MODIFICATION OF THE PROTEIN MATRIX AROUND ACTIVE- AND INACTIVE PHEOPHYTINS BY SITE-DIRECTED MUTAGENESIS, AFFECTS ON ENERGY AND ELECTRON TRANSFER PROCESSES IN PHOTOSYSTEM II

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

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Photosynthetic reaction center complexes (RCs) have two parallel and symmetry-related pathways for electron transfer from the primary electron donor to the primary electron acceptor. In photosystem II reaction centers (PS II RCs) only one of these pathways is active.

We have perturbed the protein environment of the putative active branch pheophytin (Pheo_{active}), the primary electron acceptor, by site-directed mutagenesis of the glutamate at position 130 of the D1 subunit (D1-E130). Previous results demonstrated that the D1-E130 residue hydrogen bonds (H bond) to the Pheo_{active}, similar to the glutamate residue at position 104 on the L subunit (L-E104) of the bacterial photosynthetic reaction center. The results from HF-EPR experiments indicate that replacement of the H-bond donor with histidine, glutamine, and leucine results in a shift of the g_s value towards the high-field direction. These shifts verify the predicted H-bond strength changes in the mutants. The D1-E130L mutant is able to evolve O_2 at only 10% of wild-type (WT) rates and exhibits a 90% reduction in variable chlorophyll (Chl) a fluorescence yield. This loss of variable Chl fluorescence is associated with a high initial (F_0) Chl fluorescence level indicative of closed reaction center. Significantly, the D1-E130L mutation induces a blue shift in both the ground state and transiently reduced state
of the Pheo Qx absorption band similar to the spectrum of the inactive branch Pheo. The D1-E130Q exhibits an intermediate phenotype more similar to the D1-E130L mutant than WT. The D1-E130Q mutant displays a distinct blue shift in the spectrum of transiently reduced Pheo Qx band, but not in the ground state spectrum and has intermediate rates of oxygen evolution relative to WT. In contrast, the D1-E130H mutant has near WT rates of oxygen evolution and exhibits no appreciable differences in either the ground or the reduced Pheo Qx absorption spectrum. These results are consistent with the observation that a histidine substitution leads to only a minor perturbation of the Pheo active H-bonding interaction. At cryogenic temperatures all mutants exhibit similar abilities to accumulate charge separated states including the S2-mutiline Mn signal and the QA−Fe3+ EPR signal.

In addition, there are no apparent differences in primary charge separation kinetics (Pheo reduction) between WT and the D1-E130 mutants at room temperature. These results suggest that delocalization of charge density on the Pheo active, associated with the strength of the hydrogen bonding interaction, indirectly affects QA− to QB charge separation in the D1-E130L mutant, accounting for its inability to evolve oxygen.

To determine the function of the inactive branch Pheo, which has nearly identical distance and orientation as the structurally symmetrical active branch Pheo, we have used site directed mutagenesis to provide a Mg ligand for Pheo. We mutated a leucine residue at position 210 on the D1 subunit to a metal-coordinating histidine to convert the inactive branch Pheo (Pheoinactive) potentially into a Chl. Analyses of the pigment composition of PS II RCs indicate that the histidine substitution results in the incorporation of a Chl in place of Pheoinactive, as predicted. In addition, we are able to accurately locate the Pheo active
Qx absorption band at 544 nm using the modified PS II RC complex, which verifies the
different electronic transitions between the two Pheo molecules within PS II RCs. The
Chl fluorescence emission spectrum at 77 K shows that D1-L210H RCs display a
fluorescence emission maximum at 682 nm, but the shape of emission band is narrower in
the D1-L210 mutant than in WT. On the other hand, the loss of oxygen evolution
capacity, variable Chl fluorescence, and QA accumulation indicate that electron transfer in
the D1-L210H mutant is severely perturbed. This is partially attributed to the reduced
yield of radical pair P680+Pheo- as evidenced by transient absorption spectra. These
results are discussed in terms of a photochemical model for charge separation in PS II. On
the contrary, the conserved D1-L210A mutant exhibits the same phenotype as WT.

Another objective was to investigate the dynamics of early electron transfer events
in a PS II RC in which the spectral overlap of the pigments is relatively less congested.
We mutated the D2-L210 residue, located over one face of the macrocycle center of
Pheo_{active} to a histidine to yield stably assembled RCs depleted of Pheo_{active} (a Chl occupies
the place of Pheo_{active}). Our results indicate that D2-L210H mutation has substantially
altered energy and electron transfer properties compared to WT, as expected.
Surprisingly, the potentially conservative mutant D2-L210A also has a reduced capacity to
carry out charge transfer. These results suggest that the amino acid substitutions at D2-
L210 have significant effects on electron transfer efficiency of the PS II RC complex.
To my parents
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<td>$A_0$</td>
<td>monomeric Chl a known as primary electron acceptor of photosystem I</td>
</tr>
<tr>
<td>$A_1$</td>
<td>a chlorophyll molecule</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>BChl</td>
<td>bacteriochlorophyll</td>
</tr>
<tr>
<td>Bpheo</td>
<td>bacteriopheophytin</td>
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<tr>
<td>CD</td>
<td>circular dichroism</td>
</tr>
<tr>
<td>Chl</td>
<td>chlorophyll</td>
</tr>
<tr>
<td>Cyt</td>
<td>cytochrome</td>
</tr>
<tr>
<td>D1 and D2</td>
<td>reaction center polypeptides of photosystem II</td>
</tr>
<tr>
<td>DMBQ</td>
<td>dimethylbenzoquinone</td>
</tr>
<tr>
<td>DCMU</td>
<td>3-(3,4-dichlorophenyl)-1,1-dimethyluera</td>
</tr>
<tr>
<td>DM</td>
<td>$\eta$-dodecyl $\beta$-D-maltoside</td>
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<tr>
<td>EPR</td>
<td>electron paramagnetic resonance</td>
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F₀  the minimal fluorescence
Fᵥ  variable fluorescence
HEPES  4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
I and I'  the oxidized and reduced forms of pheophytin
K₃FeCN  potassium ferricyanide
LHCII  light harvesting complex II
Mes  2-(N-morpholino) ethanesulphonic acid
NADP  nicotinamide adenine dinucleotide phosphate
P₆₈₀  the primary chlorophyll donor of photosystem II
P₇₀₀  the primary chlorophyll donor of photosystem I
Pheo  pheophytin
PS I  photosystem I
PS II  photosystem II
PC  platocyanin
PCR  polymerase chain reaction
PQ  plastoquinone
QA  primary quinone electron acceptor of photosystem II
QB  secondary quinone electron acceptor of photosystem II
RC  reaction center
SDS  sodium dodecyl sulfate
Tris  2-(N-morpholino) ethanesulfonic acid
TAP  Tris-acetate-phosphate
$Y_D$ Tyrosine 160 on the D2 polypeptides

$Y_Z$ Tyrosine 160 on the D1 protein and primary electron donor to P680
CHAPTER 1

INTRODUCTION

1.1 Photosynthesis: An overview

Photosynthesis takes place in photosynthetic bacteria, cyanobacteria, algae and green plants. It is the process by which the energy from sunlight is converted to chemical energy. It is the major pathway to fix carbon dioxide and serves as a vital link between material and energy cycling in the biosphere.

Photosynthesis can be simply summarized with the chemical equation (in green plants):

\[ 6 \text{CO}_2 + 6 \text{H}_2\text{O} + \text{Light} \rightarrow \text{C}_6\text{H}_{12}\text{O}_6 + 6 \text{O}_2 \]

In green plants, there are two phases of photosynthesis: the light reactions (the formation of principle energy currency) and Calvin cycle (the biosynthesis of carbohydrates).

The light reactions of photosynthesis mediate the conversion of solar energy into chemical energy. In higher plants and eukaryotic algae the light reactions occur in the
thylakoid membranes of chloroplasts where both photosystem II (PS II) and photosystem I (PS I) transfer electrons in series. The light reactions drive the splitting of water into molecular oxygen leading ultimately to the reduction of NADPH (a source of reducing power) and generation of ATP (the energy currency of cells). Fig. 1.1 shows the electron transport scheme of chloroplasts. The components of the electron transport pathway are essentially organized into five major protein complexes: PS II, cytochrome b₆/f and PS I complex involved in linear electron transfer, the light harvesting complex that funnels energy to the photosystems and the ATPase complex that synthesizes ATP. Plastoquinone, plastocyanin and ferredoxin are mobile electron carriers. Plastohydroquinone carries two electrons and two protons from PS II to the Cyt b₆/f complex. Plastocyanin is a lumenal soluble protein that carries a single electron from Cyt f to photosystem I and ferredoxin carries a single electron from PSI to the NADP oxidoreductase and other electron consuming pathways localized in the chloroplast.

Energy harvested by the antenna migrates excitonically to the reaction centers (RCs) of PS II and PS I in which the primary charge separation takes place. The source of the electrons is water, which is oxidized by PS II, resulting in the production of molecular oxygen. Ultimately, NADP is reduced and a transmembrane electrochemical and proton gradient are generated, which drive ATP synthesis.

In essence, all photosystems contain three major parts: 1) the RC complex that performs the photochemical charge separation between the primary donor (a bacteriochlorophyll or chlorophyll molecule associated with specific proteins) and the primary acceptor, a quinone or iron-sulfur center, 2) the proximal antenna consisting of
pigment protein complexes that are adjacent to the RC complex, and 3) the peripheral antenna. When an antenna pigment absorbs a photon, the energy is transferred from one pigment to another until it reaches the RC where the light-induced chemical reaction occurs.
Fig. 1.1 The schematic diagram relating four functional photosynthetic complexes of thylakoids.
1.2 The purple-sulfur bacteria

Photosynthetic bacteria lack chloroplasts. Light-driven photosynthesis takes place in the inner membrane of the cell. Significantly, the purple-sulfur bacteria cannot use water as a substrate and they do not evolve oxygen during photosynthesis. In fact photosynthesis is obligately anaerobic. In contrast to oxygenic photosynthetic organisms, the anoxygenic bacteria contain only one type of photosystem, which resembles either PS II (quinone-type found in purple bacteria and green filamentous bacteria) or PS I (Fe-S type found in green sulfur bacteria and heliobacteria). To date, the most fully characterized RC complex is that of the purple-sulfur photosynthetic bacteria. All purple bacteria have BChl a or BChl b, which resides in the RC complex and antenna, respectively. The antennae and RCs these make up the so-called photosynthetic unit.

The antenna complexes are pigment-proteins that bind most of the pigments found in all photosynthetic systems. Photosynthetic bacteria have several types of antenna complexes depending on the species. Purple bacteria have two types of antenna complexes: a core antenna proximal to the RC characterized by a single absorption peak at the longer wavelength 880 nm or 1015 nm known as B880 or B1015 (LHI) and the peripheral antenna complex characterized with the shorter wavelength absorbing pigments in the range of 800-850 nm known as B800-850 (LHII) (Zuber, 1993). The two classes of antenna complexes have different spectra and structures. The core antenna complex (B880 or B1015) is invariably present with the RC in all purple photosynthetic bacteria. The solar energy absorbed by the antenna complex is funneled along an excitation-energy gradient to the RC to initiate the first photochemical reaction.
Significantly, the purple bacteria are capable of altering the proportion of antenna to RC complexes in response to light changes. The size of peripheral antenna depends on the conditions of cell growth (Zuber et al., 1987).

The light-harvesting BChl a (or BChl b in a few species) and carotenoid molecules are noncovalently associated with two different low molecular weight polypeptides (5~7 kDa), designated as $\alpha$ and $\beta$, to form the LHI and LHII pigment-protein complex. Some species of purple bacteria also contain a $\gamma$ subunit (in all B1015 core antenna complex).

The structural analyses of the $\alpha$- and $\beta$-subunits show that they have three unique domains. The central transmembrane segment is made of ~20 amino acids. Infrared, UV and CD spectra indicate that a transmembrane $\alpha$-helix is formed in the center domain (Jay et al., 1983; Breton and Nabedryk, 1984). The polar N-terminus and C-termini are hydrophilic and can be digested by proteinases, suggesting that the domains project from the membrane into the cytoplasm and periplastic spaces, respectively. Resonance Raman studies demonstrate that in each subunit a conserved histidine residue situated near the hydrophobic C-terminus serves as a ligand to the Mg atom of the BChl that is critical for energy transfer (Robert and Lutz, 1985).

In the LHC II complex multiple $\alpha$-$\beta$-heterodimers are the basic building blocks and are arranged in the form of cylinder (with $\alpha$-subunit ring inside, $\beta$-subunit ring outside). The BChl a molecules are arranged into two groups: 1) having absorption bands between 820-860 nm or between 870-890 nm and adjacent to the periplastic end of the complex with their macrocycles oriented perpendicular to the membrane plane and sandwiched between $\alpha$ and $\beta$ cylinders, and 2) the other group with an absorption peak at 800 nm and
located near the cytoplasmic surface with each macrocycle sandwiched between β-subunits and parallel to the plane of the membrane. The carotenoids are in contact with BChl a to facilitate energy transfer and/or to quench the triplet-state of BChl a (Mcdermott et al., 1995; Hu and Schulten, 1998). Interestingly, the structure of LHC I displays strong similarities to LHC II (Karrasch et al., 1995).

1.2.1 Photosynthetic bacterial reaction center

Photosynthetic RCs are embedded in the membrane. The RC complex can be selectively solubilized using detergents to deplete the antenna complex. The RC preparations lacking the light-harvesting complex greatly facilitate the study of spectral and structural properties of the complex. The first RC was isolated from the mutant strain R-26 of *Rb. spbaeroides* (Reed and Clayton, 1968), and the first three-dimensional crystal structure of RC from this species was determined by Deisenhofer et al. (1985). To date, high-resolution (2.2 Å) X-ray structures of *Rps. viridis* and *Rb. spaeroides* bacterial RC (BRC) have been solved (Fritzsch et al., 1998; Stowell et al., 1997). The overall structures of RCs from these two species are very similar. The RC complex contains three intrinsic membrane-spanning protein subunits, know as L (31 kDa), M (34 kDa), and H (28 kDa) (Youvan et al., 1984), and four bacteriochlorophylls (BChl), two bacteriopheophytins (Bpheo), two quinones, and one carotenoid. Four hemes are bound to the extrinsic Cyt C subunit. The folding patterns of L and M subunits are very similar: each subunit has five transmembrane spanning α-helices and several short extrinsic α-helices. The H subunit has only one transmembranes α-helix that anchors it in the
membrane. Most of the H subunit is exposed to the cytoplasmic side of the membrane (Deisenhofer et al., 1985). In *Rps. viridis* a tetraheme, c-type cytochrome is tightly bound to the RC complex (Thornber et al., 1980). The L and M subunits are highly hydrophobic, each having ~ 70% nonploar amino acids. Most of the cofactors within the RC complex are sandwiched between the L and M subunits. The H chain does not bind any pigment, and it can be easily dissociated from the complex by washing with various kinds of detergents. The RC complex free of H subunit is functional but with decreased efficiency. It is speculated that the H subunit participates in the assembly of the complex into membrane (Chory et al., 1984).

The arrangement and orientation of cofactors bound to L and M subunits in the RC complex of the carotenoid-less mutant R-26 of *Rb. Spbaeroides* is shown in Fig. 1.2 (Chang et al., 1986). These cofactors include a BCHl special pair known as the primary electron donor P, two other BCHls designated as Chl monomers, the two BPheos (magnesium-less bacteriochlorophyll), two ubiquinones (QA and QB), and a non-heme Fe^{2+}. These cofactors are arranged with pseudo two-fold rotational symmetry. Such symmetry also relates to the L and M subunits where the five helical segments are close to parallel to the two-fold axis of symmetry. Given the nearly symmetric structure of the RC, it is remarkable that the electron transfer occurs predominantly along the branch of parallel cofactors most closely associated with the L subunit, thus only the BPheo_L absorbing at longer wavelength is photoactive (Kirmaier and Holten, 1987; Lockhart et al., 1990; Bylina et al., 1988).
Fig. 1.2 The reaction center cofactors of the carotenoid-less mutant R-26 of *Rh. sphaeroides* (Chang et al., 1986).
The absorption spectra of BRCs are clearly resolved at low temperature (Fig. 1.3). In the isolated RCs of *Rb. sphaeroides* the primary donor absorbs at the longest wavelength: 870 nm, the two monomer BChls contribute to light absorption in the region of 800 nm (Vasmel et al., 1986), and the two Bpheo absorb near 760 nm and 540 nm. In addition, the two Bpheo can be well separated in their Qx absorption bands (530-550 nm): the absorption band at 545 nm is mostly due to electronic transitions in BpheoL, whereas absorption band at 530 nm are attributed to BpheoM. The different spectral features of pigments of RCs in this optical range indicate there are different electronic transitions, which can be used to selectively study specific pigments in regard to their redox potential and structural properties.
Fig. 1.3 Ground-state absorption spectra of reaction centers from *Rb. sphaeroides* R26 at 10 K (Egorova-Zachernyck et al., 1997).
1.2.2 Electron transport

Fig. 1.4 shows the scheme of cyclic electron transport in purple photosynthetic bacteria. Light absorbed by the antenna pigments is ultimately transferred to the two special BChl a molecules (the primary electron donor, P) whereupon the excited state P loses an electron to become P+. The electron is transferred to the primary electron acceptor BpheoL within a few picoseconds (Martin et al., 1986; Breton et al., 1988; van Brederode and van Grondelle, 1999) leading to radical pair (P+Bpheo−) formation. The nature and mechanism of the primary charge separation remains controversial. The time constant for charge separation is too rapid relative to the center-to-center distance (17 Å) between P and BpheoL to be a single-step electron transfer. It was therefore suggested by Marcus that the ultrafast process of early electron transfer proceeds in two-steps, first P* donates an electron to BChlL and subsequently to BpheoL (Marcus, 1987). Several lines of experiment evidence favored this mechanism (Holzapfel et al., 1989; Holzapfel et al., 1990). Alternatively, a super-exchange mechanism is postulated, in which BChlL serves as a mediator to facilitate the electron transfer from P* to BpheoL (Bixon et al., 1989; Bixon et al., 1991). This hypothesis has also gained some experimental support (Lochart et al., 1988; Michel-Beyerle et al., 1988).
Fig. 1.4 The cyclic electron transport pathway in purple bacteria.
Consecutive electron transfer in the RC complex occurs from Bpheo\(^{-}\) to the first quinone acceptor to form the semiquinone anion of Q\(_A^-\) in about 200 ps (Parson, 1987; van Brederode and van Grondelle, 1999). Electron transfer from Q\(_A^-\) to the second quinone electron acceptor Q\(_B^-\) takes a few hundred µs. The oxidized P receives an electron from reduced cytochrome c on the µs time scale, with similar kinetics for the reduction of Q\(_B^-\) to Q\(_B^{2-}\). In the mean time, Q\(_B^{2-}\) gains two protons from the external side of the membrane. The protonated Q\(_B^2\)H\(_2\) dissociates from its binding site on the membrane and is replaced by another oxidized quinone from the quinone pool of the cell membrane. The fully reduced quinol is then oxidized by the cytochrome bc complex and the released protons accumulate in the periplasmic space to drive photophosphorylation catalyzed by the ATPase. From cytochrome c, the electron can be transferred to the soluble cytochrome c of the RC complex and ultimately to P\(^+\). The major and best-characterized cytochrome c from *Rps. viridis* is associated permanently with RCs. These cytochrome complexes consist of a 40 kDa tetraheme subunit that is tightly bound to the RC complex even at high ionic strength. A mobile cytochrome c\(_2\) was found to provide reducing power to the tetraheme subunit (Shill and Wood, 1984). In the absence of an electron donor to P\(^+\) charge recombination between P\(^+\) and Bpheo\(^{-}/Q_A^-/Q_B^-\) occurs.

Recently, new and ultrafast pathways for primary charge separation were discovered in BRCs (Marion et al., 1999). One feature worth noting is that these routes of charge separation do not involve the Chl special pair, but are directly driven by the
monomeric BChl positioned on the active branch in electron transfer. The physiological choice of path of charge separation is determined by the excitation wavelength (blue side favors electron transfer on the inactive branch) and various interactions in RC complex.

The monomeric BChl also affects the rate of initial electron transfer (Allen and Williams, 1998) and might partially contribute to the functional asymmetry of electron transfer along one-branch (Heller et al., 1995). The precise origins of directionality of the electron transfer in the RC complex are still a subject of debate. Most the proposals suggest that the direction of electron transport is controlled by the relative free energy level of the charge-separated state P⁺BChl⁻⁻⁻⁻ and P⁺BChl⁻⁻⁻⁻⁻ (Nagarajan et al., 1993; Heller et al., 1995; Gunner et al., 1996), which arises from the different protein environments of L and M subunits. Both theoretical calculations and mutagenesis studies demonstrate that the protein matrix plays key roles in the asymmetry of electron transfer (Gunner et al., 1996; Lin et al., 1999).

1.3 Photosystem I of green plants

PS I is a multi-subunit pigment-protein complex present in the thylakoids of green plants and algae. It utilizes the energy of light to generate a charge-separated state ultimately resulting in the reduction of ferredoxin on the stromal side of the membrane and the oxidation of plastocyanin on the lumenal side of the membrane. Electrons transferred to ferredoxin are ultimately used to reduce NADP⁺. The resulting NADPH is the reducing power for CO₂ fixation. The transmembrane electrochemical potential generating from the electron transport process also helps drive ATP synthesis.
Both peripheral Chls and the core Chls of the PS I RC function as antenna to capture the solar radiation energy and transfer it to the cofactors of the RC that undergo photochemistry. The peripheral light-harvesting complex of PS I called LHC I has been isolated from a variety of organisms (Mullet et al., 1980; Vainstein et al., 1989; Bassi et al., 1992). LHC I can be fractionated into two chlorophyll-protein complexes by treating thylakoids with the detergent Triton X-100, followed by separation on a sucrose-density gradient. The lighter fraction with a Chl fluorescence emission peak at 680 nm is known as LHC Ia, or LHC I-680. It contains two polypeptides with molecular weights of 23 and 22 kDa. The heavier fraction with a Chl fluorescence peak at 730 nm is called LHC Ib or LHC I-730. It has only a 20 kDa subunit. The Chl a/b ratios of both light harvesting complexes is $3.5 \pm 0.5$. There is no consensus in regard to the exact numbers of subunits present in the LHC I complex.

Unlike the BRCs and the PS II RCs of green plants, the PS I RC core contains a large number of antenna Chls (90-100) (Krauss et al., 1996). It appears that most of the chlorins in the PS I RC serve in harvesting light. The electron transfer cofactors include, the primary electron donor Chl special pair known as P700, two monomeric Chl a called A_0, another pair of Chls and a pair of phylloquinone molecules designated as A_1, and three membrane-bound iron-sulfur centers known as F_X, F_A and F_B. Most of the electron carriers are bound to the two large hydrophobic polypeptides: psaA and psaB, with exception that F_A and F_B are bound to the extrinsic polypeptide psaC. The arrangement of cofactors in the electron transfer is shown Fig. 1.5. Upon excitation, P700 is oxidized
and the electron is passed to the primary acceptor, A₀. Meanwhile, the P700⁺ is reduced by plastocyanin. The electron goes from A₀ to a second Chl monomer followed by A₁, and subsequently to the terminal electron acceptor Fx followed by the Fₐ and Fₐ iron-sulfur clusters that are located on the stroma side of the membrane.
Fig. 1.5 Schematic representation of the photosystem I complex in the thylakoid membrane.
The three-dimensional structure of PS I RC at 6 Å and 4 Å resolution indicates that the major protein subunits and associated cofactors are arranged with a two-fold axis of symmetry (Krauss et al., 1993; Kraub et al., 1996; Fromme et al., 2001), which is a common feature of all photosynthetic structures. The psaA/psaB protein heterodimer of PS I each contains eleven transmembrane α-helices. The N-terminal six transmembrane spans bind the antenna Chl a and the C-terminal five transmembrane spans bind the cofactors involved in charge separation. The iron-sulfur cluster adjacent to the lumenal surface is assigned as Fx, which is coordinated by four cysteines, two from psaA and two from psaB. As to the electron transfer sequence between FA and FB, both optical and photovoltage studies suggest that FB is the terminal electron acceptor and the reaction partner to ferredoxin (Mamedov et al., 1998; Diaz-Quintana et al., 1998). FA is the iron-sulfur cluster proximal to FX. The iron-sulfur type of PS I RCs is functionally more symmetric than the quinone-type RCs (Golbeck, 1994). Recent mutagenesis studies in Chlamydomonas show that both branches of the electron transfer cofactors in the PSI RC are active (Kuras et al., 2001).

