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UMI
TIME-RESOLVED FLUORESCENCE STUDIES OF WILD TYPE AND MUTANT PHOTOSYSTEM II REACTION CENTERS ISOLATED FROM CHLAMYDOMONAS REINHARDTII.

DISSERTATION

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

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The Ohio State University 2000

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ABSTRACT

The photosystem II reaction center of higher plants and green algae has the unique function of harvesting light energy and converting it into chemical energy utilizing a highly efficient energy transfer process. Light energy is sequestered by antennae chlorophyll and funneled into the primary electron donor, P680. Once energy is trapped on P680 an electron is quickly transferred to the primary electron acceptor, pheophytin. Numerous ultrafast spectroscopic techniques have been used to investigate the energy transfer processes and primary charge separation that occur in the photosystem II reaction center. Based on these studies attempts have been made to assign time constants to each kinetic event. However, controversy regarding these assignments has developed. Site-directed mutagenesis has already proven to be a valuable method for studying the relationship between structure and function in the photosystem II reaction center. Energy transfer and charge separation studies of photosystem II reaction centers should also benefit from site-directed mutagenesis. We have used time-resolved fluorescence spectroscopy to probe the energy transfer processes and charge separation that occur in the photosystem II reaction center. The rates of both energy and electron transfer are sensitive to the distance and orientation between the donor and the acceptor chromophores. Therefore, altering the position of chromophores involved in either energy transfer or charge separation should affect the transfer rates. In order to
clarify the assignment of time constants in the photosystem II reaction center various mutants have been generated that target the coordination site of a specific chromophore. Comparisons drawn between the decay kinetics of the wild type PS II reaction centers and the mutant PS II reaction centers should provide insight into the kinetics of the reaction centers.
Dedicated to my husband Jamie and my parents
Lynn and Bill - Thank you for all of your
love, patience and support
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CHAPTER I

AN INTRODUCTION TO THE PHOTOSYSTEM II REACTION CENTER

PHOTOSYNTHESIS

Higher plants, green algae and certain bacteria are capable of harvesting solar energy and converting this light energy into chemical energy. The energy conversion process, photosynthesis, is a major source of both food and biological fuels on earth. In addition to food and fuel, photosynthesis is also responsible for the production of O₂ in the atmosphere. Due to its vast importance, understanding the fundamental processes of photosynthesis is an active area of research.

Both light absorption and energy conversion occur in protein-chromophore complexes that are found in membranes of photosynthetic organisms. In higher plants and green algae the five protein-chromophore complexes involved in photosynthesis are embedded in the thylakoid membrane inside the chloroplast. The five complexes are known as; 1) photosystem II, 2) photosystem I, 3) cytochrome b6f, 4) ATP synthase and 5) light-harvesting chlorophyll-protein complex.¹ The five complexes work in series to absorb light energy and transfer electrons and H⁺ across the thylakoid membrane. Figure 1.1 shows the functional
organization of the five protein-chromophore complexes. However, it should be noted this is not necessarily the structural organization of the protein-chromophore complexes in the thylakoid membrane. Plastoquinones and plastocyanins present in the thylakoid membrane are mobile and function to transport electrons from one complex to the next.

The actual conversion of light energy into an electron transfer process occurs in the photosynthetic reaction centers. Although structurally there are many different photosynthetic reaction centers they can all be placed into one of two different types of photosynthetic reaction centers. The distinction between the two types of reaction centers is based on the first stable electron acceptor of the reaction center, either an Fe-S center or a quinone site. In higher plants and green algae the two different types of photosynthetic reaction centers are referred to as the photosystem I complex and the photosystem II complex. The photosystem I reaction center (PS I) is an Fe-S type that is responsible for the reduction of NADP$^+$ that occurs during photosynthesis. The photosystem II reaction center (PS II) is a quinone type reaction center that carries out water oxidation during photosynthesis. The remaining three protein-chromophore complexes also have their own unique function. Cytochrome b6f transfers electrons from PS II to PS I while pumping H$^+$ across the membrane. The ATP synthase is responsible for the synthesis of ATP and the light harvesting complex acts as an antenna for absorbing light energy.
Figure 1.1 Functional arrangement of the five protein-chromophore complexes in the Thylakoid Membrane: PS II; Cytochrome b6f; PS I; ATP Synthase; Light-Harvesting Complex (not shown).
PHOTOSYSTEM II REACTION CENTER

Structural Organization

The entire PS II complex is a large assembly of polypeptides (approximately 20) and chromophores, chlorophylls, pheophytins and carotenoids. Figure 1.2.A shows a schematic of the PS II complex consisting of several antenna chlorophyll complexes (on the order of 250 chlorophyll are present in the complex), oxygen evolving enhancers and the PS II reaction center. The reaction center of the PS II complex shown in Figure 1.2.C is a small part of the entire PS II complex.\(^1\) Although the exact structure of the PS II reaction center is still not known (currently a crystal structure with only 4Å resolution has been obtained for the PS II reaction center)\(^3\) models have been developed for its structural organization based on its homology with the purple bacterial photosynthetic reaction center (PBPRC), whose crystal structure is known (1.6 Å resolution)\(^4,5\). The PBPRC consists of four proteins; the L and M polypeptides, the H subunit and cytochrome c. The L and M polypeptides coordinate both the chromophores and two quinones involved in energy and electron transfer in the reaction center. The chromophores present in the PBPRC consist of four bacteriochlorophylls, two pheophytins and at least one carotenoid. Research has shown that the PS II reaction center also consists of four proteins; the D1 and D2 polypeptides, and the smaller cytochrome b559 and psb I.\(^6\) The D1 and D2 polypeptides have a high degree of sequence similarity to the L and M polypeptides of the PBPRC.\(^3\) Based on similarities with the L and M polypeptides the D1 and D2 polypeptides are also thought to coordinate the chromophores and two quinones involved in energy and electron transfer processes in the PS II reaction
Figure 1.2: Schematic of the PS II complex (A) and the PS II reaction center (C).
center. Two chlorophylls located in the PS II reaction center are thought to make up the primary electron donor called P680, which is similar to the two bacteriochlorophylls that make up the chlorophyll special pair in the PBPRC. However, the two bacteriochlorophylls of the PBPRC chlorophyll special pair are strongly coupled while the two chlorophylls of the PS II reaction center P680 are not. In fact there is debate as to the true nature of the primary electron donor (P680) in the PS II reaction center. As in the PBPRC another two chlorophyll monomers in the PS II reaction center, accessory chlorophylls, are located very close to the two P680 chlorophylls. Two pheophytins are also present in the PS II reaction center. These four chlorophylls together with the two pheophytins make up the PS II reaction center core that closely resembles the PBPRC. One important difference between the two reaction centers is that the PS II reaction center contains two additional chlorophylls. The additional two chlorophylls found in the PS II reaction center, peripheral accessory chlorophylls, lie outside of the PS II reaction center core, this distance is thought to be approximately 30 Å away from P680.\textsuperscript{7-9} Figure 1.3 is a schematic arrangement of the six chlorophylls and two pheophytins located in the PS II reaction center.\textsuperscript{9,10}

Many of these structural details of the PS II reaction center have been confirmed by studies on isolated PS II reaction centers. The first PS II reaction center was isolated nearly 15 years ago and consists of only the D1 and D2 polypeptides, Cytochrome b559 and chromophores coordinated to D1 and D2.\textsuperscript{6} It was found that during the isolation procedure both quinones involved in the electron transfer process are lost, however, isolated PS II reaction centers are still capable of carrying out the primary charge separation. Thus, in addition to aiding structural investigations isolated PS II reaction centers are extremely
Figure 1.3: Schematic arrangement of the six chlorophylls, two pheophytins, $Q_A$, $Q_B$, tyrosine Z, tyrosine D, and Manganese complex present in the PS II RC.
useful for studying the energy transfer and primary charge separation that occur in the PS II complex.

*Energy and Electron Transfer Processes*

The PS II reaction center has the unique role of oxidizing water during photosynthesis. Unlike other photosynthetic reaction centers the midpoint potential of the primary electron donor of the PS II reaction center, P680, is shifted approximately 370 mV from the average midpoint potential of free chlorophyll (midpoint potential is 0.78 V). The high midpoint potential of P680 (1.1 V) is able to drive water oxidation (midpoint potential is 0.86 V). Water oxidation occurs in the PS II reaction center by a highly efficient electron transfer cycle. Figure 1.4 is a diagram of the electron transfer cycle that takes place in the PS II reaction center. Electron transfer starts once the primary electron donor, P680, is excited. In the excited state *P680* is able to transfer an electron to the primary electron acceptor, one of the two pheophytins (Pheo) present in the PS II reaction center. After the primary charge separation (P680*/Pheo') the active Pheo quickly passes the electron to the first stable electron acceptor Qa, the first of the two bound quinones involved in the electron transfer. From Qa the electron is transferred to Qb, the second bound quinone which gets doubly reduced. From here the electrons are transferred to the cytochrome b6f complex through the thylakoid membrane. The oxidized P680* can now accept an electron from a nearby tyrosine (Y2) residue which then extracts and electron from the Mn - water splitting complex. To carry out water oxidation this cycle must turn over four consecutive times.

The initial excitation of P680 is achieved either by P680 directly absorbing light
Figure 1.4: Electron Transfer Cycle of the PS II Complex.¹
energy or from energy transferred from another excited chromophore in the reaction center. Chlorophyll antenna complexes and the chromophores in the PS II reaction center trap light energy and funnel it to P680 in order to initiate charge separation. However, despite extensive research efforts the energy transfer processes and primary charge separation that occur within the PS II reaction center are still not well understood. One reason for the ambiguity is the lack of a clear understanding of the primary electron donor, P680. Currently, several models for the primary electron donor, P680, exist and each model carries a different picture of energy transfer and charge separation. One model based on the similarities of the PS II reaction center with PBPRC suggests that, like the chlorophyll special pair of the PBPRC, P680 is a chlorophyll dimer, composed of two of the four chlorophylls located in the PS II reaction center core. The remaining two accessory chlorophylls of the PS II reaction center core are chlorophyll monomers involved in rapid energy equilibration with P680. In addition, electron transfer from P680 to the active Pheo may occur through one of the chlorophyll monomers, as suggested for the bacterial reaction center. The two peripheral accessory chlorophylls transfer energy to P680, providing a link between the chlorophyll antenna complexes and P680. Excitation of P680 results in an excited state transition localized over the dimer. Research has shown that the P680 does exhibit chlorophyll dimer properties. However, in contrast to the chlorophyll special pair of the PBPRC the two chlorophylls thought to be P680 are not strongly excitonically coupled. In fact P680 has also been shown to exhibit chlorophyll monomer properties. In this model the four chlorophylls in the PS II reaction center core are only weakly coupled and energy rapidly equilibrates among all four chlorophylls until it is trapped on the P680 chlorophyll. The remaining two peripheral accessory
peripheral accessory chlorophylls funnel energy into the PS II reaction center core pigments. In this case the excited state transition of P680 would be localized on only the chlorophyll monomer. In addition to the monomer and dimer models of P680, Durrant and co-workers have also proposed a "multimer" model for P680. Based on both experimental and theoretical methods they suggest that P680 is a multimer of several weakly coupled chromophores, including the four chlorophylls of the PS II reaction center core and the pheophytin electron acceptor. Energy equilibration among the chromophores occurs rapidly and is delocalized over the "multimer." Another consequence of the multimer model is that rather than a normal intermolecular electron transfer, the primary charge separation may actually be an electron transfer within a large supra molecular complex. The two peripheral accessory chlorophylls located outside of the PS II reaction center core are not part of the multimer but are still involved in funneling energy into P680.

In addition to the lack of a clear functional model for the PS II reaction center, spectral distinctions among the antenna, primary electron donor, and primary electron acceptor of the PS II reaction center are not easily accomplished. Due to the similarity of the chromophores involved in energy transfer and charge separation the pigments are nearly isoenergetic resulting in severe spectral overlap of their absorption bands. Furthermore, unlike the PBPRC, the chromophores in the PS II reaction center are only weakly coupled; as a result the absorption band of the primary donor is not split as strongly as it is in the bacterial reaction center. Although this spectral overlap ensures efficient energy transfer, interpreting the functionality of the PS II reaction center is difficult.
A variety of experimental methods, EPR, circular dichroism, FTIR, spectral hole-burning, absorbance, and fluorescence studies, have been used to investigate the structure and functionality of the PS II reaction center. However, to get understanding of the energy transfer processes and the primary charge separation that occur in the PS II reaction center, techniques capable of probing the reaction center on the same time scale as these events must be used. Among all models of the PS II reaction center there is general agreement that energy equilibration and charge separation occur on the femtosecond to picosecond time scale. Therefore, transient absorption and time-resolved fluorescence spectroscopy have been widely used to study the energy transfer processes and charge separation of the PS II reaction center and to assign time constants to each event.19-21

SPECTROSCOPIC STUDIES OF THE PHOTOSYSTEM II REACTION CENTER

Transient Absorption Studies

Transient absorption spectroscopy has been used to study the excited state dynamics of numerous systems with subpicosecond resolution.22-27 In a typical transient absorption experiment a pump pulse is used to excite the sample. A probe pulse is then used to monitor the difference in the absorbance before and at various time delays after the pump-pulse excitation. Changes in the absorbance occur due to ground state photobleaching, stimulated emission, or absorption of a transient species. Kinetic information about a system can be obtained from transient absorption spectroscopy by monitoring changes in the absorbance at a single probe wavelength as a function of time.
The energy transfer processes and charge separation of the PS II reaction center have been studied extensively by transient absorption spectroscopy. The vast majority of transient absorption experiments on the PS II reaction center concentrate on monitoring changes in the chlorophyll Q_y band region. The probe wavelengths studied are typically between 680 and 690 nm. Based on results and interpretations of transient absorption data from the chlorophyll Q_y band controversy regarding the energy transfer rates and primary charge separation in the PS II reaction center has developed. Klug and co-workers have reported measuring a 21 ps component effective rate constant for charge separation. In support of this assignment, Donovan and co-workers have also reported a 20 ps effective rate constant for the charge separation of the PS II reaction center. However, other groups report a fast, approximately 3 ps, rate constant for the primary charge separation of the PS II reaction center. It should be noted that Donovan and co-workers have also reported the presence of a fast 2-3 ps component, however, they attribute this component to singlet-singlet annihilation. This assignment was based on the dependence of the 2-3 ps component on the intensity of the excitation energy. However, there is agreement among all groups that rapid, several hundred femtoseconds, energy equilibration among chromophores occurs within the PS II reaction center. One reason for the discrepancy in assignments is the severe spectral overlap of the absorbing pigments in this region which makes distinguishing between charge separation and energy transfer processes with these data rather difficult.

Recently, groups have begun to look for other regions that may be a better representation for the charge separation process of the PS II reaction center. The pheophytin Q_x region is one such region. This band is thought to be a sensitive indicator of charge
separation because it is a more narrow, well-defined band than the chlorophyll $Q_y$ band. In addition charge separation cannot occur without seeing a bleaching of this band. Based on transient absorption data from this region Wasielewski and co-workers have assigned two different rate constants to the primary charge separation of the PS II reaction center upon excitation of red edge of the chlorophyll $Q_y$ band.\textsuperscript{20,34} They attribute an 8 ps rate constant to charge separation that occurs in a fully equilibrated PS II reaction center and a 50 ps rate constant to charge separation limited by slow energy transfer from an accessory chlorophyll to P680.\textsuperscript{34} However, other groups monitoring the bleach of the pheophytin $Q_s$ band still report a 20-21 ps rate constant for the primary charge separation of the PS II reaction center.\textsuperscript{31} Although the pheophytin $Q_s$ band does not suffer from spectral congestion like the $Q_y$ band, interpreting kinetic data from this region is still difficult. It is not a straight forward task to distinguish whether the bleaching is a result of formation of Pheo-, as a result of charge separation, or $^1$Pheo*.\textsuperscript{20,34}

**Time-Resolved Fluorescence Studies**

Time-resolved fluorescence spectroscopy has been used to study the photochemistry of a variety of systems.\textsuperscript{35-38} One reason for its wide spread use is that fluorescence measurements in the time domain contain information on the rates of inter- and intra-molecular processes. The fluorescence lifetime of a molecule is sensitive to any process that provides a competing decay pathway back to the ground state, where the lifetime is shown by equation 1-1

$$\tau_F = \frac{1}{k_F + k_{ic} + k_{uc} + k_{ec}}$$  \textsuperscript{1-1}
$\tau_p$ is the molecule's fluorescence lifetime, $k_p$ is the radiative decay rate, $k_{ic}$ is the rate of internal conversion, $k_{ic}$ is the rate of intersystem crossing, and $k_{ec}$ is the rate of external conversion.\textsuperscript{39} Both energy transfer and electron transfer are two such competing processes. Although time-resolved fluorescence does not directly measure the energy transfer and electron transfer rates it is often used to study these processes; changes in the kinetics of the molecule will be reflected in the fluorescence lifetime.

In addition to transient absorption spectroscopy, time-resolved fluorescence spectroscopy has also been used to probe the energy transfer processes and charge separation that occur in the PS II reaction center complex. Time-resolved fluorescence studies have focused on exciting the PS II reaction centers on the blue edge of the chlorophyll Q$_y$ band then monitoring the fluorescence emission around 685 nm, thus measuring the fluorescence lifetime of excited chlorophyll and pheophytin chromophores in the PS II reaction center. Energy and electron transfer rates between these chromophores can be related to measured fluorescence lifetime components.

