

Fluorescence Decay Kinetics of Wild Type and D2-H117N Mutant Photosystem II Reaction Centers Isolated from *Chlamydomonas reinhardtii*

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We compare the chlorophyll fluorescence decay kinetics of the wild type and the D2-H117N mutant photosystem II reaction centers isolated from *Chlamydomonas reinhardtii*. The histidine residue located at site 117 on the D2 polypeptide of photosystem II is a proposed binding site for one of two peripheral accessory chlorophylls located in the reaction center complex. The peripheral accessory chlorophylls are thought to be coupled with the primary electron donor, P680, and thus involved in energy transfer with P680. The conservative replacement of the histidine residue with an asparagine residue allows the chlorophyll to remain bound to the reaction center. However, slight changes in the structural organization of the reaction center may exist that can affect the energy transfer kinetics. We show that the D2-H117N mutation causes a shift in the 20–30 ps lifetime component that has been associated with energy equilibration among coupled chlorophylls in the photosystem II reaction center.

Introduction

Photosystem II is a protein–pigment complex present in the thylakoid membranes of higher plant and green alga chloroplasts that catalyzes the light-dependent oxidation of water. Chlorophylls present in the PS II complex function in energy harvesting or as the primary electron donor during charge separation. Charge separation initiates electron transfer in the PS II reaction center resulting in the removal of electrons from water and release of molecular oxygen.¹ Since it was first isolated, the PS II reaction center has been determined to consist of the D1/D2 polypeptides, the cytochrome b559 subunits, and the psb I protein.² Although the exact structure of the reaction center is still unknown, the D1/D2 polypeptides have been found to be highly homologous to the L/M polypeptides of the purple bacteria photosynthetic reaction center (PBPRC), whose structure is known.^{2–5} One difference existing between the two structures is the number of chlorophylls present. The PBPRC has four bound chlorophylls while the PS II reaction center contains approximately six chlorophylls/two pheophytin.^{4,6} Based on analogies to the PBPRC two of the six chlorophylls may function as the primary electron donor, P680.^{3,4,7} However, as many as six chlorins may be excitonically coupled.⁸ Two accessory chlorophyll monomers lying in close proximity to the primary electron donor are believed to be involved in electron transfer to pheophytin, but also are coupled to P680. Two additional chlorophylls of the PS II reaction center, the peripheral accessory chlorophylls, are located further away from P680.⁹ The peripheral accessory chlorophylls are thought to act as a link for energy transfer from the antennae chlorophylls of the PS II complex to P680.^{1,10}

The mechanism for energy transfer in the PS II reaction center is not well understood and remains an active area of research. Several models for energy transfer in the PS II reaction center have been put forth.^{11–19} One current model by Holzwarth and co-workers is that after initial excitation there is a subpicosecond equilibration of energy among the primary donor and coupled chlorophylls in the reaction center complex. They assign a longer 20–40 ps lifetime to energy equilibration between the primary electron donor, additional excitonically coupled chlorophylls, most likely the peripheral accessory chlorophylls, and any additional chlorophylls bound to the reaction center. After energy equilibration the primary charge separation (P680⁺/Pheo⁻) then occurs in ~3–8 ps.^{11,13,20,21} Another interpretation of the energy transfer between the chlorophylls present in the reaction center has been put forth by Giorgi et al.^{16,18} This model still assigns a subpicosecond energy equilibration among the primary electron donor and coupled chlorophylls. However, this model suggests that energy equilibration among all the chlorophylls present in the reaction center and the primary electron donor occurs on this subpicosecond time scale. After energy equilibration among the pigments they assign a 20–30 ps lifetime to the primary charge separation (P680⁺/Pheo⁻).

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. 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 are histidines located at site 118 on the D1 polypeptide and site 117 on the D2 polypeptide.⁴ These histidines are proposed binding sites for the peripheral accessory chlorophylls.^{22–24} A conservative replacement of the histidine residue would still allow the chlorophyll to be bound while possibly making changes in the distance and the orientation of the chlorophyll with respect to P680. A nonconservative replacement of the histidine residue

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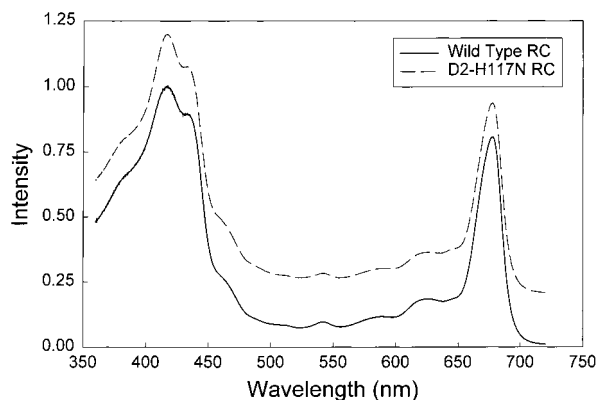


Figure 1. Absorbance spectra of the wild-type PS II reaction centers and D2-H117N mutant PSII reaction centers. The D2-H117N spectrum is offset from zero for clarity.

would likely prevent coordination of the chlorophyll, as a result the chlorophyll would be free to move away from this 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 and D2-H117N 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.

