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Subpicosecond equilibration of excitation energy in isolated photosystem II reaction centers

(energy transfer/electron transfer/P680)

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ABSTRACT **Photosystem II reaction centers have been studied by femtosecond transient absorption spectroscopy. We demonstrate that it is possible to achieve good photoselectivity between the primary electron donor P680 and the majority of the accessory chlorins. Energy transfer can be observed in both directions between P680 and these accessory chlorins depending on which is initially excited. After excitation of either P680 or the other chlorins, the excitation energy is observed to equilibrate between the majority of these pigments at a rate of 100 ± 50 fs^{-1}. This energy-transfer equilibration takes place before any electron-transfer reactions and must therefore be taken into account in studies of primary electron-transfer reactions in photosystem II. We also show further evidence that the initially excited P680 excited singlet state is delocalized over at least two chlorins and that this delocalization lasts for at least 200 fs.**

In photosynthetic organisms, solar energy is absorbed by pigments in light-harvesting complexes and then transferred in a series of ultrafast energy-transfer steps to a primary electron donor within a photosynthetic reaction center (for reviews, see refs. 1 and 2). This initiates a sequence of electron-transfer reactions within the reaction center, which results in the excitation energy being trapped in progressively longer-lived radical pair states. In the case of the photosystem II (PSII) reaction center of higher plants, these electron-transfer reactions produce an unusually high oxidizing potential of ≈1 V, approximately twice that of purple bacteria. This oxidizing potential is used to drive water splitting, which gives rise to oxygen evolution.

The primary electron donor of PSII is thought to correspond to a spectral feature at 680 nm and is referred to as P680 (3), while a pheophytin (Ph) molecule functions as an electron acceptor (4–6). Studies of PSII core complexes binding 60 and 80 antenna chlorophylls have suggested that the primary radical pair $P680^+Ph^-$ is formed at a rate of ≈100 ps^{-1} following the absorption of a photon by antenna pigments (7). A similar conclusion was reached from photovoltage studies of larger PSII complexes (8). A kinetic model, in which there is a rapid (≈1 ps) equilibration of excitation energy between the antenna pigments and P680, followed by the observed trapping of the excitation energy by radical pair formation in ≈100 ps, has been proposed for this process (refs. 9 and 10; reviewed in ref. 2). This so-called trapping limited model (11) is valid when the rate of electron transfer from the primary electron donor is slower than energy transfer back to the antenna pigments. This model has also been applied to other photosynthetic antenna/reaction center complexes (12–14). However, previous studies have not been able to time resolve the energy-transfer processes that are predicted to cause the equilibration of excitation energy between the antenna and primary electron donor pigments prior to radical pair formation.

We report here a study of excitation energy equilibration in the isolated D1/D2/cytochrome b_{559} complex, which is the reaction center of PSII. This complex is much smaller than the isolated PSII core complexes discussed above, binding only six chlorophyll a and two Ph a pigments (15, 16). While several of these pigments are presumably involved in electron-transfer processes, these pigments will also function in an energy-transferring capacity before charge separation. Time-resolved fluorescence studies have determined that at least 94% of the chlorin pigments in our PSII reaction center preparation are able to transfer excitation energy to P680, resulting in a near unity quantum yield of the primary radical pair (17, 18). In a previous study, we determined that when P680 is directly excited, Ph reduction occurs primarily at a rate of 21 ps^{-1} in isolated PSII reaction centers at room temperature (4).