As with transient absorption experiments, the spectral congestion of the chlorophyll Q$_y$ absorbance band has made interpreting time-resolved fluorescence data difficult. The decay kinetics have been found to be rather complex, exhibiting numerous fluorescence lifetime components. Fluorescence lifetime components ranging from as fast as 1-6 ps to as slow as 30-50 ns have been measured for isolated PS II reaction centers.\textsuperscript{21,40,41} Assigning each lifetime component to a specific chemical event in the reaction center has not been an easy task and the origin of several lifetime components is still uncertain. The presence and
origin of two long-lived components, 5-6 ns and 30-50 ns, have been well-established in the literature. As mentioned earlier, the isolated PS II reaction center lacks the secondary electron acceptor, plastoquinone $Q_A$, as a result the radical pair state ($P680^*/Pheo^-$) decays by charge recombination. This charge recombination has been shown to occur in 30-50 ns. Within the PS II reaction center matrix free chlorophylls that are energetically uncoupled from the PS II reaction center complex are present. The 5-6 ns lifetime component is due to fluorescence from these free chlorophylls. Attempts have been made at assigning two short-lived components; 1-6 ps and 20-40 ps. However, there are conflicting assignments for these lifetime components and debate as to their true origin exist. Holzwarth and co-workers have reported the presence of a 1-6 ps component and attributed this lifetime to the primary charge separation ($P680^*/Pheo^-$). This assignment is in agreement with the 3 ps lifetime that has been established for the primary charge separation in the bacterial reaction center. Similarly, it is consistent with low-temperature spectral hole-burning experiments on the PS II reaction center, which report a 1-2 ps intrinsic rate constant. However, it must be noted that low-temperature hole-burning experiments provide a direct measurement of the intrinsic rate constant, while time-resolved experiments measure the "effective" or observed rate constant which could include energy equilibration. These groups then assign the 20-40 ps lifetime to slow energy transfer from accessory chlorophylls to $P680$. However, other groups do not report the presence of a fast 1-6 ps lifetime component. Instead, they assign a 20-40 ps lifetime to the primary charge separation ($P680^*/Pheo^-$). This assignment is based on a "trap-limited" model where primary charge separation does not occur until after energy has equilibrated in the PS II reaction center and has
been trapped on the primary electron donor P680. The lifetime component measured is therefore considered the "effective" rate constant for charge separation that includes both the time needed for energy to be transferred to and trapped on P680 and the subsequent electron transfer to the active pheophytin. This model could still be consistent with the intrinsic rate constant of 1-3 ps for primary charge separation. Again, there is general agreement among all groups that rapid energy equilibration occurs within the PS II reaction center.

In addition to spectral congestion another factor that has hindered accurately assigning fluorescence lifetimes to kinetic events is time resolution. The majority of time-resolved fluorescence experiments on the PS II reaction center have relied on the Time-Correlated Single Photon Counting method (TCSPC) to acquire fluorescence decay curves. However, single photon counting methods typically have instrument response functions on the order of 30 - 40 ps fwhm, therefore accurately measuring a lifetime less than 8 - 10 ps is not likely. Recently, streak cameras have begun to be used for time-resolved fluorescence studies on the PS II reaction center. Although slightly better time resolution can be obtained with this method, the single photon counting method still offers a higher signal to noise ratio. However, neither method is capable of measuring femtosecond energy transfer thought to occur among chlorophylls in the PS II reaction center. Fluorescence up conversion methods have also been used to investigate the decay kinetics of the photosystem II reaction center. Fluorescence up conversion methods are capable of resolving femtosecond processes, however, getting PS II reaction center samples that are concentrated enough to acquire signal is difficult.
**Low Temperature Studies**

As mentioned previously, both transient absorption and time-resolved fluorescence measurements have been hindered by spectral congestion of the PS II reaction center absorption bands, particularly the chlorophyll Q_y band. Therefore, understanding the absorption spectrum of the PS II reaction center is crucial in interpreting the spectroscopic data. A variety of low temperature studies, spectral hole burning, site-selection fluorescence, Gaussian decomposition and theoretical line shape calculations of the absorption spectrum, have been used to resolve the spectral contributions of the individual pigments in the PS II reaction center.\(^{49,51-54}\) The advantage low-temperature studies offer is that the inhomogeneously broadened lines, due to the different local environments of the pigments, can be resolved with more detail. Some controversy exists about exact assignment of several pigments in the chlorophyll Q_y absorption band, namely, whether the red edge of the chlorophyll Q_y band contains additional contribution from a chlorophyll or a pheophytin.\(^{49,52}\) However, there is general agreement that the blue edge of the Q_y band contains contributions from the peripheral accessory chlorophylls, an accessory chlorophyll and a pheophytin.\(^{53}\) The P680 chlorophylls and active pheophytin are thought to be nearly isoenergetic around 681 nm and dominate the red edge of the Q_y band.\(^{49}\) As a result of these studies the blue edge excitation of time-resolved fluorescence experiments are thought to excite preferentially the peripheral and accessory chlorophylls while the red edge excitation of transient absorption measurements preferentially excites P680.
SITE-DIRECTED MUTAGENESIS

The lack of a known high resolution crystal structure for the photosystem II reaction center has forced researchers to develop alternative methods for probing the structure of PS II. Site-directed mutagenesis is one method that has been used to develop a better understanding of both the structure and function of the PS II reaction center.\textsuperscript{55-60} Using structural models based on the homology between the purple bacterial reaction center (PBPRC) and the PS II reaction center, specific amino acid residues in the protein complex are replaced by another amino acid residue. Structure-function relationships may then be determined by comparisons between the experimental parameters obtained from the wild type PS II reaction centers and the mutant PS II reaction centers.

Site-directed mutagenesis has recently begun to be utilized in early energy and electron transfer investigations of the PS I reaction center in order to help clarify ambiguities in transient absorption and time-resolved fluorescence experiments.\textsuperscript{61} Similar site-directed mutagenesis studies into the primary photochemistry of the PS II reaction center have been proposed.\textsuperscript{62} Coupling either transient absorption or time-resolved fluorescence with site-directed mutagenesis can provide additional information into the functionality of the PS II reaction center. For these studies ideal sites to target for mutations are residues thought to be coordinated to or to interact with the chromophores involved in the early energy transfer processes and charge separation in the PS II reaction center. Specifically, proposed binding sites of the peripheral accessory chlorophylls, P680 chlorophylls, and pheophytins are target sites for mutations. Once target sites have been identified a variety of mutations are
possible at each site, Figure 1.5 shows relevant amino acid residues. However, it should be noted that not all mutations are capable of producing a functional PS II complex that can be studied.

Mutations to the amino acid sequence are classified as either conservative or non-conservative based on the similarities in chemistry between the amino acid residue and the replacement residue, most notably is the ability to ligate. The chromophores are thought to be held firmly in the protein matrix by hydrogen bonding with or coordinating to specific amino acids along either the D1 or D2 polypeptides. Replacing an amino acid residue that is involved in hydrogen bond interactions with a chromophore or coordinating with a chromophore with an amino acid residue that is also capable of hydrogen bonding or coordinating is termed a conservative mutation; however, if the replacement is not capable of hydrogen bonding or coordinating the mutation is designated as a non-conservative mutation. Both conservative and non-conservative mutations are capable of altering the orientation or the position of the specific chromophore with respect to the other chromophores present in the PS II reaction center. Even if the changes are small the overall effect on both the structure and function of the reaction center could be quite large.

The majority of research on the energy transfer processes and charge separation of the PS II reaction center has focused on reaction centers isolated from higher plants, such as spinach. However, higher plants are not easily manipulated using genetic engineering techniques. *Chlamydomonas reinhardtii*, a unicellular green alga, has proven to been an ideal organism for site-directed mutagenesis studies of the PS II reaction center.
Figure 1.5: Amino Acid residues located along the D1 and D2 polypeptides of the PS II RC that are of importance to the mutations used.
The organism has shown to be easy to manipulate genetically and mutants of *Chlamydomonas* have proven to be very stable. In addition, PS II reaction centers can now be isolated readily from *Chlamydomonas*.1,62

**OVERVIEW OF RESEARCH**

Understanding the energy transfer processes and charge separation that occur in the PS II reaction center has been the focus of numerous research efforts. Based on the interpretations drawn from both transient absorption and time-resolved fluorescence data two conflicting models for the energy transfer and charge separation in PS II have been put forth. Figure 1.6 represents a simplified schematic for Model One. Briefly, this model assigns a 1-6 ps lifetime to the primary charge separation that occurs after P680 has been excited. Fast energy equilibration, on the femtosecond time-scale, occurs among one pool of pigments, most likely the close lying accessory chlorophylls and P680. Slow energy transfer, 20-40 ps, occurs from an additional pool of pigments, most likely the peripheral accessory chlorophylls, to P680. Figure 1.7 shows a simplified schematic for Model Two. This model varies from Model One in two important ways. First, rapid energy equilibration, on femtosecond time scale, occurs among all the pigments. Secondly, a 20-40 ps time constant is assigned to charge separation which may or may not be limited by slow energy transfer among chlorophylls.

The objective of the research presented in this dissertation is to probe the energy
Figure 1.6 Model One of the Energy Transfer and Primary Charge Separation that Occurs in the PS II RC.
Figure 1.7 Model Two of the Energy Transfer and Primary Charge Separation that Occur in the PS II RC.
transfer processes and charge separation that occur in the PS II reaction center using time-resolved fluorescence spectroscopy. Specifically, the goal is to determine if model one or model two is a better representation of the energy transfer processes and the primary charge separation that occur in the PS II reaction center. To clarify the assignment of fluorescence lifetimes site-directed mutagenesis is used to generate mutant PS II reaction centers. Comparisons drawn among the decay kinetics of wild type PS II reaction centers and mutant PS II reaction centers are used to provide insight into the kinetics of the reaction centers. Sites targeted for mutagenesis are purported coordination sites of the chromophores involved in energy transfer and charge separation. In this way structure-function relationships which may help understand energy transfer and charge separation are studied.

In chapter II the details of the experimental methods and data analysis methods used for the research presented in this dissertation are discussed. Chapter III presents the results of excitation wavelength and emission wavelength dependence studies done with the PS II reaction center complex. In chapter IV, we detail the results obtained from comparisons of the D2-H117N, D1-H118Q mutant PS II reaction centers and the wild type PS II reaction center. The D1-H117N and D1-H118Q mutations alter the coordination sites of the two peripheral accessory chlorophylls which allow us to investigate the energy transfer from these chlorophylls to P680. Chapter V presents the results of energy transfer studies of the D1-T292L mutant PS II reaction center complex. The threonine at site D1-292 has been proposed to be involved in hydrogen bond interactions with one of the chlorophylls of P680. This mutation could change the energy and/or electron transfer processes of P680. In chapter VI, we present the results of time-resolved fluorescence experiments on the D1-
E130Q, D1-E130H and D1-E130L mutant PS II reaction centers. The active pheophytin is involved in hydrogen bond interactions with the glutamic acid at site D1-E130; altering this site will allow us to study the primary charge separation that occurs in the PS II reaction center. Finally, in chapter VII we summarize the research presented in this dissertation and put forth several ideas for future studies.
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CHAPTER II

EXPERIMENTAL AND DATA ANALYSIS METHODS

EXPERIMENTAL METHODS

Photosystem II Reaction Centers

Sample Preparation

*Generation of mutants and thylakoid preparations*

All PS II reaction center mutants were generated and thylakoid membranes were prepared as described separately.¹

*PS II reaction center complex isolation*

PS II reaction center complexes of *Chlamydomonas reinhardtii* were purified from PS II, BBY-type particles prepared by Triton X-100 (TX-100) solubization of thylakoids membranes according to the procedure of Berthold *et al.*² Briefly, however, BBY-type particles were solubilized with Triton X-100 followed by centrifugation at 100,000 g for 1 hour. The PS II fraction (supernatant) then was loaded onto a DEAE-Toyopearl 650S col-
umn (Rohm and Haas) and washed until the elute had an absorbance at 675 nm of less than 0.04 OD cm⁻¹. PS II reaction center particles were eluted using a NaCl gradient, reapplied to the column and selectively eluted again. The purified reaction centers were suspended in a buffer containing 20 mM Mes-NaOH (pH 6.0), 10% (W/V) glycerol, 2 mM n-dodecyl-β-maltoside (DM), 200 mM NaCl. The chlorophyll concentration of thylakoid and PS II membranes was determined by the method of Arnon⁴ while the chlorophyll, pheophytin and carotenoid concentration of the purified PS II reaction centers was done according to Eijckelhoff et al.⁵ For chlorophyll fluorescence decay experiments PS II reaction centers having a chlorophyll/ pheophytin ratio of 6-7/2 were used.

Sample Handling

Originally, after isolating the PS II reaction center complexes, samples were stored at -80 °C. However, after the PS II reaction center complexes were thawed they would degrade in less than one hour, as indicated by a 3 - 4 nm blue shift in the Qy band (typically the Qy band would shift from 675 nm to 671 nm during data acquisition). In addition, chlorophyll fluorescence decays acquired using PS II reaction centers stored at -80 °C were not able to be fit well, as determined by high reduced chi-square values and lifetimes spread across the majority of the fitting window. We then acquired chlorophyll fluorescence decays of PS II reaction center complexes immediately after they were suspended in the buffer solution. The “fresh” PS II reaction centers displayed minimal blue shift in the Qy band during the decay collection time (typically from 676-675 nm to 674-673 nm). In addition to little degradation, the chlorophyll fluorescence decay curves from “fresh” PS II reaction centers fit well, as indicated by low reduced chi-square values and reproducible lifetime
distributions. We then stored the PS II reaction center complexes at 77 K. Samples were thawed immediately before taking the chlorophyll fluorescence decay. Similar to the “fresh” PS II reaction center complexes the Qy band showed very little blue shift throughout the decay collection time (again 675 nm to 674-673 nm) and the decay curves fit well, as indicated by low reduced chi-square and lifetime components. Most chlorophyll fluorescence decays acquired for the work in this dissertation were run using PS II reaction center complexes stored at 77K.

Prior to running chlorophyll fluorescence decays the samples were allowed to thaw in ice. Approximately 1 mL of PS II sample was combined with 2 mL of buffer solution in a long-necked cuvette. The PS II reaction center samples were then degassed by bubbling argon through the long-necked sample cuvette for 3-4 minutes. The cuvette was then sealed using a ground glass stopper and wrapping the stopper and cuvette neck with parafilm. The samples were held at approximately 4°C with a temperature controlled bath (Fisher Scientific 901) and a home built sample cell holder. To prevent interference from sample denaturing and triplet state build up the samples were continuously stirred using a Teflon magnetic stir bar. Continuous stirring would allow fresh sample to circulate into the excitation beam path over the course of sample collection.

Absorption Measurements

The absorption spectrum (Perkin Elmer Lambda 20) for each photosystem II reaction center sample was taken prior to collecting the chlorophyll fluorescence decay to determine the quality of the reaction centers. The presence of the chlorophyll Qy band peak at
675-676 nm and the lack of a large shoulder at 434 nm was indicative of "healthy" uncontaminated PS II reaction center sample. Figure 2.1 shows the absorption spectrum for "healthy" uncontaminated wild type PS II reaction centers. In addition to the chlorophyll Qy band at 675 nm, the cytochrome b559 band (415.0), chlorophyll Soret band (434 nm) and pheophytin Qx band (544 nm) can also been seen. A blue shift in the Qy band, 671-673 nm, immediately after thawing was typical for damaged samples. A large shoulder seen at 434 nm is a result of contamination from CP47; Figure 2.2 shows a comparison of the absorption spectrum of uncontaminated PS II reaction centers and PS II reaction centers that have residual CP47. The absorption spectrum was taken again after acquiring the chlorophyll fluorescence decay to determine if sample degradation had occurred. Our "healthy" samples show little or no blue shift in the Qy absorption band, 673-674 nm, which indicates little damage to the PS II reaction centers occurred during the decay collection time. The PS II reaction center samples were changed for each chlorophyll fluorescence decay curve we acquired. The pheophytin Qx absorption band at approximately 540 nm was also used to make comparisons among wild type PS II reaction centers and mutant PS II reaction centers. Peakfit 4.0 was used to fit the pheophytin Qx absorption band.

**Time-Resolved Fluorescence Measurements**

Photosystem II chlorophyll fluorescence decays were acquired using a Time-Correlated Single Photon-Counting (TCSPC) system shown in Figure 2.3. A mode-locked Nd:YAG laser (Coherent Antares 76-s) was used to pump a synchronously-pumped cavity-dumped dye laser (Coherent 700 series; DCM; tunable from 610 - 680 nm; 1-4 MHz rep
Figure 2.1: Absorption spectrum of Wild Type PS II RC. The following bands are present: cytochrome b559 (415.8 nm), Chl Soret (434.0 nm), Pheo Qx (540.6 nm), and Chl Qy (676.6 nm).
Figure 2.2: Comparison of the absorption spectra of Carotenoid uncontaminated and Carotenoid contaminated PS II RCs.
Figure 2.3: TCSPC experimental setup.
rate). The concentration of saturable absorber DQODCI was adjusted to give a temporal pulse width of approximately 3 ps. The laser pulse was directed to a beam splitter. A portion of the laser pulse was sent to a fast photodiode that initiates a “start” pulse. The pulse passed through a Constant Fraction Discriminator (CFD; Tennelec TC-455) to establish time zero and began charging a capacitor in the Time-to-Amplitude Convertor (TAC; Tennelec TC-864). The other portion of the pulse was used to excite the sample. The incident beam (~ 0.79 mm²) power was held at or under 8 mW during the entire decay collection. The PS II chlorophyll fluorescence emission was detected at 90° with respect to the sample excitation beam. The chlorophyll fluorescence emission was collected using a series of optics, including two broadband anti-reflection (BBAR) focusing lenses, a polarization analyzer (set at 54.7° with respect to the laser pulse), and a polarization scrambler, and focused on to the slit of a monochromator (American Holographic DB-10s). The collected photon then struck a Micro-Channel Plate Photomultiplier Tube (MCP-PMT; Hamamatsu R2809U-07), was amplified by a preamplifier (EG&G Ortec 9306), and sent to a picosecond timing discriminator (EG&G Ortec 9307). This “stop” pulse terminated the charging of the TAC capacitor. The signal was then sent to a Multi-Channel Analyzer (MCA; Tennelec PCA-II) that built a histogram representing the chlorophyll fluorescence decay (10,000 counts were collected in the peak channel). The instrument response function for the TCSPC system using this configuration was 38 ps fwhm.

