinone and the solvent is in plane with the $\pi$-orbitals of the carbonyl oxygen atom. Thus, the hyperfine interaction is purely anisotropic in nature with negligible isotropic hyperfine couplings. In semiquinones with substituents on the carbon atom adjacent to the carbonyl groups, as is the case for the $A_{1A}/A_{1B}$ phylloquinones, there exists the possibility that an out-of-plane H-bond could be formed (19). Calculations based on density function theory indicate that a small positive value of $a_{iso}$ is consistent with a planar H-bond, while increasing non-
planarity results in change in the sign of \( a_{iso} \). Thus, based on the negative \( a_{iso} \) value, I conclude that the degree of non-planarity is higher for H–bond I (30).

During the generation of \( A_{1A}^-/A_{1B}^- \) it is possible (rarely) to generate the \( P_{700}^+ \) radical species. A spectrum of oxidized PS I (Figure 5.8) was recorded for comparison to ensure that the contribution of \( P_{700}^+ \) to the \(^1\)H HYSCORE spectrum was negligible. As can be seen from Figure 5.8, cross peaks for \( P_{700}^+ \) is observed in the low frequency region. Furthermore, no proton ridges are observed in this spectrum. It is therefore reasonable to conclude that the proton ridges observed in the photoaccumulated PS I samples are due to protons H-bonded to the phylloquinone molecule. 

Figure 5.9a depicts the \((+, +)\) quadrant of the experimental 2D \(^{14}\)N HYSCORE spectrum of PS I photoaccumulated at pH 10.0. The two intense cross peaks that are observed with peak intensity at (2.76, 4.06) MHz and (4.06, 2.76) MHz correspond to the double quantum transitions of a \(^{14}\)N nucleus that is magnetically interacting with the \( A_{1A}^- \) and \( A_{1B}^- \) phylloquinones. The cross peaks of a non-interacting nucleus would be located on the diagonal at twice the \(^{14}\)N Zeeman frequency \((2\nu_1 = 2.127 \text{ MHz})\). The shifts along the diagonal and the antidiagonal that are observed here are due to nuclear quadrupole and electron-nuclear hyperfine interactions, respectively. The “saffron” function of the EasySpin software package (21) was used to numerically simulate the experimental \(^{14}\)N HYSCORE spectrum. The simulated \(^{14}\)N HYSCORE spectra are shown in Figure 5.9b. The values of \( a_{iso} \), \( T \), the nuclear quadrupole coupling constant, \( K \), and the asymmetry parameter of the electric field gradient, \( \eta \), are listed in Table 5.3.

The X-ray crystal structure of PS I shows presence of two nitrogen atoms in the vicinity of \( A_{1A} \) (\( A_{1B} \)) that could interact with the semiquinone anion; the amide nitrogen atoms of
L722\textsubscript{PsaA} (L706\textsubscript{PsaB}) at 2.69 Å (2.75 Å) and the indole nitrogen atom of W697\textsubscript{PsaA} (W677\textsubscript{PsaB}) at 3.32 Å (3.40 Å). Note that all distances are calculated from the nearest carbonyl oxygen atom of the respective quinones. The values of $K$ and $\eta$ obtained here are comparable to the hyperfine couplings for the peptide nitrogen of polyglycine (31), excluding the assignment of cross peak to the indole nitrogen of Trp. The large $\Delta_{iso}$ value indicates the presence of significant electron spin density on the nitrogen atom, requiring partial overlap of the electron wave function with the semiquinone. This suggests considerable (indirect) interaction between the semiquinone and the nitrogen, as is the case for the Leu residue. The ridges that are observed in the $^{14}$N HYSCORE spectrum are therefore assigned to the peptide nitrogen of L722\textsubscript{PsaA} (L706\textsubscript{PsaB}) that is hydrogen bonded to $A_{1A}^{-}$ ($A_{1B}^{-}$).

5.5 SUMMARY

Using HYSCORE spectroscopy, I confirm that each phylloquinone in PS I forms a single H-bond with the protein backbone. Tentative assignments of the stronger H-bond to the $A_{1A}$ quinone and the weaker H-bond to the $A_{1B}$ quinone are made. I propose that the difference in the H-bond strength between the two quinones accounts, to a minor extent, for the differences in midpoint potential between them. The strength of HYSCORE spectroscopy lies in its ability to map the quinone binding pocket when the bound cofactor is in its semiquinone form. I have shown that it is possible to get detailed information about the H-bonding network of $A_{1A}$ and $A_{1B}$. This is of especially valuable when no structural information is available, i.e. for the many variants of PS I that have been constructed. An extension of this study to map the binding pocket of these variants
will allow for direct correlation between function (using a variety of optical and magnetic resonance spectroscopic techniques) and structure.