Experimental Section

Sample Preparation. Generation of Mutants and Thylakoid Preparations. D2-H117N and D2-H117Q mutants were generated and thylakoid membranes were prepared as described separately.²⁵

PS II Reaction Center Complex Isolation. PS II reaction center complexes from wild type and D2-H117N mutant cells were purified from PS II, BBY-type particles prepared by Triton X-100 (TX-100) solubilization of thylakoid membranes according to the procedure of Berthold et al.²⁶ A complete description of the reaction center isolation will be provided elsewhere. Briefly, however, BBY-type particles were solubilized with Triton X-100 followed by centrifugation at 100 000 g for 1 h. The PSII fraction (supernatant) then was loaded onto a DEAE-Toyopearl 650S column (Rohm and Haas) and washed until the elute had an absorbance at 675 nm of less than 0.04 OD cm⁻¹. PSII 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 for the fluorescence decay study reported here. The chlorophyll concentration of thylakoid and PS II membranes was determined by the method of Arnon²⁷ while the chlorophyll, pheophytin, and carotenoid concentration of the purified PSII reaction centers was done according to Eijkelhoff et al.⁶ For chlorophyll fluorescence decay experiments PS II reaction centers having a chlorophyll/pheophytin ratio of 6–7/2 were used.

Fluorescence/Absorption Measurements. The absorption spectrum (Perkin-Elmer Lambda 20) for each photosystem II reaction center sample was taken prior to running the fluorescence decay to determine the quality of the reaction centers. In Figure 1 we show the absorption spectra for the wild type and D2-H117N PS II reaction centers. The presence of the chloro-

phyll Q_y band peak at 676 nm and the lack of large peak at 434 nm was indicative of a healthy reaction center.²⁸ The absorption spectrum was taken again after acquiring the fluorescence decay to determine if sample degradation had occurred. Our samples had little or no blue shift in the Q_y absorption band, 674 nm, which indicates little damage to the PS II reaction centers occurred during the decay collection time.²⁸

The samples were degassed by bubbling argon through a long-necked sample cuvette for 3–4 min. The cuvette was then sealed using a ground glass stopper and then the stopper and cuvette neck were wrapped with Parafilm. The samples were held at 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 allows fresh sample to circulate into the excitation beam path over the course of sample collection.

Photosystem II chlorophyll fluorescence decays were acquired using a time-correlated single photon-counting (TCSPC) system.^{29,30} Briefly, 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; 4 MHz rep rate) tuned to 658 nm. 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 (~0.8 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 at 684 nm using a series of optics; two broadband antireflection (BBAR) focusing lenses, a polarization analyzer (set at 54.7° with respect to the laser pulse), and a polarization scrambler, and focused on 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 are collected in the peak channel).

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. 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 decays were then fit using a distribution of lifetimes.^{31,32}

Results

Photosystem II reaction centers have complex chlorophyll fluorescence decay kinetics. In Figure 2A we present the PS II wild-type reaction center chlorophyll fluorescence decay taken using a 150 ns window. Figure 2B shows the fitted lifetime distributions for the decay in 2A. The chlorophyll fluorescence decay could be fit with four lifetime components centered at 550 ps, and 2.5, 5.5, and 35 ns. The 550 ps component is an

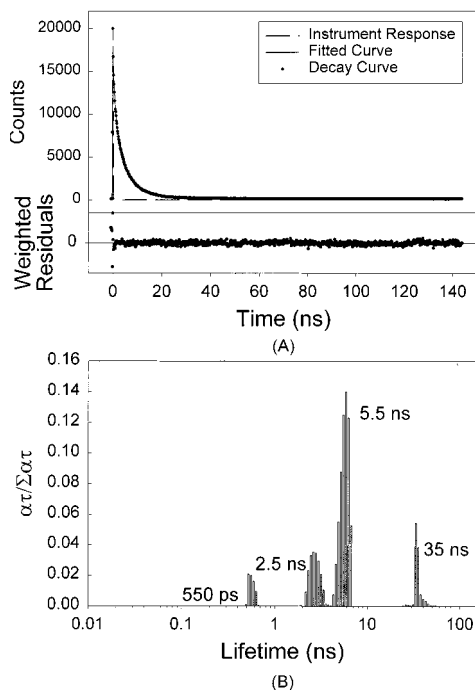


Figure 2. Chlorophyll fluorescence decay curve and exponential series method (ESM) fitted curve for the wild-type PS II reaction center in a 150 ns window (A), with the ESM lifetime distributions fit (percent contribution versus lifetime) (B).