1.4 Photosystem II of green plants

The oxygen evolving PS II core complex includes the RC and a number of antenna Chl protein complexes, which are bound to the thylakoid membranes.

The light-harvesting complex of PS II includes, the major Chl a/b complex, represented by LHC II; the minor Chl a/b complexes, known as CP 29, CP26, CP24; and the proximal antenna Chl a-containing complexes, called CP43 and CP47. The LHC II
complex has been crystallized and its structure determined at ~3.4 Å resolution by electron diffraction (Kühlbrandt et al., 1994). Each subunit of an LHC II complex has a molecular weight ~25 kDa and binds 8 Chl a, 6 Chl b, and 2 xanthophylls. LHC II is an integral membrane protein complex and is encoded by nuclear genes. As to the CP29, CP26, and CP24 proteins, they are located between the proximal core antenna and LHC II and may act as linkers between the two. The proximal antenna complexes are CP43 and CP47. These two antenna complexes are tightly associated with the RC complex, suggesting that they may function as anchors for some proteins in PS II in addition to capturing light (Ke, 2001; Zouni et al., 2001).

Excitation of the PS II RC drives charge separation between the primary electron donor, possibly a special pair of Chls known as P680, and the primary electron acceptor, a pheophytin (Pheo). Recent evidence has suggested, however, that one of the Chl momomers may be P680 (Diner and Rappaport, 2002). P680\(^+\) is one of the strongest oxidant produced in biological systems, its redox potential is high enough to oxidize water to molecular oxygen. Briefly, the highly oxidizing P680\(^+\) extracts an electron from the secondary electron donor called Y\(_Z\), a tyrosine located at residue 161 on the D1 protein. Y\(_Z\) is located at the lumenal end of the third transmembrane span in the D1 protein. The reduction of Y\(_Z\) proceeds by oxidation of the tetra-Mn water splitting complex and is coupled with proton extraction (Hoganson and Babcock, 1988; Babcock et al., 1989). On the acceptor side, the reduced Pheo formed during primary charge separation transfers an electron to next electron carrier Q\(_A\), which is a single electron acceptor. The electron then moves to Q\(_B\) that can accept up to two electrons and two
protons. The fully protonated $\text{Q}_B\text{H}_2$ undergoes an exchange with plastoquinone from the quinone pool to shuttle reducing power \textit{via} cytochrome $b_6/f$ complex to PS I, meantime, the protons released from water oxidation are sequestered into the lumen and drive photophosphorylation (Hangarter and Ort, 1986; Kramer and Crofts, 1989; Roberts and Kramer, 2001; Kramer et al., 2002). Fig. 1.6 summarizes the pathway of electron transfer in PS II from water to plastoquinone $\text{Q}_B$. 
Fig. 1.6 The pathway of PS II electron transfer from water to plastoquinone \( Q_B \).
1.4.1 Oxygen-evolving manganese-protein complex (OEC)

Although the quinone-type RC complexes of non-oxygenic bacteria, cyanobacteria and chloroplasts are quite similar, there are substantial differences. Relevant to this discussion are the differences between the PS II RC complexes of cyanobacteria and chloroplasts. As reviewed in Ruffle and Sayre (1998) several of the small polypeptides of the cyanobacterial and chloroplastic PS II complexes have different functions as demonstrated by gene deletion studies. Most obvious are the differences between the polypeptides of the water-oxidizing complex. The cyanobacterial PS II complex shares one common subunit (33 kD OEC protein) with the chloroplastic PS II complex and the two complexes have several unique subunits not found in the other type. All water-splitting complexes have a common tetra-Mn cluster that undergoes redox cycling to catalyze the oxidation of water. The close correlation between oxygen evolution activity and manganese has long been recognized (Kessler, 1955). It is well established that the four bound manganese ions are involved in oxygen evolution (reviewed in Debus, 1992). These manganese ions evidently serve as the catalytic site in the OEC cluster. Several lines of evidence from selective extraction and spectroscopic experiments show an apparent heterogeneity in the manganese redox population (Dismukes and Siderer, 1980; Yocum et al., 1981; Ono and Inoue, 1985; Bonvoisin et al, 1992; Kuzek and Pace, 2001). The structure of tetranuclear Mn cluster itself has not been clearly resolved, although possible Mn cluster structures have been proposed based on the data from EXAFS studies (Geroge et al., 1989; Yachandra et al., 1993; DeRose et al, 1994).
According to recent review (Robblee et al., 2001), the Mn cluster in PS II is made up of a pair of di-µ-oxo bridged Mn binuclear clusters linked by a mono-µ-oxo bridge.

Three extrinsic protein subunits of 17, 23, 33 kDa molecular mass, encoded by nuclear genes, are located on the lumenal side of the OEC complex of chloroplasts. These proteins are proposed to enhance the PS II oxygen evolution activity at physiological concentrations of inorganic cofactors (Ca$^{2+}$ and Cl$^{-}$) that are essential for the oxidation of water to molecular oxygen (Debus, 1992; Yocum, 1992). Of these extrinsic proteins, the 33 kDa component, is the one most extensively investigated and is also the most important subunit in the enhancement of oxygen evolution activity. Depletion of this protein from OEC results in a disassembly of manganese cluster under low Cl$^{-}$ and Ca$^{2+}$ concentrations (< 100 mM) (Miyao and Murata, 1984; Kuwabara et al., 1985). Supplying high concentrations of Cl$^{-}$ (> 100 mM) stabilizes the OEC, but the oxygen evolution rate is lower than for intact PS II complexes (Bricker, 1992). The addition of Ca$^{2+}$ can compensate for the lack of the 33 kD protein (Bricker, 1992). In addition, mutants lacking the 33 kDa protein are unable to grow autotrophically under low Ca$^{2+}$ conditions (Philbrick et al., 1991). These results reveal the central role of the 33 kDa protein in the binding of Ca$^{2+}$ in PS II. As to the other two extrinsic proteins, the overall consensus is that they may facilitate the binding of Ca$^{2+}$ and Cl$^{-}$ and / or concentrate the ions at the active site (Miyao and Murata, 1985; Waggoner and Yocum, 1987; Homann, 1988; Yocum, 1991).
The mechanism of water oxidation remains to be elucidated. The cleavage of two molecules of water is required to generate one oxygen molecule. This also requires the extraction of four electrons from water. The midpoint potential of H₂O/O₂ in the thylakoid lumen can be as high as 0.94 eV. The P680⁺/P680 couple, however, has a midpoint potential of ~1.12 eV which is sufficient to extract electrons from the OEC, tetra-Mn complex. Associated with each photochemical event, one electron must be extracted at a time. Thus, four sequential photochemical reactions are required to produce four oxidizing equivalents necessary for the release of molecular oxygen.

In the early 1970s, it was demonstrated that there was a clear period-four oscillation of oxygen evolution vs. flash number after a series of short saturating flashes (Joliot et al., 1969). Kok repeated the flash pattern and proposed a four-electron gate called the S-state model (Fig. 1.7).

In the Kok S-state model, S₀ is the most reduced state and is formed after the release of molecular oxygen from the previous cycle. The light-induced primary photochemistry reaction leads to the generation of P680⁺, which is reduced by extracting an electron from the electron donor, Y₂. The neutral radical Y₂⁺ oxidizes the OEC, driving the transition from S₀ → S₁. Successive charge separating events lead to the S-state transitions: S₁ → S₂, S₂ → S₃, and → S₄. The sequential donation of electrons one at a time by the OEC to P680⁺ with each flash ultimately leads to the formation of the unstable S₄ state, during which one oxygen molecule is evolved and S₄ reverts back to S₀ ready for another cycle. According to this model, P680 undergoes four charge separation events after absorbing four photons resulting in the release of four electrons, four protons,
and an oxygen molecule from two water molecules. The observation that the maximal quantity of oxygen is evolved following the third flash in the dark-adapted PS II membranes rather than the fourth, was attributed to the fact that the $S_1$ is dark-stable state and accounts for about 75% of the dark stable S-state population.
Fig. 1.7 The OEC S-state cycle introduced by Kok et al. (1970).
In addition to the S-state model, a number of other hypotheses have been proposed in regard to the proceeding of water splitting chemistry based on information obtained from the spectroscopy and properties of OEC components. But it is still difficult to elucidate the exact mechanism due to the limited structural information of the OEC obtained from the low-resolution crystal (3.6 Å). In general, there are two types of models: the first one called metal cluster mechanism proposes that the driving force of water oxidation is the structural changes of the tetranuclear Mn cluster, with numerous bond rearrangements occurring at different states of the cycle. The protein matrix is not directly involved in this process (Brudvig and Crabtree, 1986; Christou and Vincent, 1987). The second type known as metallo-radical mechanism, however, emphasizes the redox-active tyrosine YZ that extracts both an electron and proton from the water-oxidizing complex. The neutral radical is formed with the transfer of a proton to histidine 190 (Roffey et al., 1994; Gilchrist et al., 1995; Hoganson et al., 1997; Tommos and Babcock, 1998; Mamedov et al., 1998). In these recent models, the process of water oxidation chemistry is driven by the four turnovers of the tyrosyl radical YZ:

\[
\begin{align*}
2\text{H}_2\text{O} & \rightarrow (\text{Mn})_4\text{YZ}^+ + 4\text{e}^- + 4\text{H}^+ \\
\text{O}_2 & \rightarrow \text{P680}^+ + \text{Q}^- + \text{PSI}
\end{align*}
\]
The OEC complex, $Y_Z$ and the cofactor $Ca^{2+}$ and $Cl^-$ form the $[(Mn)_4.Y_Z.Ca^{2+}.Cl^-]$ complex. Each S-state transition involves the abstraction of a hydrogen atom from the substrate water bound at Mn-site and valence changes in the tetra-Mn cluster. The consecutive oxidations of $Y_Z$ through $P680^+$ result in the release of four protons during a cycle.

1.4.2 Photosystem II RC (reaction center)

To date, several different types of oxygen-evolving PS II preparations have been isolated including: BBY-type PS II complexes containing a number of peripheral and core antenna complexes, (Berthold et al., 1981; Kuwabara and Murata, 1982) and the PS II core complex that is the smallest unit capable of oxidizing water. PS II core preparations are depleted of most of the peripheral antenna complexes and contain four manganese atoms, the peripheral 33 kDa OEC protein, and the proximal antenna proteins CP 43 and CP 47 (Satoh et al., 1985; Yamata et al., 1987; Tang and Diner, 1994). The smallest PS II complex capable of performing charge separation is the PS II RC. Such preparations are able to transfer an electron from the singlet-excited state of the primary donor, P680, to a Pheo, a molecule known as the primary acceptor (Nanba and Satoh, 1987; Takahashi et al., 1987). However, the so-called PS II RC preparation is unable to stabilize the charge-separated state since the secondary electron transfer pathways are not functional due to the loss of $Q_A$ and inactivation of the electron donor tyrosine $Y_Z$. The
generated radical pair thus is lost by charge recombination, leading to formation of a substantial proportion of P680 excited state triplet.

1.4.2.1 Protein components and cofactors

The PS II RCs have been isolated from cyanobacteria, green algae, and green plants. This transmembrane pigment protein complex contains five subunits including D1, D2 proteins (30 and 32 kDa), cytochrome b-559 α and β subunits (10 and 4 kDa) and psbl gene product (4.8 kDa), and cofactors including six Chls, two Pheos, and one β-carotene. The major D1 and D2 proteins are encoded by chloroplast psbA and psbD genes and anchor the PS II cofactors and pigments participating in electron transfer. The D1 and D2 proteins share about 20-25% identity with the BRC L and M proteins, respectively. It is striking that the two polypeptides are highly homologous in amino acid sequence to each other and to the L and M subunits of BRC (Deisenhofer et al., 1985; Allen et al., 1987; Michel and Deisenhofer, 1988). Based on sequence similarity, together with the X-ray crystallographic structure of the BRC determined at atomic levels of resolution, it was hypothesized that the PS II RC is a heterodimer formed by the D1 and D2 subunits similar to the L and M subunits of the BRC (Michel and Deisenhofer, 1988). Models of the D1 and D2 folding patterns are shown in Fig. 1.8 (Trebst, 1986). These topological models were confirmed using peptide-specific antibodies by Sayre et al. (1986). Both the D1 and D2 subunits contain five-membrane spanning α- helices (A-E). The highest degree of sequence similarities between these two subunits is located in the putative C and D transmembrane helices. Consequently, it is speculated that these
regions are essential in coordinating cofactors of PS II RC similar to the situation in the BRC. As for the other low molecular weight proteins, previous studies revealed that a complex depleted of Cyt-b559 and \textit{psbI} gene product but retaining D1 and D2 proteins and all the cofactors of PS II RC, was still able to carry out primary charge separation (Tang et al., 1990). The possible involvement of Cyt-b559 as a cofactor involved in the control of photodamage process has been presented (Nedbal et al., 1992; Barber and De las Rivas, 1993; Poulson et al., 1995; Wang et al., 2002).
Fig. 1.8 Models of the D1 and D2 folding patterns (Trebst, 1986).
The cofactors of PS II RC preparations include Chl a, Pheo a, and β-carotene. However, the two quinones QA and QB are absent in all PS II RC preparations (Nanba and Satoh, 1987; Dekker et al. 1989; Gounaris et al., 1989; Montoya et al., 1991). All these cofactors absorb light in the visible region of the spectrum. Fig. 1.9 shows the room temperature absorption spectrum of PS II RC. The assignment of individual absorption peaks is more difficult than in the BRC due to a much greater degree of spectral overlap, particularly in the functionally important Qy region, where the six Chls and two Pheos have nearly identical absorption profiles. At cryogenic temperatures some fine structure can be observed: the absorption band centered at 675 nm is split into two peaks near 670 nm and 679 nm and a shoulder at about 683 nm (Van Kan et al., 1990). The absorption band peaking at 670 nm is attributed to at least one of the two peripheral Chls (Vacha et al. 1995). As to the red region peak (679 and 683 nm), it is established that P680 and the Pheo on the active branch (Pheoactive) are largely responsible for the absorption in this region. There is no good agreement in regard to the absorption properties of the Pheo on the inactive branch (Pheoinactive). The Pheo Qx absorption bands peaking at 543 nm are well resolved from those of Chls, but it is still hard to distinguish between the active and inactive Pheos. These spectral congestions make spectroscopic and kinetic studies of the PS II RC considerably more difficult than for BRCs where the six chlorins absorb at clearly distinguishable wavelengths. In particular, the lowest optical transition is not located on the special pair in PS II RCs.
Fig. 1.9  The absorption spectrum of PS II reaction centers from *Chlamydomonas reinhardtii* CC 2137.
1.4.2.2 The three-dimensional structure of the PS II RC

The X-ray structure of the PS II RC has not yet been determined. However, the crystal of the PS II core complex active in water oxidation has been obtained from the thermophilic cyanobacterium *Synechococcus elongatus* (Zouni et al., 2001) at 3.8 Å resolution (Fig. 1.10). The major proteins, D1 and D2, form a heterodimer, as expected (Michel and Deisenhofer, 1988). Subunits D1 and D2 can be completely distinguished from each other by the binding site of the manganese cluster. The D1 and D2 polypeptides contain five transmembrane $\alpha$-helices, which are arranged similarly to the L and M subunits of the BRC. The proximal antenna protein CP 43 and CP 47 also can be assigned based on previous results (Harrer et al., 1998; Ishikawa et al., 1999). It is noteworthy that a large numbers of the transmembrane $\alpha$-helices in the complex are arranged with a local pseudo-twofold symmetry, called pseudo-C2 axis. The cofactors involved in the electron transport are organized in two parallel arms along the pseudo-C2 axis in the crystal structure. The characteristic symmetric locations of cofactors and D1 and D2 subunits, together with the sequence homologies between D1/D2 and L/M, can be taken as a reflection that all the photosynthetic RCs probably evolved from a common ancestor.

The general features of the PS II RC indicated by the three-dimensional structure correspond to those from the two-dimensional structure of PS II complexes at 8 Å
resolution (Rhee et al., 1997). Nevertheless, the present structural understanding of PS II RC at atomic levels of resolution is dependent mostly on predicted structural models (Trebst, 1986; Sayre et al., 1986; Ruffle et al., 1992; Svensson et al., 1996, Xiong et al., 1998), which are based on the sequence resemblance of D1/D2 subunits with L/M subunits, as well as a multitude of spectroscopic analyses. Fig. 1.11 shows the three-dimensional model of PS II RC (Svensson et al., 1996). It contains D1 and D2 polypeptide chains and the cofactors including: special Chl pair P680, two accessory Chls, two Pheos.
Fig. 1.10 Crystal structure of PS II core complex from *Synechococcus elongatus* (Zouni et al., 2001)
Fig. 1.11 The three-dimensional structure model of PS II RC (Svensson et al., 1996).

Proteins are presented in ribbon style (brown: D1; blue: D2). The pigments are in stick style (green: chlorophyll; red: pheophytin.)
According to the PS II RC models, the ligands to the central Mg atoms of the P680 Chls are D1-H198 and D2-H197. The Pheo active is hydrogen bonded to the D1-E130 and possibly also to D1-Y126 and D1-Y147 residues. TyrZ is proposed to be located in a hydrophilic environment, which is partially composed of D1-Q165, D1-D170, D1-E189, and D1-H190. TyrD forms a weak hydrogen bond to D2-H189.

1.4.2.3 Excitonic coupling in PS II RC

Two or more pigments that are in close proximity to each other are likely to interact excitonically resulting in dipole-dipole couplings, which can modify the original transition dipoles (Van Grondelle et al., 1994). This excitonic interaction is distance dependent and capable of influencing the spectral features of optical transitions of various photosynthetic complexes.

Compared to the spectrum of the PS II RC, in which all the chlorin factors absorb in a narrow spectral region, the spectrum of BRC exhibits distinguishable absorption bands that can be attributed to unique cofactors absorbing at different wavelengths. In particular, the long wavelength transition in the purple BRC is largely ascribed to interactions between the BCHls of the special pair (Knapp et al., 1985). This coupling (~550 cm\(^{-1}\) for P870 and 950 cm\(^{-1}\) for P950) gives rise to a red shift of the special BCHl dimer excited state that is the lowest wavelength within the RC. Ultimately, all the excitonic energy is funneled and localized to the special pair. On the contrary, the Chl special pair in the PS II RC shows much smaller red shift relative to other pigments in the complex and thus is a more shallow energy trap for excitation energy.
Experimental studies have identified a much weaker excitonic interaction in the PS II RC than observed in the BRC (a upper limit of 140 cm$^{-1}$, and 3-4 times less than that in their bacterial counterparts) (Braun et al., 1990; Kwa et al., 1994). The PS II crystal structural studies of (Zouni et al., 2001) suggest that the separation of the two special pair Chl molecules in PS II RC is 10 Å. This large distance could contribute to the insignificant excitonic coupling between these Chls. It is believed that the reduced coupling strength of the Chl special pair of PS II enhances the charge separation efficiency relative to BRCs (~68% in PS II versus ~53% in bacteria) (Dinner and Babcock, 1996).

However, the effect of excitonic interactions between the other chlorins in the RC complex cannot be neglected since the dipolar coupling strength between the Chl special pair and monomeric Chls and adjacent Pheos are all of the similar order of 100 cm$^{-1}$ (Kwa, 1993). Taking into account the coupling between all the six central chlorins in the PS II RC, quantum mechanical calculations predict that the weak dipolar coupling results in delocalization of the excited state across several neighboring chlorins and not on the weakly coupled Chls of the special pair. Therefore, PS II RC should be viewed as a multimer of four Chl a and two Pheo a molecules (Tetenkin et al., 1989; Durrant et al., 1995, Diner, 2002).

### 1.4.2.4 Primary photochemistry in PS II RC

Experimental evidence of excitation energy transfer and trapping in isolated PS II RC indicates that primary charge separation is a multiphasic process. The faster
components is 5-8 ps and corresponds to primary charge separation from $^{1}\text{P}680$, the 20-
30 ps lifetime component is attributed to charge separation limited by slow energy
transfer from the peripheral Chl to the core pigments (Schelvis et al., 1994; Rech et al.,
1994; Greenfield et al., 1997; Donovan et al., 1997, Johnston et al., 2001; Ruffle et al.,
2001; Wang et al., 2002). Remarkably, the lifetime of charge separation is much slower
in PS II RCs than those reported in BRCs where most of the time constants are less than
10 ps. The main implication of these observations is the delocalization of the excitation
energy within the six central chlorins of RC (Van Grondelle et al., 1994), which may lead
to insignificant excitation energy transfer between pigments. Slow relaxation (~50 ps) of
radical pair $\text{P}680^+\text{Pheo}^-$, induced by conformational changes of protein surroundings,
shifts the equilibrium between the excited states and the radical pair towards the excited
states and might contribute to the slow charge separation kinetics as well (Konermann et
al., 1997). On the other hand, results from the temperature dependence studies of charge
separation differ considerably in PS II and purple bacteria RCs. In the PS II RC, $\text{P}680$
driven charge separation is slowed upon cooling (Groot et al., 1997; Greenfield et al.,
1999) while kinetics of radical pair formation in the BRC speeds up with decreasing
temperature (Fleming et al., 1988). One interpretation of these results is that the initial
step of charge separation in PS II RC needs to be activated to some extent (Diner and
Babcock, 1996).

The biradical pair, $\text{P}680^+\text{Pheo}^-$ undergoes further redox processes under normal
physiological conditions in PS II core particles including electron transfer from $\text{Pheo}^-$ to
$\text{Q}_A$, and reduction of $\text{P}680^+$ by $\text{Y}_Z$. In the isolated RCs, however, the absence of $\text{Q}_A$
facilitates P680’Pheo’ decay through a recombination reaction resulting in the formation of the spin-polarized triplet state of P680. The half time of the recombination in PS II RCs is ~10-100 ns (Danielius et al., 1987; Liu et al., 1993).

A critical aspect of electron transfer in PS II RCs still left open is the factors that determine the asymmetry of electron transfer along one branch. The spectral congestion of Qy transition of the central RC cofactors is a major obstacle to interpret the optical changes. The overlapping nature of optical band also hinders our understanding with regard to kinetics of energy transfer and primary charge separation in PS II RCs.