There have been several discussions of the similarities between the PSII reaction center of higher plants and the reaction center of purple bacteria (19, 20). However, these two complexes are likely to be very different in terms of their energy-transfer kinetics when isolated from their antenna systems. The lowest S_0–S_1 optical transition for the primary electron donor (P) of purple bacteria is at least 150 meV below those of the other accessory pigments bound to the isolated reaction center. The special pair is therefore an energetic trap for excited singlet states ($k_BT \approx 25$ meV at room temperature), and there is little thermally activated back energy transfer from P to the other pigments. It has been reported that excitation of the Q_y-absorption bands of any of the chlorins in reaction centers of purple bacteria results in localization of the excitation energy on P within 100 fs (21, 22), followed by Ph reduction at a rate of ≈3 ps^{-1} at room temperature (23, 24). In contrast, the S_0–S_1 transitions (Q_y-absorption bands) for chlorins bound to the isolated PSII reaction centers overlap, in such a way that all eight pigments have their lowest excited singlet states separated by no more than ≈30 meV (25, 26). Therefore, the P680 excited singlet state is not likely to be a deep trap for excitation energy. Extensive forward and reverse energy transfer between chlorins is therefore expected, and distinguishing these from electron-transfer reactions is a prerequisite for a meaningful study of the mechanism of primary charge separation in PSII.

Low-temperature and gaussian deconvolution studies (25, 26) have indicated that the chlorins in the isolated PSII reaction center can be approximately grouped into two pools according to their Q_y-absorption maxima: those with absorption maxima near 680 nm (referred to hereafter as C680), and the remaining chlorins with maxima near 670 nm (C670). The

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Abbreviations: PSII, photosystem two; Ph, pheophytin.

C680 absorption band is dominated by P680. The location of the Q_y-absorption maxima of the Ph are uncertain (25–29). As the 670- and 680-nm absorption bands are separated by approximately k_BT at room temperature, equilibration of excitation energy following selective excitation of either chlorin pool will result in energy transfer to the other pool.

There have been several reports of energy-transfer processes in the PSII reaction center with lifetimes ranging from several tens to hundreds of picoseconds (4, 18, 28, 30–32). In particular, Small *et al.* (28, 32) have reported that energy transfers from the C670 chlorophylls and the photoreducible Ph to P680 have lifetimes of 12 and 50 ps, respectively, at 4.2 K on the basis of spectral hole burning studies. This would imply that the trapping limited kinetic model discussed above may not be appropriate for PSII. Our own previous transient absorption studies resolved a 200-ps energy-transfer/trapping process, which we assigned to a small minority of the reaction center chlorins or to partially damaged reaction centers (4). This 200-ps component was not observed, however, when P680 was directly excited (4). We also reported the observation of two faster kinetic components with lifetimes of 400 fs and 3.5 ps after direct excitation of P680 (33). These components were both assigned to decay of an initially delocalized P680 singlet excited state, although it was not possible to determine whether these decays resulted from energy- or electron-transfer processes.

The results presented here are a continuation of our previous studies (4, 33) of isolated PSII reaction centers using femtosecond transient absorption spectroscopy. In this paper, we report a femtosecond equilibration of excitation energy between reaction center pigments, which occurs prior to those processes we have resolved previously; we also discuss further evidence for delocalization of the P680 singlet excited state.

MATERIALS AND METHODS

Experimental details were as described (4, 33). PSII reaction centers were isolated from pea thylakoid membranes (18, 34) and studied under anaerobic conditions at room temperature (295 K). PSII reaction centers were excited with 0.1-μJ pulses centered at either 665 or 694 nm. The excitation and probe pulses were parallel polarized to better than 99%. Between 5% and 10% of reaction centers in the pumped volume were excited by each excitation pulse. The instrument response of the spectrometer was estimated from the rise time of absorption changes in dye standards for each combination of excitation and probe wavelengths used in this paper. These instrument responses were assumed to have gaussian temporal profiles, an approximation that resulted in adequate, but not perfect, fits to data obtained with both the laser dyes and PSII reaction centers (see Fig. 1). The instrument responses had full width at half maximum values of 100–140 fs. Zero time delay was also determined from dye standards. The white light probe pulses were spectrally dispersed by <2 fs/nm between 660 and 695 nm.

Absorption changes were monitored as a function of time at single wavelengths between 660 and 695 nm (detection bandwidth, 2 nm). Data presented in this paper were collected over a 0- to 2-ps time scale, with a time delay of 13.2 fs between points. Data were analyzed assuming multiexponential kinetics as described by Hastings *et al.* (4), with decays being analyzed both individually and globally. These analyses included information previously obtained on relatively slow transient absorption components from data collected on longer time scales (4, 33). Data were globally analyzed either without deconvolution for time delays >100 fs or using iterative reconvolution with the instrument response. Similar lifetimes and spectra were obtained from both types of analyses, which strongly supports the validity of our fitting procedures.