5.6 TABLES

**Table 5.1** Slope and intercept determined from the analysis of the lines shape obtained after conversion to a squared plot.

<table>
<thead>
<tr>
<th>Ridge</th>
<th>$Q_{(i)}$</th>
<th>$G_{(i)}$ (MHz$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH8 H–bond I</td>
<td>−1.12229</td>
<td>476.91048</td>
</tr>
<tr>
<td>pH 10 H–bond I</td>
<td>−1.11786</td>
<td>473.79286</td>
</tr>
<tr>
<td>pH 10 H–bond II</td>
<td>−1.12804</td>
<td>471.15571</td>
</tr>
<tr>
<td>pH 10 Methyl group</td>
<td>−2.12679</td>
<td>592.53214</td>
</tr>
</tbody>
</table>

**Table 5.2** Hyperfine coupling parameters of the proton ridges.

<table>
<thead>
<tr>
<th>Ridge</th>
<th>$T$ (MHz)</th>
<th>$a_{iso}$ (MHz)</th>
<th>$A_{\perp}$, $A_{\parallel}$ (MHz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH8 H–bond I</td>
<td>3.93</td>
<td>−0.27</td>
<td>−4.20, 7.59</td>
</tr>
<tr>
<td>pH 10 H–bond I</td>
<td>3.77</td>
<td>−0.25</td>
<td>−4.02, 7.30</td>
</tr>
<tr>
<td>pH 10 H–bond II</td>
<td>3.17</td>
<td>0.18</td>
<td>−3.00, 6.54</td>
</tr>
<tr>
<td>pH 10 Methyl group</td>
<td>1.33</td>
<td>9.94</td>
<td>8.61, 12.61</td>
</tr>
</tbody>
</table>
Table 5.3 Quadrupole coupling parameters obtained from analysis of the $^{14}\text{N}$ cross peak

<table>
<thead>
<tr>
<th>Ridge</th>
<th>$a_{iso}$ (MHz)</th>
<th>$T$ (MHz)</th>
<th>$K$ (MHz)</th>
<th>$\eta$ (MHz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide nitrogen</td>
<td>0.75-1.2</td>
<td>0.15-0.19</td>
<td>0.64-0.72</td>
<td>0.5-1</td>
</tr>
</tbody>
</table>
5.7 FIGURE LEGENDS

**Figure 5.1** Pulse sequence of a typical ESEEM and HYSCORE experiment; mw, microwave pulse.

**Figure 5.2** 2D HYSCORE spectrum of PS I complexes photoaccumulated at pH 8.0 depicting the $A_{1A}^-$ phyllosemiquinone radical. The cross peaks indicate the hyperfine interactions from intrinsic ring protons and one H-bonded proton.

**Figure 5.3** Proton cross peaks observed at pH 8.0 plotted in the frequency-squared coordinate system (a). Plot of points selected from line shape obtained in the squared-frequency plot of the H-bonded protons. The straight line shows the linear fit (b).

**Figure 5.4** Experimental $^1$H HYSCORE spectrum (a) of PS I complexes photoaccumulated at pH 8.0 compared with spectra simulated (b) using the parameters listed in Table 5.1.

**Figure 5.5** 2D HYSCORE spectrum of PS I complexes photoaccumulated at pH 10.0 depicting the $A_{1A}^-$ and $A_{1B}^-$ phyllosemiquinone radicals (a) showing cross peaks assigned to hyperfine interaction of the methyl protons. Enlarged view (b) of the cross peaks indicate the hyperfine interactions from $\beta$-methylene protons of the phytol tail, intrinsic ring protons and two H-bonded proton.

**Figure 5.6** Proton cross peaks observed at pH 10.0 plotted in the frequency-squared
coordinate system (a). Plot of points selected from line shape obtained in the squared-frequency plot of the H-bonded protons. The straight line shows the linear fit (b) for H-bond I (blue) and H-bond II (brown).

**Figure 5.7** Experimental $^1$H HYSCORE spectrum (a) of PS I complexes photoaccumulated at pH 10.0 compared with spectra simulated (b) using the parameters listed in Table 5.1.

