Rate of Oxidation of P680 in Isolated Photosystem 2 Reaction Centers Monitored by Loss of Chlorophyll Stimulated Emission†

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ABSTRACT: We have continued our studies of the primary photochemistry of isolated photosystem 2 reaction centers using femtosecond transient absorption spectroscopy. Experiments were performed over a wide range of excitation and probe wavelengths, using several data collection time scales. This has enabled us to resolve five different lifetimes ranging between 100 fs and 200 ps plus a nanosecond component. We demonstrate here and elsewhere [e.g., Durrant, J. R., Hastings, G., Joseph, D. M., Barber, J., Porter, G., & Klug, D. R. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 11632–11636] that the kinetic spectra associated with all but two of these lifetimes are clearly distinguishable. We have previously reported that a 21-ps lifetime is associated with pheophytin reduction [Hastings, G., Durrant, J. R., Hong, Q., Barber, J., Porter, G., & Klug, D. R. (1992) *Biochemistry 31*, 7638–7647]. In this paper, we show that it is possible to spectrally and temporally resolve stimulated emission from PS2 reaction centers with great accuracy and that this stimulated emission is largely unaffected by those kinetic components which are faster than 21 ps. The observation of a distinct stimulated emission band allows us to distinguish charge-separated states from chlorin singlet states. In this way, we are able to show that the proportion of charge-separated states prior to the 21-ps component is between 0% and 25%. We also show that the shape of the spectrum which we obtain for the state $P680^+Ph^-$ is essentially invariant between 100 ps and 9 ns, and is the same as that previously reported for $P680^+Ph^-$ by other researchers. We therefore conclude that P680 oxidation largely occurs with an effective rate of $(21\ ps)^{-1}$, the same rate that we have previously reported for pheophytin reduction.

The primary photochemistry and photophysics of photosynthetic reaction centers have been the subject of intense study over the last 20 years. These studies have focused principally upon the reaction centers (RC's) of purple bacteria. Time-resolved optical spectroscopy of bacterial RC's has revealed complex kinetics on the femtosecond and picosecond time scales. Some of these kinetics have been variously interpreted in terms of multistep electron-transfer reactions (Holzapfel et al., 1990; Chan et al., 1991), heterogeneous electron-transfer rates (Kirmaier & Holten, 1990), vibrational coherence (Vos et al., 1991), and electronic relaxations (Vos et al., 1992). Studies of bacterial reaction centers have employed a wide range of experimental procedures in order to assist assignment of the observed kinetics to specific processes, including site-directed mutagenesis (Nagarajan et al., 1990), studies as a function of temperature (Fleming et al., 1988), and comparisons between time-resolved absorption and emission data (Du et al., 1992). In these studies, it was found to be important to conduct transient experiments using low-intensity (Holten et al., 1980) and variable-wavelength (Breton et al., 1986) excitation pulses, over a range of probe wavelengths and time scales (Holzapfel et al., 1990).

We report here some recent results from our studies of charge separation in photosystem 2 (PS2)[1] reaction centers isolated from higher plants. There have been relatively few studies of the primary photochemistry of this reaction center compared to that of purple bacteria, although the similarities between these two reaction centers have been widely discussed (Rutherford, 1986; Barber, 1987; Michel & Deisenhofer, 1988). However, we have recently demonstrated that the photochemistry of PS2 reaction centers is additionally complicated by energy-transfer processes (Durrant et al., 1992b). These energy-transfer processes are extensive, because the lowest excited singlet states of the chlorins bound to the PS2 reaction center are nearly isoenergetic at room temperature, in contrast to the situation in purple bacterial reaction centers. Time-resolved spectroscopic studies of this PS2 complex are expected to resolve several kinetic processes occurring on a range of time scales, as has indeed been observed (Durrant et al., 1992a,b; Hastings et al., 1992; Booth et al., 1991; Roeloffs et al., 1991; Wasielewski et al., 1989a,b; Jankowiak et al., 1989; Tang et al., 1990). In this study, we employ transient absorption spectroscopy at room temperature using a large range of excitation and probe wavelengths and data collection time scales, in order to distinguish and identify some of these processes.

The primary electron donor of PS2 is associated with a spectral feature at 680 nm and is referred to as P680 (van Gorkom et al., 1975; Doring et al., 1969). A pheophytin (Ph) molecule is known to act as an electron acceptor (Hastings

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[1] Abbreviations: PS2, photosystem 2; Ph, pheophytin; P680, primary electron donor of PS2; Chl *a*, chlorophyll *a*.

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et al., 1992; Danielius et al., 1987; Klimov et al., 1977) in a light-induced charge separation which produces the radical pair $P680^+Ph^-$. In large PS2 particles, this charge separation is subsequently further stabilized by secondary electron-transfer reactions which do not occur in the PS2 reaction center. One critical difference between PS2 and purple bacterial photosystems is that $P680^+$ has a 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. Thus, although the major polypeptides of the PS2 reaction center are homologous with those of purple bacteria, some modifications must exist to allow the generation and stabilization of this high oxidizing potential.

The reaction center of PS2 was first isolated in 1987 (Nanba & Satoh, 1987; Barber et al., 1987). An early study of this reaction center determined that the radical pair $P680^+Ph^-$ was formed in less than 25 ps (Danielius et al., 1987). Subsequent studies by Wasielewski and co-workers reported a lifetime of 3 ps for both the decay of the P680 excited singlet state (P680*) and the formation of features which they attributed to the state $P680^+Ph^-$ (Wasielewski et al., 1989a,b). Our own transient absorption studies of the PS2 reaction center have, however, produced very different data than those of Wasielewski and co-workers. Spectral hole burning and time-resolved fluorescence measurements could be interpreted as supporting the results of Wasielewski et al. (Jankowiak et al., 1989; Roeloffs et al., 1991); however, two other research groups have recently produced transient absorption data on PS2 reaction centers which essentially agree with our own data and its interpretation (McCauley et al., 1992; Schelvis et al., 1992).

We have used selective excitation of P680 to help resolve multiexponential transient absorption kinetics in PS2. A 21-ps component was observed and assigned to the production of reduced pheophytin (Hastings et al., 1992). This assignment was based upon the observation that this component produced a bleaching of the pheophytin Q_x absorption band at 545 nm, and the appearance of a pheophytin anion absorption band at 460 nm. At least 60% of pheophytin reduction was found to occur with this 21-ps lifetime (Hastings et al., 1992). We also found that the spectrum which develops after the 21-ps lifetime was the same as that previously reported for the state $P680^+Ph^-$ (Danielius et al., 1987; Nuijs et al., 1986; Takahashi et al., 1987). More detailed spectra of the $P680^+Ph^-$ state are presented in this paper. Two faster components with lifetimes of 400 fs and 3.5 ps have also been resolved and assigned to the decay of an initially delocalized P680 excited singlet state (Durrant et al., 1992a).