The collection time needed for each chlorophyll fluorescence decay was minimized by replacing the Micro-Channel Plate Photomultiplier Tube described above with a red sen-
tive Micro-Channel Plate Photomultiplier Tube (MCP-PMT; Hamamatsu R3809U-51). The MCP-PMT was cooled using a high performance thermoelectric cooler (Hamamatsu C4878-01). The quantum efficiency for red wavelengths is almost 100 times greater for the R3809U-51 MCP-PMT than the R2809U-07 MCP-PMT (Figure 2.4). The instrument response function for the TCSPC system with this configuration was 42 ps fwhm. Collection times were reduced from an average of four hours to an average of forty minutes per PS II decay (for the 2.5 ns window), lowering the time each sample was exposed to laser excitation.

The time window of the TCSPC instrument was adjusted to control the lifetime resolution. To observe fast components of the PS II reaction center decay we used a 2.5 ns window, the smallest available window setting for our instrument. We were able to resolve lifetimes from approximately 4 ps to 1.5 ns with a 2.5 ns window setting. We would then enlarge our time window in order to resolve longer lived components. We tried several different long window settings; 50 ns, 75 ns and 150 ns, to get the best resolution of the long-lived components. Although all three window sizes were able to resolve four separate lifetime components, only the 150 ns window was able to fit the longest lived component accurately. Both the 50 ns and 75 ns windows would fit a long-lived component equal to the size of the decay window. Figure 2.5 and Figure 2.6 show the exponential series method (ESM) fits for the 50 ns and 75 ns windows respectively. The 50 ns window displays a very broad lifetime distribution scattered around 50 ns. Similarly, the 75 ns window shows a lifetime distribution centered around 75 ns. Figure 2.7 shows the exponential series method (ESM) fit for the 150 ns window. This fit shows the longest lifetime component from the PS
Figure 2.4: Comparision of the radiant sensitivity for the Hamamatsu R2809U-07 (Bialkali) and Hamamatsu R3809U-51 (Exd. Red Multialkali) MCP-PMT.
Figure 2.5: ESM lifetime distributions for Wild Type PS II RCs obtained in a 50 ns TCSPC window setting.
Figure 2.6: ESM lifetime distribution of Wild Type PS II RCs obtained in a 75 ns TCSPC window setting.
Figure 2.7: ESM lifetime distribution of Wild Type PS II RCs obtained in a 150 ns TCSPC window setting.
II reaction center complex to be centered around 35 ns, consistent with literature values. We were able to use the 150 ns window to measure accurately lifetimes between 1 ns and 35 ns.\textsuperscript{8}

The TCSPC experiment was run in reverse mode for the 2.5 ns window. Reverse mode differs from the previously described forward mode in that the “start” pulse is provided by photons emitted from the sample. The “stop” pulse occurs when a laser pulse hits the fast photodiode. Figure 2.8 shows the configuration for the electronics of both forward and reverse modes. Reverse mode was used to minimize instrument dead time, the time required by the instrument to process data. Due to statistical limitations, the count rate of collected photons must be held at or below 1% of the laser repetition rate to prevent multiple photon events from occurring.\textsuperscript{6,7} As a result in forward mode many “start” pulses do not have a corresponding “stop” pulse. However, in reverse mode every “start” pulse has a “stop” pulse associated with it, increasing the processing efficiency and decreasing collection time.\textsuperscript{9} Running in reverse mode was important for data collection in the 2.5 ns window. The decay collection time for the 2.5 ns window was significantly longer then for the 150 ns window, allowing a large number of dark counts to build up under the decay curve. Reverse mode allowed us to minimize the number of dark counts. Reverse mode could not be used for the longer 150 ns window due the arrangement of the delay line for our TCSPC experimental setup. However, due to the faster collection time in the 150 ns window, dark counts were not a significant issue.

The chlorophyll fluorescence decay curves obtained from damaged PS II reaction centers were not able to be fit well. Figure 2.9 shows a typical exponential series method.
Figure 2.8: Configurations for the TCSPC electronics in reverse mode and forward mode.
(ESM) fit for damaged PS II reaction centers. Figure 2.10 shows the ESM fit for the same type of PS II reaction center only “healthy”. Comparison of the two fits shows only “healthy” PS II reaction centers could be used to obtain meaningful results. However, unlike damaged PS II reaction center samples, PS II reaction center complexes contaminated with CP47 would fit, giving similar lifetimes as completely purified PS II reaction centers.

DATA ANALYSIS METHODS

Lifetime Fitting Methods

The photosystem II reaction center is a complex system, therefore, choosing a data analysis model that accurately represents this environment is an important issue to be addressed. Extracting meaningful kinetic parameters from the fluorescence decay curves of a system being studied can only be accomplished if an appropriate fitting model is chosen. The most common method for fitting fluorescence decay curves is to use a discrete sum of exponentials. Typically, 1 - 6 exact lifetime components are summed to create a fitted curve of the data. Described by equation 2-1

\[ I(t) = \sum_{i=1}^{n} \alpha_i \exp(-t / \tau_i) \]  

2-1

Where \( n \) is the number of discrete lifetime components, \( \alpha_i \) is the amplitude of each component and \( \tau_i \) is the lifetime of each component. However, the discrete sum of exponentials model does not account for fluctuations in lifetime components that occur due to heterogeneous environments. We have chosen instead to use a distribution of lifetimes as a model
Figure 2.9: ESM fit for damaged PS II RCs.
Figure 2.10: ESM fit for "healthy" PS II RCs.
for the PS II chlorophyll fluorescence decay process. We believe this method more accurately reflects the physical reality of energy fluctuations of the chromophores in the protein environment. The PS II reaction center is known to have a complex matrix that can vary slightly from one reaction center to another. In addition, chlorophylls present in the PS II reaction center are located in different local environments. The exponential series method (ESM) uses a continuous distribution of lifetimes to fit fluorescence decay curves. The ESM fitting method is similar to using a discrete sum of exponentials; however, a much larger number of lifetime components, n, is used and the lifetimes, $\tau_i$, are held constant while only their amplitudes, $\alpha_i$, are adjusted to optimize the fit. For the PS II reaction center decay curves we typically chose 200 lifetime components; the lifetime components were fixed values logarithmically spaced in the fitting window. The ESM fitting method has been shown to have advantages when fitting heterogeneous environments. We have compared both fitting methods with our chlorophyll fluorescence decays in an effort to find the most appropriate model to represent the PS II reaction center.

The exponential series method and the discrete lifetimes method could be used to produce acceptable fits based on reduced chi-square values and weighted residual plots. It should be noted when a discrete sum of exponentials was used the PS II fluorescence decays in both TCSPC windows could be adequately fit by four lifetime components. Based on the precision of our data (determined by counts per peak channel, CPC) adequate fits using a discrete sum of exponentials would be expected regardless of the true physical parameters. Due to the time required for collecting to 10,000 CPC and the fragile nature of the PS II reaction centers higher precision in the data was not possible without distorting
the fluorescence decay curves as a result of sample degradation. However, we believe the ESM method held several advantages over fitting to a discrete sum of exponentials. The ESM fits were reproducible from one PS II sample to another. Fitting to a discrete sum of exponentials would not consistently yield the same values for the intermediate lifetime components. When fitting the PS II decays, the ESM method also appeared to be more robust than using a discrete sum of exponentials. Minor adjustments made to the fitting parameters in the ESM fitting routine would not cause significant changes in the lifetimes. For instance, we were able to run fits of the same PS II decay curve in different ESM fitting windows without seeing deviations in the lifetimes. We could also vary the dark count level or the starting position of the fit without any effect on the lifetimes. In contrast, we could not make similar changes in the fitting parameters while fitting to a discrete sum of exponentials without noticeable deviations in the lifetimes.

We have screened both data analysis methods to identify methods which best represent the heterogenous environment of the PS II reaction center. We compared fitting chlorophyll fluorescence decays to a discrete sum of exponentials and to a continuous distribution of lifetimes and believe that the distribution of lifetimes model provides a better representation of the PS II reaction center environment. The ESM method was more robust and was reproducible from one PS II sample to another.

The lifetime components were weighted in order to account for the biasing of short lifetime components in the fitting methods. The area under the decay curve is a better representation of a lifetime's contribution rather than using the exact amplitude of the life-
time component. For the ESM fitting method we would weight the fits as follows: the amplitude of each lifetime component is multiplied by the lifetime to give an estimated area that represents each lifetime's contribution ($\alpha \tau$). The lifetime contribution ($\alpha \tau$) was then divided by the total area under the decay curve ($\Sigma \alpha \tau$) to give the percent contribution of each lifetime component.

All chlorophyll fluorescence decays were fit to a discrete sum of exponentials or to a continuous distribution of lifetimes. Decays fit to a discrete sum of exponentials were fit using a program by Dr. James N. Demas and Seth Snyder. Decays fit to a continuous distribution of lifetimes were fit using the Exponential Series Method (ESM) program by Dr. Timothy Rhodes.
REFERENCES


CHAPTER III

TIME-RESOLVED FLUORESCENCE MEASUREMENTS OF THE PHOTOSYSTEM II REACTION CENTER OF CHLAMYDOMONAS REINHARDTII: EXCITATION AND EMISSION WAVELENGTH DEPENDENCE

INTRODUCTION

The photosystem II reaction center of higher plants and green algae is responsible for converting light energy into chemical energy during photosynthesis. Light energy is harvested by antennae chlorophyll complexes located nearby the PS II reaction center and funneled into the primary electron donor, P680, of the reaction center. Light energy can also be directly absorbed by chromophores present in the PS II reaction center, including P680 itself. However, the exact nature of the energy transfer processes and primary charge separation are not well understood. Time-resolved fluorescence spectroscopy has been used extensively to investigate the energy transfer processes and charge separation that occur in the photosystem II reaction center. One reason for the popularity of using time-resolved fluorescence in studying the kinetics of the PS II reaction center is the sensitivity of fluorescence lifetimes to both energy transfer and electron transfer. The rate of both en-
ergy transfer and electron transfer affects a molecule's fluorescence lifetime; therefore, information about both energy transfer and electron transfer processes can be obtained by measuring fluorescence lifetimes. However, controversy regarding chlorophyll fluorescence lifetime assignments has hindered developing a kinetic model for the PS II reaction center. One reason for the confusion in assigning time constants is the lack of a functional model for the PS II reaction center. As we discussed in chapter I, several different models exist for the organization of the primary electron donor, P680. Based on the different organizations of P680 several kinetic models have been derived. There is general agreement the two peripheral accessory chlorophylls function to transfer energy into the primary electron donor, however disagreement about the rate of this energy transfer does exist. In addition, energy equilibration and charge separation occur among the core pigments, the four remaining chlorophylls and two pheophytins located in the PS II reaction center. Similar to the energy transfer rate from the peripheral accessory chlorophylls into the core, the energy and electron transfer rates among the core pigments have not been well established. To further complicate assignments the decay kinetics of the PS II reaction center are extremely complex, consisting of numerous lifetime components to assign. In order to gain insight into the energy transfer processes and charge separation that occurs in the PS II reaction center an extensive lifetime study should be done.

In order to develop a functional model for the energy transfer processes and charge separation that occur in the PS II reaction center a detailed analysis of the decay kinetics of the PS II reaction center needs to be accomplished. A valid model would then have to account for all the observed kinetics. As we mentioned in chapter I, low temperature
studies have been used to resolve the spectral contributions of the chromophores present in the PS II reaction center. These studies have shown that it is not possible to excite specific chromophores selectively in the PS II reaction center. However, it is possible to excite chromophores preferentially. Decomposition of the PS II Q\textsubscript{y} band shows the two peripheral accessory chlorophylls absorb on the blue edge of the band while P680 dominates absorption on the red edge of the band\textsuperscript{7,8}. Therefore, excitation on the blue edge of the Q\textsubscript{y} band should preferentially excite the two peripheral accessory chlorophyll while more red excitation preferentially excites P680, the accessory chlorophyll and pheophytins. Time-resolved chlorophyll fluorescence experiments using blue edge excitation should result in a large contribution from energy transfer from the peripheral accessory chlorophylls and subsequent charge separation. Similarly, time-resolved chlorophyll fluorescence experiments using red edge excitation should consist largely of charge separation and energy equilibration among the core pigments. A comprehension investigation into excitation wavelength and emission wavelength dependence of the PS II reaction center decay kinetic would be useful in developing a kinetic model for the reaction center.

In this chapter we use time-resolved fluorescence to study the excitation and emission wavelength dependence of the photosystem II reaction center kinetics. Chlorophyll fluorescence decays of wild type PS II reaction centers were acquired using a variety of excitation wavelengths and emission wavelengths. We show that the PS II reaction center kinetics are extremely sensitive to excitation wavelength.
EXPERIMENTAL

Sample Preparation

Wild type photosystem II particles were generated by Dr. Jun Wang in Dr. Sayre’s laboratory. The wild type PS II reaction centers were then isolated using the procedure described in Chapter II. The PS II reaction centers were stored at 77 K until used for chlorophyll fluorescence measurements. Immediately before acquiring chlorophyll fluorescence decays the PS II reaction centers were allowed to thaw in the dark at 0 °C. For chlorophyll fluorescence decay experiments discussed in this chapter, PS II reaction centers having a chlorophyll/ pheophytin ratio of 6-7/2 were used.

Time-Resolved Fluorescence/Absorption Measurements

Thawed photosystem II reaction center samples were diluted with buffer solution before acquiring the chlorophyll fluorescence decay. Wild type PS II samples were diluted until the absorbance at 650 nm was between 0.1-0.8 a.u.. The absorption spectrum (Perkin Elmer Lamba 20) for each PS II reaction center sample was taken to determine the quality of the PS II reaction center sample. Figure 3.1 shows the absorption spectrum for a “healthy” wild type PS II reaction center sample. As mentioned previously, the presence of the chlorophyll Qy band peak between 674-676 nm and only a small shoulder at 434 nm is indicative of a “healthy” PS II reaction center sample. The wild type PS II reaction center samples used in this dissertation were typically on the red edge of this range. The absorption spectrum was taken again after the chlorophyll fluorescence decay curve was acquired to determine the extent of sample degradation. Our wild type PS II reaction center samples showed
Figure 3.1: Absorption spectrum of Wild Type PS II RCs.
very little blue shift in the chlorophyll Q<sub>y</sub> band indicating little damage had occurred to the PS II reaction centers during the collection time. Figure 3.2 compares the absorption spectra for wild type PS II reaction center sample prior to laser exposure, after 40 minutes of exposure, and after 80 minutes of exposure. Figure 3.3 compares a second wild type PS II reaction center sample before exposure and after 4 hours of exposure. Throughout the entire range of exposure time the chlorophyll Q<sub>y</sub> band shifts less than 2 nm indicating minimal sample damage.

Photosystem II chlorophyll fluorescence decays were acquired using the Time-Correlated Single-Photon Counting (TCSPC) system discussed in Chapter II. The DCM dye laser was tuned to three different wavelengths for the excitation wavelength scan. The excitation wavelengths used were 650 nm, 660 nm and 670 nm. The emission wavelength was held at 684 nm for all three excitation wavelengths used. To avoid interference from scattered light the excitation wavelength needed to be at least 10 nm away from the emission wavelength. The incident beam (≈0.8 mm<sup>2</sup>) was held at or below 8 mW at the sample. The emission wavelength scan was done using an excitation wavelength of 660 nm. Four different emission wavelengths were monitored between 679-692 nm.

The TCSPC was run in reverse mode for both the excitation wavelength scan and the emission wavelength scan. The lifetime resolution was controlled by the size of the TCSPC window. The TCSPC window size was set at 2.5 ns for both the excitation wavelength scan and the emission wavelength scan. The PS II reaction center chlorophyll fluorescence decay curves were then fit to a continuous distribution of lifetimes.
Figure 3.2: Comparison of the Wild Type PS II RCs absorption spectra after 0 minutes, 40 minutes and 80 minutes of exposure to the laser.
Figure 3.3: Comparison of the Wild Type PS II RCs absorption spectra before exposure and after 4 hours of exposure to the laser.
RESULTS

We measured the picosecond chlorophyll fluorescence kinetics of the PS II reaction center at three different excitation wavelengths ranging from 650 nm to 670 nm. All three chlorophyll fluorescence decay curves were analyzed using the ESM fitting method discussed in chapter II. Figure 3.4 shows the chlorophyll fluorescence decay taken at 684 nm in a 2.5 ns TCSPC window and the ESM fitted curve for wild type PS II reaction centers using 650 nm excitation. The weighted residuals for the ESM fit, also shown in Figure 3.4, appear to be very random which indicated the ESM method obtained a good fit. The lifetime distributions obtained from the ESM fit are shown in Figure 3.5. Five distinct lifetime components can be seen; 3-5 ps, 29-35 ps, 110-125 ps, 478-550 ps and 1.5 ns. Figure 3.6 shows the chlorophyll fluorescence decay acquired at 684 nm in the 2.5 ns TCSPC window and the ESM fitted curve and ESM weighted residuals for wild type PS II reaction centers using 660 nm excitation. Again the ESM fit obtained for the PS II reaction centers, shown in Figure 3.7, contains five distinct lifetime components; 1-2 ps, 24-28 ps, 70-80 ps, 200-236 ps and 1.5 ns. A similar set of lifetimes were obtained at 684 nm from 670 nm excitation. Figure 3.8 shows the chlorophyll fluorescence decay, ESM fitted curve, and ESM weighted residuals for the wild type PS II reaction centers using 670 nm excitation. Five distinct lifetime distributions, shown in Figure 3.9, can still be seen; 1-2 ps, 21-26 ps, 57-70 ps, 236-260 ps and 1.5 ns. Similar decay kinetics were seen in mutant PS II reaction centers using 650 nm and 660 nm excitation.