Transient absorption data collected by using 665-nm excitation have been scaled to take account of differences in the proportion of reaction centers excited by the 665- and 694-nm pulses. The scale factor was calculated to give the same final radical pair spectrum, as we have discussed (4, 33).

For each combination of excitation and probe wavelengths, transient absorption data were collected by using sample cuvettes containing the PSII reaction center suspension, the suspension buffer only, and dye standards in methanol to assess possible artifactual distortions of the data. In all the data reported in this paper, appropriate conditions were chosen to ensure that oscillations resulting from impulsive stimulated Raman scattering in the glass windows of the sample cuvette (Q. Hong, D.R.K., J.R.D., G.H., and G.P., unpublished data) were at least 10 times smaller than the transient absorption components of interest.

RESULTS

Excitation wavelengths were chosen with the intention of selectively exciting either the C680 (using 694-nm excitation) or C670 (using 665-nm excitation) chlorin pools. By comparing the spectra of the excitation pulses and the two pigment pools, it is possible to estimate that 694- and 665-nm excitation pulses should achieve an ≈75% selective excitation of the C680 and C670 pigment pools. In fact, our data indicate that the photoselection was actually better than this.

Transient absorption data presented in this paper were collected on a 0- to 2-ps time scale at probe wavelengths between 660 and 695 nm. Typical data are shown in Fig. 1. We have reported previously from global analyses of data collected on 0- to 12-ps and 0- to 80-ps time scales that a

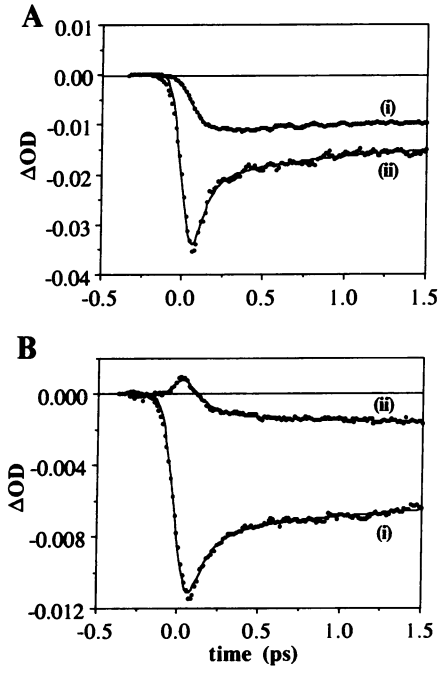

FIG. 1. (*A*) Kinetics of transient absorption changes observed at 685 nm after excitation of PSII reaction centers with 665-nm (*i*) and 694-nm (*ii*) pulses. (*B*) Kinetics of transient absorption changes observed at 670 nm using 665-nm excitation (*i*) and 667 nm using 694-nm excitation (*ii*). Circles are data points and solid lines are best fits to the data obtained using two exponential components with lifetimes of 100 and 400 fs for 665-nm excitation, and 100 and 600 fs for 694-nm excitation, and a nondecaying component. The instrument response and zero time delay were determined with laser dye standards.

minimum of three exponential components with lifetimes of 400 fs, 3.5 ps, and 21 ps were required to fit the data in addition to a nondecaying component (4, 33). When data collected on the 0- to 2-ps time scale were included in the global analyses, an additional exponential component was required to fit the data. This resulted in the 400-fs component splitting into two components with lifetimes of 100 and 600 fs; the lifetimes of the slower 3.5- and 21-ps components were unaffected.