We have recently reported the resolution of an additional component with a lifetime of 100 ± 50 fs (Durrant et al., 1992b). This component could be clearly resolved only in data collected on a 0–2-ps time scale, and was therefore not reported in our previous study (Durrant et al., 1992a). This 100-fs component results from energy transfer between P680 and accessory chlorins with absorption maxima near 670 nm. By the choice of appropriate excitation wavelengths, it is possible to drive this net energy transfer either from P680 to the accessory chlorins or vice versa. These energy-transfer processes result in equilibration of excitation energy between the majority of reaction center chlorins in 100 fs.

The primary aim of this study is to determine the effective rate of P680 oxidation in isolated PS2 reaction centers. This measurement is complicated by the fact that the excited-state absorption of $P680^+$ is broad, without any pronounced features (Barber et al., 1987; Telfer et al., 1990; Nuijs et al., 1986), while the ground-state spectrum of P680 overlaps extensively with spectra of the other chlorins bound to the reaction center (van Kan et al., 1991; Braun et al., 1990). Therefore, there are no spectral features at wavelengths accessible to our spectrometer (440–900 nm) which are unambiguous markers for the state $P680^+$. Earlier studies reported that $P680^+$ exhibits a slight positive absorption maximum near 820 nm (Mathis & Setif, 1981; van Gorkom et al., 1975); more recent studies have suggested, however, that $P680^+$ absorption is fairly flat and featureless over this region (Mathis et al., 1989; Telfer et al., 1990). Finding another method of measuring the rate of P680 oxidation is therefore highly desirable. In the results we present here, we separate and identify those spectral features associated with P680 and chlorophyll singlet excited states (P680* is in equilibrium with chlorophyll*; Durrant et al., 1992b) and those which are due to the radical pair state $P680^+Ph^-$. In this way, we can show that P680*/chlorophyll* decays at the same rate at which $P680^+Ph^-$ appears. We also show here and elsewhere that the 400-fs and 3.5-ps components (Durrant et al., 1992a) produce at most only 25% of the P680 cation and probably have nothing to do with pheophytin reduction at all, but are primarily associated with the localization of an initially delocalized P680 singlet state.

The results presented here extend our previous studies of isolated PS2 reaction centers (Durrant et al., 1992a,b; Hastings et al., 1992) to a much larger range of probe wavelengths than was initially required to determine the rate of pheophytin reduction. These recent observations are combined with more complete assignments of faster kinetic components in order to present a simplified view of the PS2 reaction center.

MATERIALS AND METHODS

Experimental details were the same as those reported previously (Durrant et al., 1992a; Hastings et al., 1992). Photosystem 2 reaction centers were isolated from pea thylakoid membranes (Chapman et al., 1991) and studied under anaerobic conditions at room temperature (295 K). This reaction center contains six chlorophylls, two pheophytins, two β-carotenes, and one cytochrome *b*-559 (Gounaris et al., 1990; Kobayashi et al., 1990). Q_A and Q_B, the secondary electron acceptors of PS2, are lost during the isolation procedure. Optical excitation of this complex results in the formation of the radical pair $P680^+Ph^-$ with a near-unity quantum yield (Booth et al., 1991).

The absolute activity of all samples was checked before and after their use. Transient absorption experiments were conducted using excitation pulses centered at either 612, 665, 685, or 694 nm. Excitation at 694 nm results in the selective excitation of P680, while excitation at 665 nm results in the selective excitation of accessory chlorins with absorption maxima near 670 nm (Durrant et al., 1992a,b). This photoselection discriminates between these two pools of pigments very effectively (Durrant et al., 1992b). Excitation at 612 or 687 nm results in the excitation of both P680 and the accessory chlorins. Only 5–10% of reaction centers in the pumped volume were excited by each excitation pulse in order to avoid multiphoton processes. One second of signal-averaging produced a noise level of $\approx 4 \times 10^{-5}$ ΔOD. In these experiments, the instrument response of the spectrometer had a 10–90% rise time of 130–180 fs over the range 440–900 nm as previously reported (Durrant et al., 1992b).

Data were collected on four time scales (0–2, 0–12, 0–80, and 0–300 ps), and globally analyzed assuming multiexpo-

nential kinetics (Hastings et al., 1992; Durrant et al., 1992b). In each global analysis, appropriate consideration was taken of data collected on both slower and faster time scales. In addition, some of the data from different time scales were globally analyzed together in order to assist in the resolution of certain kinetic components. A total of over 200 independent global analyses have been conducted of over 2000 data sets collected at ≈50 probe wavelengths, using 4 different excitation wavelengths. This has enabled us to resolve five exponential components with lifetimes of approximately 100 fs, 600 fs, 3.5 ps, 21 ps, and 200 ps in addition to a nondecaying component (Hastings et al., 1992; Durrant et al., 1992a,b). We will summarize the manner in which we can distinguish these components from each other later in this paper.

RESULTS

Spectrum of P680⁺Ph⁻. We have previously shown that Ph is reduced with a rate of (21 ps)$^{-1}$ at room temperature. Ph reduction should therefore be complete by 100 ps (five lifetimes for a 21-ps component), leaving no residual singlet states. We demonstrate that this is indeed the case by comparing radical pair spectra at 100 ps and 9 ns. Figure 1a shows the absorption difference spectrum observed 100 ps after excitation of isolated PS2 reaction centers at 694 nm. This spectrum is an extension of that previously reported by Hastings et al. (1992), and is assigned to the radical pair state P680⁺Ph⁻. The spectrum is in good agreement with spectra previously assigned to P680⁺Ph⁻ (Danielius et al., 1987; Nuijs et al., 1986; Takahashi et al., 1987; Schlodder & Brettel, 1988), and with previous steady-state and time-resolved studies of the P680 cation and pheophytin anion states (Fujita et al., 1978; Barber et al., 1987; Nanba & Satoh, 1987; Mathis et al., 1989; Telfer et al., 1990). This spectrum (Figure 1a) is, however, very different from that reported by Wasielewski et al. (1989a,b) for P680⁺Ph⁻. We have labeled several of the major spectral features shown in Figure 1a on the basis of these earlier studies. This labeling indicates which features dominate the spectrum at certain wavelengths, and is not intended to suggest that these contributions are exclusively reponsible for the absorption changes.