In addition to measuring the chlorophyll fluorescence decay kinetic of PS II reaction
Figure 3.4: Fluorescence decay curve for Wild Type PS II RCs obtained at 684 nm using 650 nm excitation.
Figure 3.5: ESM lifetime distributions for Wild Type PS II RCs obtained at 684 nm using 650 nm excitation.
Figure 3.6: Fluorescence decay curve for Wild Type PS II RCs obtained at 684 nm using 660 nm excitation.
Figure 3.7: ESM lifetime distributions for Wild Type PS II RCs obtained at 684 nm using 660 nm excitation.
Figure 3.8: Fluorescence decay curve for Wild Type PS II RCs obtained at 684 nm using 670 nm excitation.
Figure 3.9: ESM lifetime distributions for Wild Type PS II RCs obtained at 684 nm using 670 nm excitation.
centers at several different excitation wavelengths we have also obtained chlorophyll fluo-
rescence decay curves at four different emission wavelengths, ranging from 679 nm to 692
nm. The excitation wavelength was held at 660 nm for all four emission wavelengths moni-
tored. Figure 3.10 shows the chlorophyll fluorescence decay curve and ESM fitted curve
obtained from wild type PS II reaction centers emitting at 679 nm. The weighted residuals
from the ESM fit, again shown in Figure 3.10, are random indicating the ESM method was
able to obtain a good fit for the PS II chlorophyll fluorescence decay curve. The corre-
sponding ESM lifetime distributions are presented in Figure 3.11. The five lifetime compo-
nents obtained are distributed at 1-2 ps, 23-28 ps, 85-100 ps, 200-248 ps, and 1.5 ns. The
chlorophyll fluorescence decay, ESM fitted curve, and ESM weighted residuals for the wild
type PS II reaction center chlorophyll fluorescence at 683 nm is shown in Figure 3.12. The
five lifetime components; 1-2 ps, 24-30 ps, 70-85 ps, 212-236 ps, and 1.5 ns, are very
similar to the five lifetime components seen from 679 nm emission. Figure 3.13 shows the
ESM lifetime distributions obtained from collecting 683 nm emission. Figure 3.14 shows
the chlorophyll fluorescence decay curve, ESM fitted curve, and ESM weighted residuals of
the wild type PS II reaction center obtained when monitoring the emission at 687 nm. The
corresponding ESM fits for the 687 nm decay curve are presented in Figure 3.15. Again theive lifetime components; 1-3 ps, 30-40 ps, 70-80 ps, 248-300 ps and 1.5 ns, seen are very
similar to the five components seen in the 679 nm and 683 nm fits. We also detected the
chlorophyll fluorescence from wild type PS II reaction centers at 692 nm. Figure 3.16
shows the chlorophyll fluorescence decay curve, ESM fitted curve, and ESM weighted
residuals obtained. Although the percent contribution of several components was very
Figure 3.10: Fluorescence decay curve for Wild Type PS II RCs obtained at 679 nm emission using 660 nm excitation.
Figure 3.11: ESM lifetime distributions for Wild Type PS II RCs obtained at 679 nm emission using 660 nm excitation.
Figure 3.12: Fluorescence decay curve for Wild Type PS II RCs obtained at 683 nm emission using 660 nm excitation.
Figure 3.13. ESM lifetime distributions for Wild Type PS II RCs obtained at 683 nm emission using 660 nm excitation.
Figure 3.14: Fluorescence decay curve for Wild Type PS II RCs obtained at 687 nm emission using 660 nm excitation.
Figure 3.15: ESM lifetime distributions for Wild Type PS II RCs obtained at 687 nm emission using 660 nm excitation.
small, five separate lifetime distributions can still be distinctly seen, shown in Figure 3.17. Comparison of the lifetime component as a function of emission wavelength does not show any significant shifts in lifetimes, however, changes can be seen in the percent contribution of several components.

DISCUSSION

The lifetime components of the PS II reaction center exhibit a strong dependence on both excitation wavelength and emission wavelength. Comparison of the lifetime components obtained from chlorophyll fluorescence decays taken at different excitation wavelengths shows significant changes in the decay kinetics (Fig. 3.5, Fig. 3.7 and Fig. 3.9). A 478-550 ps can be seen in the decay kinetics of PS II reaction centers excited at 650 nm. However, upon going to 660 nm excitation this component is no longer present. Similarly, the 110-125 ps component seen in the decay kinetics of the PS II reaction center using 650 nm excitation is not seen when the PS II reaction centers are excited with 660 nm. However, the decay kinetics of the PS II reaction center seen using 670 nm excitation are very similar to those observed from 660 nm excitation. Although the 478-550 ps and 110-125 ps lifetime components are not present in either decay there are still five unique lifetime components seen when the PS II reaction centers are excited at 660 nm and 670 nm. There is no change in the 1-2 ps lifetime component and the 1.5 ns components when the excitation is changed from 650 nm to 660 nm or 670 nm. Similarly, there is essentially no shift in the 29-35 ps component obtained from 650 nm excitation. 660 nm excitation produces a 24-28
Figure 3.16: Fluorescence decay curve for Wild Type PS II RCs obtained at 692 nm emission using 660 nm excitation.
Figure 3.17: ESM lifetime distributions for Wild Type PS II RCs obtained at 692 nm emission using 660 nm excitation.
ps and 670 nm shows a 21-26 ps (Fig. 3.5, Fig. 3.7 and Fig. 3.9). These small variations are within the uncertainty of our fitting procedure. In addition to these three components (1-2 ps, 24-28 ps and 1.5 ns), chlorophyll fluorescence decays obtained with either 660 nm or 670 nm excitation contained two other lifetime components. A strong contribution to the chlorophyll fluorescence is from a process that occurs with a time constant of 200-236 ps. Although not as significant of a contribution as the 200-236 ps component another lifetime component was seen at 70-80 ps. Based on the spectral deconvolution of the Qy absorbance band we suggest the changes in the chlorophyll fluorescence decay kinetics due to changes in the excitation wavelength are a result of preferentially exciting different chromophores in the reaction center. However, we have not been able to determine the specific origin of several of these lifetime components. Further research is needed to assign these lifetime components to particular events in the PS II reaction center. Work done on intact PS II particles could provide insight into lifetimes associated with charge recombination processes. Intact PS II particles are capable of carrying out the entire electron transfer cycle, thus eliminating any charge recombination processes. As a result any lifetimes associate with charge recombination in the PS II reaction center should be lost in the decay kinetics of PS II particles.

Unlike changes in the excitation wavelength the PS II reaction center decay kinetics do not show any significant changes in lifetimes with changes in the emission wavelength monitored. Comparison of the lifetime components obtained for all four emission wavelengths (Fig.3.11, Fig. 3.13, Fig. 3.15 and Fig. 3.17) shows distributions all within the same
ranges; 1-2 ps, 24-34 ps, 70-100 ps, 200-300 ps and 1.5 ns. Although the positions of the lifetime distributions do not change close inspection of the components show a significant change in their relative percent contribution. Figure 3.18 shows the change in percent contribution of each lifetime for the emission wavelengths studied. Across the emission range of 679 nm to 687 nm the percent contribution of the 24-34 ps component varies slightly (Fig 3.11, Fig 3.13, Fig. 3.15). Figure 3.18 shows the 24-34 ps lifetime component has its maximum contribution to the chlorophyll fluorescence decay at 683 nm and very little contribution at 692 nm. We suggest these data support assigning the 24-34 ps component to a transfer process that occurs from a blue absorbing pigment to a red absorbing pigment. Based on the spectral deconvolution of the chlorophyll Q_y absorbance band these data would indicate assigning the 24-34 ps component to transfer processes from the peripheral accessory chlorophylls to P680 chlorophylls and accessory chlorophylls. The percent contribution of the 1-2 ps lifetime component also shows a significant change across the emission wavelengths studied. Figure 3.18 shows the maximum contribution of the 1-2 ps lifetime component to the chlorophyll fluorescence decay occurs around 687 nm with very little contribution from 679 to 683 nm. We suggest these data support assigning the 1-2 ps lifetime component to a transfer process that occurs from the red absorbing pigments of the chlorophyll Q_y band. Based on the spectral deconvolution of the chlorophyll Q_y band our data would support assigning the 1-2 ps component to transfer processes that occur from the P680 chlorophylls, accessory chlorophylls and pheophytins. Similarly, both the 70-100 ps and 200-300 ps lifetime components show significant changes in their percent contribution across the emission wavelengths studied, Figure 3.18. Much like the 1-2 ps component
Figure 3.18: Percent contribution of each lifetime component versus the chlorophyll fluorescence emission wavelength.
the 70-100 ps lifetime component has a maximum contribution to the chlorophyll fluorescence decay around 687 nm which suggests assigning this component to transfer processes from the red absorbing pigments. However, even at blue emission wavelengths the 70-100 ps component is a significant part the chlorophyll fluorescence decay. The 200-300 ps lifetime component is a significant part of the chlorophyll fluorescence decay at all emission wavelengths. Furthermore, the 200-300 ps lifetime component shows a strong contribution at both 683 nm and 692 nm which makes assigning this lifetime to specific pigments in the PS II reaction center difficult.

The origin of the picosecond lifetime components seen in the decay kinetics of the PS II reaction center are not well understood. As we have mentioned previously debate regarding the assignment of the two fastest components, 1-2 ps and 24-34 ps, does exist. The 24-34 ps component has been attributed to both charge separation and energy transfer processes. The 1-2 ps component has also been assigned to charge separation, although several groups do not report the presence of a fast 1-2 ps component.\textsuperscript{1,14,15} The remaining components seen in the picosecond decay kinetics of the PS II reaction center are attributed to energy transfer processes and/or radical pair recombination. A kinetic model developed for the functionality of the PS II reaction center must be able to explain both the excitation wavelength and emission wavelength dependence of lifetime components. We suggest our data support model one, Figure 1.6. Model one assigns the 24-28 ps lifetime component to energy transfer from the peripheral accessory chlorophylls to P680 and a 1-6 ps lifetime to the primary charge separation. Our emission wavelength scan indicated the 24-28 ps life-
time is due to a transfer process from blue adsorbing pigments to more red absorbing pigments which agrees with attributing this lifetime to energy transfer from the peripheral accessory chlorophyll to P680 rather than primary charge separation. Similarly, our emission wavelength scan suggest the 1-2 ps lifetime component is due to transfer processes from the red absorbing pigments which suggests attributing the 1-2 ps lifetime component to transfer processes from P680 and the pheophytins.

CONCLUSION

We have used time-resolved fluorescence to study the excitation wavelength and emission wavelength dependence of the kinetics of wild type PS II reaction centers. To study the excitation wavelength dependence we obtained the chlorophyll fluorescence decays of wild type PS II reaction centers at three different excitation wavelengths, 650 nm, 660 nm and 670 nm while holding the emission wavelength constant. Comparison of the ESM distributions show the PS II reaction center lifetime components are extremely sensitive to the excitation wavelengths used, especially the intermediate components. This can be seen by the large changes in lifetime components as the excitation wavelength is altered. No significant changes due to excitation wavelength changes were seen in the 1-2 ps and 1.5 ns components, however, both components push the limits of our instrumental setup. The 1-2 ps component is beyond the resolution of our instrument, while the 1.5 ns component is a limiting value due to the size of the TCSPC window. We studied the emission wavelength dependence of the PS II decay kinetics by monitoring four different emission wavelength
using one excitation wavelength. No changes in the lifetime components were seen. However, on the red edge of emission wavelengths detected the 24-34 ps component almost disappears which we suggest supports assigning the 24-34 ps lifetime component to transfer processes from the peripheral accessory chlorophylls. In addition, the 1-2 ps lifetime component shows very little contribution on the blue edge of the emission wavelengths studied which we suggest supports assigning the 1-2 ps lifetime component to transfer processes from the red absorbing pigments, P680 chlorophylls, accessory chlorophyll and pheophytins.
REFERENCES


CHAPTER IV

CHLOROPHYLL FLUORESCENCE DECAY KINETICS OF WILD TYPE, D2-H117N, and D1-H118Q MUTANT PHOTOSYSTEM II REACTION CENTERS ISOLATED FROM CHLAMYDOMONAS REINHARDTII

INTRODUCTION

A variety of ultrafast spectroscopic techniques has been used extensively to investigate the energy transfer processes and charge separation that occur in wild type photosystem II reaction centers. Based on information obtained from these studies and the structural information that is known about the PS II reaction center several models have emerged to explain the energy and electron transfer processes. As we mentioned in chapter I, the reaction center core contains four of the six chlorophylls known to be present in the PS II reaction center and the two pheophytins. Research has shown that after excitation of chromophores present in the PS II reaction center an ultrafast energy equilibration occurs, on the order of several hundred femtoseconds. Due to the close proximity of the core pigments with one another and functional models predicting weak coupling among the core pigments, this ultrafast component has been assigned to energy equilibration among the core pigments after excitation. The two additional chlorophylls located in the PS II reac-
tion center are considered peripheral accessory chlorophylls. These two peripheral accessory chlorophylls are thought to lie approximately 30 Å away from the chlorophyll special pair and function as an energy transfer link between antennae chlorophyll complexes and P680.\textsuperscript{3,5} Based on this evidence several groups have predicted the two peripheral accessory chlorophylls make up a second pool of pigments that transfer energy to P680 at a slower rate than the energy equilibration that occurs among the reaction center core pigments. Holzwarth and co-workers have assigned a 20-30 ps time constant to this energy transfer rate.\textsuperscript{6,7} However, other groups do not attribute a 20-30 ps time to this energy transfer rate, instead they suggest this transfer rate is still on the same time scale as the ultrafast femtosecond energy equilibration of the core pigments or they link it to a 20-30 ps charge separation process.\textsuperscript{8-10} As a result of these two different assignments a great deal of controversy exists as to the true nature of energy transfer among chlorophylls located within the PS II reaction center.

Site-directed mutagenesis has proven to be a valuable tool in gaining further insight into the functionality of the PS II reaction center. It is now possible to make specific changes in the amino acid sequence of the D1/D2 polypeptides. Time-resolved fluorescence studies of specific mutant PS II reaction centers should be extremely useful for investigating the energy transfer processes that occur in the PS II reaction center. The kinetics of energy transfer between pigments in mutant PS II reaction centers can then be compared to the wild type PS II reaction centers. Two sites of interest in the PS II reaction center for studying the energy transfer rate between the peripheral accessory chlorophylls and P680
are histidines located at site 118 on the D1 polypeptide and site 117 on the D2 polypeptide. Figure 4.1 is a diagram of the D2 polypeptide chain highlighting the location of the D2-117 histidine. Figure 4.2 is a diagram of the D1 polypeptide chain highlighting the location the D1-118 histidine. These histidines are proposed binding sites for the peripheral accessory chlorophylls. A conservative replacement of the histidine residues would still allow the chlorophylls to be bound while possibly making changes in the distance and the orientation of each chlorophyll with respect to P680. A non-conservative replacement of the histidine residues would likely prevent coordination of the chlorophylls, as a result the chlorophylls would be free to move away from their position. Either type of mutation would be expected to affect the energy transfer between the peripheral accessory chlorophylls and P680.

In the present study we use time-resolved fluorescence spectroscopy to probe energy transfer in wild type PS II reaction centers, D2-H117N mutant PS II reaction centers and D1-H118Q mutant PS II reaction centers isolated from Chlamydomonas reinhardtii. The D2-H117N reaction center is a conservative mutant, replacing the histidine at site 117 with asparagine. We show that this substitution results in a change in the energy transfer rate from the peripheral accessory chlorophylls to P680. The D1-H118Q mutation is also a conservative replacement, altering the proposed coordination site of the other peripheral accessory chlorophyll. However, unlike the D2-H117N mutation we do not see any significant changes in the energy transfer rate from the peripheral accessory chlorophylls to P680.
Figure 4.1: Model of the D2 polypeptide highlighting the histidine located at site 117.
Figure 4.2: Model of the D1 polypeptide highlighting the histidine at site 118.
EXPERIMENTAL

Sample Preparation

The wild type PS II particles, D2-H117N mutant PS II particles and D1-H118Q PS II particles were generated by Dr. Jun Wang in Dr. Sayre's laboratory. Both the wild type PS II reaction centers and mutant PS II reaction centers were then isolated using the procedure described in chapter II. After isolation the PS II reaction centers were stored at 77 K. Prior to acquiring the chlorophyll fluorescence decays the PS II reaction centers were allowed to thaw in the dark at 0 °C. For chlorophyll fluorescence decay experiments in this chapter, PS II reaction centers having chlorophyll/pheophytin ratio of 6-7/2 were used.