When the excitation wavelength was changed to 665 nm, a clear qualitative difference became apparent between the fastest component (having a lifetime of 100 ± 50 fs) and the slower components (lifetimes of ≈600 fs and ≈3.5 ps). The 100-fs component changed sign at all probed wavelengths when the excitation wavelength was changed (see below), while the slower components did not. The lifetime of the fastest component after excitation at either 665 or 694 nm was 100 ± 50 fs as the lifetimes were indistinguishable. Differences in the 100-fs components are clearly visible in the kinetic data shown in Fig. 1. For example, there is an ≈50-fs lag in the appearance of the transient signal at 685 nm after excitation at 665 rather than 694 nm (Fig. 1*A*), while Fig. 1*B* shows particularly clearly the change in sign of the 100-fs component, which occurs when the excitation wavelength is changed.

Fig. 2 shows spectra of the amplitudes of the 100-fs components after excitation at 665 and 694 nm [i.e., kinetic spectra (4)]. These spectra are dominated by the transient bleaching of ground state Q_y-absorption bands and stimulated emission from chlorin excited singlet states. Both spectra show features characteristic of energy transfer between pigments having different band maxima and oscillator strengths (see below). The spectrum of the ≈100-fs component after 694-nm excitation is inverted compared to that obtained after 665-nm excitation. This is what would be expected from components that are due to energy transfer in opposite directions between the two pigment pools.

The transient absorption changes resulting from the 100-fs components are further illustrated in Fig. 3. This figure shows both the transient absorption spectra at 0 ± 20 fs time delay (obtained from extrapolation of the fitted functions back to t = 0 fs) and the spectra that would result from the decay of the amplitude of the 100-fs component to zero. The transient

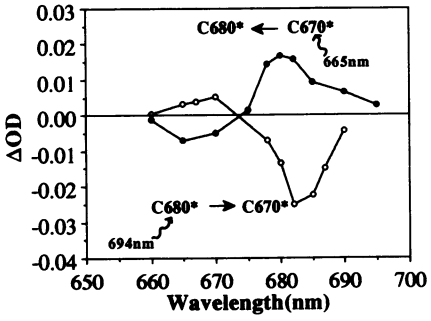

FIG. 2. Spectra of amplitudes of the ≈100-fs transient absorption components observed between 660 and 695 nm after excitation at 665 (●) and 694 (○) nm. These kinetic spectra (sometimes called decay-associated spectra) were determined from global analyses of kinetic data such as those shown in Fig. 1. The spectrum observed using 665-nm excitation has a negative maximum at 670 nm corresponding to a recovery of the initial C670 bleach/stimulated emission and a positive maximum at 680 nm corresponding to production of C680 bleach/stimulated emission. The positive 680-nm maximum is larger than the negative 670-nm maximum due to the larger mean oscillator strength of the C680 chlorins relative to the C670 chlorins (see *Discussion*). The spectrum obtained using 694-nm excitation is inverted with respect to the spectrum obtained using 665-nm excitation. C670* and C680* are lowest excited singlet states of C670 and C680 chlorins, respectively.