1.4.2.5 Protein engineering of the PS II RC

Site-directed mutagenesis is a powerful tool for identifying amino acid residues of the protein matrix that affect electron and energy transfer reactions in RCs. A majority of the protein engineering studies in PS II has employed *Synechocystis* sp. PCC6803, a unicellular cyanobacterium. It is readily amenable to genetic engineering. The transforming exogenous DNA molecule undergoes homologous recombination and is integrated into the cyanobacterial chromosome (William, 1988; Carpenter and Vermass, 1989). All of the site-directed mutagenesis studies that have been carried out on chloroplastic PS II complexes have been done using the conditional heterotrophic (acetate supports growth in the absence of photosynthesis) eukaryotic alga, *Chlamydomonas reinhardii*. The chloroplast genomes of most green plants and eukaryotic algae are highly conserved throughout evolution. Compared to green plants, it is easier to obtain stable, homoplasmic chloroplast transformants in *Chlamydomonas*. 
The best feature of this unicellular alga for chloroplast DNA transformation is the well-characterized chloroplast and nuclear background (Harris, 1989; Chunaev, 1990). Furthermore, Chlamydomonas has only one large (~ 40%-60% of cell volume), cup shaped chloroplast containing ~80 copies of chloroplast DNA. The copy number of the chloroplast DNA can be reduced even further using fluorodeoxyuridine, which selectively inhibits thymidine synthesis in chloroplasts. An established algal mutant collection is also an advantage. Thus, it is an attractive system for studies of chloroplastic PS II complexes.

Many mutagenesis studies have been done to investigate the central redox components in the PS II RC core. Based on the analogy with purple bacteria, D1-H198 and D2-H198 have been proposed to coordinate the Mg atom of the two Chls of P680 (Trebst, 1986; Sayre et al., 1986; Michel and Deisenhofer, 1988). The results from site-directed mutagenesis studies in Synechocystis 6803 suggest that the non-conservative mutations, such as D2-H197Y or D2-H197L (D2-H197 corresponds to the D2-198 in spinach), cause a loss of PS II function and a disassembly of the PS II complex (Vermaas et al., 1987; Pakrasi and Vermass, 1992). The conservative mutations D2-H197Q or D2-H197D, partially affects PS II function (Vermaas, 1993). These observations confirm the concept that D1-H198 and D2-H198 serve as ligands to P680. In purple bacteria, the corresponding residues binding to the primary donor are L-H173 and M-H200. Interestingly, the mutants L-H173L and M-H200L form a BChl/Bpheo heterodimer as the special pair upon removal of the ligand provided by histidine residue. Biochemical analyses the M-H200L mutant indicates that the BRC activity is still partially retained
(Kirmaier et al., 1988; Bylina and Youvan, 1988). It thus appears that the P680 binding site in PS II is more sensitive to the structural changes than in bacteria. Mutagenesis studies in *Chlamydomonas* also show that the D1-H198L mutation results in the disassembly of PS II complex (unpublished data). Another residue shown to play a critical role in charge separation in bacteria is M-Y210 (Finkele et al., 1990). Its homolog in PS II is residue D2-L205. In the PS II model proposed by Ruffle (1992), this residue is located near both accessory Chls on the active branch and P680 pair. The conversion of D2-L205 to D2-L205Y in *Chlamydomonas* did not increase the rate of charge separation as expected, indicating that the difference in residues at similar position in bacteria and PS II does not apparently contribute to different rates of charge separation between these two systems (Andronis et al., 1999).

The first electron acceptor in PS II is Pheo a, which is proposed to H-bond to D1-E130. This is similar to the situation in purple bacteria where the L-E104 forms a H-bond to BpheoL (Michel and Deisenhofer, 1988). The spectroscopic studies reveal that protein matrix of the primary electron acceptor binding site are well conserved between the bacterial and PS II RC (Lubitz et al., 1989; Nabedryk et al., 1990). Site-directed mutagenesis experiments have been applied to the homologous residue D1-Q130 in *Synechocystis* PCC 6803 (Giorgi et al., 1996). The substitution of D1-Q130 with glutamate or leucine caused a shift in the Pheo Qx band shift and a modified quantum yield of radical pair formation. The result is comparable to the analogous mutation of L-E104 in purple bacteria (Bylina et al., 1988). Another interesting residue is D2-L210, which corresponds to M-L214 in *Rb.spaeroides*. The M-L214H mutation was shown to
provide a ligand to Bpheo on the functional branch and to convert it to a BChl (Kirmaier et al., 1991). The introduction of a similar mutation in PS II might facilitate the resolution of Pheo on different branches of electron transfer by selective depletion of one Pheo from its binding pocket. Furthermore, such replacements may be useful to investigate the kinetics of primary photochemical reaction.

It is evident from the emerging PS II crystal structure that a Chl molecule is located between the Chls of the special pair and Pheo. This Chl monomer is positioned 30° tilted relative to the membrane plane similar to its homolog in bacteria (Vanmieghem et al., 1991). However, the two histidine residues in the L and M subunits (L-H153 and M-H180) that ligate each accessory Chl on both sides do not have analogs at the comparable positions in PS II. The presence of two conserved histidine residue in both D1 and D2 protein at residues D1-H118 and D2-H118 suggested their possible participation in the binding of Chl (Michel and Deisenhofer, 1988). Mutagenesis studies from *Chlamydomonas* indicate that these two symmetrical residues coordinate the peripheral accessory Chls, 30 Å away from P680 (Roelofs et al., 1991; Hutchison et al., 1996; Zouni et al., 2001), and are involved in energy transfer from proximal antenna to the RC core pigments (Ruffle et al., 1999; Johnston et al., 2000; Ruffle et al.; 2001; Wang et al., 2002).

In regard to the protein environment of the secondary electron acceptor, QA, it is believed that this region in PS II possesses high structural similarity to that in purple bacteria. However, there is no consensus on the structure of QA and QB binding pockets among the various PS II models. To elucidate the function of the protein environment in
plastoquinone chemistry, more experiments are needed to understand the orientation and structure around the quinone.

Site-directed mutagenesis studies have demonstrated that the donor to P680⁺ are two Tyr residues, known as Y_Z and Y_D (Metz et al., 1989; Buser et al., 1990). This electron donation pathway is very different from bacteria where the oxidized BChl special pair is reduced by cytochrome c. In the PS II model, Y_Z and Y_D are located near the lumenal ends of helix C on the D1 and D2 subunits. Mutagenesis experiments in *Synechocystis* 6803 and spectroscopic investigations suggest that D2-H189 forms a weak H-bond with Y_D (Tang et al., 1993; Tommos et al., 1993; Rodriguez et al., 1987), which fits well with predictions from PS II models (Xiong, et al., 1998). The analogous position to D2-H189 in the D1 protein is D1-H190. However, the distance between Y_Z and D1-H190 is out of the range for the H-bond interaction (~ 4 Å) in the PS II structural model. In addition, the EPR spectrum of oxidized Y_Z in D1-H190F mutant in *Chlamydomonas* is identical to that in WT, indicating that the mutation retains the structural integrity close to Y_Z and D1-H190 residue is not adjacent to the donor Y_Z (Kramer et al., 1994; Roffey, et al., 1994).

1.5 This work

This thesis describes a detailed study of the effect of the protein environment near the two Pheos that participate in electron transfer process in PS II. In Chapter 2 the influence of the H-bond between carboxyl group of D1-E130 and 13¹-carbonyl group of Pheo_{active} on primary charge separation, and electron transfer on the donor and acceptor side of P680 is described. The results from genetically modified PS II complexes are
compared to wild-type PS II complexes. Chapter 3 and 4 deal with PS II RCs where Pheo\textsubscript{inactive} and Pheo\textsubscript{active} are replaced with Chls by introducing a histidine ligand to coordinate the Mg of the tetrapyrrole macrocyclic ring. In Chapter 3 we report on energy transfer and primary photochemistry in isolated PS II RCs depleted of Pheo\textsubscript{inactive}. The results are discussed in the context of pigment arrangements and interactions within the RC complex. Chapter 4 presents the results of the manipulation of the protein environment surrounding Pheo\textsubscript{active}. The energy and electron transfer processes in mutated RC are compared to that in WT and in homologous mutations in BRCs.
CHAPTER 2

THE MODULATION OF PS II ELECTRON TRANSFER BY THE D1-E130 RESIDUE

2.1 Introduction

The Photosystem II reaction center (PS II RC) is the smallest PS II structural complex that is capable of carrying out primary photochemistry. The isolated RC contains six chlorophylls (Chls), two pheophytins (Pheos), 1-2 croteinoids, and a non-heme iron (Nanba and Satoh, 1987). All of the cofactors with the exception of the cytochrome b559 heme are coordinated by the D1 and D2 polypeptides. One of the important functions performed by PS II RC is the generation of a charge-separated state, P680⁺Pheo⁻, on a picosecond time-scale upon absorbing light (Wasielewski et al., 1989; Booth et al., 1990; Wiederrecht et al., 1994; Schelvis et al., 1994; Greenfield et al., 1997).

The recent low-resolution (3.6 Å resolution) crystal structure of the PS II complex demonstrates that four of the six Chls, two Pheos and presumably the quinones (which are typically missing in PS II RC preparations) are sandwiched between the D1 and D2
proteins. These cofactors are arranged with a two-fold axis of symmetry and form two parallel pathways that are mirror images of each other for the transfer of electrons across the photosynthetic membrane (Zouni et al., 2001). Moreover, the organization and orientation of electron carriers in PS II is essentially identical to that of the bacterial RC (BRC). The structure, spectra and redox potential of these cofactors is demonstrated to be substantially influenced by a variety of cofactor-protein interactions, such as ligand interactions, van der Waals contacts, and hydrogen bonding interactions (H-bond). The H-bond interactions between protein side chains and pigments have been extensively studied in the BRC by mutagenesis, resonance Raman, and IR spectroscopy (Bylina et al., 1988; Lutz, 1984; Bocian et al., 1987; Mäntele et al., 1988). Although there are striking structural similarities between the two types of RCs, the PS II RCs display considerable spectral congestion in the range of 670-680 nm unlike that of the functionally related BRC where the lowest optical transition of the special BChl dimer is readily distinguishable from the other chlorins. The weak energy coupling between the core pigments in PS II is generally believed to contribute to the spectral degeneracy, which to some extent causes the present controversy with regard to the mechanism of primary charge separation. Overall, the mutual interactions between cofactors and the protein environment have not been well established in PS II compared to the BRC due in part to the lack of a high-resolution crystal structure and the similarities between corresponding chlorin environments on both branches of the potentially parallel electron transfer pathways.

In spite of the highly overlapping nature of the chlorin Qy optical transitions, the two Pheos of the RC have a unique Qx absorption at approximate 542 nm, thereby allowing us
to examine the spectral changes of these pigments associated with changes in their surrounding protein matrix and local geometry. We have focused our attention on identifying local environmental factors that affect the Pheos and the channeling of electron transfer down exclusively on one branch of the parallel pathways by application of various genetic and spectroscopic approaches. In addition, we are interested in studying the effect of modified chromophore-amino acid interactions on the primary photochemistry in genetically engineered PS II RCs.

So far, the atomic structure of the Pheo binding sites remains unclear (in 3.6 Å resolution crystal structure). Protein sequence alignment of the crystallographically characterized L/M subunits of the BRC and the D1/D2 subunits of the PS II complex indicates that the glutamate residue at position of 104 on the L subunit (L-E104) corresponds to the PS II D1-130 residue and that their carboxyl groups H-bond to the ring V 131 carbonyl group of Bpheo/Pheo on the active branch (Michel and Densenhofer, 1988). PS II model structures (Ruffle et al., 1992; Svensson et al., 1996) also suggest that glutamate residues at L-104 of BRC and at D1-130 of the chloroplastic PS II RC occupy similar positions. It is noteworthy that on the inactive branch one of the potential H-bond interactions is between Pheo and the D2-Q130 residue, which is equivalent to a valine residue on the BRC M subunit. Apparently, the protein environment of the Pheo of the alternate pathway is less homologous between the two photosynthetic systems. Resonance Raman, infrared, and ENDOR studies are in good agreement that the 131 carbonyl group (ring V) of the functional bacteriopheophytin (BpheoL) H-bonds to the L-E104 residue of the BRC (Bocian et al., 1987; Nasedryk et al., 1988; Feher et al., 1988).
The data from mutagenesis studies in *Rhodobacter capsulatus* RCs showed that the breakage of H-bond (L-E104L) caused a blue shift in the Qx absorption peak of BPheoL. However, the quantum yield of primary radical pair accumulation was the same as that of WT, and the time constant for initial charge separation was only moderately affected (3.4 ps versus 4.8 ps). It is surprising that the direction of electron transfer remains unchanged after the potential energetic inequivalence between the two structurally equivalent, symmetry-related Pheos was eliminated by mutagenesis (Bylina et al., 1988). Interestingly, the corresponding amino acid residue in cyanobacterial PS II complex is also located at residue D1-130, but it is glutamine instead of glutamate. Substitution of the D1-Q130 residue with a glutamate in the cyanobacterium *Synechocystis* PCC 6803 resulted in 2-3 nm red shift of the Pheo Qx bleaching band, and a 20-30% increase in the quantum yield of charge separation (Giorgi et al., 1996). Such results demonstrate that D1-Q130E mutation in cyanobacteria can alter the spectral features and quantum yield of radical pair (P680+/Pheo−) formation to the same extent as that in PS II complex of higher plants.

Taking into account these observations, we introduced a series of subtle protein environmental changes in close proximity to the putatively active Pheo in the chloroplastic PS II complex. Site-directed mutagenesis was used to generate mutations at position D1-E130 (its carboxylic oxygen is ~2.6 Å from the 131 carbonyl oxygen on Pheo based on the PS II structural model constructed by Svensson et al., 1996) in the PS II complex of *Chlamydomonas reinhardtii* (*C. reinhardtii*). We converted residue D1-E130 to semi-conservative histidine and glutamine (cyanobacterial equivalent) residues, and a non-
conservative leucine residue. We further characterized these manipulated PS II complexes.

Our data from high-field EPR studies demonstrated that the H-bond strengths were altered in magnitude proportional to their predicted H-bonding interactions. Significantly, the removal of H-bond interaction in D1-E130L mutant was found to cause nearly a complete loss of both oxygen evolution capacity and variable Chl a fluorescence. In addition, the apparent blue shift of Pheo Qx band relative to that of WT was observed in both the ground state and transient state absorption spectra of the D1-E130L RC. However, the time constant of ultrafast primary charge separation, the S2-multiline signal, and the steady-state yield of QAFe²⁺ at cryogenic temperatures were unchanged in this mutant. On the other hand, both D1-E130H and D1-E130Q mutants have electron transfer efficiencies that are comparable to WT. The steady state and reduced state absorption spectra of D1-E130H are similar to WT, but a clear blue shift of the Pheo Qx band is seen only in the transient absorption spectrum of D1-E130Q mutant, indicating possible modulation of electron transfer direction.

2.2 Materials and Methods

2.2.1 DNA constructs

A description of plasmid PBA155 PME I, which contains the aadA gene conferring spectinomycin/streptomycin resistance and the intron-less psbA gene, is available in Minagawa and Croft, (1994), and Ruffle et al., (2001).
Three different point mutations were introduced into the intron-less psbA gene at position 130 where glutamate was replaced with histidine, glutamine, and leucine by changing the codon from GAG to CAT, CAA, and CTT, respectively. Each mutation generated a new Sal I endonuclease restriction site in the chloroplast genome by introduction of a silent mutation at residue D1-R129 (CGT to CGA) along with codon alterations. The mutagenic oligonucleotide primers for E130H, E130Q and E130L were: 5’-CTACATGGGTCGAC\text{CAT}TGGGAATTTC-3’ (H+), \\
5’-CTACATGGGTCGAC\text{CAA}TGGGAATTTC-3’ (Q+), \\
and 5’-CTACATGGGTCGAC\text{T}TTGGGAATTTC-3’ (L+), respectively.

Site-directed mutagenesis was performed as follows. First, the intron-free psbA gene on plasmid PBA155 PME I was amplified by PCR using primers CRD1PME I- (5’TTTAGTTTAAACAAAAATTAGTTGTTTGAGC-3’) and one of the mutagenic primers (+). The product of this first PCR reaction was gel purified using the QIAEX II gel extraction kit and used as a primer for a second PCR reaction. The second PCR reaction was carried out using PBA 155 PME I as a template, and the first PCR product along with the CRD1PAC+ (5’TTAAAACTTTAAATTTAACATATGACAGC-3’) primer. The second PCR product was gel purified and used as a template for a third round of PCR amplification, in which CRD1PAC+ and CRD1PME I- were used as primers. The resultant fragment was digested with Hind III and PME I and inserted into a complementary site on the similarly digested plasmid PBA155 PME I, followed by ligation and transformation into \textit{E.coli} strain DH5-\textalpha by electroporation.
2.2.2 C. reinhardtii chloroplast transformation

Plasmids containing mutagenized psbA genes were transformed by particle gun bombardment into the psbA deletion strain, CC 741, as described by Roffey et al., (1994). Briefly, a CC 741 (provided by the Chlamydomonas genetics center, Duke university) cell culture was grown in Tris-Acetate-Phosphate (TAP) media (Gorman and Levine, 1965) to a density of $\sim 3 \times 10^6$ cell/mL. Then the cells were concentrated to a density of $\sim 2 \times 10^7$ cell/mL by centrifugation. The concentrated cells were poured onto the center of a TAP agar plate ($\sim 1$ mL) and dried in a sterile-transfer hood (total $\sim 5$ mL concentrated cells are needed to load on 5 TAP agar plates each time). Transformation was carried out using a helium inflow gun as described in Roffey et al., (1993). DNA-coated tungsten particles were accelerated using pressurized helium in a partial vacuum chamber. Tungsten particles (M-11, 1.1 $\mu$M in diameter) were sterilized in ethanol and precipitated with DNA as follows: 3-5 $\mu$L of 1-1.5 mg DNA/mL, 50 $\mu$L of $\sim 30$ mg/mL tungsten suspension, 50 $\mu$L of 2.5 M CaCl$_2$, and 20 $\mu$L 0.1 M spermidine were mixed gently and centrifuged for 1 min in a microfuge at room temperature. Most of the supernatant was removed and the tungsten-DNA pellet was resuspended in residual $\sim 10$ $\mu$L aliquot. This DNA-coated tungsten suspension was pipetted onto a nylon screen positioned $\sim 15$ cm above a TAP agar plate which was under a vacuum of 25 inches of Hg. The particles were then propelled into the target cells when the helium pressure (80 psi) was released.

The shot cells were then resuspended in 3-5 mL TAP medium, and were spread on 2-3 TAP plates containing 50 $\mu$g/mL ampicillin and 100 $\mu$g/mL spectinomycin and put in 21°C incubator under dim light. Colonies resistant to spectinomycin appeared 2 weeks
later, and were transferred to fresh TAP plates containing antibiotics. Following 2-3 rounds of re-steaking on alternate antibiotics (TAP plates with ampicillin/spectinomycin or streptomycin), colonies were grown in TAP liquid medium for DNA analysis.

2.2.3 Preparation of cellular DNA

Cells were cultured in 10-15 mL TAP medium to a density of ~5 × 10^6 cell/mL, then pelleted 4,000 × g for 10 min and washed once with TE buffer containing 10 mM Tris 8.0, 1.0 mM EDTA. The cells were resuspended with 0.35 mL TE and rapidly frozen in liquid nitrogen. 50 µL of 2% (w/v) SDS and 25 µL of proteanase K were added to the frozen cells and incubated for 2 hr at 55°C, followed by adding 50 µL 5 M of ammonium acetate and incubated for another 30 min on ice. The mixture was then centrifuged at room temperature for 10 min. The resulting supernatant was transferred to a fresh tube and extracted with 0.8-1 mL phenol/chloroform/isoamyl alcohol (24:24:1) three times. The total DNA was precipitated with ethanol (with final concentration of 70%) on ice for 1-2 hr and washed once with 1 mL 70% ethanol. The pellet was vacuum dried and resuspended in 20 µL TE buffer containing 10 µg/mL RNase. Using the resulting total DNA as a template, mutants were initially identified by restriction enzyme digestion of PCR-amplified psbA fragment using the CRD1PAC+ and CRD1PME- primers, and confirmed by DNA sequencing.
2.2.4 *C. reinhardtii* strains and growth conditions

Wild-type (WT) cells were *C. reinhardtii* CC-2137 provided by Spreitzer, R. (University of Nebraska). All cell cultures were grown in TAP medium at 25°C in continuous light (40-100 µmol photons m⁻² s⁻¹) on a shaker. For all analyses, cells were harvested at about 5× 10⁶ cells/mL by centrifugation at 4,000 × g for 10 min. To make PS II thylakoids and membrane particles, the cells were washed once in buffer A: 0.35 M sorbitol, 20 mM Hepes pH 7.5, 2 mM MgCl₂, and resuspended at a concentration of 1 mg Chl/mL in buffer A. For Chl fluorescence decay analysis, concentrated cell samples were diluted with TAP medium to 20 µg Chl/mL and kept in the dark until analyzed usually within 20 min.

2.2.5 Chl analysis

The Chl a concentration was determined by mixing 10 µL sample with 490 µL methanol, and then centrifuged to pellet proteins. The optical absorption of supernatant at 663 and 645 nm was recorded and calculated according to Arnon (1949).

2.2.6 Sample preparation

Thylakoids of *C. reinhardtii* CC-2137 and D1-E130 mutants were prepared essentially as in Roffey et al., (1994). *Chlamydomonas* cells were pelleted and resuspended in buffer A containing 0.35 M sorbitol, 20 mM Hepes pH 7.5, 2 mM MgCl₂ to give a final Chl concentration of 1.0 mg Chl/mL. The cells were then broken in a bionebulizer (Glas-Col, Bloomington, IN) at a nitrogen pressure 110 psi. Generally, the
cells were passed through the bionebulizer twice, and the suspension was centrifuged for 1 min at 1,200 × g to remove unbroken cells and cell debris. Thylakoids membranes were pelleted by centrifugation for 30 min at 40,000 × g. The thylakoids were resuspended in buffer A, homogenized using a glass-teflon homogenizer and pelleted at 40,000 × g for 15 min. The final preparation of thylakoids was resuspended in buffer A at 2 mg Chl/mL, rapidly frozen in liquid nitrogen and stored in –80°C for future use.

The preparation of PS II-enriched membranes (BBY) was done as described by Shim et al. (1990). Briefly, fresh Triton X-100 (Calbiochem) (stock 20%, w/v) was used to solubilize the thylakoids (1.5-2 mg Chl/mL) at a Triton X-100/Chl ratio of 25:1 for 30 min at 4°C. Then membrane fragments were pelleted by centrifugation for 30 min at 40,000 × g, and washed twice in SMN buffer (20 mM Mes-NaOH pH 6.0, 100 mM NaCl, 10% (w/v) glycerol) to remove the detergent. The resulting pellet was resuspended at 3-4 mg Chl/mL in buffer containing 20 mM Mes-NaOH pH 6.0, 0.4 M sucrose, 15 mM NaCl, 5 mM MgCl₂ and stored in –80°C. All preparation procedures were carried out in the dim green light, and the samples were kept on ice throughout the process.