Our assignment of the 100-ps transient spectrum to a pure P680⁺Ph⁻ state (with no chlorin singlet states) was verified by the transient spectrum at 9 ns (Figure 1b). Chlorin singlet excited states decay with lifetimes of ≈6 ns, and therefore any such states which contribute to the 100-ps spectrum would largely have decayed by 9 ns. Essentially identical spectra are observed at time delays of 100 ps and 9 ns (Figure 1a,b), and it can therefore be concluded that chlorin singlet excited states (which have a maximum lifetime of 6 ns) do not contribute significantly to either transient spectrum. We estimate that an upper limit of 10% excited chlorin singlet states contributes to the 100-ps transient spectrum.

Transient Spectra of Chlorophyll a. Figure 2a shows the transient spectrum of chlorophyll *a* in ether 10 ps after excitation with 665-nm pulses. Similar spectra were obtained for chlorophyll *a* in methanol [data not shown; see also Shepanski and Anderson (1981)] and have also been reported for isolated light-harvesting chlorophyll *a*/*b* complexes (Nuijs et al., 1986; Klug, 1987). These spectra all represent Chl *a* excited singlet states.

The spectrum of the Chl *a* or P680 excited singlet state can be readily distinguished from that of P680⁺Ph⁻ in the near-infrared (700–860 nm), as is illustrated by the following description. P680⁺Ph⁻ seems to produce a fairly flat positive absorption change between 710 and 810 nm when parallel

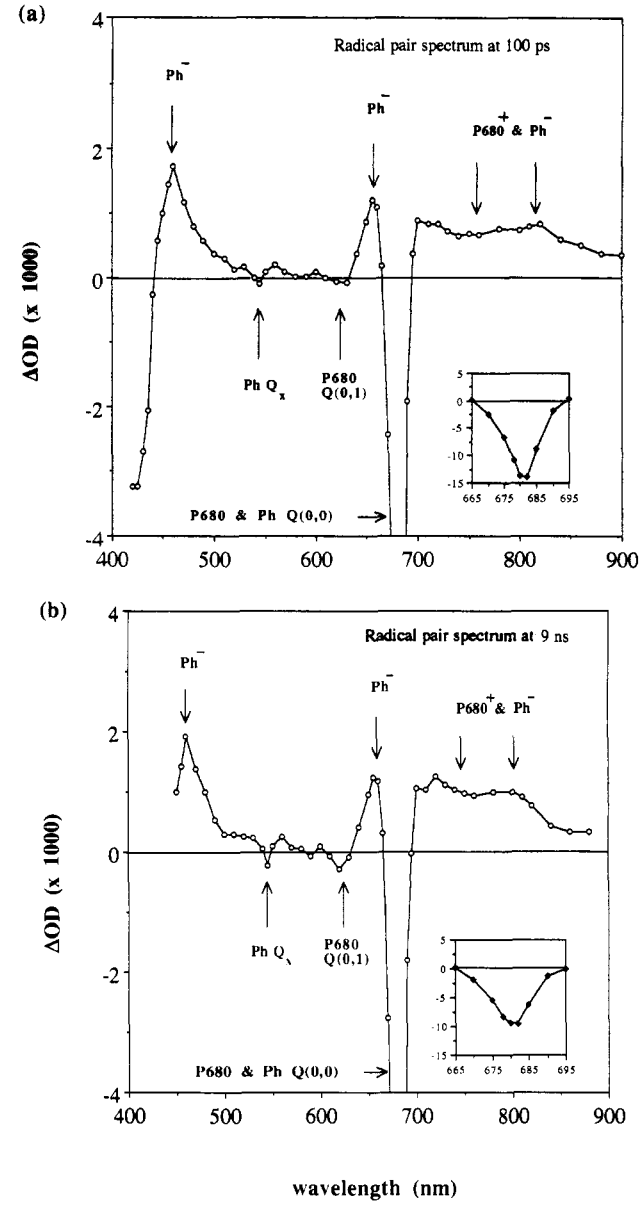

FIGURE 1: Absorption difference spectra observed at time delays of (a) 100 ps and (b) 9 ns following photoselective excitation of P680 in isolated PS2 reaction centers using 694-nm pulses. The two spectra have essentially the same shape and are both assigned to the state P680⁺Ph⁻. The smaller amplitude of the spectrum observed at 9 ns compared to 100 ps is consistent with the 37-ns mean lifetime obtained previously for the P680⁺Ph⁻ state (Booth et al., 1991; Danielius et al., 1987; Crystall et al., 1989). Similar spectra were obtained using 665-nm excitation (data not shown). Complete 100-ps spectra were obtained in two ways: first, by directly measuring the transient spectrum at 100 ps; second, by kinetic analyses of data collected on 0–80- and 0–300-ps time scales. The spectra obtained by these two procedures were indistinguishable.

polarizations are used for pump and probe beams (see, for example, Figure 1). The difference spectra of P680⁺ and Ph⁻ are thought to be somewhat similar in this spectral region (Fujita et al., 1978; Mathis et al., 1989; Telfer et al., 1990), with P680⁺ having a ≈30% greater extinction coefficient at 820 nm than Ph⁻ (Fujita et al., 1978; Mathis & Setif, 1981). In contrast to the radical pair, the transient spectrum of the Chl *a* excited singlet state shows a net negative absorption change between 715 and 740 nm (Figure 2b). Chl *a* has no ground-state absorption in the near-infrared; therefore, all features are due to the combination of excited-state absorption (positive contribution to a transient spectrum) and stimulated emission (negative contribution to a transient spectrum). The