Time-Resolved Fluorescence/Absorption Measurements

The absorption spectrum (Perkin Elmer Lambda 20) for each photosystem II reaction center sample was taken prior to running the chlorophyll fluorescence decay to determine the quality of the reaction centers. PS II reaction centers were diluted until the absorbance at 650 nm was 0.1-0.6 a.u. Figure 4.3 shows the absorption spectrum for the wild type PS II reaction centers. The presence of the chlorophyll Q_y band peak at 676 nm and the lack of large peak at 434 nm was indicative of a “healthy” reaction center. The absorption spectrum was taken again after acquiring the chlorophyll fluorescence decay to determine if sample degradation had occurred. Our samples had little or no blue shift in the chlorophyll Q_y absorption band, 674 nm, which indicates little damage to the PS II reaction centers occurred during the decay collection time. Figure 4.4 shows the absorbance spectra of the D2-H117N PS II reaction center before the chlorophyll fluorescence decay was acquired
Figure 4.3: Absorbance spectra of wild type and D2-H117N PS II RC taken immediately before acquiring the fluorescence decay.
Figure 4.4: Absorbance spectra of D2-H117N PS II RC taken before and after collecting the fluorescence decay curve.
and after the chlorophyll fluorescence decay was taken. The chlorophyll Qy band blue
shifted only 1.7 nm during the acquisition time. The chlorophyll fluorescence count rate
was also monitored to determine the extent of sample degradation. All PS II reaction
centers showed a drop in the chlorophyll fluorescence count rate during the first 10 minutes
of laser exposure. The chlorophyll fluorescence count rate for damaged PS II reaction
centers would continue to drop throughout the entire decay collection time. The wild type
PS II reaction centers and both mutant PS II reaction centers were stable during the acqui­
sition time, showing only an initial drop in the chlorophyll fluorescence count rate and
holding constant during the rest of the collection time.

All PS II chlorophyll fluorescence decays were taken using the Time-Correlated
Single Photon Counting (TCSPC) method described previously in Chapter II. The DCM
dye laser was tuned to give an excitation wavelength of 660 nm. The incident beam power
(≈0.8 mm²) was held at or below 8 mW for both the wild type PS II reaction centers and
mutant PS II reaction centers. The chlorophyll fluorescence emission was collected at 684
nm. The time window of the TCSPC instrument was adjusted to control the lifetime resolu­
tion. To observe fast components of the PS II reaction center decay we used a 2.5 ns
window. We were able to resolve lifetimes from approximately 4 ps to 1.5 ns with this
window setting. We would then enlarge our time window to 150 ns in order to resolve
longer lived components. The longer time window was used to measure lifetimes between
1 ns and 35 ns. The TCSPC was run in forward mode for the 150 ns window setting and in
reverse mode for the 2.5 ns window setting. The decays were then fit using a distribution of
lifetimes.16,17

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RESULTS

In Figure 4.5 we present the PS II wild type reaction center chlorophyll fluorescence decay taken using a 150 ns window. Figure 4.6 shows the fitted lifetime distributions for the decay in Figure 4.5. The chlorophyll fluorescence decay could be fit with four lifetime components centered at 550 ps, 2.5 ns, 5.5 ns and 35 ns. The 550 ps component is an artificial value. Owing to the size of the observation window all short-lived components are fit to this limiting value. In order to observe directly the short-lived components we obtain decays with a 2.5 ns window. In Figure 4.7 we show the PS II wild type reaction center chlorophyll fluorescence decay taken with a 2.5 ns window. Figure 4.8 shows the corresponding lifetime distributions for the decay. Five lifetime components at 1-2 ps, 24-28 ps, 60-70 ps, 200-236 ps, and 1.65 ns were needed to fit the 2.5 ns window chlorophyll fluorescence decay. Similar to the longer-time window, the component at ~1 ps is likely a limiting value for components faster than our time resolution. And the 1.6 ns component represents all the long-lived components. The two windows give rise to seven separate, identifiable components in the chlorophyll fluorescence decay of PS II.

To probe the energy transfer of the peripheral accessory chlorophylls we compared the fast decay kinetics of the D2-H117N PS II reaction centers and D1-H118Q PS II reaction centers with the wild type PS II reaction centers. The chlorophyll fluorescence decay of the D2-H117N mutant reaction center taken in the longer window (~150 ns) gave similar lifetime components to those obtain from the wild type reaction centers taken in the longer window. The only significant difference between the lifetime components of the wild type
Figure 4.5: Chlorophyll fluorescence decay curve of the Wild Type PS II RC obtained at 684 nm using 660 nm excitation in a 150 ns TCSPC window setting.
Figure 4.6: ESM Lifetime components for the Wild Type PS II RC chlorophyll fluorescence decay curve obtained at 684 nm using 660 nm excitation in a 150 ns window.
Figure 4.7: Chlorophyll fluorescence decay curve of Wild Type PS II RC obtained at 684 nm using 660 nm excitation in a 2.5 ns TCSPC window setting.
Figure 4.8: ESM lifetime components for the Wild Type PS II RCs chlorophyll fluorescence decay curve obtained at 684 nm using 660 nm excitation in a 2.5 ns window.
reaction centers and D2-H117N mutant reaction centers is seen in the shorter window (~2.5 ns). Figure 4.9 shows the chlorophyll fluorescence decay of the D2-H117N mutant reaction center taken in the 2.5 ns window. The chlorophyll fluorescence decay for the D2-H117N PS II reaction center also fit to five lifetime distributions in this window (Figure 4.10). The lifetimes were centered at 1-2 ps, 10-12 ps, 60-70 ps, 200-236 ps and 1.75 ns. A comparison of the decay components of the wild type PS II reaction center and of the D2-H117N mutant PS II reaction center show a significant change in only one of the five components. In the wild type PS II reaction center there is a fast component at 24-28 ps, however in the D2-H117N PS II reaction center there is no contribution from a 24-28 ps component but rather we observe a 10-12 ps lifetime distribution. Figure 4.11 shows the chlorophyll fluorescence decay curve and ESM fitted curve of the D1-H118Q mutant PS II reaction center obtained in the 2.5 ns TCSPC window setting. Similar to both the wild type PS II reaction centers and the D2-H117N mutant PS II reaction centers the chlorophyll fluorescence decay curve of the D1-H118Q mutant PS II reaction centers acquired using the 2.5 ns window also fit to five lifetime components. The ESM lifetime distributions for the D1-H118Q mutant PS II reaction center are presented in Figure 4.12. Five unique components can be seen at 1-2 ps, 24-28 ps, 70-80 ps, 212-236 ps, and 1.5 ns. Unlike the D2-H117N mutant PS II reaction centers no significant shift was seen in the 24-28 ps lifetime component. Instead, the decay kinetics of the D1-H118Q mutant PS II reaction centers are almost identical to the decay kinetics of the wild type PS II reaction centers.
Figure 4.9: Chlorophyll fluorescence decay curve for the D2-H117N PS II RC obtained at 684 nm using 660 nm excitation in a 2.5 ns TCSPC window setting.
Figure 4.10: ESM lifetime components for the D2-H117N PS II RC chlorophyll fluorescence decay curve obtained at 684 nm using 660 nm excitation.
Figure 4.11: Fluorescence decay curve of D1-H118Q PS IIIRCs obtained at 684 nm using 660 nm excitation in a 2.5 ns TCSPC window setting.
Figure 4.12: ESM lifetime distributions for D1-H118Q PS II RCs
fluorescence decay curve obtained at 684 nm using 660 nm excitation.
DISCUSSION

The time resolved chlorophyll fluorescence decays of the wild type PS II reaction centers show nine lifetime components in the two time windows (Fig. 4.6 and 4.8), of these nine there are seven unique components; 1-2 ps, 24-28 ps, 60-70 ps, 200-236 ps, 2.5 ns, 5.5 ns, and 35 ns. The origin of the two long-lived components has been well established in the literature and our lifetimes of 5.5 ns and 35 ns (Fig. 4.6) agree well with lifetimes seen by other groups. The presence of the 5.5 ns component is due to chlorophylls that are energetically uncoupled from the reaction centers. To confirm this assignment in our PS II decays and with the ESM fitting method we measured the fluorescence decay of chlorophyll in methanol, Figure 4.13. We obtained a single ESM lifetime at 5.6 ns (Figure 4.14). We obtained the chlorophyll for this measurement by extracting chlorophyll from leaves using methanol. The presence of the very long-lived component has been assigned to recombination of the primary charge separated state P680+/Pheo-. The intermediate lifetime components (in our decays 60-80 ps, 200-236 ps and 1.5 ns) have been attributed to energy transfer and/or radical pair relaxation processes.

The presence of a 1-6 ps component has previously been reported by Holzwarth and co-workers based on time-resolved chlorophyll fluorescence measurements of PS II reaction centers isolated from spinach. They assigned this lifetime to the primary charge separation (P680+/Pheo-). However, debate about this assignment exists. It is agreed that ultrafast energy transfer among chlorophylls in the PS II reaction centers does occur on the femtosecond time scale. Our time-resolved chlorophyll fluorescence measurements show
Figure 4.13: Fluorescence decay curve of extracted chlorophyll in methanol obtained at 684 nm using 660 nm excitation.
Figure 4.14: ESM lifetime distribution for chlorophyll fluorescence decay obtained at 684 nm using 660 nm excitation.
the presence of an extremely fast component. The chlorophyll fluorescence decays we acquired for both the wild type and D2-H117N mutant PS II reaction centers were consistently fit with a 1-2 ps lifetime distribution (Fig. 4.8 and 4.10). This lifetime is beyond the resolution of our TCSPC system, therefore, we will not attempt to assign an exact lifetime value or attribute this component to a specific kinetic event. We suggest that this lifetime distribution is a combination of the extremely fast events resolved into one lifetime component, much like the 550 ps component we see in the 150 ns window fit. To ensure this component was not a fitting artifact we used the wild type PS II decay curve seen in figure 4.7 to generate synthetic decay curves that lacked the two fastest components, 1-2 ps and 24-28 ps (Figure 4.15). The ESM fit for the synthetic decay curves were identical to the wild type PS II fit seen in figure 4.7 except neither short lifetime component appear (Figure 4.16).

In addition to the 1-6 ps component we see a lifetime component at 24-28 ps in the wild type reaction centers. Comparison of this component of the two PS II reaction centers shows a change from 24-28 ps for the wild type PS II reaction center (Fig. 4.8) to 10-12 ps for the D2-H117N mutant PS II reaction center (Fig. 4.10). The remaining six lifetimes for the two PS II reaction centers reveal no significant deviations. This would indicate the 24-28 ps component and the 10-12 ps component arise from the same process. The histidine replaced in the D2-H117N reaction center is a proposed binding site for one of the two peripheral accessory chlorophylls located in the PS II reaction center. Histidine is capable of coordinating the peripheral chlorophyll. The D2-H117N mutant makes a conservative replacement of the histidine with asparagine so the chlorophyll may still be bound by the
Figure 4.15: Comparison of the authentic WT PS II RC decay curve versus a synthetic WT PS II decay curve missing the two short lived components
Figure 4.16: ESM lifetime components for the Wild Type PS II RCs synthetic chlorophyll fluorescence decay curve in a 2.5 ns Window.
amino acid. However, the native binding site has been altered. As a result a change in the bond strength is expected, leaving two different possibilities for the structural organization of the D2-H117N reaction centers. First, the peripheral accessory chlorophyll is still bound to the D2-H117N residue. This organization would result in slight changes to both the distance and the orientation of the peripheral accessory chlorophyll with respect to P680. These factors are both important contributors in the Förster energy transfer rate, shown in equation 4-1

\[
k_{r} = \frac{9(\ln 10)\kappa^{2}Q_{d}J}{128\pi^{5}n^{4}N\tau_{d}R^{6}}
\]

where \(\kappa^{2}\) is the orientation factor for the dipole-dipole interaction (determined by the angle between the donor and acceptor dipoles), \(Q_{d}\) is the fluorescence quantum yield of the donor without the presence of the acceptor, \(n\) is the refractive index of the medium between the donor and acceptor, \(N\) is Avogadro's number, \(\tau_{d}\) is the fluorescence lifetime of the donor without the presence of the acceptor, \(R\) is the distance between the center of the acceptor and donor chromophores, and \(J\) is the normalized spectral overlap. The distance between the two chromophores has an inverse sixth order dependence. Thus, even small changes in either the distance could have a large effect on the energy transfer rate. Similarly, even small changes in the orientation of the donor and acceptor chromophores would have a large effect on the energy transfer rate. Although the exact value of \(\kappa^{2}\) is difficult to determine in solution its value can range from 0 to 4.0.22 The minimum value is obtained when the dipoles of the donor and acceptor chromophores are perpendicular to one another and the
maximum value is obtained when the donor and acceptor dipoles are parallel to one another.\textsuperscript{22} Table 4-1 shows how the energy transfer rate would change if the mutation resulted in only a change in the orientation. Similarly, Table 4-2 shows the change in the energy transfer rate if the mutation resulted in only a change in the distance between the donor and acceptor chromophores. Table 4-3 shows how the energy transfer rate is affected by a change in both the orientation and distance between the donor and chromophores due to the mutation. Although the numbers used for the calculations in Table 4-1, Table 4-2 and Table 4-3 are only experimental calculations based on a 29.1 Å distance between the peripheral accessory chlorophyll and P680 and random $\kappa^2$ values it can be seen that even slight changes can result in an energy transfer rate that is over 4 times faster or slower than the original energy transfer rate. As a result of the D2-H117N mutation we saw an increase in the lifetime of almost 2.5 times that of the wild type PS II reaction center. Because Förster energy transfer is a competing pathway for deexcitation to the ground state the fluorescence lifetime of the chromophore is affected, shown by equation 4-2

$$\tau_F = \frac{1}{k_F + k_{ic} + k_T + k_{isc}} \quad 4-2$$

where $\tau_F$ is the molecule's fluorescence lifetime, $k_F$ is the rate of fluorescence, $k_{ic}$ is the rate of internal conversion, $k_T$ is the Förster transfer rate and $k_{isc}$ is the rate of intersystem crossing.\textsuperscript{22} Thus, if there is a change in the energy transfer rate between chlorophylls in the D2-H117N reaction center, one would expect to see a change in the fluorescence lifetime of the chromophore. The only significant change we see in the decay kinetics is a change in the
Wild Type PS II RC
(ratio of $\kappa^2/R^6$) | Mutant PS II RC
(ratio of $\kappa^2/R^6$) | Factor by which $K_T$
changes due to mutation
--- | --- | ---
3.294E+51* | 8.234E+50 | 4
3.294E+51* | 2.470E+51 | 1.333
3.294E+51* | 3.294E+51 | 1
3.294E+51* | 4.117E+51 | 0.8
3.294E+51* | 4.940E+51 | 0.667
3.294E+51* | 5.764E+51 | 0.571
3.294E+51* | 6.587E+51 | 0.5

*A value of 29.1 Å is assumed for R (the distance between the donor and acceptor) for both wild type and mutant PS II RC.

(*) Indicates a value of 2.0 was assumed for $\kappa^2$ for wild type PS II RC.

Value of $\kappa^2$ was increased by 0.5 from 0.5 to 4.0 for the mutant PS II RC.

Table 4-1: Comparison of the energy transfer rate for wild type and mutant PS II RC. All values in the energy transfer equation are held constant except for $\kappa^2$. $\kappa^2$ is assumed to change due to the mutation.
<table>
<thead>
<tr>
<th>Wild Type PS II RC (ratio of $\kappa^2/R^6$)</th>
<th>Mutant PS II RC (ratio of $\kappa^2/R^6$)</th>
<th>Factor by which $K_T$ changes due to mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.294E+51*</td>
<td>3.294E+51*</td>
<td>1</td>
</tr>
<tr>
<td>3.294E+51*</td>
<td>2.974E+51*</td>
<td>1.1076</td>
</tr>
<tr>
<td>3.294E+51*</td>
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<td>1.3520</td>
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<tr>
<td>3.294E+51*</td>
<td>2.210E+51*</td>
<td>1.4901</td>
</tr>
</tbody>
</table>

*Value of 29.1 Å was used as the value of $R$ for the wild type PS II RC (the distance between donor and acceptor).

* (* ) Indicates a value of 2.0 was assumed for $\kappa^2$.

*For Mutant PS II RC the value of $R$ is increased by 0.5 Å up to 2.0 Å.

Table 4-2: Comparison of the energy transfer rates for wild type and mutant PS II RC
All terms of the energy transfer rate are held constant and only the distance is assumed to change due to the mutation.
<table>
<thead>
<tr>
<th>Wild Type PS II RC  (ratio of $\kappa^2/R^6$)</th>
<th>Mutant PS II RC  (ratio of $\kappa^2/R^6$)</th>
<th>Factor by which $K_T$ changes due to mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.294E+51*</td>
<td>6.09034E+50</td>
<td>5.407924</td>
</tr>
<tr>
<td>3.294E+51*</td>
<td>1.8271E+51</td>
<td>1.802641</td>
</tr>
<tr>
<td>3.294E+51*</td>
<td>2.4361E+51</td>
<td>1.351981</td>
</tr>
<tr>
<td>3.294E+51*</td>
<td>3.04517E+51</td>
<td>1.081585</td>
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<tr>
<td>3.294E+51*</td>
<td>4.87227E+51</td>
<td>0.675991</td>
</tr>
</tbody>
</table>

- A value of 29.1 Å is assumed for $R$ (the distance between the donor and acceptor) for wild type.
- Mutation was considered to change the distance $R$ by approximately one bond length (1.4 Å).
- (*) Indicates a value of 2.0 was assumed for $\kappa^2$ for wild type PS II RC.
- Value of $\kappa^2$ was increased by 0.5 from 0.5 to 4.0 for the mutant PS II RC.