The PS II RCs were prepared from PS II-enriched membranes as in (Wang et al., 2002) with some modifications. The PS II membranes (about 150 mg of Chl) were diluted to 0.5 mg Chl/mL and solubilized with Triton X-100 at a final concentration of 4% (w/v) for 1 hr while stirring gently in the dark at 4°C. The solubilized material was subjected to centrifugation at 150,000 × g for 1 hr at 4°C. The supernatant was diluted six times with buffer B (20 mM Mes-NaOH pH 6.0, 0.05% Triton X-100 (w/v), 10% glycerol) before being applied to a column (50 × 100 mm) of TSK DEAE-650 (S) (TosoHAAS)
equilibrated with buffer C containing 20 mM Mes-NaOH pH 6.0, 0.35% (w/v) Triton X-100, 10% glycerol, and 25 mM NaCl at 4°C. The sample was loaded at a flow rate of 4 mL/min and washed overnight with approximately 4000 mL of buffer C at 3.0 mL/min, until the absorbance of the eluate at 675 nm was reduced to 0.02. A 25-180 mM NaCl gradient in buffer C was then applied to the column at a rate of 2.0 mL/min. The absorption spectra of the greenest fractions were recorded, and those with spectral features diagnostic for RCs (the ratio of Soret peaks at 435 nm/417 nm was less than 0.6) were pooled. The eluant was diluted four times with buffer B and loaded at 2.0 mL/min on the second small column (15 × 150 mm) of TSK DEAE-650 (S), which has been equilibrated the same as for the first column. After washing with about 30 mL buffer C, the column was subjected to further wash at 2 mL/min with 20 mL of buffer D containing 50 mM Tris-HCl pH 7.5, 2 mM DM (η-dodecyl β-D-maltoside), 10% (w/v) glycerol. PS II RCs were eluted with buffer D containing 150 mM NaCl, and stored in liquid nitrogen. The preparations of D1-E130 mutant RC were essentially the same as that of WT. But for D1-E130 mutants, the Triton X-100 concentration in washing buffer C was 0.20% (w/v). The relative amount of Chl a, Pheo a, and carotenoids in purified PS II RCs complex was quantified by measuring the absorption at 412, 431, 460, 480 nm after the pigments in PS II RCs were extracted with 80% acetone and the proteins was pelleted, according to the method of Eijckelhoff and Dekker (1997). The pigment ratios were thereby calculated. Samples having a Chl a/Pheo a ratio of 6.5-8 were used in our experiment with the exception of D1-E130L mutant, which had a Chl a / Pheo a ratio of 7-9.
2.2.7 Oxygen evolution measurements

Oxygen evolution activity was measured under continuous illumination at 25°C as previously described (Hutchison et al., 1996). Steady-state rates of oxygen evolution in WT and D1-E130 mutants were measured using a Hansatech oxygen electrode with thylakoid membranes having a Chl concentration of 8-10 µg in 1.0 mL buffer A: 0.35 M sorbitol, 20mM Hepes pH 7.5, 2 mM MgCl₂. The artificial electron acceptors, 20 µM 2,6 dimethylbenzoquinone (DMBQ) and 2 mM potassium ferricyanide, as well as uncoupler of phosphorylation (30 mM methylamine) were added to the assay buffer. Saturating light (~1000 µmol photons m⁻²s⁻¹) was provided by a Hansatech LS2 light source.

2.2.8 Microsecond Chl a fluorescence decay kinetics

The growth and decay of Chl a fluorescence on the microsecond time scale in dark-adapted cells was measured at 25°C after a single turn-over saturating flash with a home-built modulated LED fluorometer as described by (Kramer et al., 1990). The weak monitoring light pulses from a bank of red LEDs were applied following a saturating actinic flash (2 µs) provided by a xenon flash lamp. Concentrated cell cultures were diluted with TAP medium to a Chl concentration of 20 µg Chl/mL. To determine S₂QA⁻ charge recombination kinetics after a single actinic flash, the samples were incubated for 5 min in the dark with addition of 3-(3,4-dichloro)-1,1-dimethylurea (DCMU) at a concentration of 20 µM prior to measurements.
2.2.9 Electron paramagnetic resonance (EPR) spectroscopy

The $Q_A^-\text{Fe}^{2+}$ accumulation was measured using a Bruker ESP-300 spectrometer equipped with an Oxford Instruments helium cryostat and temperature controller. PS II membranes were incubated with 100 mM sodium formate for 15 min to enhance the size of the $Q_A^-\text{Fe}^{2+}$ signal, and then subjected to illumination at 200 K for 10 minutes. The light-induced generation of $Q_A^-\text{Fe}^{2+}$ signal was rapidly trapped at 77 K and measured as reported (Hutchison et al., 1996). The instrument settings were: Microwave power, 22 mW; microwave frequency, 9.47 GHz; magnetic field modulation frequency, 100 kHz; magnetic field modulation amplitude, 2.0 mT; sample temperature, 4 K. All samples were scanned 10 times. The signal was normalized on the basis of the TyrD signal. For the measurement of TyrD signal, the same dark-adapted sample was warmed to room temperature, illuminated at 4°C for 2 min and immediately frozen in liquid nitrogen. The instrument settings for the TyrD were: microwave power, 0.2 mW; microwave frequency, 9.47 GHz; magnetic field modulation frequency, 100 kHz; magnetic field modulation amplitude, 0.4 mT; sample temperature, 150 K.

In experiments where the S₂ state multiline EPR signals of the Mn complex were measured, the PS II membranes were incubated in the dark for 30 min on ice to equilibrate the PS II complex in a S₁ state. At the end of dark incubation, the artificial electron acceptor phenyl-$\rho$-benzoquinone (PpBQ) was added to give a final concentration of 50 $\mu$M. To observe the multiline signal in the S₂ state of PS II, the samples were illuminated at 200 K for 3 min, and then frozen quickly at 77 K (Deák et al., 1999). The instrument settings were: microwave power, 3.2 mW; microwave frequency, 9.47 GHz; magnetic
field modulation frequency, 100 kHz; magnetic field modulation amplitude, 2.0 mT; sample temperature, 4 K. The Mn content of EDTA-washed PS II membranes was determined according to Roffey et al., (1994).

The approach to generate the Pheo radical in PS II membranes of *C. reinhardtii* WT and D1-E130 mutants was similar to that described by Dorlet et al., (2000), except that the sodium dithionite-treated samples were illuminated at 0 °C for 5 min. The HF-EPR spectrometer settings as well as the procedure for simulating the spectra were the same as those reported by Dorlet et al. (2000). We used Mn(II) (g=2.00101) as a standard to calibrate and accurately measure g values.

### 2.2.10 Steady state optical absorption and time-resolved spectroscopy

The room temperature ground state absorption measurements of PS II RC were recorded in the dark using a Cary 3E UV-visible spectrophotometer.

The PS II RC concentration was diluted using buffer D (50 mM Tris-HCl pH 7.2, 2 mM DM (η-dodecyl β-D-maltoside), 10% glycerol) to give a room-temperature absorbance of 1.2 at the Qy absorption band (676 nm) using a 1 cm path length cell. Samples were kept at 4°C for the following measurements. Femtosecond transient absorption measurements were carried out as described previously (Greenfield et al., 1997). Briefly, narrow-bandwidth sub-200 fs low excitation pulses (200 nJ/pulse) with a wavelength of 685 nm were applied to selectively excite the “red” side of the composite Qy band. The transient absorption kinetics was measured at the peak of Pheo Qx band.
(542 nm) and typical scans extended to 2 ns. Transient spectra were recorded over the 500-600 nm regions.

Time-resolved Chl fluorescence decay kinetics measurements were performed according to Johnston et al., (2000). Briefly, the Chl fluorescence decay of PS II RCs was determined using a time-correlated single photon-counting (TCSPC) system. A mode-locked Nd:YAG laser (Coherent Antares 76-s) was adopted to pump a dye laser (Coherent 700 series, DCM, 4 MHz rep rate). The sample was diluted using buffer containing 50 mM Tris-HCl pH 7.2, 2 mM DM (η-dodecyl β-β-d-maltoside), 10% (w/v) glycerol to give an absorbance of 1.2 at 676 nm, and was degassed by bubbling argon through a cuvette for 5 min. The cuvette was then sealed with Parafilm. The sample was held at 4 °C with a temperature-controlled bath. To prevent the sample from denaturing and to reduce the build up of Chl triplet states the sample was continually stirred using a Teflon magnetic stir bar. This also allows fresh sample to circulate into the excitation beam path during the measurement.

The sample was excited at 660 nm to selectively excite the “blue” side of the Chl Qy band. The Chl fluorescence emission was detected at 684 nm. The data collection time window was adjusted to 2.5 ns to observe the decay of fast lifetime components in RCs. The sample quality was monitored following the experiment by analysis of the blue shift of Qy absorption band (< 1 nm), suggesting no significant damage of our samples during the decay collecting time.
2.3 Results

2.3.1 Identification of D1-E130 mutants

Total DNA was extracted from WT and D1-E130 mutants to determine whether the mutations were introduced into the chloroplast genome by homologous recombination. Fig. 2.1 shows the results of Sal I digestion of amplified PCR fragments using primers CRD1PAC+ and CRD1PME I-, and template DNA from WT and putative transformants. All transformants carrying E130 mutation contained a new Sal I restriction site as indicated by the presence of two new bands (~0.39 and 0.41 kb together, and ~0.69 kb). Subsequent sequencing of the DNA using the primer D1-H118A+ (5’-ACCTAGAAGGAATGC CGCAAACGATAAG-3’) corroborated the presence of the introduced mutations (Fig. 2.2).
Fig. 2.1 Detection of the D1-E130 mutations by PCR. DNA fragments amplified with primers CRDPAC+ and CRD1PME I- were digested with Sal I and analyzed on a 1% agarose gel. Templates employed for PCR reaction were as follows: Wild-type (lane 1), D1-E130H (lane 2), D1-E130L (lane 3) and D1-E130Q (lane 7). Both lane 5 and 6 are markers, lane 4 was digested plasmid PBA155 PME I.
Fig. 2.2 DNA sequence showed the nucleotide substitution at residue D1-E130. The bases changes for introducing the restriction enzyme Sal I along with codon alterations are underlined.
2.3.2 PS II electron transport properties of D1-E130 mutants

All three D1-E130 mutants were able to grow photosynthetically. In order to study the effects of mutations on the light-driven electron transfer reaction of PS II, we measured the maximum rates of oxygen evolution of thylakoids from WT and D1-E130 mutants in the presence of artificial PS II electron acceptors. In sharp contrast to the D1-E130H and D1-E130Q mutants, which maintained DMBQ-supported oxygen evolution rates comparable to WT, the D1-E130L mutant exhibited a remarkably lower electron transport rate (only 10% of WT), indicating a severe blockage in electron transfer somewhere between water and the site of reduction of the artificial electron acceptor, DMBQ (Table 2.1).
<table>
<thead>
<tr>
<th></th>
<th>Oxygen evolution (μ mol O₂ mg Chl⁻¹ hr⁻¹)</th>
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<tr>
<td>WT</td>
<td>180 ± 20</td>
</tr>
<tr>
<td>D1-E130H</td>
<td>115 ± 10</td>
</tr>
<tr>
<td>D1-E130Q</td>
<td>90 ± 5</td>
</tr>
<tr>
<td>D1-E130L</td>
<td>10 ± 5</td>
</tr>
</tbody>
</table>

Table 2.1 Oxygen evolution rates for wild-type and D1-E130 mutant thylakoids. Values are the average of three to four separate measurements.
Microsecond Chl fluorescence decay kinetics of light-grown cells provided additional information on the status of electron transfer in the D1-E130 mutants. Fig. 2.3 shows the polyphasic Chl a fluorescence rise and decay kinetics induced by a single-turnover saturating flash. The maximum value of variable Chl a fluorescence, represented by $F_V/F_0$, is believed to reflect the amount of $Q_A^-$ in the sample (Philbrick et al., 1991). Both D1-E130H and D1-E130Q mutants showed substantial rise in Chl fluorescence induction similar to WT, indicating the electron transfer from S states of water-splitting complex to $Q_A$ was not severely impacted. But for the D1-E130L mutant, there was more than a 90% reduction in variable Chl fluorescence yield. These data corroborated the results from oxygen evolution measurements. The low $F_{v}/F_{0}$ values of the D1-E130L mutant were largely attributed to an unusually high $F_0$ value (Fig. 2.3, inset). We further measured the Chl fluorescence decay kinetics of D1-E130 mutants after an actinic flash in the presence of herbicide DCMU, which was used to block the electron transfer from $Q_A$ to $Q_B$ (Fig. 2.4). In the presence of DCMU, $Q_A^-$ oxidation typically is dominated by charge recombination between $Q_A^-$ and oxidized donor side of P680. A slightly slower decay of the high fluorescence state was observed in D1-E130Q and D1-E130L mutants than in WT (Fig. 2.4). On the other hand, D1-E130H mutation exhibited a faster Chl fluorescence decay.
Fig. 2.3 Chlorophyll fluorescence decay kinetics of light-grown wild-type and D1-E130H, D1-E130Q, and D1-E130L cells. Inset: the raw data from chlorophyll fluorescence decay kinetics of light-grown wild-type and D1-E130 mutant cells.
Fig. 2.4 Chlorophyll fluorescence decay kinetics of light-grown wild-type ____ , and D1-E130H ____ , D1-E130Q ____ and D1-E130L ____ cells measured in the presence of 20 µM DCMU.
The first relatively stably reduced species generated in PS II after initial charge separation is QA-. The photoreduced semiquinone anion radical interacts with nearby Fe\(^{2+}\) to give rise to a well-characterized EPR signal (Miller and Brudvig, 1991). To determine whether the electron transfer processes around PS II were influenced by mutations, we directly monitored the EPR signal of QA- formation. The sodium formate-enhanced QA-Fe\(^{2+}\) signals in WT and D1-E130 mutants were generated upon illumination at 200 K. Fig. 2.5 shows the steady-state QAFe\(^{2+}\) EPR signal at g = 1.82. The QAFe\(^{2+}\) to Y\(_D\) signal intensity ratios in PS II particles of WT and E130 mutants are summarized in Table 4. The steady state levels of QAFe\(^{2+}\) in D1-E130H and D1-E130L mutants were approximately equal to that of WT. However, the QAFe\(^{2+}\) signal intensity in D1-E130Q mutant was increased by 20-30% (Table 2.2). The modest enhancement of QAFe\(^{2+}\) signal might be attributed to the reduced back reaction from QA- to the S\(_2\) state (Figure 2.4), and/or accelerated forward electron transfer from Pheo- to QA. It is interesting to note that the yield of the QAFe\(^{2+}\) signal in the D1-E130L mutant approached WT levels, particularly since it has a high F\(_0\) level and very low rates of oxygen evolution.
Fig. 2.5  The sodium formate enhanced $Q_A\text{Fe}^{2+}$ EPR signal in PS II particles from wild-type— , and D1-E130H— , D1-E130Q—but and D1-E130L— mutants.
<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>D1-E130H</th>
<th>D1-E130Q</th>
<th>D1-E130L</th>
</tr>
</thead>
<tbody>
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<td>Normal</td>
<td>1</td>
<td>0.9</td>
<td>1.35</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Table 2.2 Normalized yield of sodium formate-enhanced $Q_A$ Fe$^{2+}$ EPR signals from wild-type and D1-E130 mutant PS II particles. (The yields are normalized on the basis of the $Y_D$ signal intensity. The error in estimation is about ±0.2).
2.3.3 S$_2$ state multiline EPR signal of D1-E130 mutants

To determine whether the donor side of PS II, particularly the oxygen evolving machinery, accounts for the observed impairment of electron transfer efficiency in the D1-E130L mutant, we measured S$_2$ state multiline EPR signal centered at around $g = 2$. The broad, hyperfine-structured multiline signal in the spectra (after subtraction of the dark spectra) is ascribed to the S=1/2 ground spin state of an anti-ferromagnetically exchange-coupled Mn tetramer (Hansson et al., 1987; Pace et al., 1991; Britt et al., 1992). Fig. 2.6 shows that the line shapes of Mn-derived multiline signal in WT and all D1-E130 mutants are identical, with each having a well-resolved line width of about 800 Gauss. Furthermore, the approximate Mn contents of PS II particles isolated from light-grown D1-E130 mutant cells were $\sim 4 \pm 0.2$ Mn /200 Chl which is essentially identical to that of WT (Table 2.3). From these results, we concluded that the water-oxidizing complex was intact and similar to WT.
Fig. 2.6 The S₂-mutiline signal of PS II particles from wild-type and D1-E130 mutants.
<table>
<thead>
<tr>
<th></th>
<th>Mn / 200 Chl</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>4.2 ± 0.2</td>
</tr>
<tr>
<td>D1-E130H</td>
<td>4.4 ± 0.1</td>
</tr>
<tr>
<td>D1-E130Q</td>
<td>3.9 ± 0.3</td>
</tr>
<tr>
<td>D1-E130L</td>
<td>3.9 ± 0.1</td>
</tr>
</tbody>
</table>

Table 2.3  Manganese contents from PS II membranes of wild-type and D1-E130 mutant light-grown cells. The standard deviation from two determinations of each quantity is shown.
2.3.4 Ground state optical absorption spectra of D1-E130 mutants

Like other D1-E130 mutants, the D1-E130L mutant was capable of assembling intact RCs, although both the oxygen evolution rates in thylakoids and variable Chl a fluorescence kinetics of cells revealed a functional lesion in PS II performance. However, the D1-E130L mutant RC had poor stability relative to WT RC and the other mutants. The D1-E130L mutant was more sensitive to detergent (Triton X-100) and more liable to denaturation during light-induced measurements of photosynthetic activity. The room-temperature steady-state absorption spectrum of each D1-E130 mutant RC exhibits a peak Qy absorption band at $675.5 \pm 0.5$ nm, similar to that of WT (Fig. 2.7). An important feature was that all thee mutants showed various spectroscopic blue shifts of the Pheo Qx transition relative to WT (541.8, 541.5, 540 nm versus 542 nm). A significant blue shift (approximately 2 nm) of Pheo Qx absorption maximum was observed in D1-E130L mutant RCs (Fig. 2.7, inset).
Fig. 2.7 Room temperature ground state absorption spectra of wild-type ——, and D1-E130H ———, D1-E130Q ——— and D1-E130L ——— reaction centers. Inset: absorption spectra of Pheo Qx band of PS II RCs in wild-type and D1-E130 mutants.
To resolve the individual Pheo Qx absorption peaks, we deconvoluted the RC spectra of WT and D1-E130 mutants in the Qx absorption region, assuming that the extinction coefficients of the two Pheos were the same. The results from deconvolution of WT were consistent with previous work (Mimuro et al., 1995): The Pheo Qx band, peaking at ~542 nm, could be resolved into two Gaussian-shaped bands with absorption maxima at 544 nm and 539 nm, respectively. The Pheo absorbing at the longer wavelength was ascribed to the photoactive Pheo, as was its counterpart Bpheo_L in purple bacteria (Bylina et al., 1988). This interpretation was also supported by our following studies on the location of Pheo Qx band using D1-L210H mutant where the removal of Pheo on the inactive branch resulted in an apparent loss of the Pheo Qx absorption by 45-50% and a red shift of the Qx Pheo band to 543.5-544 nm resulting from the active Pheo (Chapter 3).

Fig. 2.8 shows the deconvolution of the ground state Pheo Qx band in D1-E130 mutants. Comparison of the deconvolution spectra suggested that the observed blue shift of the Qx absorption maximum could be attributed predominantly to the Pheo absorbing at the longer wavelength. These results were in accordance with previous observations (Bylina et al., 1988) in BRCs and implied that alteration of the H-bond strength was a main contributor to the spectral shift of the Qx band. In contrast to the 6 nm blue shift of the Pheo Q_X absorption peak of Bpheo_L (active Pheo) observed in the BRC L-E104Q mutant, only a ~1 nm blue shift of Pheo Q_X band was observed when an analogous substitution was introduced into PS II RCs. These results confirm predictions from
models that the protein environment around the two Pheos is more homologous in PS II RCs than in BRC.

Fig. 2.8 Gaussian deconvolution of Pheo Qx absorption peak in wild-type and D1-E130 mutants. The Pheo Qx peak was fit to two separate Gaussian bands using Peakfit v4 software. The ratio of Gaussian amplitude of two peaks is 1:1, with standard error of ± 10%.
2.3.5 High field EPR (HF-EPR) study of Pheo anion radical in D1-E130 mutants

To verify whether alterations of the D1-E130/Pheo H-bond strength contribute to the blue shift of the Pheo Qx band and lowered electron transfer efficiency, we applied HF-EPR to measure the g value of the Pheo$^*$ radical, which is an effective probe of its molecular structure and the local protein microenvironment. Of the three principal magnetic vectors ($\alpha, \beta, \gamma$), the $g_\alpha$ direction was demonstrated to be oriented along the ring V carbonyl bond of Pheo and thus should be most sensitive to the presence of H-bond (Dorlet et al., 2000). The EPR spectra of light-induced Pheo$^*$ radical of WT and D1-E130 mutants are shown in Fig. 2.9. The spectra of Bpheo$^*$ from *R. viridis* and Pheo$^*$ from spinach also are displayed for comparison. Table 2.4 gives the g values of Pheo$^*$ and BpHeo$^*$ from a simulation of the experimental data. Like tyrosyl and semiquinone radicals (Fasanella and Gordy, 1969; Burghaus et al., 1993), the $g_\alpha$ value decreases with increasing H-bond strength. Replacement of the H-bond donor with leucine induced a significant shift in the $g_\alpha$ value, which is caused by a red shift of the n→π* electronic excitation upon the loss of H-bond on the ring V carbonyl group. There were similar shifts towards the high field direction for $g_\alpha$ value in both D1-E130H and D1-E130Q mutants compared to WT (2.00429 and 2.00431 versus 2.00420). It was therefore suggested that for a given geometry, glutamine and histidine residues were weaker H-bond donors than glutamate.
Fig. 2.9  HF-EPR spectra of the Pheo<sup>x</sup> in PS II from spinach, wild-type and D1-E130 mutants of <i>C. reinhardtii</i> and Bpheo<sup>x</sup> in <i>R. viridis</i>. <i>Unbroken line, experiment; broken line, simulation</i>. The <i>vertical lines</i> give the positions of the wild-type <i>g</i><sub>x</sub> and <i>g</i><sub>z</sub> values.

Experimental conditions: nominal microwave frequency 285 GHz; modulation amplitude 2.0 mT (PS II) and 1.0 mT (<i>R. viridis</i>); temperature 4.2 K except for D1-E130L taken at 3.0 K.
<table>
<thead>
<tr>
<th></th>
<th>gx</th>
<th>gy</th>
<th>gz</th>
<th>ΔH&lt;sub&gt;x&lt;/sub&gt;</th>
<th>ΔH&lt;sub&gt;y&lt;/sub&gt;</th>
<th>ΔH&lt;sub&gt;z&lt;/sub&gt;</th>
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<tr>
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<tr>
<td>Bacterial Bpheo&lt;sup&gt;+&lt;/sup&gt;</td>
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<tr>
<td>R. viridis&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.00437</td>
<td>2.00340</td>
<td>2.00239</td>
<td>2.0</td>
<td>2.1</td>
<td>2.2</td>
</tr>
</tbody>
</table>

Table 2.4  g values of Pheo<sup>+</sup> and Bpheo<sup>+</sup> from simulation of experimental data and ADF-ZORA calculations.
2.3.6 Primary charge separation in PS II of D1-E130 mutants

We conducted transient absorption measurements to determine whether changes in the protein environment in the proximity to the photochemically active Pheo would elicit any modifications in initial charge separation. Fig. 2.10 shows the transient absorption spectra of the RCs in the Qx bleach region (542 nm) recorded 2 ns after a 200 fs, 200 nJ pulse at an excitation wavelength of 685 nm. As presented earlier (Greenfield et al., 1997), there should be negligible contribution of $1^\text{a}$Pheo to the bleach of Pheo Qx band at 2 ns time scale since the decay of the excited state is substantially faster than electron transfer kinetics. Therefore, the development of Pheo Qx bleach principally results from the formation of the radical anion. The elicitation and growth of the bleach in the Qx band of Pheo at 278 K, was centered at 541.5±0.5 nm in WT RCs. However, in the D1-E130Q mutant, the peak wavelength of Pheo Qx bleach was distinctly blue shifted (1.5-2 nm) with respect to WT. This was in contrast to its ground state absorption spectrum, in which only a marginal shift (~0.5 nm) of Pheo Qx band was seen relative to WT. The most evident blue shift of the Pheo Qx bleach band was again observed in D1-E130L mutant, entirely corresponding to its steady state absorption spectrum. D1-E130H mutant RC displayed no distinct shift of Pheo Qx bleach band compared to WT.
Fig. 2.10 Transient absorption spectra of PS II RCs of wild-type and D1-E130 mutants measured at 2 ns after excitation. Wild-type – , and D1-E130H – , D1-E130Q – – – and D1-E130L – – . The spectra were normalized at 558 nm.
The decay kinetics of RCs in the Pheo Q\textsubscript{x} transition region are shown in Fig. 2.11. The PS II RC complex was excited on the red edge of composite Qy chlorin absorption band to preferentially excite P680 and the active Pheo. Fits were begun after 400 fs to exclude instrument artifacts. Three decaying lifetime components and a nondecaying long-lived component were needed to adequately fit the data. Table 2.5 shows the results of a sum of exponential fits to the raw data. Analysis of the kinetics data from WT confirmed that charge separation was a strongly biphasic processes as reported by Greenfield et al., (1997). But here, we focused our attention only on the first lifetime component of about 2-5 ps, which has been assigned to primary charge separation. The initial electron transfer lifetimes were similar for WT and D1-E130 mutants, indicating that the primary charge separation process was not distinctly affected by these mutations (Table 2.5). However, the lifetime of the small nondecaying component (shelf) differed among the D1-E130 mutants. This component presumably corresponds to charge recombination and has a lifetime of 36 ns in WT RCs at room temperature (Greenfield et al., 1996; den Hartog et al., 1998), though our fitting data were truncated at 2 ns. Notably, its lifetime in D1-E130L RCs was much slower (13.4 ns versus 3.5 ns), but in the other two mutants was slightly faster than that in WT (about 1 ns versus 3.5 ns).
Fig. 2.11  Pheophytin transient absorption decay kinetics wild-type and D1-E130 mutant RCs measured at the Pheo Qx bleach peak (542 nm). Inset: kinetic behavior before 20 ps.
Table 2.5 Wild-type and D1-E130 mutant Pheo reduction kinetics. The pump and probe wavelengths were 542 nm. The excitation energy was kept constant at 200 nJ.