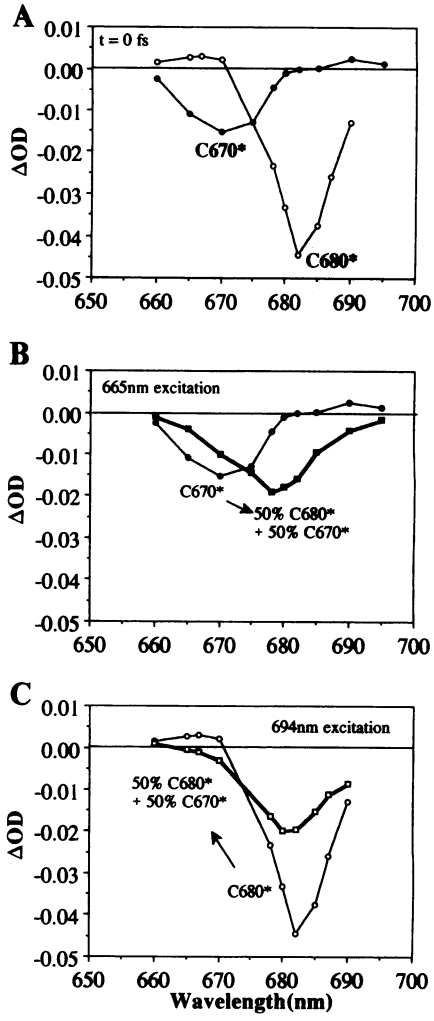

FIG. 3. Transient absorption spectra before (○, ●) and after (□, ■) the 100-fs components. Excitation was at 665 (●, ■) or 694 (○, □) nm. These spectra were calculated from the sum of amplitudes of all the kinetic components resolved in the global analyses, either including (○, ●) or excluding (□, ■) contributions from the 100-fs components. The spectra before the 100-fs components correspond to the spectra at t = 0 ± 20 fs and are shown together in *A*. These data do not take account of any components with lifetimes of ≪100 fs.

spectra at t = 0 (Fig. 3*A*) clearly demonstrate the effectiveness of the photoselection achieved with both the 665- and 694-nm excitation pulses. This also confirms the validity of grouping the PSII reaction center chlorins into these two pools. Fig. 3 *B* and *C* is discussed in more detail below.

DISCUSSION

We have shown that it is possible to excite selectively the C670 or C680 chlorin pools in the isolated PSII reaction center. After excitation of either pigment pool, complex multiexponential transient absorption kinetics are observed. The fastest component has a lifetime of 100 ± 50 fs after excitation of either pigment pool and can be qualitatively distinguished from the slower components (lifetimes of ≈600 fs and ≈3.5 ps) as follows. The spectrum of the 100-fs components between 660 and 690 nm completely inverts when the excitation wavelength is changed from 694 to 665 nm, while the spectra of the slower components do not. This 100-fs component is assigned to energy transfer between the C670 and C680 chlorin pools, presumably resulting in excitation energy equilibration between the C670 and C680 pigments. The slower 600-fs and 3.5-ps components are as-

signed, as previously (33), to decay of a delocalized P680 excited singlet state.

Our observation that the 100-fs energy-transfer processes can be completely reversed by changing the excitation wavelength indicates that all of the states involved are optically accessible singlet excited states. Moreover, neither chlorin cation nor anion states are present at these early times (unpublished data). The observation that the spectra of these two components are opposite (Fig. 2) indicates that neither polarization effects nor complications of excited state absorption significantly affect the observation of this equilibration.

Composition of the C680 and C670 Pigment Pools and Delocalization of P680. The structural identity of P680, the primary electron donor of PSII, is currently the subject of some controversy (25, 26, 35–38), and the extent of the analogy between P680 and the special pair of bacteriochlorophylls found in reaction centers of purple bacteria is unclear. The results presented here, however, do allow us to determine the degree of delocalization of the P680 excited singlet state. This can be achieved by comparing the absorption changes resulting from energy transfer between the C670 and C680 pigment pools (Fig. 3). Energy transfer from C670 pigments to C680 pigments results in a 60% increase in the area of the transient spectrum (bleached oscillator strengths), while energy transfer in the reverse direction results in a 40% reduction in the area.

These large changes cannot be attributed to differences in excited state absorption. The excited state absorption (S_1–S_n) of chlorophyll a is known (39). The extreme case of C680* exhibiting no excited state absorption can only account for a quarter of the observed changes.

From the data described above, it can therefore be concluded that the C680 pigments exhibit a significantly higher Q_y-band oscillator strength than the C670 pigments. The relative magnitudes of the C670 and C680 oscillator strengths can be quantified by using the 0-fs spectra shown in Fig. 3*A* to simulate the kinetic spectra of the 100-fs components shown in Fig. 2. Alternatively the areas of these 0-fs spectra following 665- and 694-nm excitation can be compared directly, as these spectra have been normalized to take account of differences in excited reaction center populations (see *Materials and Methods*). Both estimates indicate that C680 excited singlet states result in an absorption change 2.5 ± 0.6 times larger than those of C670 pigments. The 670-nm-absorbing chlorins are primarily monomeric chlorophylls (25, 26), and we therefore conclude that the C680 pigments exhibit a Q_y-band oscillator strength at least twice that of monomeric chlorophyll species. This observation presumably results from P680 dominating the C680 absorption changes observed here, with the excited singlet state(s) of P680 being delocalized over at least two chlorin molecules.