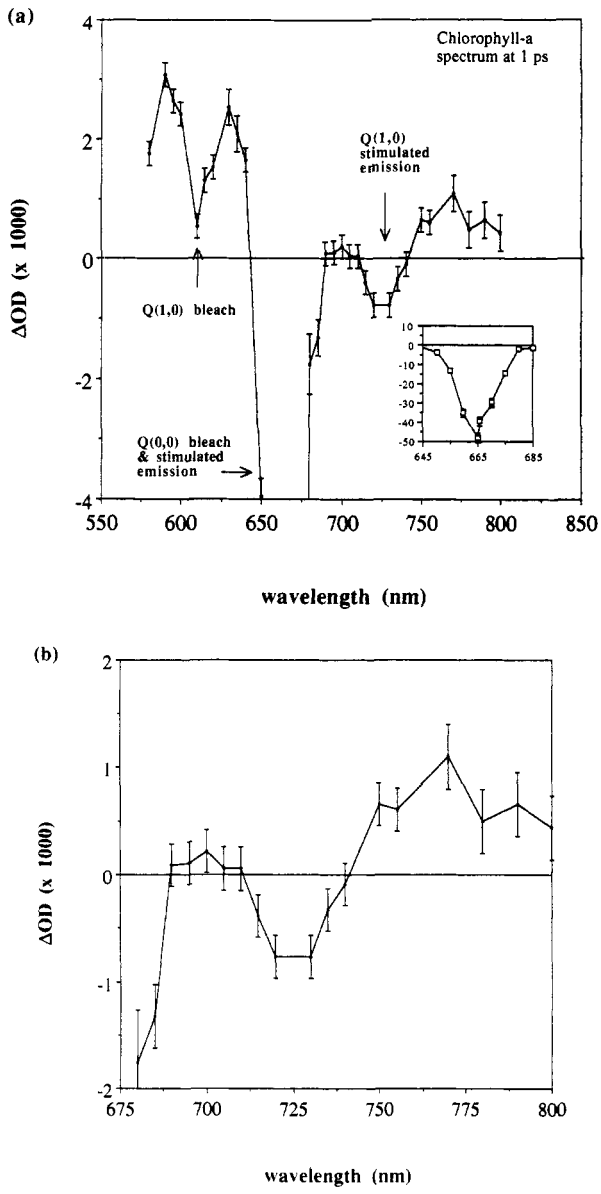

FIGURE 2: Transient absorption spectra 1 ps after excitation of chlorophyll *a* in ether using 665-nm pulses. Panel a shows the spectrum between 575 and 800 nm, and panel b shows an expansion of this spectrum between 680 and 800 nm. The stimulated emission side band is clearly seen to dominate excited-state absorption around 725 nm.

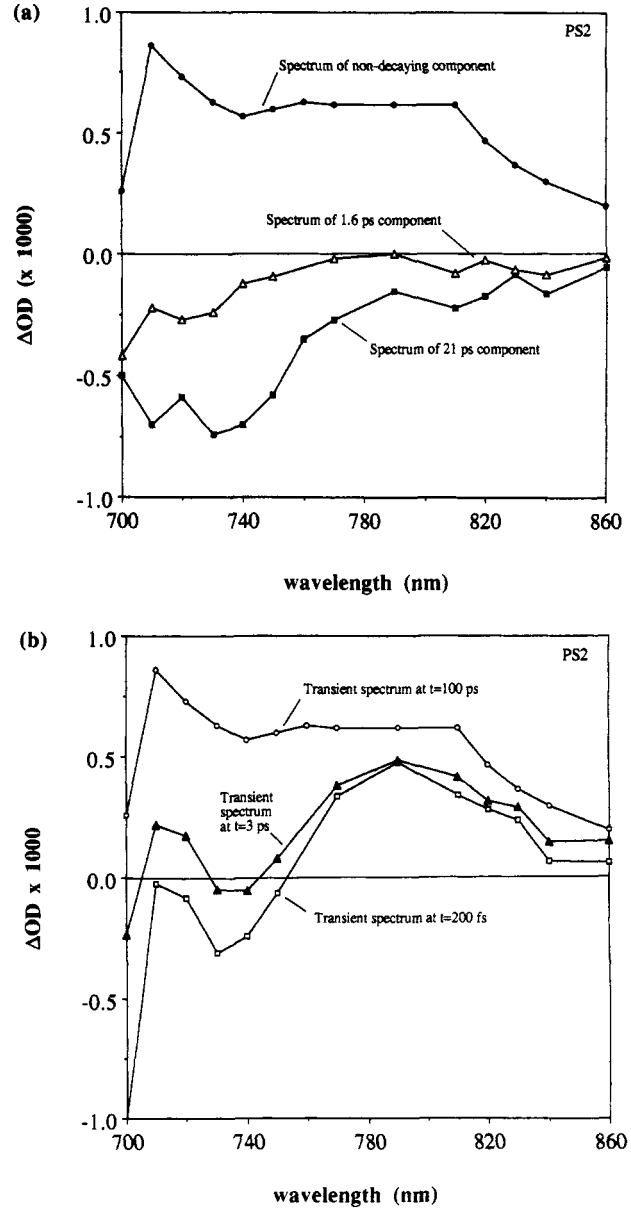

FIGURE 3: (a) Kinetic spectra of the amplitude of 1.6-ps (△), 21-ps (■), and nondecaying (●) components (components 2, 3, and 5, respectively) observed following selective excitation of P680 in isolated PS2 reaction centers using 694-nm pulses. These spectra were determined from global analyses of data collected on 0–13- and 0–80-ps time scales. (b) Absorption difference spectra at time delays of 200 fs (□), 3 ps (▲), and 100 ps (○) calculated from the kinetic spectra shown in (a).

negative feature centered around ≈725–730 nm is due to stimulated emission dominating the transient spectrum at this wavelength. This feature can therefore be used as a marker for the presence of fluorescent chlorin excited states, i.e., Chl *a* or P680 singlet states. The steady-state absorption spectrum of Chl *a* in ether shows an approximately 6.5:1 ratio of Q(0,0): Q(0,1) peak extinction coefficients [Q(0,0) refers to the main Q band of Chl *a*, while Q(0,1) refers to the vibrational side band]. After taking account of the fact that the negative feature at 665 nm has a 50% contribution from the stimulated emission (the Stokes shift of Chl *a* is 6 nm), we find that a ratio of the Q(0,0) bleach to the Q(0,1) bleach is ≈(6–7):1 once the excited-state absorption of Chl *a* has been taken into account. The shape and amplitude of the excited-state absorption spectrum which we estimate are rather similar to those determined previously (Shepanski & Andersson, 1981). Despite the distorting influence of the excited-state absorption which pulls the base line for the Q(0,1) bleach above that of the Q(0,1) stimulated emission, the reflective symmetry between absorption and emission spectra of the Chl *a* Q(0,1) transition is seen in Figure 2a to be well-preserved.