<table>
<thead>
<tr>
<th></th>
<th>( A_{\text{fast}} )</th>
<th>( \tau_{\text{fast}} ) (ps)</th>
<th>( A_{\text{int}} )</th>
<th>( \tau_{\text{int}} ) (ps)</th>
<th>( A_{\text{slow}} )</th>
<th>( \tau_{\text{slow}} ) (ps)</th>
<th>( A_{\text{shelf}} ) (e^-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>(26 ± 1)%</td>
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<td>(41 ± 1)%</td>
<td>40 ± 2</td>
<td>(31 ± 1)%</td>
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<td>(40 ± 10)%</td>
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<td>(39 ± 10)%</td>
<td>380 ± 180</td>
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<tr>
<td>D1-E130Q</td>
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<td>(45 ± 5)%</td>
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<tr>
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</tbody>
</table>
We further applied time-resolved Chl a fluorescence kinetics at 278 K, an approach complementary to transient absorption measurement, but which allows us to study energy transfer processes within the PS II RC complexes as well as Pheo reduction. The 660 nm excitation pulse was chosen to preferentially excite the peripheral accessory Chls, and Chl monomers in our Chl fluorescence decay studies. Fig. 2.12 shows the Chl fluorescence decay kinetics and corresponding lifetime distributions (exponential series method) of decay for WT and two D1-E130 mutant RCs. It can be seen that both of the D1-E130 mutants exhibited faster Chl fluorescence decay kinetics than WT. The Chl fluorescence decay kinetics of the WT PS II RCs were further fit by exponential serial methods (ESM) with five lifetime components: 1.2 ps, 28 ps, 67 ps, 226 ps, 1.65 ns, and 2.5 ns (Johnston et al., 2000). The 1-2 ps component is believed to reflect primary charge separation, and the 20-30 ps lifetime is due to the energy equilibration between peripheral Chls and central core part of the PS II RC. A comparison of the decay components of WT and two D1-E130 mutant RCs suggested no pronounced lifetime differences in any of the five lifetime components (Table 2.6), consistent with the data from the ultrafast transient absorption experiment, although the excitation pulses were located at different sides of the Qy band in these two studies. However, the amplitudes of the ultrafast lifetime components (< 50 ps) for charge separation in both mutants was significantly lower (over 50% reduction) than that of WT, demonstrating the decreased efficiency of energy transfer to lowest excitation multimer state and energy transfer from peripheral Chls to core pigments of RC. The fluorescence decay kinetic traces for the D1-E130L mutant is not yet available due to the extremely unstable nature of RCs of this mutant. In general, these
time-resolved data demonstrated that a change in the H-bond strength of the D1-130 residue and the active-branch Pheo results in drastic changes of electron transfer yield in PS II, but the time course for charge separation was not distinctly influenced by the mutations.
Fig. 2.12 Chlorophyll fluorescence decay curve and exponential series method fitted curve for PS II RCs of wild-type and two D1-E130 mutants.
Continued

WT

D1-E130H

D1-E130Q
Table 2.6 Time-resolved Chl fluorescence decay lifetime distributions of wild-type and D1-E130 mutant RCs.
2.4 Discussion

Sequence alignment of the L and M subunits of BRC and the D1 and D2 subunits of PS II indicated that the glutamate residue at position L-104 corresponds to the same residue at D1-130 (Michel and Densenhofer, 1988). Consequently, the D1-E130 was proposed to H-bond to the ring V 13¹ carbonyl group of the photochemically active Pheo in PS II. This hypothesis was supported by results from FTIR and ENDOR measurements (Nabegryk et al., 1990; Lubitz et al., 1989).

In this work we have generated three mutants at residue 130 on the D1 protein in the unicellular eukaryotic green algae, C. reinhardtii, in which the D1-E130 residue was substituted with a histidine, glutamine and leucine. As noted from their room temperature ground state optical absorption spectra (Fig. 2.8), all D1-E130 mutants showed remarkably similar electronic transitions around 675-676 nm, suggesting that mutation of the glutamate residue at D1-130 did not introduce major structural perturbations in the vicinity of central core chromophores of the RCs. However, the Pheo Qx absorption bands of mutants showed various degrees of blue shifts in their absorption spectra. It was estimated that there was a close correlation of the peak position of Qx band and the strength of H-bond between the ring V carbonyl group of Pheo and amino acid residues; the more polar residue at position D1-130 (or L-104 in bacteria) the greater the red-shift of the Pheo Qx absorption band (Svensson et al., 1996). The replacement of L-E104 with glutamine and leucine introduced an apparent blue shift of only the photoactive Bpheo (Bylina et al., 1988). Substitution of residue D1-Q130 to glutamine and leucine in cyanobacteria introduced a different spectroscopic shift in the peak of Pheo Qx band with
respect to that of WT (~ 544 and 540 nm versus 543 nm) (Giorgi et al., 1996). Our observation of shifts in the Pheo Qx region caused by mutations constitutes direct evidence that a similar H-bond is present in the PS II RC complex of higher plants. The H-bond has a substantial effect on the position of Pheo Qx absorption maximum.

Because of the nearly symmetrical protein environment adjacent to the Pheo binding it was difficult to resolve their unique and characteristic Qx transitions at low temperatures, even at 6 K (Chang et al., 1994). This is contrary to BRC where two Bpheos absorb at clearly distinct spectral regions. We could not conclude from the optical absorption spectra that the blue shifts of the Pheo Qx band in D1-E130 mutants resulted from a peak shift of the photoactive Pheo. Nevertheless, our deconvolution data, which concurred with the previous results from the fluorescence excitation spectroscopy and MCD spectra (Mimuro et al., 1995) suggested that the decrease of the H-bond strength due to D1-E130 mutations gave rise to a blue shift of only the functional Pheo. It was therefore concluded that the D1-E130 residue, the equivalent of L-E104 in the BRC, is a binding site for the photoactive Pheo in higher plants.

To determine whether changes in the H-bond strength between Pheo and D1-130 were responsible for the observed spectral shift in the D1-E130 mutants, we measured the subtle g-anisotropy modifications induced by site-directed mutagenesis to probe the electronic structures of Pheo° radical using high field EPR spectroscopy. For a particular geometry, the strength of the H-bond corresponds with changes in the gs value, an indicator of local electrostatic environment changes around Pheo° (Dorlet et al., 2000). Our measurements suggested that gs values were higher in each D1-E130 mutant
compared to WT, especially in the D1-E130L mutant (Dorlet et al., 2001). This is compelling evidence that glutamate is a stronger H-bond donor than histidine and glutamine. These results are in excellent agreement with the above conclusions and previous view that the presence of a more polar side chain at D1-130 gives rise to the longer wavelength absorption of Pheo Qx peak, and a stronger H-bond interaction between Pheo and its polypeptide environment (Svensson et al., 1996).

The flash-induced Chl a fluorescence kinetics suggested that the D1-E130L mutant lost its ability to form the high Chl fluorescence state, P680Q\textsubscript{A}\textsuperscript{-}, on the microsecond timescale. Since there were no evident changes with regard to the main lifetime components for primary charge separation, as well as the steady-state Q\textsubscript{A}Fe\textsuperscript{2+} formation in this mutant, we hypothesized that substituting glutamate with leucine might have affected electron transfer on the donor side. The substantially lower rate of DMBQ-supported oxygen evolution in the D1-E130L mutant appeared to support this hypothesis. However, the S\textsubscript{2}-mutiline EPR signal, a fingerprint of the fine environment around oxygen-evolving complex, was the same in all D1-E130 mutants as that in WT, so as was the Mn content per PS II. Thus, we attribute the reduced rates of photosynthesis in the D1-E130L mutant to the following two reasons; first, an incomplete or incorrect assembly of PS II complex occurred in this mutant, as implied by higher F\textsubscript{0} value in the Chl a fluorescence kinetics measurement. Second, assuming that no global perturbation occurred in the mutant, the D1-E130L mutation may have affected Q\textsubscript{A} reduction on short time scale by impacting Pheo/Pheo\textsuperscript{-} structure, and/or involving Q\textsubscript{A} electronic structural changes. This effect would still potentially allow for the near wild-type accumulation of Pheo\textsuperscript{-} as indicated by EPR.
under steady state conditions where $Q_B$ reduction is blocked. In *Rh. spaeroides*, the L-E104L mutation (homologous to D1-E130L), which breaks the H-bond between ring V carbonyl group of $Bpheo_L$ and L-104, altered the electron transfer rate from $Bpheo_L^-$ to $Q_A$ (Bylina et al., 1988). Moreover, ENDOR and Special TRIPLE spectroscopy indicated that in the L-E104L mutant, the relaxation of two distinct radical anion states, a $Bpheo$, differed from that in WT (Müh et al., 1998). This observed difference was attributed to the altered structural relaxation behavior of $Bpheo^-$, which resulted from the rotation of the 3-acetyl group of $Bpheo_L$ upon the formation of charge-separated state. Calculations again suggested that the removal of this H-bond could change the redox potential of $Bpheo_L$ (Hanson et al., 1987; Michel-Beyerle et al., 1988). On the other hand, previous observations from L-E104L mutation showed that the breakage of this H-bond led to the conformational changes of the 10a-ester C=O of $Bpheo_L$, which was partially conjugated with ring V and was speculated to be involved electrostatically in $Q_A$ reduction (Breton et al., 1997; Breton et al., 1992). As for the situation in PS II, the prediction from hybrid density functional calculations indicated that H-bond interaction between 131 carbonyl group of Pheo and glutamate residue at D1-130 was able to stabilize the anion-radical form of Pheo (O’Malley, 2000). Though corresponding evidence for PS II is unavailable as of yet, it is quite conceivable that similar outcomes resulting from the removal of the H-bond could take place. For example, in the D1-E130L mutant, the much slower time constants for charge recombination derived from transient absorption (13.4 ns versus 3.5 ns) kinetics could be attributed to reorganization or reorientation changes of Pheo/Pheo$^-$.
relative to other central pigments in RC, and/or to the alteration of redox potential of Pheo due to fine adjustment of the structure around it.

The Pheo Qx bleach band in the D1-E130Q mutant PS II RCs, measured 2 ns after the 685 nm excitation pulse, exhibited a blue shift (1.5-2 nm) relative to that of WT (Fig. 2.11). Such an apparent blue shift was not obtained in the ground state transition of the Pheo Qx band in this mutant. The greatest blue shift, however, was shown in both ground state and reduced state spectra of D1-E130L mutant. A transient blue shift might be due to the direct effect of the H-bond modulations on the active Pheo Qx transition (as seen in ground state spectra), and/or partial reduction of the inactive Pheo that absorbs at the lower-wavelength. We also cannot rule out the possibility that Pheo- has rotated in its binding pocket relative to that of WT, thus resulting in different angular orientation of Pheo- in this mutant. Based on the clear difference observed in the ground state and reduced spectra of D1-E130Q RC, it is tempting to speculate that the electron transfer directionality is modified by this mutation. In this context, it is difficult to investigate whether this occurred in the D1-E130L mutant, since a blue shift was observed in both the ground state as well as in the transiently reduced state.

Many studies have been made using BRC complexes to determine the basis for asymmetric electron transfer from the primary donor to the primary electron acceptor. It was reported that local variances in the internal dielectric constant, arising from global effects of multiple amino acid contributions, control the balance of electron transfer between the two Pheos (Steffen et al., 1994). Differences in the local amino acid
environments around the two Pheos also may account for the higher energy level (0.1-0.3 eV) of P+ BpheoL− relative to P+BpheoM− (Parson et al., 1990; Gehlen et al., 1994; Blomberg et al., 1998). It is thus concluded that the functional symmetry between the two potential electron transport pathways is associated with significant differences in the microenvironment of the cofactors. The theoretical calculations also indicated that the fine environment around the two Pheos contributed to their orientation asymmetry (Hasegawa and Nakatsuji, 1998), which might be a factor affecting electron transfer preference of the L branch over the M branch. In the BRC, the most striking structural difference between the BpheoL and BpheoM sites is that the BpheoL has a glutamate side chain near its ring V carbonyl group, while valine is found to close to BpheoM. However, mutagenesis experiments (Bylina et al., 1988) and computational analyses of Blomberg et al., (1998) shared a finding that the H-bond interaction between Pheo and its specific protein environment did not play a crucial role in the controlling directionality of electron transfer, although a H-bond to BpheoL can modify its redox potential and makes it easier to reduce by ~50 meV (Hanson et al., 1987; Michel-Beyerle et al., 1988). On the other hand, a double mutation that also modified the midpoint potential of BChl in Rh. capsulatus (M-G201D/M-L212H) gave rise to ~15% electron transfer to the BPheo inactive. Thus, BChl is believed to be partially involved in the asymmetry of electron transfer (Heller et al., 1995).
It was well known that the D1 and D2 polypeptides possess a higher degree of sequence homology than the L and M subunits (Michel and Densenhofer, 1988). In contrast to the BRC, the primary electron donors of PS II RC complexes have not been identified and charge separated state could be formed from excited states delocalized on any of the chlorin pigments (Durrant et al., 1995). In view of the reduced energy gap of initial electron transfer process, reduced dielectric strength asymmetry, we postulate that the unidirectional electron transport is more easily perturbed in PS II than in the BRC. The PS II complex is more sensitive to changes in the distance and relative orientation of one pigment relative to that of another. As one of the components of P680 multimer (Durrant et al., 1995), Pheos can’t be excluded from the candidates that regulate the inherent directionality of electron transfer. In spite of the fact that the protein environment adjacent to Pheo binding sites is more symmetric than their analogous Bpheos (Mimuro et al., 1995; Chang et al., 1994; Xiong and Subramaniam, 1998; Germano et al., 2002), the pigment exchange studies in PS II RCs of spinach demonstrated that the active Pheo was more inaccessible and difficult to modify than the one on the inactive branch (Shkuropatov et al., 1999). One obvious difference between the protein matrix of two Pheos is the amino acid residues in close proximity: it is glutamate on D1 protein, but glutamine on D2 protein. Our D1-E130Q mutation made the protein matrix on each side identical, but whether this alteration induces the intrinsic reduction of Pheo on the inactive branch is still a puzzle, though the difference between ground and transit state spectra implies such possibility. Overall, we expect that the association of the chromophores with
their environment is critical in determining the direction of electron transfer in PS II RCs of higher plants.
CHAPTER 3

SUBSTITUTION OF A CHLOROPHYLL INTO THE INACTIVE PHEOPHYTIN BINDING SITE; EFFECTS ON ELECTRON AND ENERGY TRANSFER PROCESSES

3.1 Introduction

The isolated Photosystem II PS II RC complex has five protein subunits, including the D1 and D2 polypeptides, the Cyt b559 polypeptides, and the psbI gene product. In addition, the purified PS II RC contains six chlorophylls Chls, two Pheos and one heme, and is missing the quinones at the QA and QB sites and the tetra-Mn complex (Nanba and Satoh, 1987). As a result, the isolated PS II RC is not capable of forming a stable charge-separated state and can only reduce Pheo, which then back reacts with P680⁺ forming a high proportion of P680 triplet. Significantly, the lack of the proximal antennae Chl protein complexes makes it possible to follow spectroscopically the primary charge separation events.

Based upon the low resolution crystal structure (Zouni et al, 2001) and electron crystallography of PS II (Rhee et al, 1998), it was found that the D1 and D2 proteins of PS
II exhibit substantial tertiary structural similarity to L and M proteins of the BRC. The cofactors, which are coordinated to D1 and D2 heterodimer and involved in PS II electron transfer, form two parallel electron transfer pathways with a pseudo-C2 axis of symmetry similar to those in BRC. Interestingly, light-driven primary electron transfer in the BRC occurs almost exclusively (90%) along only one of the two potential branches that span the photosynthetic membrane, the one more associated with L subunit (Kirmaier and Holten, 1993). Based on the structural similarities between BRCs and PS II RCs and some spectroscopic results (Klimov et al., 1980; Nanba and Satoh, 1987), it is believed that such functional asymmetry in electron transfer takes place in PS II as well. Namely, the photo-induced charge separation occurs only between P680 (possibly the Chl special pair, although recent evidence suggests the ChlM on the active branch pathway; see review by Diner and Rappaport, 2002) and the Pheo on the active side, know as Pheo active, although the Pheo on the inactive branch (Pheo inactive) has approximately the same environment and orientation as Pheo active.

At present, it is difficult to experimentally monitor the directionality of electron transfer in PS II. The severe spectral overlap of the chlorins, especially in the Qy absorption band, makes it difficult to assign single pigments to unique spectral features. Currently, the spectroscopic analysis of primary photochemical events in PS II is limited to the bleaching in the Pheo Qx band and the bleaching associated with P680 oxidation, although the assignment of P680 to a particular chlorin remains controversial (Greenfield et al., 1997; Jankowiak et al., 2002). Even at 6 K it is not possible to resolve spectrally the
two Pheos in the Qx region of ground state absorption spectra of PS II RCs (Chang, et al., 1994).

Work has been done to break the cofactor symmetry by introduction of a metal-coordinating residue His over one side of BPheoactive, in *Rh. sphaeroides* (M-L214H) and in *Rb. capsulatus* (M-L212H) (Kirmaier et al., 1991; Heller et al., 1995). The result has been the replacement of BPheoactive with a BChl. This incorporated BChl is designated as β and these mutant RCs is called β-type RCs. Extensive studies of engineered β-type RCs indicated that they shared some common features: reduced quantum yield of P*QA−, and lengthened P* lifetime (~ 2 times longer) relative to native RCs, but the yield of forward electron transfer from P* has not been altered in β-type RCs. The interpretation for the altered photochemistry in the β-type RCs was an enhanced rate of charge recombination (P*β− → P) due to the decreased free energy gap (between P* and P*β−).

To explore the contribution of the free energy of cofactors to the kinetics and yield of charge separation in PS II, we attempted to make the β-type PS II RCs. We have replaced the Pheoinactive with a Chl, which has a structure similar to that of Pheo but with very different redox properties and electron affinity, and no absorption in the Pheo Qx absorption region (542 nm) of the spectrum. These modified RCs, with altered pigment composition, are expected to help resolve the contribution of Pheoinactive to the Pheo Qx band. The substitution of the inactive branch Pheo with a Chl might enhance electron transfer along the active branch since it would be even less favorable for electron transfer along the inactive branch due to the higher midpoint potential of Chl than Pheo (~ 200-300 meV higher in solution).
According to three-dimensional PS II structure model the leucine at residue D1-210 occupies a position over the center of Pheo\textsubscript{inactive} macrocycle. We use site-directed mutagenesis and chloroplast transformation to incorporate a Chl into the binding site of Pheo\textsubscript{inactive} of \textit{Chlamydomonas} by introducing a His ligand at position D1-L210. As a result, we were able to identify the spectral contributions of Pheo\textsubscript{inactive} in the Pheo Qx region. We also made another conservative mutant, D1-L210A, for comparison. Surprisingly, our results suggest that replacement of the Pheo on the inactive path (D1-L210H mutant) with a Chl greatly hindered electron transfer on the active branch pathway. More importantly, the yield of radical pair P680\textsuperscript{+}Pheo\textsuperscript{−} was reduced, and energy transfer was perturbed in the D1-L210H mutant but apparently enhanced in the D1-L210A mutant. These results have significant implications for the mechanisms of charge separation in PS II RCs as well as in intact PS II complexes capable of water oxidation. It is apparent that in wild-type PS II RCs (lacking QA and QB) that primary charge separation occurs with equal yield along both the active and inactive branch pathways leading to the reduction of both Pheos. The replacement of the Pheo\textsubscript{inactive} with a Chl results in the reduction of only the Pheo\textsubscript{active}. Chl CD spectroscopy of the D2-L210H mutants suggests, however, that the structure and/or identity of P680 has been changed in the D1-L210H mutant.

This is reflected by a substantially impaired ability to carry out charge separation in intact PS II complexes that have a QA electron acceptor. These results are interpreted in terms of a redistribution of the delocalized excited state in mutant PS II complexes which results in the formation of either an alternate primary donor (P680) following excitation or
a less stable P680* that is more likely to deactivate to the ground state, thereby effectively inhibiting PS II electron transfer.

3.2. Material and Methods

3.2.1 Generation of the Mutants

The point mutations at D1-L210 were introduced into the intron-less psbA gene in plasmid PBA155 PME I as described by Minagawa and Croft, (1994) and Ruffle et al. (2001) using oligonucleotide-directed mutagenesis. Changing codon psbA 210 from TTA to GCA and CAC led to the substitution of a leucine residue for an alanine or histidine residue, respectively. The mutagenic oligonucleotide primers for D1-L210A and D1-L210H were: 5’-TTCGGTGGTTCAGCATTCTCAGCTATGCATGGTTCTT-3’, and 5’-TTCGGTGG TTCACACTTCTCAGCTATGCATGGTTCTT-3’. Each mutation created a new Nsi I endonuclease restriction site in the chloroplast gene by introducing a silent mutation at residue D1-H215. The plasmids containing mutagenized psbA fragments were transformed into a psbA deletion strain, CC 744 as described in Chapter 2. The preliminary identification of spectinomycin and streptomycin resistant transformants was performed by restriction site analysis of PCR-generated psbA fragments using CRD1PAC+ and CRD1PME I- (as indicated in Chapter 2) as primers, followed by DNA sequencing using a standard DNA sequencing kit (Pharmacia). The control strain of C. reinhardtii in our study is the 2137 strain (CC-2137), referred as WT.
3.2.2 Membrane Sample Preparation

Control and mutant strains of *C. reinhardtii* were grown in Tris-Acetate-Phosphate (TAP) media (Gorman and Levine, 1965) at 25°C in low light (5-15 μphotons.m⁻².s⁻¹) to avoid photoinhibition. Cells were harvested as described by Roffey et al. (1994). PS II thylakoids and membranes of *C. reinhardtii* WT and the D1-L210H mutants were prepared as reported in Chapter 2. PS II RCs were prepared from membranes essentially the same way as described in Chapter 2 with minor modifications. For the D1-L210H mutant the washing buffer contained 0.2% (w/v) Triton X-100. The relative amount of Chl a, Pheo a, and caroteinoids in purified PS II RC complex was calculated according to Eijckelhoff and Dekker, (1997).