Our conclusion that P680 comprises at least two coupled chlorin molecules is in agreement with a previous study by us (33) that was based on a comparison of data collected using 612- and 694-nm excitation. This conclusion is supported by absorption spectroscopy of the P680 triplet state (26, 37, 38), circular dichroism (25, 40), fluorescence anisotropy (41), and gaussian deconvolution (25, 26) studies of PSII reaction centers. Kwa *et al.* (41) interpreted their fluorescence anisotropy measurements as indicating that the exciton coupling between the P680 chlorins is broken sometime after the initial excitation. Our results indicate that the P680 singlet state remains delocalized for at least a few hundred femtoseconds. It is possible, however, that the initially excited delocalized P680 singlet state may localize on a single pigment on a slower time scale. Our conclusion that the initially excited P680 singlet state is delocalized over at least two pigments, however, does not necessarily imply that these pigments are structurally analogous to the special pair of bacterial reaction centers.

We have concluded that the C680 pigment pool is dominated by P680, in agreement with previous gaussian deconvolution studies (25, 26). The large amplitude of the 100-fs components observed here after excitation of either the C670 or C680 pigments indicates that the majority of the chlorins in the PSII reaction center participate in these femtosecond energy-transfer processes. Several other studies, including our own, have observed slower energy-transfer processes in PSII reaction centers with lifetimes ranging from several tens to hundreds of picoseconds (4, 28, 30, 31). It must be concluded that, at least at room temperature, these slower energy-transfer processes are associated with only a minority of chlorins in the reaction center, in agreement with our previous studies (4). We have not yet determined whether the two Ph in the PSII reaction center are included in the femtosecond equilibration of excitation energy we have observed.

Establishment of a Boltzmann Equilibrium? The spectra of the excitation pulses indicate that 694-nm excitation will produce vibrationally cool C680 pigments, while 665-nm excitation will excite C670 pigments with excess vibrational energy. However, the same rate of equilibration of the excitation energy is observed after excitation of either pigment pool. Moreover, we have observed an endothermic energy transfer from C680 chlorins to C670 chlorins. These observations imply that some thermalization of vibrational energy occurs within 100 fs, which suggests that the equilibrium produced by the 100-fs energy-transfer processes may be a Boltzmann equilibrium.

If there is indeed a Boltzmann equilibrium between the C670 and C680 pigment pools, the equilibrium constant can be determined from the relative amplitudes of the 100-fs components after excitation at 665 and 694 nm (the amplitudes of these components reflect the difference between the initial excitation energy distributions and those at equilibrium). This yields an equilibrium constant of 1.0 ± 0.5 between the C670 and C680 pigment pools. This equilibrium constant is consistent with that expected for such a Boltzmann equilibrium, given the 10-nm separation of the pools' Q_y-absorption bands and assuming that a single species (P680) dominates the C680 pool and that the C670 pool comprises two to four chlorins.

Fig. 3 *B* and *C* shows the transient spectra before and after the 100-fs energy-transfer equilibration is complete. Similar transient spectra are obtained after equilibration with either 665- or 694-nm excitation (Fig. 3 *B* and *C*; square symbols). The observation of similar spectra supports our conclusion described above that excess excitation energy is largely thermalized on this time scale. The transient spectra at equilibrium are dominated by P680 bleach/stimulated emission due to the higher oscillator strength of P680 compared with those of the C670 chlorins. Therefore, energy transfer from C670 chlorins to P680, thereby forming the equilibrium state, results in a large shift in the peak of the transient spectrum from ≈670 to ≈678 nm (Fig. 3*B*), whereas energy transfer from P680 to the C670 chlorins to form an equilibrium results in only an ≈2-nm shift in the peak of the transient spectrum in the opposite direction (Fig. 3*C*). Nevertheless, there is a large change in the amplitude of the transient spectrum at 680 nm and a change in sign at 670 nm (see Fig. 3*C*).