We find that P680 also exhibits a Q(0,1) stimulated emission side band, which can be seen in the 200-fs transient spectra of Figure 3b or Figure 6. This allows us to use the P680 stimulated emission side band to time-resolve the loss of P680 singlet states. We presume that the Q(0,0) band of P680 produces stimulated emission as well as the Q(1,0) band. Unfortunately, the Q(0,0) region of the reaction center absorption spectrum is highly congested with many Gaussian bands overlapping over a relatively narrow wavelength range. The small Stokes shift in chlorophyll means that the Q(0,0) P680 stimulated emission band will overlap with the P680 bleach. This makes it very difficult to assign any spectral changes in the Q(0,0) region unambiguously to stimulated emission, which is why the conclusions of this paper are partly based on observations of the Q(1,0) band.

Table I: Exponential Components Resolved in Global Analyses of Transient Absorption Data from PS2 Reaction Centers

component	central excitation wavelength (nm)			
	612	665	685	694
1	not present[a]	100 ± 50 fs	not present	100 ± 50 fs
2	1.6 ± 0.5 ps	1.5 ps (400 fs + 2.6 ps)[b]	1.5 ps (900 fs + 3.5 ps)[b]	1.6 ps (600 fs + 3.7 ps)[b]
3	21 ± 3 ps	27 ± 4 ps	[c]	21.5 ± 2 ps
4	200 ± 100 ps			not present
5	nondecaying[d]	nondecaying	nondecaying	nondecaying

[a] Not present: the amplitude of this component was too small to be resolved. [b] Significantly better fits were obtained when an additional component was included, yielding the lifetimes shown in parentheses. It can be concluded that the lifetimes quoted for component 2 are the mean lifetimes of more than one component, or possibly a distribution of lifetimes. [c] Data were not collected on time scales appropriate for the resolution of this component. [d] Nondecaying: a component which did not decay on the time scale of the experiment (lifetime ≫300 ps).

Rate of P680 Oxidation. Table I summarizes the lifetimes obtained for the components resolved in our studies of isolated PS2 reaction centers so far [see also Hastings et al. (1992) and Durrant et al. (1992a,b)]. Table I also demonstrates that approximately the same lifetimes are recovered for each component using any of the four different excitation wavelengths, although the amplitudes of some components are strongly dependent upon excitation wavelength. Two components reported previously with lifetimes of 600 fs and 3.5 ps are grouped together as a single 1.6-ps component in this table. These two components have similar spectra and similar dependence on excitation wavelength (in contrast to all the other components), and it is not clear if they are produced by two distinct physical processes or a single process with a distribution of lifetimes centered on a mean of 1.6 ps (Durrant et al., 1992a). Good fits were obtained when they were combined into a single component with a lifetime of 1.6 ps, except between 650 and 690 nm, where these 600-fs and 3.5-ps components have large enough amplitudes to be distinguished and must be included as separate lifetimes in the analysis.

Figure 3 shows absorption changes in the near-infrared (700–860 nm) observed following selective excitation of P680 in isolated PS2 reaction centers. Similar data were obtained using 665-nm excitation (data not shown), which would be expected from our previous observation that equilibration of excitation energy between the majority of reaction center chlorins occurs in 100 fs (Durrant et al., 1992b). Figure 3a shows "kinetic" spectra of the amplitudes of the 1.6-ps, 21-ps, and nondecaying components between 700 and 860 nm. Figure 3b shows the transient spectra at time delays of 200 fs, 3 ps, and 100 ps calculated from the kinetic spectra. These transient spectra can be considered to correspond to spectra taken prior to the 1.6-ps component (t = 200 fs), prior to the 21-ps component (t = 3 ps), and after the 21-ps component (t = 100 ps). Typical kinetic data are shown in Figure 4.

Comparison of Figures 1, 2, and 3 allows an assignment of the 21-ps component. The spectrum at t = 100 ps in Figure 3 is assigned to the radical pair state as discussed above [the peak at 710 nm is probably produced by pheophytin anion absorption (Fujita et al., 1978)]. The spectrum at t = 200 fs (Figure 3b) is similar to that observed for the excited singlet states of chlorophyll (Figure 2). However, the key result is that the spectrum after the 1.6-ps component but before the 21-ps component is still essentially that of chlorin singlet excited states, and moreover is clearly distinguishable and different from the spectrum of the radical pair state. For example, the 3-ps spectrum clearly exhibits the stimulated emission side band at 730 nm, and does not exhibit the broad positive absorption changes between 710 and 810 nm which is characteristic of the $P680^+$ and Ph^- states. The same lifetime (21 ± 3 ps) was recovered from analyses of data collected in the 730-nm region (decay of the stimulated emission side band), 800–860 nm (production of $P680^+Ph^-$ absorption), and 540–550 nm (bleaching of the pheophytin ground-state Q_x band).

Figure 5 compares and contrasts the kinetic spectra of the 1.6- and 21-ps components between 600 and 850 nm. These two components are most clearly distinguishable between 650 and 700 nm. The amplitude of the 21-ps component is relatively small in this region. The spectrum of this component has a narrow positive feature at 682 nm which probably represents the bleaching of a pheophytin Q_y absorption band, while the broad negative features are due to loss of stimulated emission and changes in excited-state absorption (Hastings et al., 1992). In contrast, the 1.6-ps component is dominated by a large negative feature with a maximum at 682 nm which corresponds to 50% recovery of the total bleach/stimulated emission [see also Durrant et al. (1992a)] which we assign to exciton localization. It is clear from these spectra (Figure 5) that the 21- and 1.6-ps components are due to completely different physical processes.

Figure 6 is an attempt to summarize our results concerning the rate of radical pair formation in PS2. The transient spectra of Figure 6 have subtle differences which are best demonstrated using spectra of kinetic components [e.g., see Hastings et al. (1992) and Durrant et al. (1992b)]. This is because the assignable features are ≈20 times smaller than the bleach of the Q(0,0) bands, which is why very high signal to noise is required to interpret these data properly. The similarity of the features in the Q(0,0) region, and the congestion of the spectral features of P680, Chl a, and pheophytin a, requires any assignments to be based on the more subtle and smaller features such as those around 545 nm (Hastings et al., 1992) which can be used to identify bleaching of pheophytin, the stimulated emission side band of P680 at 730 nm, and the radical pair absorption spectrum between 700 and 860 nm. Despite the problems in using the Q(0,0) region to unambiguously identify $P680^+$ or Ph^-, by studying the Q(0,0) bands we have been able to monitor energy equilibration between P680 and the accessory Chl a's via forward and reverse energy transfer (Durrant et al., 1992b), and observe another two components whose average lifetime is 1.6 ps when observed in the Q(0,0) region (Durrant et al., 1992a), but which we have tentatively assigned to a localization process.