3.2.3 Oxygen evolution measurements

The maximum rate of oxygen evolution was measured as previously described in Chapter 2. Samples of PS II membranes containing 10 μg Chl in 1 mL assay mixtures were illuminated with saturating white light (1000 μmol photons m⁻² s⁻¹).

3.2.4 Spectroscopic characterization of WT and D1-L210 mutants

Room temperature absorption spectra were recorded using Cary 3E UV-Vis spectrophotometer.

The flash-induced microsecond Chl a fluorescence decay kinetics was performed on a home-built instrument as described by (Kramer et al, 1990). The concentrated cells were
diluted with TAP medium to a concentration of 20 µg Chl/mL and incubated in the dark for 5 min in the presence of or absence of 20 µM DCMU prior to measurements.

X-band EPR spectra were recorded with Bruker ESP-300 spectrometer equipped with an Oxford Instruments helium cryostat and temperature controller. For the detection of the QA−Fe2+ signal, PS II membranes were illuminated at 200 K for 10 minutes in the presence of 100 mM sodium formate. All samples were scanned 10 times. The instrument settings are the same as described in Chapter 2. The signals were normalized on the basis of the TyrD signal. For light-induced generation of pheo− signal, the PS II RC preparations were illuminated in the presence of sodium dithionite (2 mg/mL) for 3 min at 4°C by a white light source through a heat-absorbing filter, and rapidly frozen in 77 K for EPR measurements. EPR conditions were: microwave power, 10 µW; microwave frequency, 9.47 GHz; magnetic field modulation frequency, 100 kHz; magnetic field modulation amplitude, 0.2 mT; and sample temperature, 15 K.

Light-induced difference absorption spectra were recorded using HP-8452a spectrophotometer. The PS II RC preparations were diluted with a buffer containing 10% glycerol, 20 mM Mes-NaOH, pH 6.0 and 2.0 mM n-dodecyl-β-D maltoside (DM). The diluted samples (~ 4.8 µM Chl) were supplemented with sodium dithionite (2 mg/mL) and 1 µM methyl viologen, and incubated in dark at 4°C for 5 min prior to measurement. To photo-accumulate reduced Pheo a, the PS II RC were illuminated using a heat-filtered white light (about 2800 µ mol photons m−2 s−1) for 10 seconds on the top of cuvette prior to measurements. The samples were kept at 4°C during data collection with a circulated refrigerated water bath.
Steady-state 77 K Chl fluorescence emission spectra of PS II RC preparation were recorded on a Fluoro-Max spectrometer with a home-made liquid nitrogen dewar. The samples were excited at 436 nm. The excitation and emission slits were 5 and 2 nm, respectively. PS II RCs were diluted to a final concentration of 5 µg Chl/mL with a buffer containing 50% glycerol, 20 mM Mes-NaOH pH 6.0, and 2.0 mM n-dodecyl-β-D-maltoside (DM). All spectra were normalized at 750 nm.

Circular dichroism (CD) spectra were recorded at 4°C on an Aviv CD spectrometer (model 40DS/UV-VIS-IR) provided with a circulating water bath. The bandwidth is 2 nm. The PS II RC concentrations were adjusted to 20 µg/mL Chl in buffer (50mM Tris-HCl, pH 7.2, 10% glycerol (w/v), 2.0 mM DM (n-dodecyl β-D-maltoside).

Femtosecond transient absorption measurements were carried out using PS II RCs as described in Chapter 2. The sub-200 fs excitation pulses were centered at 685 nm. Different samples were used in the experiment to access the reproducibility of the data. During data collection, samples (typically 40-50 µM Chl) were maintained at 4°C and were rotated in a cuvette.

3.3 Results

3.3.1 Identification of D1-L210 mutants

Site-directed mutagenesis followed by chloroplast DNA transformation was used to generate D1-L210 mutants. To facilitate the genetic analysis, an Nsi I restriction site was created along with the D1-L210 mutation. Following the transformation of strain CC 744, spectinomycin-resistant colonies were obtained and analyzed by PCR after a few cycles of
screening on selective media containing 100 \( \mu g/mL \) spectinomycin and 50 \( \mu g/mL \) ampicillin. Fig. 3.1 shows restriction site analysis of PCR-amplified \( psbA \) fragments (using CRD1PAC+ and CRD1PME I- as primers as described in Chapter 2) from WT and transformed cells. It can be seen that the putative transformants have the new Nsi I restriction site associated with the D1-L210 mutations as indicated by the appearance of two new DNA bands (~0.5 kb and 0.7 kb) when the PCR products were digested with Nsi I. The DNA sequence analysis using primers D1-H195S (5’-CAACATCCTCATGTTCCCATTCCAC-3’) confirmed the presence of the mutations (Fig. 3.2).
Fig. 3.1 Detection of the D1-L210 mutations by PCR. DNA fragments amplified with primers CRDPAC+ and CRD1PME I- were digested with Nsi I and analyzed on a 1% agarose gel. Templates employed for PCR reaction were as follows: wild-type (lane 1), D1-L210A (lane 2), D1-L210H (lane 3).
Fig. 3.2 DNA sequence showed the nucleotide substitutions at residue D2-L210. The base changes for introducing the restriction enzyme Fsp I along with codon alterations are underlined.
3.3.2 Pigment analysis and room temperature optical absorption spectra of D1-L210H PS II RCs

Quantitative analysis of the pigment composition of the genetically modified PS II RCs indicated that the molar ratio of Chl a to Pheo a had been changed significantly in the D1-L210H RCs (Table 3.1). The replacement of leucine with a histidine residue at position 210 in the D1 protein resulted in the replacement of Pheoinactive with a Chl. This result was further supported by the room temperature ground state optical absorption spectrum of D1-L210H mutant (Fig. 3.3, inset). After the RC spectra was normalized at 623 nm isosbestic point, the amplitude of the Pheo Qx absorption peak was shown to be 50% less than that of WT. No apparent spectral changes were observed in the Qy regions of the RC spectra (Fig. 3.3). Importantly, an apparent red shift of the Pheo Qx absorption band (~1.5-2 nm) was observed in the D1-L210H mutant PS II RCs relative to WT (Fig. 3.3, inset). The two Pheos in PS II RCs were believed to have different absorption maximum similar to their counterparts in the BRC, the long- wavelength absorbing form of Pheo is presumably located on the active branch (Bylina et al, 1988). The observed red shift in the Qx region of the D1-L210H mutant is therefore, attributed to the loss of Pheoinactive absorbing at lower wavelengths (539 nm). Unfortunately, since the chlorins in PS II RC all have similar Qy absorption spectra we still cannot unambiguously verify whether a Chl was incorporated in the binding pocket of Pheoinactive.
Table 3.1 Pigment composition of the wild-type and D1-L210H mutant PS II reaction center preparations. The standard deviation from four determinations is shown.

<table>
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<tr>
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<th>Chl a</th>
<th>Pheo a</th>
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<tr>
<td>WT</td>
<td>6.5 ± 0.5</td>
<td>2</td>
</tr>
<tr>
<td>D1-L210H</td>
<td>7.5 ± 0.5</td>
<td>1</td>
</tr>
</tbody>
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Fig. 3.3 Ground-state absorption spectra of PS II RCs isolated from the wild-type and D1-L210H mutant strains. The spectrum for wild-type —; D1-L210 mutant, ---. Inset: absorption spectra of Pheo Qx band of PS II RCs in wild-type and D1-L210 mutants
3.3.3 PS II electron transport of D1-L210H mutant

To determine whether the mutation affected the electron transfer properties of PS II, the flash-induced Chl a fluorescence rise and decay in dark-adapted WT and D1-L210H cells was measured in the presence or absence of DCMU. The rapid rise of Chl fluorescence from F₀ to Fₘ is indicative of the reduction of QA (Philbrick et al, 1991). The significantly reduced variable Chl a fluorescence induction suggests that D1-L210H mutant is incapable of forming the high fluorescence state, P₆₈₀/ QA⁻ (Fig. 3.4). This data contradicted our expectation that the replacement of Pheoinactive with a Chl would not dramatically influence PS II electron transfer processes, if the ligation to Chl was only related to the presence of adjacent His. In the presence of DCMU (Fig. 3.4, inset), there was no apparent Chl fluorescence decay suggesting that there was a limited back reaction from QA⁻ to the donor side of PS II.
Fig. 3.4 Chlorophyll fluorescence decay kinetics of light-grown cells from the wild-type and D1-L210H mutant strains. Wild-type, —; D1-L210 mutant, ---. Inset: measurements carried out in the presence of 20 µM DCMU.
Oxygen evolution rate measurements provided further evidence that electron transfer in the D1-L210H mutant was impaired. Table 3.2 shows that the D1-L210H mutant has very little evolving oxygen activity even under saturating light conditions in the presence of the artificial PS II electron acceptor DMBQ. These results indicate a block in electron transfer somewhere between water and the primary quinone acceptor.
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<th>Oxygen evolution (µ mol O₂ mg Chl⁻¹ hr⁻¹)</th>
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<tbody>
<tr>
<td>WT</td>
<td>270 ± 20</td>
</tr>
<tr>
<td>D1-L210H</td>
<td>10 ± 5</td>
</tr>
<tr>
<td>D1-L210A</td>
<td>280 ± 10</td>
</tr>
</tbody>
</table>

Table 3.2 Oxygen evolution rates of PS II particles isolated from wild-type and D1-L210 mutant strains. Values are the average of three separate measurements using isolated PS II particles.
The first stable charge-separated state generated in PS II after the primary photochemical reaction is known as $S_2Q_A^-$. We thus used EPR spectroscopy to measure the steady state yield of $Q_A^-$. Due to the magnetic interaction between $Q_A^-$ and the nearby Fe$^{2+}$ atom a diagnostic EPR signal at $g=1.82$ is generated. The yield of sodium formate-enhanced $Q_A^-Fe^{2+}$ signal in D1-L210H mutant was dramatically lowered upon illumination at 200 K compared to WT (Fig. 3.5). Using $Y_D$ as an internal standard the $Q_A^-Fe^{2+}$ signal amplitude in the mutant was determined to be only 6% of that of WT. Thus, it was apparent that charge separation to form $Q_A^-$ was substantially impaired in the D1-L210H mutant.
Fig. 3.5 The sodium formate enhanced $Q_A\text{Fe}^{2+}$ EPR signal (g=1.82 and 1.67) in PS II particles from wild-type and D1-L210H mutant strains. Wild-type, —; D1-L210 mutant, -. EPR conditions: sample temperature, 4 K; microwave power, 32 mW; microwave frequency, 9.48 GHz; field modulation frequency, 100 kHz; magnetic field modulation amplitude, 2.0 mT.
3.3.4 Accumulation of Pheo$^\circ$ in PS II RCs of the D1-L210H mutant

The Pheo$^\circ$ EPR signal is generated in sodium dithionite-treated PS II RC preparations upon illumination at 4°C. Fig. 3.6 shows the light-minus dark EPR spectra of reduced Pheo in WT and the D1-L210H mutant recorded at 15 K. The line shape and linewidth of the Pheo$^\circ$ EPR signal in the D1-L210H mutant was identical to that of WT. However, the steady-state yield of the g = 2.003 Pheo radical was significantly reduced in the D1-L210H mutant (approximate 25% ± 5% of WT).
Fig. 3.6 The photoaccumulated Pheo EPR signal from wild-type and D1-L210H mutant strains. Wild-type, —; D1-L210 mutant, ---.
3.3.5 Light-induced difference absorption spectrum of D1-L210H mutant

The intact PS II RCs could be isolated from the D1-L210H mutant as shown in Fig. 3.3. We then measured the photoaccumulation of reduced Pheo in PS II RC preparations by light-induced difference absorption spectra. Fig. 3.7 shows the absorption difference spectra recorded at 4°C upon illumination for 10 seconds in the presence of 1 µM methyl viologen and 2.0 µg/mL sodium dithionite. The light-induced difference absorption spectrum of WT RCs resembled that from spinach (Yruela et al, 2001), with negative peaks at 515, 543, 682 nm and positive peaks at 595, 656, 672 nm. The bleach bands in Qx and Qy region attributed to Pheo− (Nanba and Satoh, 1987; Yruela et al, 1994) were developed in the WT. Moreover, Fig. 3.7 shows a pronounced spectroscopic red shift in both regions compared to that of WT. Considering that Pheoactive absorbs at a longer wavelength (Fig. 3.3), such result implied that both Pheoinactive and Pheoactive were photoreduced in our WT RC preparation. In addition, a 70% decrease in the amplitude of the 543 nm Pheo Qx bleach band was apparent in the mutant when normalized at 624 nm. This observation suggested that the photochemical activity was altered in the D1-L210H mutant.
<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
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<tr>
<td>500</td>
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</tr>
<tr>
<td>550</td>
<td>0.00</td>
</tr>
<tr>
<td>600</td>
<td>0.02</td>
</tr>
<tr>
<td>650</td>
<td>0.04</td>
</tr>
<tr>
<td>700</td>
<td>-0.003</td>
</tr>
<tr>
<td>750</td>
<td>-0.002</td>
</tr>
<tr>
<td>800</td>
<td>-0.001</td>
</tr>
</tbody>
</table>

Fig. 3.7 Light-induced difference absorption spectra of PS II reaction centers isolated from the wild-type and D1-L210H mutant strains. Wild-type, —; D1-L210 mutant, ---.

Inset: the spectra of Pheo Qx absorption region. PS II reaction center preparations were illuminated with white actinic light (2500 µmol photons m$^{-2}$s$^{-1}$) through a heat absorbing filter for 10 s.
3.3.6 Femtosecond transient absorption measurements of D1-L210H mutant

The Pheo$^-$ Qx bleach kinetics were measured at two wavelengths 542.2 nm for WT and 543.6 nm for the D1-L210H mutant. Fig. 3.8 shows the transient absorption kinetics in Pheo Qx region following excitation pulses centered at 685 nm. Our fits were started at 0.5 ps to exclude the instrument response limited components, which presumably include the response to the buffer as well as the simultaneous excited-state absorption of chlorins (Greenfield et al., 1997). Three exponential decay components are required to adequately fit the data. Table 3.3 summarizes the results of fit to the bleach growth data using a non-linear least squares Marquardt fit. As for the initial electron transfer, the time constant for this step is 5-6 ps in the mutant, compared with approximately 4 ps in WT. There is no significant change for the slower component (50-60 ps), which was attributed to charge separation limited by energy equilibration within the RC complex, however, the amplitude of this component increases by a factor of ~ 1.5 in the mutant relative to WT.

Fig. 3.9 displays the Pheo$^-$ transient absorption spectra of D1-L210H and WT RCs observed 1.5 ns after a 200 nJ, 685 nm excitation pulse. These data have been normalized to 558.5 nm. The Pheo Qx bleach at 1.5 ns is predominantly attributed to the formation of charge-separated state P680$^+$Pheo$^-$ (Greenfield et al., 1999). It was apparent from analysis of the Pheo$^-$ Qx bleach spectra of WT and D1-L210H mutants that there was no bleach contribution from the Pheo$_{inactive}$ in the mutant. These results are consistent with the replacement of the Pheo$_{inactive}$ with a Chl and further support the hypothesis that both Pheos can be reduced in PS II RCs lacking Q$_A$, unlike intact PS II complexes.
Fig. 3.8 Transient absorption kinetics at 542.2 nm (wild-type) and 543.6 nm (D1-L210H) for the isolated PS II RCs at 4 °C. Inset: the kinetics at early time.
<table>
<thead>
<tr>
<th></th>
<th>$\tau_{\text{fast}}$ (ps)</th>
<th>$A_{\text{fast}}$</th>
<th>$\tau_{\text{intermediate}}$ (ps)</th>
<th>$A_{\text{intermediate}}$</th>
<th>$\tau_{\text{slow}}$ (ps)</th>
<th>$A_{\text{slow}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>$3.6 \pm 0.5$</td>
<td>$39 \pm 2 %$</td>
<td>$59 \pm 2$</td>
<td>$38 \pm 3 %$</td>
<td>$429 \pm 10$</td>
<td>$22 \pm 5 %$</td>
</tr>
<tr>
<td>D1-L210H</td>
<td>$5.2 \pm 1$</td>
<td>$32 \pm 4 %$</td>
<td>$62 \pm 4$</td>
<td>$57 \pm 5 %$</td>
<td>$538 \pm 5$</td>
<td>$11 \pm 5 %$</td>
</tr>
</tbody>
</table>

Table 3.3 Averages and estimated errors for the fit parameter to the data at the peak of the Pheo Qx band (542.2 nm for wild-type and 543.6 nm for D1-L210H mutant).
Fig. 3.9 Transit absorption spectra of isolated PS II RCs at 4 °C recorded 1.5 ns following a 200 nJ, 685 nm excitation pulse. Wild-type, —; D1-L210 mutant, ---.
3.3.7 Low temperature fluorescence emission spectrum of D1-L210H mutant

To investigate whether the efficiency of energy transfer within D1-L210H PS II RCs was altered, we analyzed the 77 K Chl fluorescence emission yield of PS II RC complexes. The Chl a fluorescence emission spectrum of D1-L210H mutant was almost identical to that of WT when excited at 436 nm (Fig. 3.10). Both the mutant and WT RCs had a Chl fluorescence emission maximum at 682 nm, (Kwa et al, 1992; Yruela et al, 1994; Freiberg et al, 1994). The Chl fluorescence emission spectrum of the D1-L210H mutant was narrower than that of WT, indicating that the electronic states of the fluorescing species were perturbed.
Fig. 3.10 Chlorophyll fluorescence emission spectra at 77 K from wild-type and D1-L210H mutant strains excited at 436 nm. Wild-type, —; D1-L210 mutant, ---. The spectra were normalized at 750 nm.
3.3.8 Circular dichroism (CD) spectra of WT and D1-L210H mutant RCs

To further probe the energy coupling in D1-L210H mutant, we measured the visible CD spectra of WT and D1-L210H mutant RCs. This spectroscopic method is very sensitive to pigment interactions within RC complex. The CD spectra of WT and D1-L210H RCs measured at 4°C are shown in Fig. 3.11. The Chl CD spectrum of WT RCs had a strong positive signal centered at 682 nm, and a smaller negative band peaking at 669 nm. However, those two peaks were inverted in the D1-L210H RCs. The positive-negative band pair in the red region has been demonstrated to be associated with excitonic interaction involving P680 (Tang et al, 1990; Finzi et al, 1998; Finzi et al, 1999). Therefore, it was possible the mutation considerably altered excitonic interaction between core chlorins in the RC, particularly the interaction of P680 and its adjacent cofactors. However, recent studies indicate that the CD signal of PS II RC does not exclusively arise from P680, but also from interaction between other RC chlorins and contributions belonging to pigment-protein interactions (Vácha et al., 2002). This would suggest that the resulting significant spectral shape changes in D1-L210H mutant might be attributed to new exciton interactions established between the incorporated Chl and the rest of the RC core pigments and more importantly, the new pigment-protein interactions.
Fig. 3.11 Visible light CD spectra of PS II reaction centers isolated from the wild-type and D1-L210H mutant strains. Wild-type, —; D1-L210 mutant, ---.
3.3.9 Electron transfer properties of D1-L210A mutant

There was very little apparent effect of the D1-L210A mutation on overall rates of electron transfer. Table 2 shows the electron transfer rates of the D1-L210A mutant. Interestingly, the D1-L210A mutant actually had a slightly higher rate of oxygen evolution than WT.

Similarly, the flash-induced Chl fluorescence decay kinetics of the D1-L1210A mutant were similar to WT (Fig. 3.12). The rapid formation of high fluorescence state (QA⁻), demonstrated that electron transport from the S state of water splitting complex to QA was not affected by mutation, in accordance with results from oxygen evolution assays. The Chl fluorescence decay curve is fit by two exponentials (Table 3.4). It is clear that QA⁻ of D1-L210A mutant decays slower than that of WT. Normally, the QA⁻ oxidation occurs by forward electron transfer to QB, and the contribution of the back reaction of S₂QA⁻ is minimal. In the presence of DCMU, the forward electron transfer is blocked at the QB site, the Chl fluorescence decay thus reflects the rate of charge recombination between QA⁻ and the S₂ state. There were no significant changes of QA⁻ decay kinetics of the D1-L210A mutant (Fig. 10, inset) relative to WT, suggesting that the observed slower QA⁻ decay in the absence of DCMU could be attributed to a slightly decreased rate of electron transfer to QB.
Fig. 3.12 Chlorophyll fluorescence decay kinetics of light-grown cells from the wild-type and D1-L210A mutant strains. Wild-type, —; D1-L210 mutant, ---. Inset: measurements carried out in the presence of DCMU.
<table>
<thead>
<tr>
<th></th>
<th>Time constants of decay</th>
<th></th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>118 µs (0.83)</td>
<td>678 µs (0.17)</td>
</tr>
<tr>
<td>D1-L210A</td>
<td>150 µs (0.77)</td>
<td>1114 µs (0.23)</td>
</tr>
</tbody>
</table>

Table 3.4 The Chlorophyll fluorescence decay time constants and amplitudes of wild-type and D1-L210A mutant cells. Values in parentheses are amplitudes.
In addition, the yield of the $Q_A\text{Fe}^{2+}$ EPR signal for the D1-L210A mutant was similar to WT (Fig. 3.13). Quantification of EPR signal normalized to $Y_D$ signal size demonstrated that the D1-L210A mutant generated a somewhat even higher yield of the $Q_A\text{Fe}^{2+}$ signal (approximate 15 ± 5 %) compared to WT. These results were consistent with the higher Chl fluorescence maximum observed in the Chl fluorescence decay kinetics.
Fig. 3.13 The sodium formate enhanced $Q_A Fe^{2+}$ EPR signal ($g=1.82$ and $1.67$) in PS II particles from wild-type and D1-L210A mutant strains. Wild-type, —; D1-L210 mutant, -. EPR conditions: sample temperature, 4 K; microwave power, 32 mW; microwave frequency, 9.48 GHz; field modulation frequency, 100 kHz; magnetic field modulation amplitude, 2.0 mT.
3.3.10 Room temperature optical absorption spectra of D1-L210A mutant

The ground state absorption spectrum of the D1-L210A mutant was similar to WT (Fig. 3.14), in sharp contrast to that from D1-L210H mutant. This result further supports the conclusion that the substitution of the leucine with histidine at D1-210 caused the replacement of Pheo\textsubscript{inactive} with a Chl.
Fig. 3.14 Ground-state absorption spectra of PS II RCs isolated from the wild-type and D1-L210A mutant strains. Wild-type, —; D1-L210 mutant, ----.
3.4 Discussion

We demonstrated that the replacement of leucine residue at D1-210 with a histidine resulted in the incorporation of a Chl in the Pheo\textsubscript{inactive} binding pocket. This conclusion is based on analysis of the pigment composition and the ground state optical absorption spectrum. Significantly, the Pheo Qx band was red shifted to 543.5-544 nm with respect to WT (542 nm), indicating that only the active branch Pheo was present in the RC. Furthermore, it is concluded that Pheo\textsubscript{inactive} absorbs maximally around 540 nm in the Qx region of its spectrum. This is first direct evidence that two Pheo molecules in RC complex of higher plants differed not only in their accessibility, but also in their electronic transition peaks. The location of Pheo\textsubscript{active} at longer wavelength relative to Pheo\textsubscript{inactive} at 77 K is similar to the situation in the BRC (Bylina et al, 1988). This was in good accordance with the results from MCD spectra of PS II RC from spinach (Mimuro et al, 1995), and recent studies of excitonic calculations (Jankowiak et al., 2002). However, our results are not consistent the finding that both Pheos absorb at 542 nm as determined by pigment exchange experiments in PS II RCs of spinach (Shkuropatov et al, 1997) and Pheo measurements made at 10 K (Breton, 1990).