Table 4-3: Comparison of the energy transfer rate for wild type and mutant PS II RC. All values in the energy transfer equation are held constant except for $\kappa^2$ and $R$. Mutation is assumed to alter both orientation and distance between donor and acceptor.
24-28 ps component (Fig. 4.7) to 10-12 ps (Fig. 4.9). This would suggest a relationship between the peripheral accessory chlorophyll and the 24-28 ps lifetime component, more specifically the 24-28 ps component is due to an energy transfer process involving the peripheral accessory chlorophyll and P680. Alternatively, the peripheral accessory chlorophyll may no longer be bound to site 117 in the D2-H117N PS II reaction centers. The PS II reaction center preparations we used, however, had 6-7 chlorophylls/2 pheophytins suggesting that the peripheral accessory chlorophyll was still bound to the asparagine residue. If, however, the peripheral accessory chlorophyll coordinated by the D2-H117N mutant was not bound then energy transfer from this peripheral accessory chlorophyll to P680 would be lost. We did not see a loss of any lifetime component, only a shift in the 24-28 ps component. However, if we consider an energy equilibration model rather than a direct energy transfer we may still expect to see a change in the lifetime. Energy equilibration occurs among a pool of pigments, if one chlorophyll is removed there could be an effect on the overall equilibrium position for energy transfer among the pigment pool. As a result a change in the energy equilibration time would be expected.

The D1-H118Q mutant PS II reaction center makes changes to the coordination site of the other peripheral accessory chlorophyll. Similar to the D2-H117N mutant the D1-H118Q mutant makes a conservative replacement of a histidine residue, however, a different amino acid residue has been substituted into the D1 chain. The histidine residue is replaced by a glutamine residue. In addition to replacing the histidine with glutamine several other mutations to the D1-H118 site were studied. However, L, R and N mutations, corresponding to leucine, arginine, and asparagine, were unstable in Chlamydomonas
reinhardtii. In the Q mutant PS II reaction center the chlorophyll can still be coordinated to the site, however, the mutation may alter both the distance and orientation of the chlorophyll with respect to P680. Comparison of the circular dichroism (CD) spectra of the wild type PS II reaction center with the D1-H118Q PS II reaction center did not show a change in the Q_y band of the two reaction centers. The CD spectra of the D2-H117N PS II reaction center showed a significant difference in the Q_y band compared to both the wild type PS II reaction center and the D1-H118Q PS II reaction center. This would indicate the D1-H118Q mutation does not alter the orientation of any of the absorbing pigments of the Q_y band, which includes the peripheral accessory chlorophyll located at site 118. However, the D2-H117N mutation does appear to make a significant change in the orientation of a pigment absorbing in the Q_y band, the peripheral accessory chlorophyll located at site 117 on the D2 polypeptide. Our time-resolved chlorophyll fluorescence data support this. As mentioned previously, both distance and orientation are important contributors in the Förster energy transfer rate. Any change, even small changes, in distance or orientation of the peripheral accessory chlorophyll with respect to P680 would be expected to alter the energy transfer rate between chlorophylls. Due to the similarities between the asparagine residue and the glutamine residue we would expect to see changes in the slow energy transfer rate of the peripheral accessory chlorophyll much like we did in the D2-H117N PS II reaction center. However, unlike the D2-H117N mutant PS II reaction center, comparison of the decay kinetics for the wild type PS II reaction center (Fig 4.8) with the D1-H118Q mutant PS II reaction center (Fig. 4.12) shows no significant shifts in any of the lifetime components. We feel this indicates that no significant change in the orientation of the peripheral
accessory chlorophyll with respect to P680 has occurred as a result of the D1-H118Q mutation, however, the D2-H117N mutation does cause a change in orientation between the peripheral accessory chlorophyll and P680. As a result of this change in either distance or orientation due to the D2-H117N mutation we see a change in the energy transfer rate of the D2-H117N mutant PS II reaction center. Therefore, we still attribute the 24-28 ps lifetime component to energy transfer among coupled chlorophylls.

Other time-resolved chlorophyll fluorescence and transient absorption studies done with the wild type PS II reaction centers isolated from spinach also have reported the presence of a 15 - 35 ps lifetime. Time-resolved chlorophyll fluorescence studies have typically used an excitation wavelength on the blue edge of the chlorophyll Q, absorption band, preferentially exciting the peripheral accessory chlorophylls. Based on decay associated spectra this component has often been attributed to energy transfer among chlorophylls in the reaction center. However, transient absorption studies have mainly excited on the red edge of the chlorophyll Q, band, preferentially exciting P680. Many of the groups exciting on the red edge have assigned the 15 - 35 ps lifetime to the primary charge separation, P680'/Pheo'. Our chlorophyll fluorescence studies have focused on preferentially exciting the peripheral accessory chlorophylls. Based on our results, we suggest the 15 - 35 ps lifetime component is due to energy transfer among coupled chlorophylls in the reaction center and P680. However, we cannot rule out that the 15 - 35 ps component seen using red edge excitation is the result of a different process.
The time-resolved chlorophyll fluorescence data we obtained for the wild type PS II reaction center and the D2-H117N mutant PS II reaction center suggest that energy transfer from the peripheral accessory chlorophylls to P680 should be assigned a time constant of 24-28 ps. Altering the proposed coordination site of the peripheral accessory chlorophyll resulted in a significant shift in only the 24-28 ps lifetime component of the PS II reaction center. However, a similar change to the proposed coordination site of the second peripheral accessory chlorophyll, the D1-H118N mutation, did not show a significant change in the 24-28 ps component or any other lifetime component of the PS II reaction center. Based on the CD spectra of the wild type, D2-H117N and D1-H118Q PS II reaction centers we attribute the lack of lifetime changes in the D1-H118N PS II reaction center to the type of mutation. Replacing the histidine located at site D2-117 with asparagine affects the orientation of the a pigment absorbing in the chlorophyll Qy band which can be seen by the large change in the chlorophyll Qy band of the D2-H117N PS II reaction center CD spectrum. However, replacing the histidine at site D1-118 with glutamine does not appear to affect the orientation of a pigment that absorbs in the chlorophyll Qy band. The wild type PS II reaction center and the D1-H118Q PS II reaction center have identical chlorophyll Qy bands in their CD spectra. Similarly, we saw a significant change in the decay kinetics of the D2-H117N PS II reaction center in comparison to the wild type PS II reaction center, while the D1-H118Q PS II reaction center showed no differences from the wild type PS II reaction center.
Although our time-resolved fluorescence data support assigning the 24-28 ps lifetime component to energy transfer from the peripheral accessory chlorophylls we cannot as yet establish if the 20-30 ps component seen in blue edge excitation experiments is due to the same phenomena as the 20-30 ps component seen in experiments using red edge excitation. In addition, we cannot yet establish if the 24-28 ps component is due to energy transfer from the peripheral accessory chlorophyll to P680 or due to energy equilibration among a pool of chlorophylls that include the peripheral accessory chlorophyll.
REFERENCES


CHAPTER V

ENERGY TRANSFER AND CHARGE SEPARATION STUDIES OF D1-T292L MUTANT PHOTOSYSTEM II REACTION CENTERS

INTRODUCTION

In chapter IV we discussed the energy transfer from the peripheral accessory chlorophylls into the reaction center core, specifically P680. The peripheral accessory chlorophylls are thought to be a link for energy transfer from the antennae chlorophyll complexes into the primary electron donor, P680. In addition to the peripheral accessory chlorophylls, the accessory chlorophylls and pheophytins located in the reaction center core are also capable of transferring energy to P680 to initiate charge separation. Once excited P680 is capable of transferring an electron to the primary electron acceptor, the active pheophytin. This primary charge separation initiates the electron transfer cycle used to drive water oxidation in the photosystem II reaction center. The energy and electron transfer processes of P680 have been the subject of numerous studies. Based on the results and the interpretation of these studies controversy has developed regarding the rate at which both energy transfer and charge separation occur within the PS II reaction center. It is generally agreed that energy transfer among the core pigments occurs very fast, on the
femtosecond time scale. However, as we have previously mentioned there is considerable
debate as to the rate of primary charge separation. Several groups have reported the pri-
mary charge separation occurs in approximately 3 ps while other groups have reported 21
ps. A time constant of 3 ps is consistent with hole-burning experiments done at low
temperatures which have established the direct time constant to be 3 ps. In addition, the
time constant for primary charge separation in the purple bacterial reaction center has been
established to be 2-3 ps. However, a time constant of 21 ps for the effective primary
charge separation is consistent with the trap-limited model that has been put forth for PS II.

In chapter I we mentioned that the true nature of P680 is not well understood and
different models have been put forth. Based on the similarities with the PBPRC one model
put forth is that P680 consists of two chlorophylls excitonically coupled. It has been
proposed that these two chlorophylls are coordinated to the conserved histidines located at
sites D1-H198 and D2-H197. Attempts to isolate PS II reaction centers that have made
mutations at these positions has not yet been successful. However, in addition to these sites
other residues have been proposed to interact with the two P680 chlorophylls. One particu-
lar location is threonine residue located at site 292 on the D1 polypeptide. The threonine at
site D1-292 has been proposed to be involved in hydrogen bond interactions with a keto
group on one of the two chlorophylls of the special pair. This interpretation has been
challenged recently by the work of Noguchi et al., who have shown by FTIR that the keto
C=O group of P680 is free from hydrogen bond interactions. Site-directed mutagenesis
studies at this site might provide insight into the energy and electron transfer processes of
P680. If P680 is bound to this site altering the residue could change either the distance or
orientation of the P680 chlorophyll with respect to the other chromophores present in the PS II reaction center.

In this chapter we present the time-resolved chlorophyll fluorescence results of another mutant reaction center isolated from *Chlamydomonas reinhardtii*; D1-T292L. The D1-T292L mutant PS II reaction center is a non-conservative mutation that replaces the threonine located at site 292 with leucine. The threonine at site 292 that was altered can be seen highlighted in Figure 5.1. We show there are no significant changes in the decay kinetics of the D1-T292L mutant PS II reaction centers compared to the wild type PS II reaction centers.

**EXPERIMENTAL**

*Sample Preparation*

The wild type PS II particles and D1-T292L mutant PS II particles were generated by Dr. Jun Wang in Dr. Sayre's laboratory. The PS II reaction centers were isolated from the PS II particles using the procedure described in Chapter II. Prior to use the PS II reaction centers were stored at 77 K. The frozen samples were allowed to thaw in the dark at 0 °C immediately before acquiring the chlorophyll fluorescence decay. The chlorophyll fluorescence decays studied in this chapter were obtained from PS II reaction centers that contained 6-7 chlorophylls/2 pheophytins.
Figure 5.1: Model of the D1 polypeptide highlighting the threonine at site 292.
Time-Resolved Fluorescence/Absorption Measurements

To determine the quality of each PS II reaction center sample the absorption spectrum (Perkin Elmer Lambda 20) was measured prior to taking each chlorophyll fluorescence decay. The PS II reaction center samples were diluted with buffer until the absorbance at 650 nm was 0.2-0.4 a.u. Figure 5.2 shows the absorption spectrum of the PS II wild type reaction center and the D1-T292L mutant PS II reaction center. A “healthy” reaction center is indicated by the presence of the chlorophyll Qy band at 674 - 676 nm and only a slight shoulder at 434 nm. The absorption spectrum of the PS II reaction center samples were repeated after the chlorophyll fluorescence decay was taken to determine the extent of sample degradation. Our samples showed only a slight blue shift in the chlorophyll Qy band indicating little damage occurred to the reaction center samples during the fluorescence collection time. The chlorophyll fluorescence count rate was also monitored to determine the extent of sample degradation. The wild type PS II reaction centers were very robust showing only the initial 500 counts per second drop in the chlorophyll fluorescence count rate. Similarly, the D1-T292L PS II reaction center appeared to be stable during the collection time, showing only a slight drop in the chlorophyll fluorescence count rate (approximately 500 counts per second).

All PS II chlorophyll fluorescence decays were taken using the Time-Correlated Single Photon Counting (TCSPC) method described previously in chapter II. The excitation wavelength was tuned to 660 nm for both the wild type PS II reaction centers and mutant PS II reaction centers. The chlorophyll fluorescence emission was monitored at 684 nm. The TCSPC was run in reverse mode for all chlorophyll fluorescence decays discussed.
Figure 5.2: Comparison of the absorbance spectra of the Wild Type PS II RCs and D1-T292L PS II RCs.
in this chapter. The lifetime resolution was controlled by adjusting the TCSPC window setting. A 2.5 ns window setting was used to monitored the picosecond chlorophyll fluorescence decay components. All chlorophyll fluorescence decays were fitting using a distribution of lifetimes (Exponential Series Method).^{16,17}

RESULTS

To investigate the effects the D1-T292L mutation has on the energy and electron transfer processes of P680 we have focused on only the picosecond chlorophyll fluorescence decay kinetics of the wild type and D1-T292L PS II reaction centers. In Figure 5.3 we present the chlorophyll fluorescence decay curve for wild type PS II reaction centers. The lifetime distributions obtained for the wild type PS II reaction center decay are shown in Figure 5.4. The ESM fit resulted in five lifetime components distributed around 1-2 ps, 24-28 ps, 70-83 ps, 212-236 ps, and 1.5 ns. In chapter IV, we proposed assigning the 24-28 ps component to energy transfer from the peripheral accessory chlorophylls into P680. However, other groups have assigned lifetime to the primary charge separation. To probe the electron transfer that occurs from P680 to pheophytin and further support our assignment we have compared the decay kinetics of the wild type PS II reaction center and D1-T292L mutant PS II reaction center. Figure 5.5 shows the chlorophyll fluorescence decay curve for the D1-T292L mutant PS II reaction center. The ESM lifetime components we obtained from the fit for the D1-T292L mutant PS II reaction center are presented in Figure 5.6. The lifetime components are similar to those obtained for the wild type PS II reaction center.
Figure 5.3: Fluorescence decay curve for Wild Type PS II RCs obtained at 684 nm using 660 nm excitation.
Figure 5.4: ESM lifetime distribution for Wild Type PS II RCs fluorescence decay curve obtained at 684 nm using 660 nm excitation.
Figure 5.5: Fluorescence decay curve of D1-T292L PS IIRCs obtained at 684 nm using 660 nm excitation.
Figure 5.6: ESM lifetime distributions for the D1-T292L PS II RCs fluorescence decay curve obtained at 684 nm using 660 nm excitation.
centers showing no significant shifts in any lifetime component. The five lifetime components are distributed around 1 - 2 ps, 24 - 28 ps, 67 - 80 ps, 212 - 236 ps and 1.5 ns.

DISCUSSION

We have shown that site-directed mutagenesis can be a valuable technique in investigating the energy and electron transfer processes that occur in the PS II reaction center. Both time-resolved fluorescence and transient absorption spectroscopy have been used extensively to study the PS II reaction center. However, assigning time constants to specific events that occur in the PS II reaction center based on only studies of wild type PS II reaction centers has led to considerable controversy. Comparisons made between the decay kinetics of wild type PS II reaction centers and mutant PS II reaction centers should enable us to clarify the origin of many of the lifetime components. Site-directed mutagenesis allows us to alter the coordination sites of specific chromophores located in the PS II reaction center. As we discussed in chapter I and IV both energy transfer and electron transfer are extremely sensitive to both the distance and the orientation between the donor chromophore and the acceptor chromophore. If a mutation alters either the distance or the orientation of the coordinated chromophore it will be reflected in any lifetimes associated with that specific chromophore. In chapter IV we assigned the 24-28 ps lifetime component seen in time-resolved fluorescence studies of the PS II reaction center to an event involving the peripheral accessory chlorophylls, specifically to energy transfer from the peripheral accessory chlorophylls to P680. To investigate the energy and electron transfer processes
of the primary electron donor, P680, it is necessary to study mutant PS II reaction centers that alter the position of the P680 chlorophylls.

The D1-T292L mutant PS II reaction center is a non-conservative mutation. The threonine residue located at site 292 on the D1 polypeptide is replaced by a leucine residue. It has been proposed that this threonine is involved in hydrogen bond interactions to a keto group (C\(_\text{=O}\)) located on one of the two chlorophylls of the special pair. If threonine is involved in hydrogen bonding, replacing it with leucine would disrupt any hydrogen bond interactions at this site. The chlorophyll could then be free to rotate, affecting the orientation of the chlorophyll with respect to the remaining pigments in the reaction center. Thus, the energy transfer processes or charge separation that occurs between this chlorophyll and any other pigments in the PS II reaction center would be expected to change. Comparison of the decay kinetics of the wild type PS II reaction center with the D1-T292L PS II reaction center shows no significant shifts in any of the lifetime components. If the threonine located at site 292 on the D1 polypeptide is coordinated to one of the chlorophylls of the special pair disrupting the bond does not affect the kinetics of the PS II reaction center. We suggest that the D1-T292L mutation does not alter the orientation of the chlorophyll. However, we cannot say unequivocally whether or not threonine is involved in hydrogen bond interactions with one of the chlorophylls of the special pair.
CONCLUSIONS

Comparison of the decay kinetics between mutant PS II reaction centers and wild type PS II reaction centers should provide insight into the relationship between structural organization and function of the PS II reaction center. Previously in chapter IV, we have shown altering the distance or the orientation of reaction center pigments involved in energy transfer and charge separation changes the decay kinetics of the reaction center. In this chapter we have discussed the time-resolved chlorophyll fluorescence decay of the D1-T292L mutant PS II reaction center. The time-resolved chlorophyll fluorescence decay we obtained for the D1-T292L PS II reaction center did not show changes in any lifetime component. Although we feel this suggests the mutation does not alter the orientation of either of the special pair chlorophylls we cannot establish if the threonine at site 292 is involved hydrogen bond interactions with one of the chlorophylls.
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CHAPTER VI

COMPARISON OF THE DECAY KINETICS OF WILD TYPE AND D1-E130 MUTANT PHOTOSYSTEM II REACTION CENTERS: AN INVESTIGATION OF THE PRIMARY CHARGE SEPARATION

INTRODUCTION

In the previous chapters, IV and V, we have focused on the energy transfer processes that occur between the chlorophylls located in the PS II reaction center. However, the two pheophytins located in the PS II reaction center are also involved in energy equilibration with P680. Furthermore, the active pheophytin is the electron accepting chromophore involved in the primary charge separation with P680. Although energy equilibration among the pheophytins and P680 is thought to be included in the femtosecond energy transfer component along with the accessory chlorophylls, time-resolved chlorophyll fluorescence studies could still provide insight into the primary charge separation. Despite debate as to the true time constant for primary charge separation there is general agreement that it occurs on the picosecond time scale. As mentioned in previous chapters, Holzwarth and co-workers have assigned a 3 ps time constant to primary charge separation while Klug and co-workers assigned a 21 ps time constant to the primary charge separation in the PS II reac-
Like chlorophylls found in the PS II reaction center both pheophytins are thought to be coordinated to the D1 and D2 polypeptide chains. The location of the active pheophytin is proposed to be homologous to the active pheophytin in the PBPRC. In the PBPRC the active pheophytin is coordinated to a glutamic acid residue located at site 103 on the L polypeptide. This site has been found to be conserved at site 130 on the D1 polypeptide chain of *Chlamydomonas reinhardtii*. Based on this evidence the active pheophytin is thought to be coordinated by the glutamic acid residue located at site 130 on the D1 polypeptide. Site-directed mutagenesis studies at this site have provided further structural evidence that the active pheophytin is coordinated at site D1-130. Figure 6.1 is a diagram of the D1 polypeptide chain highlighting the glutamate at site D1-130. Additional site-directed mutagenesis studies at site D1-E130 may provide insight into the primary charge separation between P680 and the active pheophytin.