**Figure 5.8** 2D HYSCORE spectrum of $P_{700}^+$ species in the chemically oxidized PS I sample.

**Figure 5.9** Enlarged view of the $^{14}$N cross peak in the experimental HYSCORE spectrum (a) and the simulated spectrum (b).
Figure 5.1

Two-Pulse ESEEM

Three-Pulse ESEEM

HYSCORE
Figure 5.3
Figure 5.4
Figure 5.5
Figure 5.6
Figure 5.7
Figure 5.8
Figure 5.9
Chapter 6

Concluding Remarks
The work presented in this dissertation unequivocally establishes the hydrogen bonding as an integral part of the phylloquinone binding pocket in Photosystem I. At first sight, the H-bond to the A_{1A} and A_{1B} phylloquinones appears at odds with the requirement that PS I produce an extremely low redox potential on the acceptor side. A H-bond stabilizes the reduced form of the (semi)quinone relative to the non-reduced form, thereby raising the redox potential to a higher (i.e. more positive) value. Because every H-bond to a quinone carbonyl group will shift the redox potential to a more positive value (1), it is appropriate that Q_{A} and Q_{B} in PS II are bound by two H-bonds, rather than just one, to the protein. However, in PS I, the protein environment must drive the midpoint potential of each phylloquinone even lower (i.e. more reducing) than would otherwise be necessary to compensate for the effect of the H-bond. The work described in this dissertation addresses the question of why a H-bond even exists between the phylloquinones and the protein environment in PS I.

In recent years, the H-bond has been under scrutiny beginning with the number of H-bonds between the quinone and the protein. The single H-bond between the protein and the phylloquinone depicted in the 2.5 Å X-ray crystal structure of cyanobacterial PS I spans a distance of 2.69 Å from the backbone nitrogen of L722_{PsaA} to the carbonyl oxygen at C_{4} of the A_{1A} phylloquinone ring. Similarly, a H-bond spans 2.75 Å from the backbone nitrogen of L706_{PsaB} to the carbonyl oxygen at C_{4} of the phylloquinone ring in the A_{1B} site. However, the X-ray crystal structure depicts the ground state; ENDOR studies of photoaccumulated PS I have suggested a second H-bond to the photoaccumulated phyllosemiquinone anions in the A_{1A} and A_{1B} sites (2-4). Could there be movement of the phylloquinone so that a second H-bond is formed after reduction?
The work described in this thesis suggests otherwise. Experiments involving the incorporation of quinones selectively labeled with $^{13}$C into the binding pocket and the mapping of the electron spin density around the quinone ring conclusively proved that each quinone was involved in only one H-bond.

The perturbation of the H-bond does not affect the quinone’s affinity to the binding pocket, indicating that the H-bond is structurally dispensable. However, a dramatic increase in the electron transfer rate from A$_1^-$ to F$_X$ is observed due to a shift in the redox potential of the quinone to a more reducing value. In the absence of the H-bond, the quinone is more susceptible to protonation during the formation of the phyllosemiquinone radical. Were protonation to occur, a second electron reduction would become energetically feasible, resulting in a phyllohydroquinone that would be trapped in a high potential well and not able to pass the electron against a large thermodynamic gradient in either the forward or backward direction. If this line of thinking is correct, the purpose of the H-bond is to tie up the carbonyl group so that double reduction/protonation cannot occur during periods of high intensity illumination.

Perhaps the most interesting outcome of the studies describes in this dissertation is the observation of the B-branch quinone. Although, it is widely accepted that electron transfer proceeds via both branches in cyanobacterial PS I, the spectroscopic evidence for electron transport via the B-branch is not explicit. Biochemical and biophysical studies of several B-branch site-directed mutants suggest the involvement of this branch in forward electron transfer. However, no direct spectroscopic evidence of participation had ever been obtained for the B-branch cofactors. By breaking the H-bond and protonating the A-branch quinone, it was possible to unambiguously observe electron transfer via the B-
branch. The unique spectroscopic marker obtained for A_{1B} is the first direct evidence indicating that cyanobacterial PS I is capable of electron transfer via both branches. Although both quinones are involved in electron transfer, they have significantly different redox properties. In an attempt to identify the protein determinants that cause these seemingly large differences in the midpoint potentials of the phylloquinones in the A- and B-branches, a novel pulsed magnetic resonance technique was applied to PS I and a complete map of the quinone-protein interactions was charted. The results outlined herein indicate that A_{1A} might be involved in a stronger H-bond with the surrounding protein environment than A_{1B}. The relative difference in H-bond strengths between the two quinones is however not sufficient to account for the large differences in their redox properties. The H-bond is therefore one of the many contributing factors that determine the difference in the midpoint potential between the two quinones.

This dissertation has provided invaluable information on the ability of the protein to modulate the thermodynamic properties of biological cofactors. If true understanding of a phenomenon means the ability to predict the consequence of a change, then we are edging ever closer to that goal in understanding phylloquinone function in PS I. Armed with this type of structural, thermodynamic, and mechanistic information, it should soon be possible to the tailor the A_{1A} and A_{1B} sites by introducing non-native quinones and by selectively altering the protein environment to confer a predetermined set of thermodynamic properties to these important biological cofactors.


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