The light-induced difference spectra indicated that the Pheo Qx bleach band of WT results from contributions of both the blue (inactive branch) and red (active branch) forms of Pheo. This interpretation is consistent with previous results from Yruela et al., (2001). It was well established that the Qy absorption maximum of Pheo\textsubscript{active} is close to 680 nm. The Qy absorption maximum of Pheo\textsubscript{inactive} remains more controversial, absorption bands
at 668.3 nm (Jankowiak et al, 1999, 2002) 670 nm (Braun et al, 1990; Mimuro et al, 1995; Stewart et al, 2000), and 680 nm (Shkuropatov et al, 1997) have been attributed to Pheoinactive. The wavelength of the bleaching in the Qy region was 1.5 ± 0.5 nm red-shifted in D1-L210H mutant relative to WT, indicating that the Qy transition of Pheoactive was around 682 ± 0.5 nm and thus Pheoinactive absorbed at 681 ± 0.5 nm in the native RCs assuming that only Pheo− contributes to the Qy bleach and both Pheo− contribute equally.

As to the electron transfer efficiency, the rate of oxygen evolution and Chl fluorescence decay kinetics suggested that it is significantly lower in D1-L210H mutant compared to WT and the D1-L210A mutant. The accumulation of QA− also was greatly reduced in D1-L210H mutant PS II complexes under cryogenic conditions. The data from light-induced difference absorption spectra further suggested that the photochemical activity of D1-L210H mutant RC preparation diminished 70%, which we attribute to the loss of reduced Pheoinactive and decreased reduction of Pheoactive. In addition, the intensity of Pheo anion EPR signal accumulated by illumination at 4°C in PS II RC complexes was much lower in the D1-L210H mutant than in WT. These results suggest that decreased photochemical activity contributes to the overall reduction of PS II electron transfer in the D1-L210H mutant.

The data from transit absorption spectra recorded at 4°C indicated the time constant for charge separation is slower in the D1-L210H mutant than in WT (5.2 ps versus 3.5 ps). In addition, the amplitude of Pheo Qx bleach in the isolated D1-L210H RCs (1.5 ns) is reduced (~ 40-50%) relative to WT. At this time point, we attribute the observed bleach to the radical pair P680+Pheo−. Thus it is concluded that in D1-L210H RCs the steady-state
yield of radical pair formation is about half of that WT PS II RCs, consistent with the steady-state optical experiments. However, recent studies indicate that a distinct blue shift of Pheo Qx bleach was observed with time, thus at longer time intervals (i.e. few hundred ps) following excitation contributions from the energy equilibration with Pheo inactive cannot be ignored, (Jankowiak et al., 2002). Greenfield et al (1999) also suggest the possibility of Pheo as energy trap. If we take into account the contribution of Pheo inactive, which is missing in D1-L210H mutant, to the final bleach of Pheo at 1.5 ns (less than 25 %, according to Jsnkowiak et al., 1999), the yield of radical pair in mutant is still less than WT. On the other hand, in the D1-L210H mutant the red shift observed at 1.5 ns time region, relative to WT, clearly demonstrated the contributions of Pheo inactive to the final bleach. It is very difficult to determine whether the slow energy transfer between Pheo inactive and other central core pigments of RC or the reduction of Pheo inactive actually contribute to the bleach seen at nanoseconds in PS II RCs.

As discussed above, based upon the similarities of BRCs and PS II RCs, it is believed that light-induced charge separation occurs preferably between the cofactors of one branch of the native PS II RC. A Chl and a Pheo located on the alternative branch thus appear not to be involved in charge separation in intact PS II complexes (but not in RCs). As to the physiological function of the inactive branch, the results from recent experiments in BRCs suggest that the inactive branch participates in the photoprotection. The rapid formation and decay of reduced Pheo inactive radical after excitation with blue light seems to support the hypothesis that the function of the alternate pathway is to
dissipate extra energy in the BRC (Lin et al, 2001). In addition the Pheo_{inactive} may affect the distribution of excitonic interactions among the central pigments (Germano et al, 2001). Here, comparison of the CD spectra of WT and D1-L210H RCs clearly indicates that there were changes in the excitonic interactions between pigments in D1-L210H mutant. This result indicates that introducing a Chl in place of Pheo_{inactive} alters the inherently delicate energy equilibrium of the highly optimized system, and/or changes the pigment-protein interactions. Interestingly, the D1-L210H mutant Chl Qy CD features have the appearance of a derivative spectrum relative to WT. Such spectra may result from phase interactions between multiple components of the delocalized excited states across the six core chlorins. In the absence of QA the excited state distribution among the chlorins may be altered in the mutant relative to WT. Alternatively, the substitution of a Chl for a Pheo may affect the spectrum.

Theoretically, it is shown that the small orbital energy shifts belonging to one pigment relative to that of another can give rise to significant effects on the interaction of matrix elements (Scherer and Fischer, 1989). The Mg orbitals of Chl cause changes in charge distribution within the pigment. It is therefore conceivable that substitution of a Pheo with a Chl results in changes of energy coupling of “multimer” and maybe the (de)localization of excited state on pigments, which is attributed to the altered functional behavior of modified RC.
4.1 Introduction

Photosystem II (PS II) is a multi-subunit membrane protein complex consisting of over 20 different polypeptides. It is the only photosystem that is capable of generating a strong enough oxidant to extract electrons from water. At the core of the complex is the PS II RC, made up of D1 and D2 proteins and their bound cofactors that participate in light-driven electron transfer reactions. Based on the low-resolution PS II structure (Zouni et al., 2001), it is clear that the cofactors display a two-fold axis of symmetry, the same as those in purple BRC. The symmetry does not extend to the donor side that is exclusively involved in oxygen evolution, however, the redox-active tyrosyl residues $Y_Z$ and $Y_D$ are organized symmetrically along the C2-axis near the lumen side of the membrane.
Substantial information about the process of electron transfer in PS II has been obtained using various spectroscopic techniques. The pathway of electron flow through the PS II RC involves: 1) A light-induced charge separation involving the oxidation of a chlorophyll (Chl) called P680. Oxidation of P680 results in the reduction of the primary electron acceptor, pheophytin (Pheo) within a few picoseconds (Wasielewski et al., 1989; Chang et al., 1994; Schelvis et al., 1994), 2) The reduced Pheo then passes an electron to a tightly bound plastoquinone molecule (QA) in about 200 ps (Nuijs et al., 1986; Eckert et al., 1988). In the meantime, the highly oxidizing P680$^+$ is reduced in nanoseconds by a redox active tyrosine YZ located at residue 161 on the D1 protein (Debus et al., 1992; Barry, 1993), 3) The radical YZ is then reduced by the tetra-Mn water splitting complex. This results in the release of molecular oxygen, 4) A second plastoquinone at the Q$_B$ binding site is reduced and protonated, and when doubly reduced and protonated, dissociates from the bound site. In the isolated PS II RC, where QA is invariably absent, the radical pair P680$^+$Pheo$^-$ cannot advance and instead decays by recombination, largely to the triplet state of P680 (Rutherford et al., 1981) which ultimately returns to the ground state with the emission of light (luminescence). In the BRC, electron transfer occurs along only one branch of the two parallel electron transfer pathways (Kirmaier and Holten, 1987; Kirmaier and Holten, 1993; Heller et al., 1995). It is reasonable to predict that the light-driven electron transfer in PS II RC occurs exclusively along one branch of cofactors given the striking structural and functional homology between the PS II RC and BRC.
High-resolution three-dimensional structures have been elucidated (Michel and Deisenhofer, 1988; Stowell et al., 1997; Fritzsch et al., 1998) in the BRC. The four BChls and two Bpheo which participate in electron and energy transfer processes have well-separated absorption peaks. This fact facilitates characterization of the primary charge-transfer processes in the BRC. On the contrary, the PS II RC structure and function has been more challenging to characterize. It is not possible, however, to spectrally resolve the individual chlorins of the PS II RC even at cryogenic temperatures. As a result, it has been difficult to elucidate the nature and mechanism of the primary photosynthetic reactions in PS II. Since all chlorins in PS II RC absorb at nearly identical wavelengths in the Qy region, it has been inferred that there is weak coupling between the pigments and little effect of the protein environment on the energy absorption profiles of the pigments. As a consequence, charge separation in PS II RC occurs while the excited state is equilibrated between all the chlorins of the RC, leading to slower rates of primary electron transfer than that in the BRC where the excitation energy is rapidly localized on the lowest optical transition of the BChl special pair and charge separation between special Chl dimer and Bpheo is rapidly completed in < 10 ps.

We are interested in investigating the dynamics of early electron transfer events in a PS II RC in which the spectral overlap of the pigments is relatively less congested. Here, we employed the unicellular green alga *Chlamydomonas reinhardtii* as a model system to identify the residue(s) of the PS II RC proteins that interact with cofactors involved in primary electron transfer, particularly the residue (s) close to functional Pheo molecule (Pheo_{active}).
In the previous studies, we have shown that substitution of D1-L210 residue, presumably located over the macrocycle center of Pheo\textsubscript{inactive}, with a histidine residue (to provide the fifth ligand for the central Mg atom of Chl), yielded stably assembled RCs in which Pheo\textsubscript{inactive} was replaced by a Chl. We also located the Qx absorption peak of Pheo\textsubscript{active} at 543.5 ± 0.5 nm. The red shift of the Pheo Qx band observed in the D1-L210H mutant compared to WT indicates that the electronic transitions between the two Pheos in PS II are still different, although there is a more symmetrical protein matrix around these pigments than in bacteria. In this study, we introduced the same mutation at its symmetrical site in the D2 protein. The D2-L210 residue is analogous to the conserved Leu 214 on the M subunit in \textit{Rb. sphaeroides}, and thus is expected to be located over the center of Pheo\textsubscript{active}. Previously it was shown that 85% of the RC contained a BChl molecule in the native Bpheo\textsubscript{L} site in the M-L214H mutant (Kirmaier et al., 1991), and electron density values at the center of Bpheo\textsubscript{L} ring indicated the presence of a BChl instead of a BPheo in crystallized RCs (Chirino et al., 1994). Further analysis of this particular mutant revealed that the accumulation of QA is reduced due to the enhanced rate of charge recombination (P$^+$BChl$^-$→ P). Interestingly, in spite of the replacement of the Bpheo\textsubscript{L} with a Chl that has a higher inherent redox potential, the unidirectional asymmetry of electron transfer remained unchanged. In this chapter, we characterized the D2-L210H mutant in \textit{C. reinhardtii}. Our results indicate that D2-L210H mutation has substantially altered energy and electron transfer properties compared to WT. We also introduced a conservative mutation, D2-L210A, for
comparison. Surprisingly, this potentially conservative mutant also has a reduced capacity to carry out charge transfer.

4.2 Materials and Methods

4.2.1 Generation of the psbD L210H and L210A mutants

Site-directed mutations were made using the system originally developed by Minagawa (personal communication): Plasmid PBD202 carries the aadA gene conferring resistance to the antibiotics spectinomycin and streptomycin, and the psbD gene. Mutation of the D2-210 residue, converting it from a leucine to a histidine or alanine, was introduced by changing the TTA (Leu) codon to CAC (His) or GCA (Ala), respectively, in the psbD gene. The primer also incorporated a silent mutation at residue D2-C211 and D2-A212 by changing the codon TGT to TGC and GCT to GCA, respectively. These mutations maintained the cysteine and alanine residues but introduced a new Fsp I restriction site. The sequences of the mutagenic oligos are: 5’-GGTGCTGCTTTACACTGCACATTC-3’, and 5’-GGTGCTGCTTTAGCATGCACATTC-3’ for the D2-L210H and D2-L210A mutations. Plasmids containing the point mutations were constructed by oligo-nucleotide-directed mutagenesis according to procedures described in Chapter 2. The nature of the mutation was identified by testing for the alteration of the restriction enzyme site added in the mutagenesis step, and confirmed by dideoxy sequencing through the region of interest (Chapter 2). The modified psbD genes were then delivered via helium gun as described in Chapter 2 into a psbD-deficient strain of C.
reinhardtii strain, CC Δ2 (Minagawa, personal communication). After several cycles of alternative selection (100 µg/mL spectinomycin/streptomycin and 50 µg/mL ampicillin), candidate transformants were identified by restriction site analysis of PCR-amplified psbD fragments using transgenic alga as template and ~250 base pairs upstream and ~250 base pairs downstream of the mutation site was resequenced. The isolation of cellular DNA from C. reinhardtii was the same as described in Chapter 2.

4.2.2 Photosynthetic membrane fraction preparations

Wild-type (WT) CC-2137 and D2-L210H mutants of C. reinhardtii were grown in Tris-Acetate-Phosphate (TAP) media (Gorman and Levine, 1965) at 25°C in continuous light (40-100 µmol photons m⁻² s⁻¹) on a shaker. For all analyses, cells were harvested at about 5× 10⁶ cells/mL by centrifugation at 4,000 × g for 10 min. Thylakoids of WT and D2-L210 mutants were prepared in dim green light as in Chapter 2, suspended in buffer A containing 0.35 M sorbitol, 20 mM Hepes pH 7.5, 2 mM MgCl₂, and stored in –80°C in the same buffer with final Chl concentration of over 2 mg Chl/mL. PS II-enriched membranes were prepared by solubilization of thylakoids with Triton X-100 as described by Shim et al., (1990). All procedures were done in dim green light in the cold room. The PS II membranes were stored at –80°C in 0.4 M sucrose, 15 mM NaCl, 5.0 mM MgCl₂, 20 mM Mes pH 6.0 at 3-4 mg of Chl/mL.

4.2.3 Low-temperature Chl a fluorescence emission spectroscopy
PS II membranes frozen in buffer containing 0.4 M sucrose, 15 mM NaCl, 5 mM MgCl, 20 mM Mes pH 6.0 were diluted to 5 µg Chl/mL and 77 K Chl fluorescence emission spectra were recorded on a Fluro-Max spectrometer using an excitation wavelength of 436 nm. The excitation and emission slit widths were 5 and 2 nm, respectively. The spectra were normalized at 685 nm.

4.2.4 Oxygen evolution measurements

Steady-state rates of oxygen evolution were measured with a Hansatech oxygen electrode using thylakoids (10 µg) in 1.0 mL assay buffer containing: 0.35 M sorbitol, 20 mM Hepes pH 7.5, 2 mM MgCl₂, 30 mM methylamine, 20 µM 2,6 dimethylbenzoquinone (DMBQ) and 2 mM potassium ferricyanide. Saturating light (~1000 µmol photons m⁻²s⁻¹) was provided by a Hansatech LS2 light source using a heat filter.

4.2.5 Chl a fluorescence decay kinetics

Cells were spun down and Chl fluorescence analyses were completed within 1 hour of resuspending the cells. The cells concentration was adjusted to 20 µg Chl/mL using TAP medium. Chl a fluorescence decay measurements were performed in the absence and presence of 20 µM 3-(3,4-dichloro)-1,1-dimethylures (DCMU) on a laboratory version of the instrument reported by (Kramer et al., 1990).

4.2.6 EPR spectroscopy
X-band EPR spectra at liquid helium temperatures were measured with a Bruker ER 300 spectrometer equipped with an Oxford liquid helium cryostat. The PS II membranes (4-5 mg Chl/mL) were incubated with 100 mM sodium formate for 15 min at 4°C in the dark. The QA\Fe^{2+} EPR signal was induced by illumination (~1000 µmol photons m^{-2}s^{-1}) for 10 min at 200 K. The resulting QA\Fe^{2+} signal was trapped by quickly freezing the samples at 77 K for EPR analysis. EPR spectra were recorded using the same instrument settings as described in Chapter 2. The quantification of QA\Fe^{2+} radical was done by double integration of the first derivative signal and normalized on the basis of Y_D. As for the measurement of Y_D, the same sample was warmed to room temperature, illuminated at 4°C for 2 min and immediately frozen in liquid nitrogen. For quantification of Pheo^-, the PS II membranes were incubated with 40 mM sodium dithionite for 10 min in the dark at ice to chemically reduce QA prior to 3 min illumination (~1000 µmol photons m^{-2}s^{-1}) at 4°C. The EPR conditions are the same as described in Chapter 3.

4.3 Results

4.3.1 Construction of the D2-L210 mutations in a psbD-deficient background

Site-directed mutagenesis was employed to generate the D2-L210H mutation in vitro. A Fsp I restriction site was introduced by silent mutation at residues D2-C211 and D2-A212 along with the D2-L210H mutation. Following transformation of the CC- ΔD2, D2-deletion strain, using helium-driven particle infusion gun, antibiotic resistant colonies were obtained. The total DNA of these putative transformants was extracted and subjected to PCR analysis using D2- JW93 (5’-ATGAGAATTGCATCGGTACAT
ATCAAGAG -3’) and D2-JW94 (5’-ATATTATAGACGTCGGTAATCTTCC-3’) as primers. Fig. 4.1 shows that the D2-L210 mutations carries Fsp I restriction site, as indicated by the appearance of new 0.7 and 0.36 kb bands when the PCR-derived psbD fragments were digested with Fsp I enzyme. The ultimate verification of the presence of the target mutations was performed by DNA sequencing using D2-Y161H (5’-TTGACCTAATGGGTGGATCAGGAATAC-3’) as a primer (Fig. 4.2).
Fig. 4.1 Detection of the D2-L210A and D2-L210H mutations by PCR. DNA fragments amplified with primers D2-JW93 and D2-JW94 were digested with Fsp I and analyzed on a 1% agarose gel. Templates employed for PCR reaction were as follows: Wild-type (lane 1), D1-L210A (lane 2), D1-L210H (lane 3).
Fig. 4.2 DNA sequence showed the nucleotide substitutions at D2-L210 residue. The bases changes for introducing the restriction enzyme Fsp I along with codon alterations are underlined.
4.3.2 77 K Chl fluorescence emission spectra

Chl fluorescence emission spectroscopy is an effective and sensitive way to monitor energy transfer and trapping in PS II. At 77 K, the Chl fluorescence emission spectrum of PS II membranes exhibits two distinct band centered at 685 and 695 nm, respectively. It has been proposed that the principle contributor to the emission at 695 nm is a low-energy Chl bound to H-114 of the CP47 protein (Shen et al., 1994). The origin of maximum at 685 nm emission band is complicated, and includes contributions from charge recombination (Braun et al., 1990) and PS II antennae complex (LHC-II) (Funk et al., 1998).

A rough comparison of the Chl fluorescence emission spectra between WT and D2-L210 mutants (Fig. 4.3) suggest that there is no substantial deviation of the F685 emission band in the mutant from WT. However, the F695 emission band undergoes a remarkable red shift (about 8-9 nm) in both mutants, and the intensity of this band relative to F685 is enhanced significantly.

The two peripheral accessory Chls are believed to serve as links of energy transfer between RC and antenna complex (Seibert, 1993; Foelofs et al., 1993; Johnston et al., 2001). These two Chls are located 30 Å away from P680 (Föster, 1947; Roelofs et al., 1991; Zouni et al., 2001). One of the peripheral Chls known as ChlZ is involved in a low quantum yield electron transfer pathway around PS II and is identified to be associated with fluorescence quenching of the F695 emission peak (Wang et al., 2002). We suggest that the D2-L210 mutations influence the fluorescence of the Chl attached to H-114 of
CP47 by affecting energy equilibration among several chlorins, which could potentially trap the excited state within RCs.
Fig. 4.3 Fluorescence emission spectra of PS II particles from wild-type and D2-L210 mutant strains measured at 77 K. Excitation was at 436 nm. Wild-type, —; D2-L210A mutant …; D2-L210H mutant ---. The spectra were normalized at 660 nm.
4.3.3 Electron transfer properties of D1-L210 mutants

Measurement of oxygen evolution rates under continuous illumination is a simple way to monitor the efficiency of electron transfer in PS II. Steady-state rates of oxygen evolution were measured at saturating light intensities with 20 µM DMBQ as a PS II (Q_B) electron acceptor. Table 4.1 indicates that both D2-L210H mutants have only approximately 20% of the oxygen evolving activity of WT under saturating light intensities. Similarly, the rate of oxygen evolution for the D2-L210A mutant was substantially lower than WT. The substantial reduction in the rate of PS II electron transfer could be caused by the direct impairment of primary photochemistry due to the mutations.
<table>
<thead>
<tr>
<th></th>
<th>Oxygen evolution (µ mol O₂ mg Chl⁻¹ hr⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>270 ± 20</td>
</tr>
<tr>
<td>D2-L210A</td>
<td>65 ± 5</td>
</tr>
<tr>
<td>D2-L210H</td>
<td>45 ± 5</td>
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</tbody>
</table>

Table 4.1 Oxygen evolution rates of PS II particles isolated from wild-type and D2-L210 mutant strains. The standard deviation from four determinations is shown.
4.3.4 Chl a fluorescence induction and decay

The yield of variable Chl fluorescence, defined as Fv, reflects the formation of charge separated state P680/QA− and is important indicator of PS II activity (Krause and Weis, 1991; Dau, 1994) or PS II content (Chu et al., 1994). The Fv/F0 ratio, representing the maximal Fv, was measured. The rise and decay of Chl a fluorescence for the WT and mutants is shown in Fig. 4.4. The Chl a fluorescence in the D2-L210H mutants is reduced by approximately 30% - 40% relative to that of WT. Considering that F0 value in the D2-L210H mutant is not higher than WT (Fig. 4.4, inset), the lowered Fv/F0 ratio that is proportional to the QA− formation might be attributed to slowed forward reaction and/or faster back reaction. On the other hand, the D2-L210A mutant shows the same high yield of variable fluorescence as WT.
Fig. 4.4 Chlorophyll fluorescence decay kinetics of light-grown cells from the wild-type and D2-L210H mutant strains. Wild-type, —; D2-L210A mutant . . .; D2-L210H mutant -. Inset: the raw data of wild type and D2-L210 mutants.
The flash-generated high Chl fluorescence state decays primarily by forward electron transfer to the second quinone acceptor, Q_B, which is relatively stable and in the absence of an electron acceptor decays by charge recombination. However, when a PS II herbicide such as DCMU is bound, Q_B is displaced from its site and electron transfer from Q_A to Q_B is blocked. Thus, Q_A^- oxidation depends on charge recombination with the donor side of P680. In our study (Fig. 4.5), no significant difference of Chl fluorescence decay is observed in the D2-L210A mutation, but the fluorescence decays faster in the D2-L210H mutant than in WT, as expected. The faster charge recombination in this mutant should thus contribute to the reduced yield of variable fluorescence observed in Fig. 4.4. It is likely the donor side of P680 is involved in the faster fluorescent decay in D2-L210H mutant, but it is conceivable that faster charge recombination between Q_A^- and P680^+ accounts for the observed changes based upon the results of the analogous M-L214H mutation in BRCs.
Fig. 4.5 Chlorophyll fluorescence decay kinetics of light-grown cells from the wild-type and D2-L210H mutant strains. Wild-type, —; D2-L210A mutant …; D2-L210H mutant --- in the presence of 20 μM DCMU.
4.3.5 Quantification of the semiquinone-iron complex: QA Fe$^{2+}$

The quinone in the QA site is tightly bound to the protein. It undergoes a single electron reduction to form a semiquinone anion under normal physiological conditions. It is the first cofactor of the PS II electron transfer chain in which the charge separated state is stable in the microsecond time scale assuming no forward electron transfer to QB. In addition, the functional intactness of the primary quinone molecule can be easily monitored since the singly reduced quinone QA$^-$ magnetically interacts with its nearby non-heme iron to generate a 40 mT wide EPR signal at $g=1.82$ and 1.67 (Miller and Brudvig, 1990). After illumination below 230 K, where the forward electron transfer from QA to QB and beyond is very slow, the resulting QA Fe$^{2+}$ can be trapped by rapid cooling.