DISCUSSION

Discrimination of Six Kinetic Components. We briefly review the evidence supporting our ability to distinguish six kinetic components from each other, and moreover to assign them to distinct physical processes. The evidence is as follows:

(1) Independent global analyses of data collected at different probe wavelengths yielded the same lifetimes for each

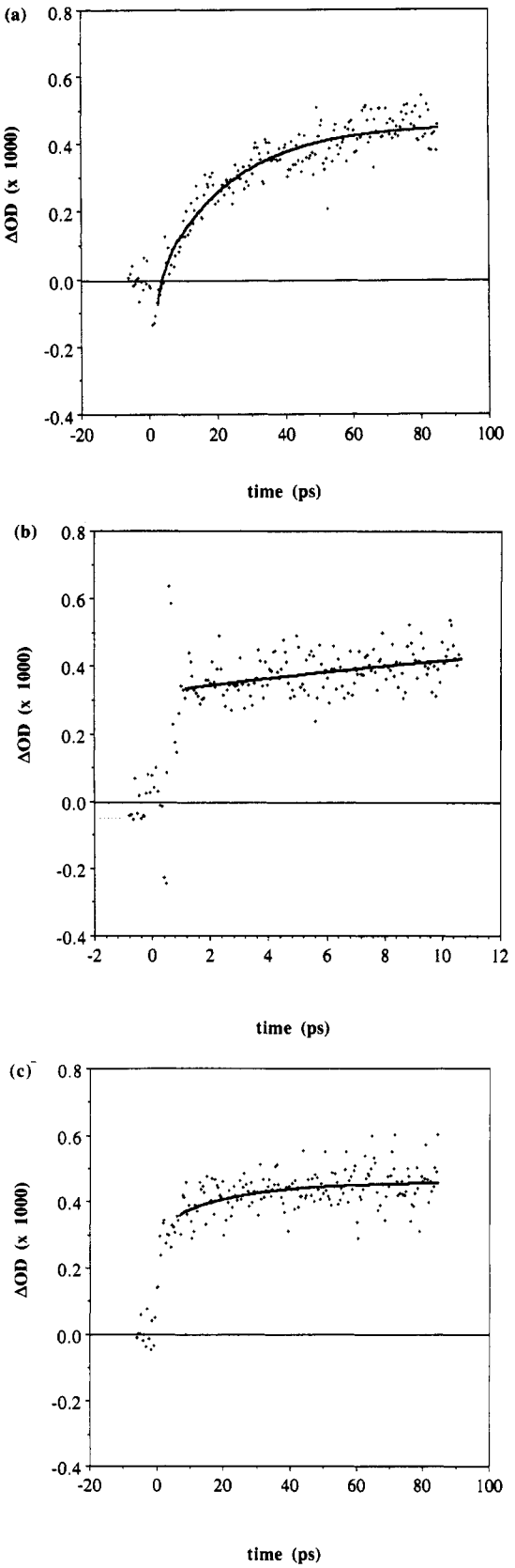

FIGURE 4: Examples of PS2 kinetics at (a) 740 nm and (b, c) 820 nm using 694-nm photoselective excitation of PS2 reaction centers. Kinetic data were collected on both 0–13- and 0–92-ps time scales for all 48 probe wavelengths shown in Figure 1. The solid lines are fitted functions. Data shown here were only analyzed for time delays greater than 200 fs; it was therefore not necessary to include component 1 (lifetime of ≈100 fs) in these analyses. The oscillatory signal observed near zero time delay in (b) comes from impulsive stimulated Raman scattering from the glass windows of the sample cuvette (Hong et al., 1993).

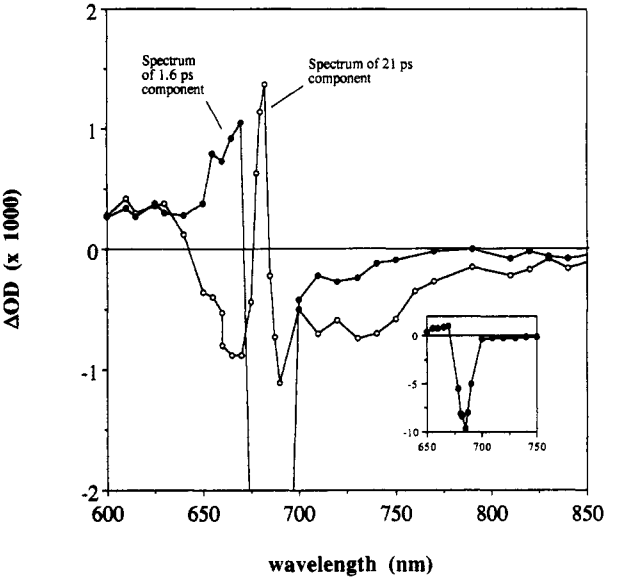

FIGURE 5: Kinetic spectra of the amplitude of the 1.6- (●) and 21-ps (○) components shown between 600 and 850 nm following excitation of PS2 reaction centers at 694 nm. Data collection and analysis details are the same as in Figures 3 and 4. These kinetic spectra are very different from each other which indicates that they are due to quite distinct physical processes.

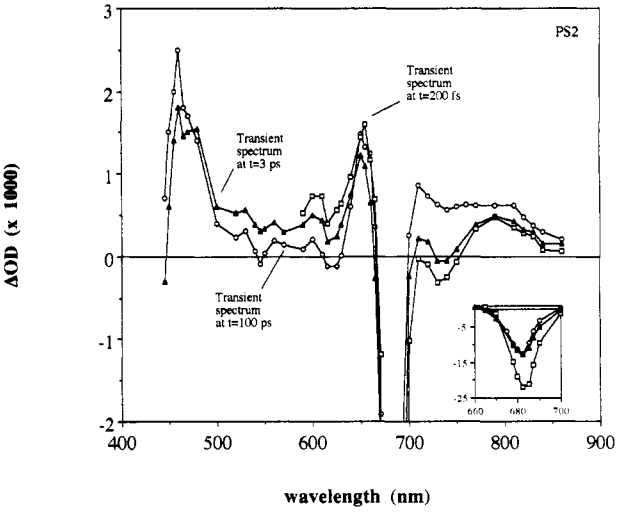

FIGURE 6: Transient spectra at a range of times which are included to give the reader a rough overview of the spectral changes which occur. This figure demonstrates the similarity of the Q(0,0) features at 3 and 100 ps. Only be carefully examining the much smaller changes (factor of 20) in the 700–860- and 660–450-nm regions does the nature of the 21-ps component become clear. The large changes in the Q(0,0) region between 200 fs and 3 ps might mislead one to believe that this component is related to charge separation, but the changes in key spectral regions such as the 730-nm stimulated emission Q(0,1) side band show that P680 is still largely in its singlet state at 3 ps. Small changes in a large transient spectrum cannot be seen in this figure which is why we generally prefer to show kinetic spectra such as those shown in Figure 5. It is possible to see much more detail in the spectra of Figure 5 than can be clearly represented in the transient spectra shown in this figure.

component [for example, see Table I in Hastings et al. (1992)]. Each component has a different isosbestic point (see, for example, Figure 5; isosbestic points appear as points of zero ΔOD in a kinetic spectrum), and analyses of data at these isosbestic points recovered the same lifetimes for the remaining kinetic components.