In this chapter we use time-resolved fluorescence spectroscopy to probe the decay kinetics of several D1-E130 mutant PS II reaction centers: D1-E130H, D1-E130Q and D1-E130L. Both the D1-E130H and the D1-E130Q PS II reaction centers are conservative mutations. Like glutamate both histidine and glutamine are capable of hydrogen bond interactions with the pheophytin. However, the D1-E130L PS II reaction center is a non-conservative mutation; leucine is not able to coordinate the pheophytin. As a result the active pheophytin would be free to move away from this position. All three mutations would be expected to affect the overall rate of the primary charge separation (P680+/Pheo-).
Figure 6.1: Model of the D1 polypeptide highlighting the Glutamic acid residue at site 130.
EXPERIMENTAL

Sample Preparation

The wild type PS II particles were generated by Dr. Jun Wang and the D1-E130 mutant PS II particles were generated by Xiong Ling in Dr. Sayre's laboratory. The PS II reaction centers were then isolated using the procedure described in chapter II and frozen at 77 K until used for acquiring chlorophyll fluorescence decay curves in our laboratory. Prior to running chlorophyll fluorescence measurements the PS II reaction center samples were allowed to thaw in the dark at 0 °C. The wild type PS II reaction centers discussed in this chapter had a concentration of 6-7 chlorophylls/2 pheophytins. The D1-E130H and D1-E130Q mutant PS II reaction centers contained 7-9 chlorophylls/2 pheophytins and the D1-E130L mutant PS II reaction centers contained 9-11 chlorophylls/2 pheophytins. It is possible that a pheophytin had been lost due to mutating the binding site. However, EPR analysis of the photo accumulation of Pheo\(^{-}\) indicate less than a 30% reduction in Pheo\(^{-}\) accumulation. (Ling Xiong, private communication)

Time-Resolved Fluorescence/Absorption Measurements

The absorbance spectrum (Perkin-Elmer Lambda 20) of each wild type PS II reaction center sample and each D1-E130 mutant PS II reaction center sample was measured before the chlorophyll fluorescence decay was acquired to determine the quality of the reaction center samples. The PS II reaction center The PS II reaction centers were considered "healthy" if the absorbance peak of the chlorophyll Q\(_{y}\) band was between 674 - 676 nm. The wild type PS II reaction center chlorophyll Q\(_{y}\) band typically was on the higher
wavelength end of the range (675-676 nm), however, the D1-E130 mutant PS II reaction center chlorophyll Q\_\text{y} band was normally on the lower wavelength end (674-675 nm). Figure 6.2 compares the absorbance spectra of the wild type PS II reaction center and the D1-E130Q PS II reaction center. All PS II reaction center samples were diluted with buffer so that the absorbance maximum at 650 nm was between 0.1 - 0.3 a.u. The absorbance spectrum of each PS II reaction center sample was taken again after the chlorophyll fluorescence decay was run to determine the extent of sample degradation. The wild type PS II reaction center samples were very robust, showing very little blue shift in the chlorophyll Q\_\text{y} band (less than 2 nm) and only a small drop in the chlorophyll fluorescence count rate, 500 counts per second, as discussed in chapter II. The D1-E130 mutant PS II reaction centers were more susceptible to degradation. In order to minimize degradation of the D1-E130 mutant PS II reaction centers the laser power was reduced to less than 3 mW. The D1-E130 PS II reaction centers exposed to lower laser power showed smaller blue shifts in the chlorophyll Q\_\text{y} band. Initially, using 8 mW of excitation power the D1-E130 mutant PS II reaction centers would have a blue shift of almost 3 nm in the Q\_\text{y} band. After the incident power was lowered the blue shift seen in the Q\_\text{y} band was typically 2 nm. However, even at lower laser power the D1-E130L mutant PS II reaction centers was still not as stable as the wild type PS II reaction centers. The D1-E130L mutant PS II reaction centers showed a more pronounced drop in the chlorophyll fluorescence count rate, by the end of the collection the fluorescence count rate had dropped to only 500-1000 counts per second. Sample degradation could also be seen in the decay kinetics for all of the D1-E130 mutant PS II reaction centers. While the wild type PS II reaction centers were stable for up to four hours of
Figure 6.2: Comparison of the absorbance spectra of Wild Type PS II RC and D1-E130Q PS II RC
exposure the D1-E130 mutant PS II reaction centers could only be exposed for 30-50 minutes. Figure 6.3A and Figure 6.3B compare the chlorophyll fluorescence lifetimes obtained from wild type PS II reaction centers after 40 minutes and 80 minutes of laser exposure. Very little difference in the lifetimes can be seen between the two. Figure 6.4A and Figure 6.4B compare the chlorophyll fluorescence lifetimes of the D1-E130Q PS II reaction centers after 40 minutes and 80 minutes of laser exposure. Although the ESM fits are fine after 40 minutes of sample exposure the decays were not able to be fit after 80 minutes of sample exposure. Furthermore, the resulting lifetimes from the fits after 80 minutes of exposure look very similar to the lifetimes of damaged PS II reaction centers shown in Figure 2.9.

All PS II chlorophyll fluorescence decays were taken using the Time-Correlated Single Photon Counting (TCSPC) method described previously in chapter II.9,10 The DCM dye laser was tuned to give an excitation wavelength of 660 nm. The incident beam (=0.8 mm²) power was held at or under 3 mW during the entire decay collection. The collected chlorophyll fluorescence emission was monitored at 684 nm. The TCSPC was run in reverse mode for the wild type PS II reaction centers and all three D1-E130 mutant PS II reaction center chlorophyll fluorescence decays. The lifetime resolution was controlled by adjusting the TCSPC window setting. A 2.5 ns window was used for all chlorophyll fluorescence decays in this chapter. All chlorophyll fluorescence decays were fitting using a distribution of lifetimes (Exponential Series Method).11,12
Figure 6.3A: ESM lifetime distributions for Wild Type PS II RCs fluorescence decay curve after 40 minutes of laser exposure.
Figure 6.3B: ESM lifetime distributions for the Wild Type PS II RCs fluorescence decay curve after 80 minutes of laser exposure.
Figure 6.4A: ESM lifetime distributions for D1-E130Q PS II RCs fluorescence decay after 40 minutes of laser exposure.
Figure 6.4B: ESM lifetime components for the D1-E130Q PS II RCs fluorescence decay after 80 minutes of laser exposure.
RESULTS

In this chapter we focused on the picosecond chlorophyll fluorescence decay components of the PS II reaction center. Figure 6.5 shows the chlorophyll fluorescence decay of the wild type PS II reaction center taken in a 2.5 ns TCSPC window along with the ESM fitted curve and the weighted residuals from the fit. The ESM fit gave five distinct lifetime distributions plotted in Figure 6.6. The five lifetime components were distributed around 1-2 ps, 24-30 ps, 70-85 ps, 212-236 ps, and 1.5 ns. We again attribute the very fast 1-2 ps component to a limiting value for all kinetics processes that occur faster than the time resolution of our instrument. Rapid energy equilibration among several chromophores in the PS II reaction center is known to occur in 100 - 600 femtoseconds would be included in this component. In addition to ultrafast energy equilibration a time constant of 1-6 ps has been proposed for charge separation which would also be included in our 1-2 ps component.

To probe the energy transfer from pheophytins to P680 and/or charge separation from P680 to the active pheophytin we compared the decay kinetics of the wild type PS II reaction center with all three D1-E130 mutant PS II reaction centers. The decay curves of the three mutant PS II reaction centers looked very similar to the decay curves of the wild type PS II reaction centers and also fit to distributions of lifetimes. Figure 6.7 shows the chlorophyll fluorescence decay curve of the D1-E130H PS II reaction center along with the ESM fitted curve and the weighted residuals of the ESM fit. The ESM fit for the D1-E130H PS II reaction center also gave five separate lifetime components. The five lifetime
Figure 6.5: Fluorescence decay curve for Wild Type PS II RCs obtained at 684 nm using 660 nm excitation.
Figure 6.6: ESM lifetime distributions for the Wild Type PS II RCs fluorescence decay curve obtained at 684 nm using 660 nm excitation.
distributions are 1-2 ps; 28-32 ps, 73-90 ps, 264-314 ps and 1.5 ns are plotted in Figure 6.8. Although several distributions are not identically centered, comparison of the wild type PS II reaction center and D1-E130H PS II reaction center lifetime distribution reveals only a significant shift in the 264-314 ps lifetime component. Figure 6.9 shows the chlorophyll fluorescence decay curve of the D1-E130Q PS II reaction center along with the ESM fitted curve and weighted residuals. Again, the five lifetime components from the ESM fit of the D1-E130Q PS II reaction center are very similar to the wild type PS II reaction center lifetime components. Figure 6.10 shows the ESM lifetime distributions for the D1-E130Q PS II reaction center. The lifetimes, 1-2 ps, 32-35 ps, 73-93 ps, 200-248 ps, and 1.5 ns, are almost identical to the lifetimes obtained from the D1-E130H PS II reaction center. However, the lifetimes obtained for the D1-E130L PS II reaction centers are slightly different from the components obtained from both the D1-E130H and D1-E130Q PS II reaction centers. Figure 6.11 shows a chlorophyll fluorescence decay curve acquired for the D1-E130L PS II reaction center along with the ESM fitted curve and the weighted residuals. Unlike the other PS II reaction centers the D1-E130L PS II reaction center only fit to four lifetime components; 26-33 ps, 67-83 ps, 212-242 ps, and 1.5 ns. Figure 6.12 shows the ESM lifetime distributions for the D1-E130L PS II reaction centers. Although the four components are identical to components seen for the wild type, D1-E130H and D1-E130Q PS II reaction centers the extremely fast component, 1-2 ps, is missing. In addition to the fit shown in Figure 6.12, the D1-E130L PS II reaction centers samples fit to a second ESM distribution. Figure 6.13 shows a second chlorophyll fluorescence decay curve for the D1-E130L PS II reaction centers along with the ESM fitted curve and weighted residuals ob-
Figure 6.7: Fluorescence decay curve for D1-E130H PS II RCs obtained at 684 nm using 660 nm excitation.
Figure 6.8: ESM lifetime components for the D1-E130H PS II RCs fluorescence decay curve obtained at 684 nm using 660 nm excitation.
Figure 6.9: Fluorescence decay curve for D1-E130Q PS II RCs obtained at 684 nm using 660 nm excitation.
Figure 6.10: ESM lifetime distributions for the D1-E130Q PS II RCs fluorescence decay curve obtained at 684 nm using 660 nm excitation.
Figure 6.11: Fluorescence decay curve for D1-E130L PS II RCs obtained at 684 nm using 660 nm excitation.
Figure 6.12: ESM lifetime distributions for the D1-E130L PS II RCs fluorescence decay curve obtained at 684 nm using 660 nm excitation.
tained from this chlorophyll fluorescence decay curve. The lifetime distributions obtained from this fit are almost identical to the previous D1-E130L fit except for early times. Figure 6.14 shows the ESM lifetime distributions obtained for the second D1-E130L PS II reaction centers. Again we see the 29-35 ps, 45-63 ps, 204-232 ps, and the 1.5 ns components. However, at early time we see an extremely fast 1-2 ps component as we have seen for our wild type and other PS II mutant reaction centers and another 3-6 ps component. The 45-63 ps component is shifted slightly, however, we attribute this shift to difficulties in resolving the 29-35 ps and 45-63 ps components due to their close proximity and small percent contribution. We believe one reason we have obtained two different fits for the D1-E130L PS II reaction center is due to the difficulty in acquiring a chlorophyll fluorescence decay curve. As we have previously discussed the D1-E130L PS II reaction centers were not as stable as the wild type PS II reaction centers and other mutant PS II reaction centers. As a result, the chlorophyll fluorescence decay curves we have may be due to degraded D1-E130L PS II reaction center samples.

DISCUSSION

Groups studying the energy transfer processes and charge separation of the PS II reaction center using either transient absorption or time-resolved fluorescence studies consistently report very similar results. In fact, using 660 nm excitation Holzwarth and co-workers report the presence of five lifetimes from their picosecond chlorophyll fluorescence experiments, 6 ps, 20 ps, 67 ps, 330 ps and 4.3 ns, that are quit similar to the five
Figure 6.13: Fluorescence decay curve for D1-E130L PS II RCs obtained at 684 nm using 660 nm excitation.
Figure 6.14: ESM lifetime distributions for the D1-E130L PS II RCs fluorescence decay curve obtained at 684 nm using 660 nm excitation.
lifetime distributions we have obtain for our wild type PS II reaction centers using 660 nm excitation; 1-3 ps, 24-28 ps, 70-80 ps, 206-236 ps and 1.5 ns. Despite reporting similar time constants, groups do not assign these time constants to the same kinetic events. Specifically, disagreement as to the origin of the 1-6 ps component and the 20-30 ps component has developed. Holzwarth and co-workers have assigned the 1-6 ps component to primary charge separation and the 20-30 ps component to energy transfer among pigments based on decay associated spectra they have derived from their time-resolved chlorophyll fluorescence data. In support of this work, Wasielewski and co-workers attributed a 5-8 ps component to primary charge separation and a 22 ps component to charge separation limited by energy transfer from the blue peripheral accessory chlorophylls based on their transient absorption data. Although Durrant and co-workers also report the presence of a 3-4 ps component in their transient absorption data, they attribute this to energy localization on P680. In addition, they assign their 21 ps component to the primary charge separation. Similarly, Donovan and co-workers also have measured a 2-3 ps component in their transient absorption data which they attribute to either singlet-singlet annihilation processes or energy relaxation processes. Furthermore, they assign a 20-22 ps component to charge separation although they do not rule out that energy equilibration may be included in this rate. Clearly confusion as to the true nature of energy transfer and charge separation in the PS II reaction center exist.

Currently, extensive energy transfer and charge separation studies have been done using only wild type PS II reaction centers. We have shown that additional insight into the
functionality of the PS II reaction center can be obtained by studying the energy transfer and charge separation processes of site-directed mutant PS II reaction centers. In chapter IV, comparison of the decay kinetics of the wild type PS II reaction center and the D2-H117N PS II reaction center shows a significant shift in only the 27 ps lifetime component. Based on this data we suggested the 24-28 ps component is due to energy transfer between the peripheral accessory chlorophylls and P680. However, we could not rule out that the 24-28 ps component is due to charge separation limited by slow energy transfer from the peripheral accessory chlorophylls to P680.