Close inspection of Fig. 3 *B* and *C* indicates a spectrum after the 100-fs component using excitation at 665 nm is blue shifted ≈2 nm relative to the spectrum observed using 694-nm excitation. This small difference may result from the exclusive use of parallel polarized excitation and probe pulses, as this will have the effect of underestimating the absorption changes of pigments different in orientation to those directly excited (this may also explain the small differences between the two spectra shown in Fig. 2). Alternatively, the 2-nm blue shift could result from an incomplete thermalization of excess vibrational energy or from the

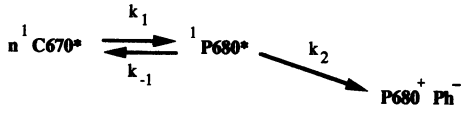

FIG. 4. Simple kinetic model of isolated PSII reaction centers including energy transfer between P680 and the C670 pigment pool and charge separation resulting in the formation of $P680^+Ph^-$. k_1 and k_{-1} are energy-transfer rate constants, and k_2 is an electron-transfer rate constant. This model is correct only if there are no intermediate states between P680* and $P680^+Ph^-$ (see text).

presence of some 670-nm-absorbing chlorins, which are unable to transfer excitation energy to the other pigments on this time scale.

One of the decay pathways for the primary radical pair state is via charge recombination and radiative decay to the ground state. It follows from our observation of an ≈1:1 equilibrium between P680 and the C670 chlorins that the charge recombination or delayed fluorescence produced by this decay pathway will be emitted by both P680 and C670 chlorins. This conclusion provides an explanation for the broad width of the time-resolved emission spectrum for the charge recombination fluorescence measured by Booth *et al.* (18).

Tentative Kinetic Model for Trapping Excitation Energy in the PSII Reaction Center. We have reported elsewhere that Ph reduction is observed with a lifetime of 21 ± 3 ps following excitation of PSII reaction centers at 612 or 694 nm (4). It can therefore be concluded that equilibration of the excitation energy between the majority of pigments precedes Ph reduction. Therefore, it is appropriate to describe the kinetics within the PSII reaction center in terms of the trapping limited model (7, 9, 11), as illustrated in Fig. 4. Using the equilibrium constant (k_1/k_{-1}) of 1.0 ± 0.5 for distribution of excitation energy before radical pair formation and an observed rate of radical pair formation of 21 ps^{-1}, we obtain a value of 10.5 ± 3 ps^{-1} for the intrinsic rate of primary charge separation (k_2) in PSII. However, it must be appreciated that this model assumes that the only trapping process is the 21-ps Ph reduction. As other kinetic components are observed with lifetimes of a few picoseconds, this assumption may not be valid. Identification of these unassigned components will allow development of a more complete kinetic model for the electron- and energy-transfer pathways within the isolated PSII reaction center.

Solution of the kinetic model shown in Fig. 4 determines that the observed rate of equilibration of the excitation energy is approximately the sum of the two energy-transfer rates k_1 and k_{-1}. As we have determined the equilibrium constant k_1/k_{-1} to be 1 ± 0.5, it follows that $k_1 \approx k_{-1} \approx 200 \pm 100$ fs^{-1}. It should be noted that k_1 and k_{-1} correspond to the mean rates of energy transfer between the two pigment pools rather than to rates of energy transfer between two specific chromophores.

The C680 pool appears to be dominated by P680 alone. We can therefore conclude that the average energy-transfer rate from individual C670 chlorins to P680 is ≈200 fs^{-1}. The separation in energy between the C670 and C680 S_1 levels yields a rate of 600 fs^{-1} for energy transfer from P680 to each C670 chlorin. These energy-transfer rates are of the same order of magnitude as those previously estimated for antenna/reaction center complexes of PSI (12, 13) and PSII (9), and within LHC2 (42), and are also consistent with Förster energy transfer (42).

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