(2) Changing the excitation wavelength has the effect of changing the distribution of excited states initially populated. If the system thermalizes prior to any of the observed kinetics

[see Durrant et al. (1992b) for evidence supporting the validity of this assumption in our experiments], then any change in the excitation wavelength will alter only the amplitude of each component, without altering their lifetimes. Independent analyses of data collected with four different excitation wavelengths recovered the same lifetimes for each kinetic component observed (Table I) despite large changes in the amplitudes of some components.

(3) Five of the six components can be shown to result from physically distinct processes as their amplitudes exhibit very different dependencies on excitation and/or probe wavelengths. The amplitudes of the 100-fs and 200-ps components were strongly dependent upon excitation wavelength, while the amplitudes of the other components were not [see Table I and Durrant et al. (1992a)]. For example, while the spectrum of the 100-fs component between 660 and 690 nm completely inverted when the excitation wavelength was changed from 665 to 694 nm, the spectra of the 1.6- and 21-ps components were essentially unchanged. Different components also exhibited clearly different dependencies upon probe wavelength. This is illustrated for the 1.6- and 21-ps components in Figure 5. The kinetic spectra of these components are clearly distinguishable, and they must therefore be assigned to different physical processes. Components 2a and 2b (lifetimes of 600 fs and 3.5 ps), however, have similar kinetic spectra and dependence upon excitation wavelength (Durrant et al., 1992a; unpublished data); therefore, these components could not be qualitatively distinguished and are discussed together as a single component with an average lifetime of 1.6 ps in this paper.

Development of a Kinetic Model. The central problem in developing a kinetic model is that there is not in general a direct one to one relationship between exponential components observed in kinetic analyses and the physical steps which underlie them. We will present elsewhere a proper kinetic model for the photosystem 2 reaction center; however, there are aspects of the data which allow us to simplify discussion for the purposes of this paper. Optical excitation of isolated PS2 reaction centers results ultimately in the formation of one product state ($P680^+Ph^-$) with a 100% yield on the picosecond time scale. Moreover, all of the components discussed here have lifetimes separated by at least an order of magnitude. Assuming that the assignments which we have made are essentially correct, then it is clear that all of the kinetic components which we have detected directly produce large interconversions between chemical states which can be ascertained from their amplitudes. In this case, as a first approximation, it is possible to consider each kinetic component separately and sequentially, with each one directly related to a different process. However, this simple kinetic scheme for PS2 becomes more complicated if equilibrium mixtures of states are formed prior to the final radical pair formation. We already know that such a mixture is created by the flow of excitation energy between P680 and the accessory chlorins with a 100-fs experimentally determined time constant, to produce an $\approx$1:1 equilibrium between these two pools of pigments (Durrant et al., 1992b). If this is the only significant event prior to charge separation, then one would be inclined to suggest a microscopic electron-transfer rate of $\approx$10.5 ps from the 21-ps lifetime associated with the loss of stimulated emission and the reduction of pheophytin.

Assignments of Kinetic Components. Our previous studies of isolated PS2 reaction centers have reported the existence of several kinetic components with lifetimes between 100 fs and 200 ps (Durrant et al., 1992a,b; Hastings et al., 1992). These kinetic components are summarized in Table I. We have presented additional data here which confirm that the processes associated with these components produce the state $P680^+Ph^-$. The spectrum which we obtain for $P680^+Ph^-$ is in good agreement with that obtained in many previous studies of PS2 (see above); such agreement is particularly important, as it demonstrates that when we apply femtosecond transient absorption spectroscopy we see the same radical pair state as that observed by many other groups using different but related techniques.

The fastest kinetic component which we have resolved so far (100 fs) is due to energy transfer, which results in the equilibration of excitation energy between P680 and those accessory chlorins with absorption maxima near 670 nm (Durrant et al., 1992b). This equilibration process is essentially complete prior to all processes considered in this paper. The slowest (200 ps) component is not observed when P680 is directly excited using 694-nm pulses, and is assigned to a slow energy-transfer/trapping process involving a minority of reaction center chlorins (Hastings et al., 1992). It can therefore be concluded that P680 oxidation must be associated with components 2 and/or 3 and we show above that it is component 3 (the 21-ps component) which produces the majority of $P680^+$.

A (Chl *a*)* transient spectrum is shown in Figure 2 which allows us to estimate the shape and intensity of the stimulated emission contribution between 700 and 800 nm. The transient spectrum of $P680^+Ph^-$ is shown in Figure 1. From these data, it is possible to differentiate the contributions of stimulated emission and $P680^+Ph^-$ excited-state absorption in the data of Figure 3. Comparison of the amplitudes of the 1.6- and 21-ps components (Figure 3a) shows that the 21-ps component accounts for 70 $\pm$ 30% of the loss of the stimulated emission at 730 nm, and for 75 $\pm$ 15% of the gain of the positive absorption between 710 and 860 nm. We therefore conclude that it is the 21-ps component rather than the 1.6-ps component which accounts for the majority of the decay of chlorin singlet excited states and production of the radical pair state $P680^+Ph^-$.

The 21-ps component has been shown previously to be associated with the production of reduced pheophytin (Hastings et al., 1992). In our previous studies, we were unable to determine the identity of the precursor state(s) to this 21-ps pheophytin reduction, but we can now draw some conclusions as the transient spectrum at 3 ps is effectively that of the precursor state(s). The data we have presented here demonstrate that this 3-ps transient spectrum is dominated by contributions from excited chlorin singlet states (Figure 3).