As we mentioned in Chapter I, electron transfer is also a competing decay pathway that can be studied by time-resolved fluorescence experiments. The measured fluorescence lifetime is affected by the rates of all decay pathways competing with fluorescence, shown in equation 1-1. One competing pathway is external conversion, such as electron transfer. Therefore, although we are not directly measuring the electron transfer rate we can draw conclusions about electron transfer based on the results of time-resolved fluorescence experiments. Similar to the energy transfer rate, the rate of electron transfer is also dependent on the distance and orientation between the donor and the acceptor. The electron transfer rate derived from quantum mechanical theory is expressed as:
Frank-Condon factor. Although both the electronic coupling element and the Frank-Condon factor are influenced by the distance between the donor and acceptor, the electronic coupling element usually dominates. The electronic coupling element is known to be exponentially proportional to distance, as a result the electron transfer rate is thought to fall off exponentially with an increase in distance between donor and acceptor. Based on the distance dependence of the electron transfer rate site-directed mutagenesis studies on the PS II reaction center could be used to study charge separation in the reaction center. In order to investigate the primary charge separation of the PS II reaction center, mutations altering the coordination sites of P680 and/or the active pheophytin need to be studied. Altering the coordination site of P680 or the active pheophytin would be expected to change both the distance and orientation between the donor, P680, and acceptor, the active pheophytin, which in turn should affect the rate of charge separation. The glutamic acid at site 130 along the D1 polypeptide is the proposed coordination site of the active pheophytin in the PS II reaction center. Based on this assignment we have studied the decay kinetics of three different D1-E130 PS II reaction centers, D1-E130H, D1-E130Q and D1-E130L, in order to investigate the primary charge separation. The D1-E130H and D1-E130Q PS II reaction centers are very similar to the wild type PS II reaction center, making only a conservative replacement of the glutamic acid residue. However, the D1-E130L PS II reaction center makes a non-conservative replacement of the glutamic acid residue. To determine if the mutations altered the binding site of the active pheophytin in the PS II reaction center we compared the level oxygen evolution from the mutant PS II particles and wild type PS II particles. Both D1-E130H and D1-E130Q mutant PS II particles had similar (although
slightly lower) levels of oxygen evolution as wild type PS II particles, however, the D1-E130L mutant PS II particles had almost no oxygen evolution. The lack of oxygen evolution indicated the electron transfer process was blocked in the D1-E130L mutant PS II particles. However, because the level of oxygen evolution was measured from PS II particles rather than PS II reaction centers, we could not determine at which point the electron transfer cycle the process was blocked. In addition, using the levels of oxygen evolution of the D1-E130 mutant PS II particles to determine the extent to which the mutations altered the binding site of the active pheophytin, we examined the pheophytin Q x band of the D1-E130 mutant PS II reaction centers. Figure 6.15 shows the pheophytin Q x absorbance bands of the D1-E130H, D1-E130Q, D1-E130L and wild type PS II reaction centers taken by Xiong Ling. The pheophytin Q x band of the D1-E130H PS II reaction center is identical to the pheophytin Q x band wild type PS II reaction center. The D1-E130Q mutant PS II reaction center shows a slight blue shift in the pheophytin Q x band while the D1-E130L PS II reaction center shows a large blue shift in the pheophytin Q x band when compared to the pheophytin Q x band of the wild type PS II reaction center. The pheophytin Q x band can be fit with two bands due to the slight change in environment of the inactive and active pheophytins. The blue edge of the pheophytin Q x band is dominated by absorbance due to the inactive pheophytin while the red edge is due to absorbance due to the active pheophytin. Table 6-1 shows the percent contribution of the inactive pheophytin (539.1 nm) and active pheophytin (544.5 nm). Both the D1-E130H and D1-E130Q mutant PS II reaction centers have similar contributions from the inactive and active pheophytin as the wild type PS II reaction centers. However, the D1-E130L PS II shows a significant decrease in the contri-
6.15: Absorbance spectra of the pheophytin Q_x band for Wild Type, D1-E130H, D1-E130Q, and D1-E130L PS II RCs.
<table>
<thead>
<tr>
<th></th>
<th>Inactive Pheo (539.1 nm)</th>
<th>Active Pheo (544.5 nm)</th>
<th>(532.0 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>60%</td>
<td>40%</td>
<td></td>
</tr>
<tr>
<td>D1-E130H</td>
<td>52%</td>
<td>48%</td>
<td></td>
</tr>
<tr>
<td>D1-E130Q</td>
<td>56%</td>
<td>44%</td>
<td></td>
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<tr>
<td>D1-E130L</td>
<td>64%</td>
<td>22%</td>
<td>14%</td>
</tr>
</tbody>
</table>

Table 6-1: Percent contribution of both the inactive and active pheophytins to the pheophytin $Q_x$ absorbance band of the D1-E130H D1-E130Q, D1-E130L and Wild Type PS II reaction centers.
bution of the active pheophytin. Furthermore, a third band at 532 nm is required to fit the pheophytin Q<sub>x</sub> absorbance band. Based on the data obtained from the comparisons of oxygen evolution and pheophytin Q<sub>x</sub> band both the D1-E130H and D1-E130Q mutant PS II reaction centers are very similar to the wild type PS II reaction centers. However, the D1-E130L mutation alters the PS II reaction centers, blocking electron transfer and possibly forcing charge separation to go through the inactive pheophytin.

Although the D1-E130H mutant PS II reaction centers are very similar to the wild type PS II reaction centers the mutation could make a slight change in the distance and/or orientation between P680 and the active pheophytin and alter the rate of charge separation. A comparison of the picosecond decay kinetics of the wild type PS II reaction center, Figure 6.6, and D1-E130H PS II reaction center, Figure 6.8, shows only a significant shift in the 200-248 ps lifetime component to 264-314 ps. Based on the lifetime shift we see due to the D1-E130H mutation we suggest the 200-248 ps lifetime is due to a charge recombination process in the reaction center. Energy transfer from the two pheophytins into P680 and charge separation are thought to occur much faster, on the femtosecond to early picosecond time scale, then 200-248 ps. Therefore, our data support assigning the 200-248 ps lifetime component to a back processes involving the active pheophytin, likely a charge recombination process. The D1-E130Q PS II reaction center is also very similar to the wild type PS II reaction centers, however, the mutation could still alter the distance between P680 and the active pheophytin affecting the rate of charge separation. Comparison of the decay kinetics of the D1-E130Q PS II reaction center, Figure 6.10, with the wild type PS II
reaction center, Figure 6.6, shows no significant shifts in any lifetime component. The D1-E130L mutant PS II reaction centers are significantly different from the wild type PS II reaction centers. Replacing the glutamic acid residue with leucine should allow the active pheophytin to move freely from this site. However, comparison of the decay kinetics of the wild type PS II reaction center, Figure 6.6, and the first D1-E130L PS II reaction centers decay, Figure 6.12, shows no significant shift in any of the longer-lived lifetime components (>10 ps) but the 1-2 ps component is lost. Comparison of the decay kinetics of the wild type PS II reaction center and the second D1-E130L PS II reaction center, Figure 6.14, again shows no significant shift in any of the lifetime components but an addition of another extremely fast, 3-7 ps, lifetime component. Although the kinetics of both D1-E130L PS II reaction centers are slightly different from the wild type PS II reaction centers it is only in the 1-8 ps region that we see any significant changes. The lack of a significant shift in any of the longer-lived lifetime components (>10 ps) of the three D1-E130 mutant PS II reaction centers suggests that the rate of primary charge separation is faster than the resolution of our instrument and is not the dominate component of the four lifetimes we are able to resolve. To further support this claim, it is only at extremely fast times, 1-8 ps, that we see any lifetime changes in the D1-E130L PS II reaction center. This also supports the claim of Holzwarth and co-workers that primary charge separation in the PS II reaction center occurs on the order of 1-6 ps. Furthermore, this provides additional support to our claim in Chapter IV that the 24-28 ps component is due energy transfer between the peripheral accessory chlorophylls and P680. Only a change in the position of the peripheral accessory chlorophyll with respect to P680 results in a change in the 24-28 ps component, altering the
position of the primary electron acceptor has no affect on this component. However, as we also mentioned in Chapter IV, we still cannot rule out that the 22 ps component seen in experiments using red edge excitation is due to a entirely different process as the 24-28 ps component we see from blue edge excitation.

CONCLUSIONS

 Numerous time-resolved chlorophyll fluorescence studies have been done to examine the kinetics of both energy and electron transfer in the PS II reaction center. However, considerable debate has risen regarding the assignment of time constants for the energy transfer and electron transfer processes of the PS II reaction center. Previously we have shown that site-directed mutagenesis can provide further insight into the dynamics of the PS II reaction center than time-resolved fluorescence alone. Altering the position of the chromophores involved in energy and electron transfer in the PS II reaction center changes the rates of the transfer processes, therefore changing the chlorophyll fluorescence lifetimes. A correlation can then be made between a specific lifetime component and kinetic event. We have obtained the time-resolved chlorophyll fluorescence decays of three different D1-E130 mutant PS II reaction centers and compared them to the time-resolved chlorophyll fluorescence decay of wild type PS II reaction centers. In order to investigate the primary charge separation of the PS II reaction center we have developed PS II reaction centers that alter the position of the primary electron acceptor, pheophytin. We did not seen any significant changes in the 24-34 ps lifetime component for any of the D1-E130 mutant PS II reaction
centers compared to wild type PS II reaction centers, it is only at time extremely fast times (1-8 ps) that we see changes in the early decay kinetics of the D1-E130 mutant PS II reaction center compared to wild type PS II reaction centers. We suggest that the time constant for primary charge separation in the PS II reaction center is beyond the resolution of our instrument. More than likely the rate of charge separation is a significant contribution of the extremely fast component we see at 1-3 ps in all of our chlorophyll fluorescence decays, however, we are unable to assign an exact value to this event. Furthermore, we believe these data add additional support to our claim in Chapter IV that the 24-28 ps lifetime component is dominated an energy transfer process and not an electron transfer process. In addition, we see a significant change in the 200-248 ps lifetime component in the D1-E130H PS II reaction center. Both energy transfer and primary charge separation involving the active pheophytin occur much faster than 200 ps. We suggest this supports assigning the 200-248 ps lifetime component to a charge recombination processes involving the active pheophytin.
REFERENCES


CHAPTER VII

CONCLUSIONS AND FUTURE STUDIES

CONCLUSIONS

In this dissertation we have presented the results of energy transfer and charge separation studies on the Photosystem II reaction center of *Chlamydomonas reinhardtii* using time-resolved fluorescence spectroscopy. Time-resolved fluorescence spectroscopy allowed us to follow energy migration through the PS II reaction center by monitoring the lifetime of each excited state along the pathway. We found the chlorophyll fluorescence decay kinetics of the PS II reaction center to consist of nine unique lifetime components ranging from several picoseconds to 30-50 nanoseconds. As we discussed in chapter I, spectral congestion of the chlorophyll Q_y band has hindered assigning lifetimes to energy or electron transfer from a specific chromophore to another in the reaction center. Although low temperature studies have shown that it is possible to excite preferentially particular chromophores it is impossible to excite selectively a chromophore in the PS II reaction center. Therefore, a single fluorescence decay is composed of numerous lifetime components due to energy and electron transfer among all the pigments present in the PS II reaction center. In this work
we have focused on using excitation wavelengths on the blue edge of the Q_y band, preferentially exciting the two peripheral accessory chlorophylls found in the PS II reaction center. Excitation of the peripheral accessory chlorophylls enabled us to monitor the energy transfer into the primary electron donor, P680, and the subsequent charge separation, P680'/Pheo'.

We have shown that the chlorophyll fluorescence decay curves for the wild type PS II reaction centers consist of seven unique lifetime components; 1-3 ps, 24-33 ps, 65-85 ps, 200-248 ps, 1.5-2.5 ns, 4.5-5.5 ns, and 30-40 ns. The origin of the two long-lived components has already been well established in the literature, however, the assignment of the remaining five lifetimes is still not certain and there exists considerable debate as to the origin of several of these components. We have demonstrated that site-directed mutagenesis can be a powerful tool in studying the functionality of the PS II reaction center and in assigning lifetimes to transfer processes in the PS II reaction center. Both energy transfer and electron transfer are extremely sensitive to the distance and the orientation between the donor chromophore and the acceptor chromophore. As a result, changing the coordination site of either the donor chromophore or acceptor chromophore would be expected to affect the rate of any transfer processes due to the altered chromophore. Site-directed mutagenesis allows us to make such environmental changes to the chromophores present in the PS II reaction center. We have obtained the chlorophyll fluorescence decay kinetics from six different PS II mutant PS II reaction centers. Comparison of the decay kinetics of these six mutant PS II reaction centers with wild type PS II reaction centers has allowed us to assign
time constants to energy transfer from the peripheral accessory chlorophylls to P680 and charge separation.

In order to study energy transfer among chlorophylls located in the reaction center we have presented the results from two different mutant PS II reaction center that alter the coordination sites of the peripheral accessory chlorophylls. The two peripheral accessory chlorophylls located in the PS II reaction center have been proposed to be bound to histidine residues located on the D1 and D2 polypeptide chains at site 118 and 117 respectively. In chapter IV we discussed the decay kinetics obtained for the D2-H117N PS II reaction center and in chapter V we discussed the decay kinetics obtained for the D1-H118Q PS II reaction center. We saw a significant shift from 24-28 ps for the wild type reaction center to 10-12 ps for the D2-H117N reaction center. However, the decay kinetics from the D1-H118Q PS II reaction centers did not show a similar shift in the 24-28 ps lifetime component. Comparison of the circular dichroism spectra of the three PS II reaction centers it appears the D2-H117N PS II reaction center alters the position of a pigment absorbing in the Qy band, however, the D1-H118Q PS II reaction center does not. Based on the CD spectra and our kinetic studies it appears the D2 mutation alters the position of the peripheral accessory chlorophyll coordinated at site 117 and as a result affects the rate of energy transfer while the D1 mutation does not alter the position of the peripheral accessory chlorophyll coordinated at site 118 and therefore we see no effect on the rate of energy transfer. These results would indicate the 24-28 ps component was due to energy transfer from the peripheral accessory chlorophylls to P680. However, we cannot rule out that the 24-28 ps
component is due to charge separation limited by slow energy transfer among chlorophylls. In order to investigate the primary charge separation we looked at mutant PS II reaction centers that altered coordination sites of the chromophores involved in the primary charge separation, P680 and the active pheophytin. The active pheophytin has been proposed to be coordinated to a glutamic acid residue located at site 130 on the D1 polypeptide. Based on this assignment we investigated the decay kinetics of three different mutant PS II reaction centers. In chapter VI we presented the results from time-resolved chlorophyll fluorescence studies of D1-E130H, D1-E130Q and D1-E130L PS II reaction centers. The decay kinetics of the D1-E130H and the D1-E130Q PS II reaction centers show no significant changes in any of the early lifetime components compared to the wild type PS II reaction centers. Comparison of the lifetime components for the D1-E130L PS II reaction centers and wild type PS II reaction centers only showed significant changes at early times less then 10 ps. These data suggest that the rate of electron transfer is faster then the resolution of our instrument and adds additional support to our claim that the 24-28 ps component is a result of energy transfer from the peripheral accessory chlorophylls and not charge separation. In addition the D1-E130H PS II reaction center showed a significant shift in the longer lifetime component, 200-248 ps. We suggest this data supports assigning the 200-248 ps lifetime component to a charge recombination processes involving the active pheophytin.

In conclusion, we have presented a comparison of the decay kinetics of wild type PS II reaction centers and six different mutant PS II reaction centers in an effort to investigate the energy transfer processes and charge separation that occur in the reaction centers. We have demonstrated that site-directed mutagenesis can be a valuable technique in studying
the dynamics of the PS II reaction center. Furthermore, based on our data we suggest that model two, Figure 1.7, is a more accurate representation of the early energy transfer processes that occur in the PS II reaction center.

FUTURE STUDIES

As we have discussed throughout this dissertation one of the main reasons for the difficulty in assigning lifetimes to specific events that occur in the PS II reaction center is spectral congestion of the chlorophyll Q_y band. Low temperature studies have focused on deconvoluting the chlorophyll Q_y band and although they have shown it is possible to excite preferentially specific chromophores in the PS II reaction center their energy level lie too close to excite selectively any chromophore. Many transient absorption and time-resolved fluorescence studies have focused on exciting on the blue edge of the chlorophyll Q_y band in order to excite preferentially the peripheral accessory chlorophylls and monitoring energy migration. However, a large number of studies have centered on using excitation on the red edge of the chlorophyll Q_y band to excite preferentially P680 and therefore primarily investigate charge separation. Both methods have produced similar time constants particularly the 20-30 ps lifetime. However, as we have previously mentioned the assignment of this time constant has varied. Based on our time-resolved excitation studies using blue edge excitation we have attributed this lifetime to energy transfer. Recently, it has been proposed that although a 20-30 ps component is seen in experiments using both blue edge and red edge excitation the component may be due to entirely different phenomena depending on
the excitation wavelength. In order to investigate this possibility we propose acquiring the chlorophyll fluorescence decays of the wild type PS II reaction centers and the six mutant PS II reaction centers we have discussed in this dissertation using red edge excitation. Although the chlorophyll fluorescence intensity would be much lower it is be possible to excite the PS II reaction centers at 695 nm and monitor the emission at 684 nm. Comparison of the decay kinetics for the wild type PS II reaction centers and the six mutant PS II reaction centers using excitation on the red edge of the chlorophyll Qy band should determine if the 20-30 ps component is due to the same source as the 20-30 ps component seen using blue edge excitation.

The six mutant PS II reaction centers we have discussed in this dissertation have centered on altering the coordination site of the two peripheral accessory chlorophylls and the active pheophytin. The D1-T292L PS II reaction center we discussed in chapter V was a non-conservative mutation to a site proposed to have hydrogen bond interactions with one of the side keto groups of a P680 chlorophyll. However, this has been challenged recently by the work of Noguchi et al. Based on models derived from the PBPRC the P680 chlorophylls have been proposed to be coordinated to two conserved histidines located along the D1 and D2 polypeptides at site 198 for both. Figure 7.1 highlights the location of both histidines along the D1 and D2 polypeptides. Site-directed mutagenesis to these sites have lead to insights in the structural organization of P680. We believe that time-resolved chlorophyll fluorescence studies done using these mutant PS II reaction centers could also provide insight into the kinetics of energy transfer and charge separation in the PS II reaction center.

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Figure 7.1: Model of the D1 polypeptide highlighting the histidine at site 198. The proposed binding site for one of the P680 chl.
Site-directed mutagenesis has proven to be a powerful tool in studying the energy transfer and charge separation processes that occur in the PS II reaction center. Additional investigations using other mutant PS II reaction centers could prove to be important in assigning the remaining lifetime components seen from the PS II decay kinetics. The origin of the intermediate lifetime components; 65-85 ps, 200-248 ps, 1.5 ns, has not yet been established. Most often these lifetimes are attributed to additional energy transfer processes and/or radical pair recombination. Further site-directed mutagenesis studies could be useful in making these assignments.
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