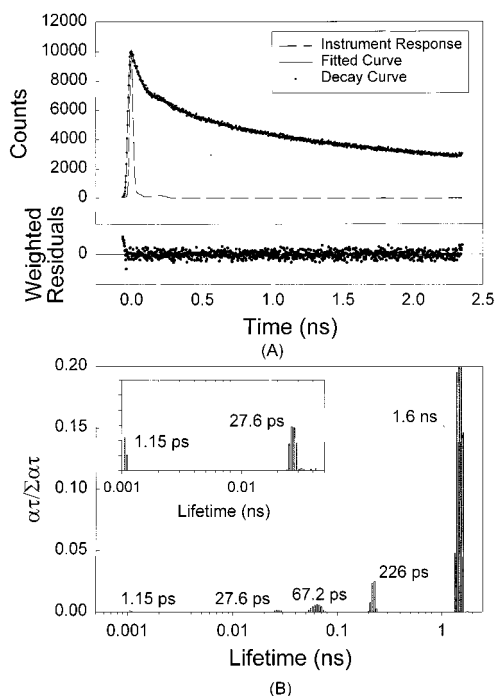


Figure 3. Chlorophyll fluorescence decay curve and exponential series method (ESM) fitted curve for the wild-type PS II reaction center in a 2.5 ns window (A); with the ESM lifetime distributions fit (percent contribution versus lifetime) (B).

artificial value. Owing to the size of the observation window all short-lived components are fit to this limiting value. To observe directly the short-lived components we obtain decays with a 2.5 ns window. In Figure 3A we show the PS II wild-type reaction center chlorophyll fluorescence decay taken with a 2.5 ns window. Figure 3B shows the corresponding lifetime distributions for the decay. Five lifetime components centered at 1.15, 27.6, 67.2, and 226 ps, and 1.65 ns were needed to fit

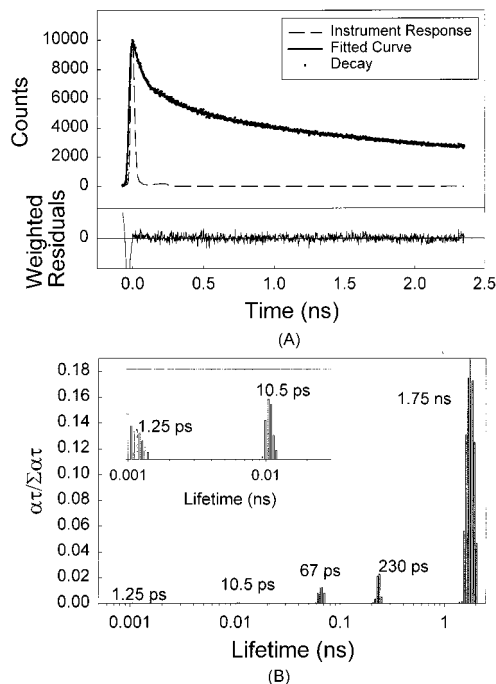


Figure 4. Chlorophyll fluorescence decay curve and exponential series method (ESM) fitted curve for the D2-H117N PS II reaction center in a 2.5 ns window (A); with the ESM lifetime distributions fit (percent contribution versus lifetime) (B).

the 2.5 ns window 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 fluorescence decay of PS II.

To probe the energy transfer of the peripheral accessory chlorophylls we compared the fast decay kinetic of the D2-H117N PS II reaction center with the wild type. The D2-H117N PS II reaction centers also gave complex chlorophyll fluorescence decays very similar to 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 obtained from the wild type reaction centers taken in the longer window. The only significant difference between the lifetime components of the wild type reaction centers and D2-H117N mutant reaction centers is seen in the shorter window (~ 2.5 ns). Figure 4A 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 4B). The lifetimes were centered at 1.15, 10.5, 67, and 230 ps and 1.75 ns. A comparison of the decay components of the wild type PS II reaction center and of the mutant PS II reaction center shows a significant change in only one of the five components. In the wild-type PS II reaction center there is a fast component centered at 27.6 ps, however in the D2-H117N PS II reaction center there is no contribution from a 27.6 ps component but rather we observe a 10.5 ps lifetime distribution.