Although chlorophyll and pheophytin anions exhibit similar excited-state absorption bands in the near-infrared (Fujita et al., 1978), these species can be distinguished in other spectral regions (Fujita et al., 1978; Hastings et al., 1992). The states $P680^+Chl^-$ and $P680^+Ph^-$ should have similar absorption spectra between 700 and 850 nm. We estimate from the data shown in Figure 3 that the proportion of $P680^+Chl^-$ or $P680^+Ph^-$ states present at 3 ps after excitation is 25 $\pm$ 15%. The same results were obtained using both 694-nm (selective excitation of P680) and 665-nm excitation (selective excitation of accessory chlorins), as would be expected from our previous studies (Durrant et al., 1992b). It can therefore be concluded that a *lower limit* of 75 $\pm$ 15% of P680 oxidation occurs with the same $(21\ ps)^{-1}$ rate which we observed previously for a *lower limit* of 60% pheophytin reduction (Hastings et al., 1992).

The percentages which we quote are not meant to imply that an individual process exhibits more than one time constant due to sample heterogeneity, but are limitations imposed by imprecise knowledge of excited-state absorption spectra, overlap of multiple spectral features, finite signal to noise, and ambiguities of the kinetic scheme. For example, it is easy to imagine a kinetic scheme whereby there is some loss of P680 stimulated emission prior to the formation of $P680^+Ph^-$ through formation of a species such as an intra-P680 charge-transfer state. Similarly, the upper limit of 60% quoted for pheophytin reduction is simply due to the apparently immediate bleaching of some pheophytin by the pump pulse [see Hastings et al. (1992) for a more complete discussion].

Further data support our assignment of the majority, and possibly all, primary radical pair formation in PS2 reaction centers to the 21-ps component. The 21-ps component produces an increase in a positive band at 650 nm (see Figures 1 and 5) which is usually associated with pheophytin reduction, while the 1.6-ps component is clearly distinguished by having the opposite sign at this wavelength (see Figure 5 for a comparison of the kinetic spectra). This means that the two components represent different physical processes which would imply that no pheophytin is reduced during the 1.6-ps phase. Finally, the spectrum of the 21-ps component is essentially independent of excitation wavelength (Hastings et al., 1992; unpublished data), which confirms that this component cannot be assigned to an energy-transfer process.

We have recently found that the P680 excited singlet state is apparently highly delocalized with an oscillatory strength 2–3 times that of a Chl *a* molecule (Durrant et al., 1992b), which supports an earlier study where we reported that the processes associated with the 1.6-ps component result in decay and possible localization of an initially delocalized P680 excited singlet state (Durrant et al., 1992a). The results we present here show that during this localization process, an *upper limit* of 25% of the chlorin excited states become cation/anions (i.e., an upper limit of 25% of the stimulated emission side band is lost). We are not yet able to determine whether the 1.6-ps component produces any radicals at all (e.g., Chl^-), as the loss of stimulated emission may simply reflect the reduced oscillator strength of the localized state, but work is currently in progress to address this issue more carefully.

Wasielewski et al. (1989a) have previously reported a lifetime of 3 ps for formation of $P680^+Ph^-$ in isolated PS2 reaction centers. This conclusion was partly based upon the observation of a 3-ps lifetime for the appearance of absorption changes assigned to the state $P680^+Ph^-$ at 820 nm. However, Figure 2 shows that chlorophyll excited singlet states also exhibit a positive absorption band in this region as has also been reported by other groups (Nuijs et al., 1986; Hansson et al., 1988). The observation of positive absorption changes at 820 nm is therefore by itself ambiguous and insufficient for the purpose of making an assignment. Figure 4b,c shows our own data obtained at 820 nm following the direct excitation of P680. We have also collected kinetic data at 14 wavelengths between 700 and 860 nm, and from the clear presence of a high proportion of stimulated emission (see Figures 4a and 3b) are able to assign the initial ($t = 0$) positive transient signal at 820 nm (Figure 4b) to chlorin excited singlet states. This initial signal subsequently increases in amplitude by 30% with a 21-ps lifetime (Figure 4c) due to the formation of $P680^+Ph^-$.

The stimulated emission side band at 730 nm can be used as a general and useful marker for the presence of fluorescent chlorin excited states. For example, localization of the initially delocalized P680 excited singlet state will result in a reduction in the oscillatory strength of the $S_0 \rightarrow S_1$ transition, and therefore a net loss of stimulated emission. Radical pair formation would cause the remaining stimulated emission to be lost. Our data indicate that the 1.6- and 21-ps components account for 30 ± 20% and 70 ± 20%, respectively, of the loss of this stimulated emission side band. These percentages are consistent with our assignments of the 1.6- and 21-ps components to localization associated with an as yet unassigned mechanism and to charge separation processes, respectively.

Roeloffs et al. (1991) have reported the observation of a 3-ps fluorescence component using picosecond time correlated single photon counting, which they assign to radical pair formation. Their conclusion is not consistent with our results reported here using fermtosecond transient absorption. It is difficult to compare results from such different techniques, but the result of Roeloffs et al. was obtained using an instrument response function of 70 ps compared with the 150-fs instrument response which we have used. We have previously reported that PS2 reaction centers show a number of lifetimes shorter than 21 ps (Durrant et al., 1992a,b). In fact, both the 1.6-ps and 100-fs components might well show up strongly in fluorescence measurements. Although it is difficult to predict exactly how these lifetimes would affect the results of time-resolved data using a 70-ps instrument response function, it is likely that if these multiple short components are not taken into account then the fluorescence lifetime could appear to be shorter than the 21-ps value which we report in this paper for loss of stimulated emission.

It has been suggested that in reaction centers of purple bacteria it takes two sequential electron-transfer reactions to reduce pheophytin (Holzapfel et al., 1990). We have reported here that the production of $P680^+Ph^-$ in isolated PS2 reaction centers is associated primarily with a single kinetic component with a 21-ps lifetime. However, we are not yet able to determine whether this component results from a single electron-transfer reaction or is due to a multistep process.

CONCLUSION

The results which we present here demonstrate that P680 oxidation in isolated PS2 reaction centers occurs primarily with the same effective 21-ps lifetime as reported previously for pheophytin reduction (Hastings et al., 1992). We have also demonstrated that faster processes with a mean lifetime of 1.6 ps, associated with localization of the P680 excited singlet state (Durrant et al., 1992a), do not produce a high proportion of either $P680^+Ph^-$ or $P680^+Chl^-$ as the majority of stimulated emission is still present after the 1.6-ps component has finished. Experiments are currently in progress to determine which of these processes (1.6 or 21 ps), or a combination of both, corresponds to the primary trapping step in photosystem 2.

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