In our experiment, 100 mM sodium formate is also added which competes with bicarbonate for the binding of non-heme iron and blocks electron transfer from QA to QB (Petrouleas and Diner, 1990). A large QA Fe$^{2+}$ EPR signal is induced from the PS II membrane of WT upon illumination at 200 K (Fig. 4.6). In contrast to WT, the sodium formate enhanced EPR signal is significantly decreased in the D2-L210H mutant, with an amplitude corresponding to about 25-30% of that in WT, indicating again the suppression of electron transfer activities of PS II. Such results are in good agreement with results from the M-L214H mutation in purple bacteria. It is noted that the signal size of QA$^-$ in D2-L210A mutant was also reduced by 70% relative to WT.
Fig. 4.6  The sodium formate enhanced $Q_A\text{Fe}^{2+}$ EPR signal ($g=1.82$ and 1.67) in PS II particles from wild type and D2-L210H mutant strains. Wild-type, —; D2-L210A mutant …; D2-L210H mutant ---. EPR conditions: sample temperature, 4 K; microwave power, 32 mW; microwave frequency, 9.48 GHz; field modulation frequency, 100 kHz; magnetic field modulation amplitude, 2.0 mT.
4.3.6 The Pheo anion racial in WT and D2-L210 mutants

To determine whether the primary photochemistry could be attributed to a reduced efficiency of electron transfer, we tried to isolate D2-L210 mutant RCs. However, attempts to isolate PS II RC from D2-L210 mutants gave poor results, 1% the normal yield and questionable spectra, indicating that D2-L210 mutations were structurally unstable or failed to assemble. Then we measured the EPR signal of reduced Pheo in PS II membranes pre-treated with 40 mM sodium dithionite upon illumination at 4°C (Fig. 4.7). The yield of Pheo\(^*\) of both mutants was only about 50-60% of WT. These results supported our expectation that the decreased photochemical activity in D1-L210H mutant is partially responsible for the observed changes of electron transfer. It is unclear which of the two Pheos was reduced, the active or the inactive branch Pheo. However, since it may be supposed that a Chl in the D2-L210H mutant is substituted for the active branch Pheo it is possible that only the inactive branch pheo was reduced.
Fig. 4.7 The photoaccumulated Pheo\textsuperscript{\textast} EPR signal from wild type and D2-L210 mutant PS II particles. Wild-type, —; D2-L210A mutant …; D2-L210H mutant ---.
4.4 Discussion

We have introduced point mutations at residue D2-L210 in the psbD gene of *Chlamydomonas reinhardtii*. The availability of a psbD\textsuperscript{-} genetic background greatly facilitates construction of D2 mutants by homologous recombination.

In the BRC the replacement amino acids that cannot (leucine) coordinate the central Mg of Chl with those that potentially can (histidine), yields RCs in which the BChl has been converted into Bpheo (Bylina and Youvan 1988; Chirino et al., 1994), and *vice versa* (Kirmaier et al., 1995). The RCs with modified pigment compositions provide valuable samples to investigate the interactions between chromophores and the function of individual pigment molecules. This is particularly important for PS II RCs where all pigments crowd in the red spectral region. The less distinct spectra of cofactors in the PS II RC also make primary photochemistry difficult to investigate. Pigment exchange approaches have been extensively used in BRC in which site-directed exchange of the special pair BChls, two monomeric BChls, and two Bpheos has been achieved with externally added pigment analogs (Scheer and Struck, 1993; Scheer and Hartwich, 1995). However, the application of such methods in PS II is still challenging, since the experimental procedure to introduce external pigment requires an elevated temperature (or at least room temperature), which makes PS II RC more liable to denaturation. Furthermore, the controversy and uncertainty of individual pigment absorption characteristics in PS II RC renders the spectroscopic features of modified chromophores difficult to identify.
The M-L214H mutation in the BRC resulted in the replacement of the BPheo with a BChl. Analysis of this mutant further suggests that the lifetime of electron transfer from P* to Bpheo active doesn’t rely heavily on the free energy gap between P* and P+ BChl-. reflected by the fact that considerable changes in -ΔG° resulting from the exchange of Bpheo with BChl (the free energy gap in WT is 160 meV and in M-L214H mutant is –75 meV) only slightly slowed the forward electron transfer rate (15 ps in WT versus 30 ps in M-L214H mutant). The decrease in the yield of QA- (about 60% of WT) in this mutant was attributed to dramatically enhanced charge recombination (P+ BChl- → P), which competes effectively with forward electron transfer to QA. These data demonstrate that the midpoint potential of P+ BPheo in the native RC lies well below the P+ BChl. Therefore, the presence of Bpheo instead of BChl as a primary electron acceptor in all photosynthetic system reduces potential charge recombination and favors reduction of QA. This is especially meaningful for all RCs where the radical pair recombination results in not only the reduction of the quantum yield of electron transfer in the forward direction, but also the formation of triplet states, the source of toxic singlet oxygen that can trigger the photodamage (Durrant et al., 1990; Telfer et al., 1994).

In our study, our major objective was to mutate residue D2-L210 to a histidine to obtain PS II RCs with modified pigment compositions. We substituted a hydrophobic leucine residue at D2-210 with a metal-coordinating histidine residue to facilitate the binding of a Chl at the site of Pheo active. In the low-temperature fluorescence emission spectra of D2-L210H mutant, the F695 emission band, known to be very sensitive to
temperature and detergent, exhibits a significant red shift and broadening, while there were no obvious changes for the F685 emission band. Based on their origin and characteristics, we conclude that energy transfer between the distal antenna pigments and the PS II core is unperturbed by mutation, as indicated by the striking similarity of F685 fluorescence emission band between WT and mutant. However, the D2-L210 mutations affected Chl fluorescence from CP47 (as seen in F695 emission band). Interestingly, the Chl fluorescence $F_0$ value of the D2-L210H mutant was similar to that of WT. On the other hand, the PS II electron transfer properties of D2-L210H mutant were severely affected as evident by the following. (1) The oxygen evolution rate of the mutant is much lower than WT. (2) The flash-induced $Q_A^-$ formation from Chl fluorescence decay kinetics is only 60% of WT. (3) The steady-state $Q_A^-$ accumulation indicates ~30% reduction in the mutant relative to WT. (4) The Pheo$^-$ radical anion generated by the mutant following illumination is 60% of WT. Overall, we attribute the decreased electron transfer efficiency to faster back reaction between Pheo$^-$ and P680$^+$ relative to WT. The faster decay of high the fluorescence state, P680$Q_A^-$, in the presence of DCMU in the D2-L210H mutant indicates an increased back reaction compared to WT.

The phenotype of the PS II D2-L210H mutant is generally comparable to the structurally equivalent M-L214H mutant in purple bacteria in the sense that both have a reduced yield of $Q_A^-$ and faster charge recombination compared to WT. However, our ability to further explore the kinetics of charge separation and radical pair formation (P680$^+$Chl$^-$) was hampered by the inability to obtain RCs from the D2-L210H mutant. The lack of isolated PS II RCs from the D2-L210H mutant complicates the process of
interpreting the Pheoinactive Qx band as well. Attempts to isolate RC from a similar type of mutation (L-F121H to incorporate a BChl in place of BPheoL in *Rh. capsulatus*) failed unless L-F97 (or another adjacent residue) was replaced with smaller amino acid such as cysteine or valine (Heller et al., 1995). It is argued that the role of L-H121 in the ligation to BChl is accommodated by changes in neighboring residues. We speculate that the analogous changes in PS II may also be mandatory to allow ligation to the central Mg of Chl by introducing minor structural rearrangement of protein that accompanies the D2-L210H mutation. Moreover, it was proposed that there was a rotation associated with the ring of the introduced BChl anion in the bacterial mutant (Schenck et al., 1993), indicating that the local structure was perturbed to accommodate coordination by the histidine at M-L214. However, the crystal structure of M-L214H mutant did not show distinct changes in the positions of BpheoL at 3.3 Å resolution (Chirino et al., 1994). Therefore, it is possible that when a Chl is forced into Pheoactive binding pocket by the presence of histidine at D2-210 alone, structural changes occur, and/or no pigment occupies Pheoactive site (in this case, the observed accumulation of Pheo− and QA− was from native RC carrying Pheoactive), which leads to the loss of the stability of RC and reduced electron transfer efficiency in the mutant.

On the other hand, if no local protein structural perturbations are involved in the coordination of D2-L210H to Chl, the clear difference of midpoint potential between Chl and Pheo (Chl is ~300 meV higher than Pheo in solution) should contribute to the altered functional behavior of electron transfer in the mutant, as happened in the BRC. In PS II the electron transfer rate and yield of radical pair (P680+Pheo−) formation were very
sensitive to the free energy gap between excited P680* and P680*Pheo− (Merry et al., 1998). It is conceivable that smaller free energy gaps between P680* and P680*Chl in mutant RC gives rise to a lower yield of reduced primary and secondary acceptors by theoretically increased charge recombination and/or the slowed forward reaction. The results from the Chl fluorescence decay kinetics in the presence of DCMU support this hypothesis. However, the effect of the pigment-dependant midpoint potential shifts of pigments on the charge separation in PS II RC might be different from the BRC considering that the chlorins have weak coupling and the nature of primary photochemistry is not completely delineated in PS II.

It is surprising that the potentially conservative mutant D2-L210A also had a similar phenotype to the D2-L210H mutant. The only interpretation we can draw is that the alanine residue induces substantial local structural changes. It also may allow a water molecule to fit into the space formerly occupied by the leucine side chain, which may provide a ligand to the central Mg of Chl a. Overall, based on the results obtained to present, it is clear that the amino acid substitutions at D2-L210 have significant effects on electron transfer efficiency of PS II within RC complex. Whether the observed changes in D2-L210H mutant result from local structural dislocation of protein, and/or the midpoint potential modulations accompanied by chlorin exchange is still under investigation.
CHAPTER 5

SUMMARY

The photosynthetic reaction center (RC) is an integral pigment-protein complex that carries out charge separation across the photosynthetic membrane upon illumination. There are two types of RCs in cyanobacteria and higher plants: the pheophytin (Pheo)-quinone type (such as PS II), and the iron-sulfur type (such as PS I). Anaerobic photosynthetic bacteria contain only one type of photosystem, which belongs to either the Pheo-quinone type (purple bacteria and green filamentous bacteria) or the iron-sulfur type (green sulfur bacteria and heliobacteria).

To date, the PS II type RC of purple bacteria has attracted the greatest research attention. In the early 1980s, the three-dimensional X-ray structures in the RC of Rps. viridis and Rb. sphaeroides were resolved at high resolution (Fritzsch et al, 1998; Stowell et al, 1997). It is found that the two major proteins, L and M, are arranged symmetrically along a local pseudo-C2 axis of symmetry. These proteins coordinate the cofactors that are involved in light-induced electron transfer. Despite the striking structural symmetry of the two (parallel) potential electron transfer pathways, the electron transfer in this type...
of RC proceeds exclusively along the branch more closely associated with L subunit. As for the primary photochemistry, it is established that the first stable radical pair is formed in about 3-5 ps at room temperature.

The low-resolution crystal structure of PS II demonstrates that the D1 and D2 proteins also have a two-fold rotational axis of symmetry related. Similar to the L and M subunits of the BRC, the D1 and D2 proteins bind the cofactors that participate in electron transfer in PS II. Some experiments support (Klimov et al., 1980; Nanba and Satoh, 1987) that PS II also has functional asymmetry with respect to the directionality of electron transfer.
Fig. 5.1 A schematic representation of the electron transport in PS II

\[ 2H_2O \rightarrow 4e^- + 4H^+ + O_2 \]
Although the BRC and PS II have apparently similar structures, the spectroscopic properties of the two reaction center types have remarkable differences. In the BRC, all six chlorin pigments (four BChl and two Bpheo) absorb at well-separated wavelengths and the lowest optical transition of the Chl special pair is substantially red shifted compared to the other chromophores within RC complex. Such is not the case for PS II. In the PS II RC, all six Chls and two Pheos absorb in a very narrow region and have substantial spectral overlap. In contrast to the BRC, the red shift of the primary donor, P680, possibly a Chl special pair in PS II, is not as great relative to other pigments because the reduced excitonic coupling between the Chls associated with the greater distance of separation between these two Chls (Zouni et al, 2001). As a result, P680 is a much weaker energetic trap than its homolog in the BRC. This seems to be an evolutionary strategy to increase energy conversion efficiency of oxygenic organisms since the energy gaps between pigments are lower (Diner and Babcock et al, 1996). Overall, it is difficult to probe the details of PS II function using spectroscopic and kinetic approaches due to the spectral congestion and the large number of chlorin pigments (8 chlorins in PSII versus 6 in the BRC).

A consensus is emerging that the kinetics of charge separation in the PS II RC is a mutiphasic process including the intrinsic fast time constant of 3-5 ps reflecting charge separation and extending to 20-30 ps for energy equilibration between the core pigment
molecules (Roelofs et al, 1993; Rech et al, 1994). We have focused on the primary events in charge separation or the reduction of the primary electron acceptor Pheo. One advantage of studying Pheo reduction is that the Pheo Qx transition is well separated from that of Chl, with a peak at 542 nm.

The overall aim of this work presented here is: 1) to characterize the protein binding microenvironment around the photochemically active Pheo (Pheo_{active}), 2) determine whether the protein interaction with the active Pheo contributes to the directionality of charge separation, 3) determine the role of the Pheo located on the inactive branch (Pheo_{inactive}) in the energy and primary electron transport, and 4) characterize the effects of substituting the Pheo_{active} with a Chl, on the charge separation process. We describe here the energy and electron transfer processes in mutagenized PS II RC using various spectroscopic techniques on the unicellular green alga, *Chlamydomonas rehardtii*.

*Chapter 2* is a general introduction to the structure and function of Pheo-quinone-type photosystems including that of purple bacteria and higher plants. In *Chapter 2* the results of perturbation of the protein environment in close proximity to the putative active branch Pheo are presented. A conserved E130 residue in the D1 protein is proposed to H-bond to the Pheo ring V carbonyl group (Swensson et al, 1996), as does the homologous residue (L-E104) in purple bacteria. Thus, mutations at this site may weaken or break this H-bond. As we show from high field EPR studies the $g_x$ values shifted in D1-E130 mutants in response to the H-bond strength of the amino acid substitution. This observed trend in $g_x$ value was in excellent agreement with the
expectation that histidine and glutamine were weaker H-bond donors than glutamate, and that a leucine substitution at residue 130 breaks the H-bond.

Further characterizations indicated that the thylakoids of D1-E130L mutant had substantially reduced ability to evolve oxygen. This was associated with a 90% reduction in variable Chl a fluorescence yield, which reflects the reduced fraction of PSII RCs that generated a stabilized charge separated state. In addition, room temperature steady state spectrum of the D1-E130L mutant showed a remarkable blue shift (2 nm) in the Pheo Qx band relative to WT. The Gaussian deconvolution of ground state spectra of WT and D1-E130 mutants confirmed that the blue shift resulted from perturbation of the photochemically active Pheo, which absorbs at a longer wavelength (~543.5-544 nm) as does its counterpart in bacteria. A similar spectroscopic shift was also observed in the transiently (2 ns) reduced Pheo spectrum, suggesting that changes in the active branch Pheo H-bond strength, and/or a redirection of the electron to the inactive Pheo absorbing at lower wavelength (539-539.5 nm).

In contrast, the D1-E130H mutant had oxygen evolution rates comparable to WT and a relatively high yield of variable Chl a fluorescence (90% of WT). There were no noticeable differences from WT in the ground state or the reduced state Pheo Qx spectrum. These results were consistent with the observation that histidine substitution leads only to minor perturbations of the H-bond interaction.

Oxygen evolution and Chl a variable fluorescence yield were both slightly reduced for the D1-E130Q mutant relative to the D1-E130H mutant. Significantly, the transiently reduced spectrum of D1-E130Q mutant showed a distinct blue shift (1.5-2 nm) in the
Pheo Qx bleach band. Significantly, no corresponding blue shift was observed in ground state Pheo spectrum of this mutant. These observations suggested that not only the H-bond strength, but also the directionality in electron transfer might be modified by this mutation.

We also investigated the effects of these mutations on the integrity of the water-oxidizing complex by monitoring the S₂-multiline EPR signals. All mutants exhibited the same Mn-derived multiline line EPR spectrum as WT. In addition, the steady-state Qₐ⁻Fe²⁺ EPR signals generated at 200 K were essentially the same as in all the D1-E130 mutants and WT. Interestingly, the data from transient absorption kinetics recorded after 685 nm excitation pulse indicated that the lifetime for charge separation was similar for all three mutants. But the Chl a fluorescence decay kinetics obtained after excitation at 660 nm showed that the amplitude of the fast component varied between the mutants and WT, although no significant changes of initial rate were observed.

The work presented in Chapter 3 deals with the function of the inactive branch Pheo. Selective modification of the pigments in RC is demonstrated as a valuable approach to study the role of any individual pigment and its interactions with the protein environment. I employed site-directed mutagenesis to provide a Mg ligand for Pheo to convert it into a Chl, with substantially altered redox potential. Since Chl has no absorption in the Pheo Qx region, this substitution should facilitate identification of the Pheoactive absorption peak. According to the PS II structural models, a leucine residue at position D1-210 occupies a position over the center of macrocycle ring of Pheoinactive. D1-L210 was initially mutated to alanine (D1-L210A) and then to the potential metal
coordinating residue, histidine (D1-L210H) to replace the Pheo\textsubscript{inactive} with a Chl. Analyses of the pigment compositions of the histidine mutant indicate that the Pheo was converted to a Chl.

PS II electron transfer yield in D1-L210A mutant was marginally lower than WT, as implied by its oxygen evolution rate and data from Chl a fluorescence kinetics. The steady state EPR QA\textsuperscript{−} signal generated at 200 K, however, was \(\sim20\%\) greater than that of WT. In addition, no changes were found in the Pheo Qx band D1-L210A PS II RC spectrum.

On the other hand, the PS II membranes of D1-L210H mutant were characterized by a 90% reduction in the oxygen evolution rate. In addition, the variable Chl a fluorescence was substantially decreased (\(\sim5\%\) of WT). The nearly unnoticeable EPR signal of QA\textsubscript{−}Fe\textsuperscript{2+} further confirmed that electron transfer in the D1-L210H mutant was severely perturbed. Nevertheless, it still was able to assembly RCs. Analyses of the pigment composition of PS II RCs indicated that the histidine substitution resulted in the removal of Pheo\textsubscript{inactive} as predicted. This was further supported by the absorption spectra of WT and D1-L210H RC where the D1-L210H mutant had a \(\sim40-50\%\) reduction in the Pheo Qx absorption compared to WT. In addition, the Pheo Qx band was red shifted to 543.5-544 nm, which demonstrated that the remaining Pheo was Pheo\textsubscript{active}. The experiment using this modified PS II RC complex not only directly verified the different electron transitions between the two Pheos, but also accurately identified the Pheo\textsubscript{active} Qx absorption band. As a result, it was concluded that Pheo\textsubscript{inactive} absorbs light maximally around 540 nm.
At present, it is difficult to identify experimentally whether the deletion of Pheoinactive introduces a concomitant incorporation of a Chl. No matter what happened within D1-L210H RC, it is interesting to know the effect of depleting Pheoinactive on the energy and electron transfer of modified PS II. The light-induced difference absorption spectra obtained at 4°C in the presence of sodium dithionite and methyl viologen suggested that the photochemical activity of D1-L210H was reduced compare to WT since there was over a 50% decrease in the Pheo Qx bleach band, as well as a corresponding decrease in the Qy bleach band. A distinct red shift was found in both the Pheo Qx and Qy bleach region in the mutant, indicating the contribution of Pheoinactive reduction to the spectrum of WT in our experimental conditions, and the presence of only Pheoactive in the mutant. The photo-accumulated steady state Pheo− EPR signal in D1-L210H RCs was 20% of WT, in agreement with its decreased photochemical activity.

Comparison of the Chl CD spectra of WT and D1-L210H RCs recorded at 4°C revealed that a positive band peaking at 682 nm and negative lobe centered at 669 nm in WT were reversed in the mutant. This observation demostrated the excitonic interaction of pigments was changed in the mutant, particularly energy coupling to P680. The result from Chl a fluorescence emission spectrum at 77 K showed that D1-L210H RCs displayed a fluorescence emission maximum at 682 nm that was identical to that of WT, but the narrower shape of spectrum in the mutant reflected marked changes in energy transfer.

Based on the data collected, two interpretation are presented for the Chl substitution of Pheoinactive: first, the D1-L210H mutation has likely caused minor structural
modifications relative to WT, which induced the chain of observed changes. Second, no apparent tertiary structural perturbation occurred in mutant. But the introduced Chl changes the excitonic coupling between pigments in RC complex. The incorporated Mg changes the charge distribution in the tetrapyrrole macrocycle affecting pigment energy interactions.

Chapter 4 describes the results of characterizing modified PS II RC complexes with a putative Chl in place of Pheo\textsubscript{active} binding pocket. According to PS II models, the D2-L210 residue corresponds to M-L214 in purple bacteia \textit{Rb. sphaeroides}, its symmetrical side is D1-L210, which we discussed in Chapter 3. Thus, the D2-L210 residue is roughly located over the center of macrocyclic ring of Pheo\textsubscript{active}. Site-directed mutagenesis was employed to replace the conserved leucine residue on D2 protein with histidine to provide a ligand for a central Mg ion in Chl. This substitution was expected to incorporate a Chl in place of Pheo\textsubscript{active}.

The initial analysis of D2-L210H mutant Chl a fluorescence decay kinetics showed a 70% reduction in variable Chl fluorescence compared to WT. Under continuous light, the D2-L210H mutant exhibited a substantially reduced DMBQ-supported oxygen evolution rate (27% of WT). In agreement with these results the magnitude of QA\textsubscript{Fe}^{2+} was decreased by 75% relative to WT, indicating the suppression of electron transfer in the mutant.

To further study the effect of this mutation on charge separation, we attempted to isolate RCs from this mutant. Unfortunately, we failed to isolate an intact RC indicating that D2-L210H mutation was structurally unstable. Such results suggest that the histidine
side chain perturbed the structure of the RC. The unsuccessful isolation of D2-L210H RC also precludes our ability to characterize the optical transitions of Pheo_{active}.

We also observed that the intensity of steady-state EPR Pheo− signal in D2-L210H PS II membrane was only 60% of WT. The blockage of initial electron transfer was expected since a Chl has a much higher intrinsic midpoint potential than the native Pheo, which decreases the energy gap between P680* and P680^{+Chl}, and thus thermodynamically favors charge recombination.

Interestingly, the 77 K Chl a fluorescence emission spectrum of D2-L210H mutant had emission bands at 685 nm and 702 nm, whereas WT has emission bands at 685 nm and 695 nm bands. The unchanged 685 nm emission band is attributed to the antennae complex of PS II. On the other hand, the shift of 695 nm band, may reflect altered energy coupling between CP47 and P680.
LIST OF REFERENCES


Ono, T., Inoue, Y. (1985) S-state turnover in O2-evolving system of CaCl2-washed photosystem II particles depleted of three peripheral proteins as measured by