Discussion

Lifetime Fitting Methods. The photosystem II reaction center is a complex system, therefore, choosing a data analysis model that accurately represents this environment should be addressed. The most common method for fitting fluorescence

decay curves is to use a discrete sum of exponentials. Typically, anywhere from one to six discrete lifetime components are summed to create a fitted curve of the data. We have chosen instead to use a distribution of lifetimes as a model for the PS II chlorophyll fluorescence decay process. We believe that this method more accurately reflects the physical reality of the energy fluctuations of the chromophores in the protein environment. A discrete sum of exponentials does not take into account minor fluctuations in energy levels due to a heterogeneous environment. The PS II reaction center is known to have a complex matrix that can vary slightly from one reaction center to another. The exponential series method (ESM) uses a continuous distribution of lifetimes to fit fluorescence decay curves.^{31–33} A chosen number of lifetimes, typically between 100 and 200, are logarithmically spaced in the fitting window and only their amplitudes are adjusted until the fit is optimized. 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-squared 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. 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.

Time-Resolved Fluorescence. The time-resolved fluorescence decays of the wild-type PS II reaction centers show nine lifetime components in the two time windows (Figure 2B and 3B), of these nine there are seven unique components; 1.15, 27.6, 67.3, and 226 ps, and 2.5, 5.5, and 35 ns. The origin of the two long-lived components has been well established in the literature and our lifetimes of 5.5 and 35 ns (Figure 2B) agree well with lifetimes seen by other groups.^{10,28,34–36} 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. We obtained a single ESM lifetime at 5.6 ns (not shown). 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 67 and 226 ps and 2.5 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.^{11,34,37} 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 center does occur on the femtosecond time scale. Our time-resolved chlorophyll fluorescence measurements show 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 (Figure 3B and 4B). 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 feel we can say 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 generated synthetic decay curves that lacked the two fastest components, 1.15 and 27.6 ps. The ESM fit for these decay curves did not produce either lifetime component.

In addition to the 1–6 ps component we see a lifetime component at 27.6 ps in the wild-type reaction centers. Comparison of this component of the two PS II reaction centers shows a change from 27.6 ps for the wild-type PS II reaction center (Figure 3B) to 10.5 ps for the D2-H117N mutant PS II reaction center (Figure 4B). The remaining six lifetimes for the two PS II reaction centers reveal no significant deviations. This would indicate the 27.6 ps component and the 10.5 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 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, therefore a change in the energy transfer rate would be expected in the mutant reaction center.³⁸ The only significant change we see in the decay kinetics is a change in the 27.6 ps component (Figure 3B) to 10.5 ps (Figure 4B). This would suggest a relationship between the peripheral accessory chlorophyll and the 27.6 ps lifetime component, more specifically the 27.6 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 27.6 ps component. However, if we consider an energy equilibration model rather than a direct energy transfer we may still expect 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. Other time-resolved chlorophyll fluorescence and transient absorption studies done with the wild-type PS II reaction centers isolated from spinach have also reported the presence of a 15–35 ps lifetime.^{11,15–19,34,37} Time-resolved fluorescence studies have typically used an excitation wavelength on the blue edge of the chlorophyll Q_y absorption band, preferentially exciting the peripheral accessory chlorophylls. Based on decay associated spectra derived from these studies the 15–35 ps lifetime 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_y band, preferentially exciting P680. Many of the groups exciting on the red edge have assigned a 15–35 ps lifetime to the primary charge separation, P680⁺/Pheo⁻. Our 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.

Conclusions

We have screened various data analysis methods to identify methods which best represent the heterogeneous 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; we 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 time-resolved chlorophyll fluorescence data we have obtained for the wild type PS II reaction center and the D2-H117N mutant PS II reaction center provide further evidence that the histidine at site 117 on the D2 polypeptide is a binding site for one of the two peripheral accessory chlorophylls. The replacement of the histidine residue with an asparagine residue showed a significant change in the fluorescence kinetics of the chlorophylls present in the PS II reaction center. In addition, we suggest that the 27.6 ps lifetime component in the chlorophyll fluorescence decays of the wild type reaction center should be assigned to the energy transfer involving the peripheral accessory chlorophyll and P680, rather than to only the primary charge separation (P680⁺/Pheo⁻). However, we cannot as yet establish if this 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.

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