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Determination of the primary charge separation rate in Photosystem II reaction centers at 15 K

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Abstract

We have measured the rate constant for the formation of the oxidized chlorophyll a electron donor ($P680^+$) and the reduced electron acceptor pheophytin a^- (Pheo a^-) following excitation of isolated Photosystem II reaction centers (PS II RC) at 15 K. This PS II RC complex consists of D_1, D_2, and cytochrome b-559 proteins and was prepared by a procedure which stabilizes the protein complex. Transient absorption difference spectra were measured from 450–840 nm as a function of time with 500 fs resolution following 610 nm laser excitation. The formation of $P680^+$–Pheo a^- is indicated by the appearance of a band due to $P680^+$ at 820 nm and corresponding absorbance changes at 490, 515 and 546 nm due to the formation of Pheo a^-. The appearance of the 490 nm and 820 nm bands is monoexponenital with $\tau = 1.4 \pm 0.2$ ps. Treatment of the PS II RC with sodium dithionite and methyl viologen followed by exposure to laser excitation results in accumulation of Pheo a^-. Laser excitation of these prereduced RCs at 15 K results in formation of a transient absorption spectrum assigned to ^{1*}P680. We observe wavelength-dependent kinetics for the recovery of the transient bleach of the Q_y absorption bands of the pigments in both untreated and pre-reduced PS II RCs at 15 K. This result is attributed to an energy transfer process within the PS II RC at low temperature that is not connected with charge separation.

Abbreviations; PS I - Photosystem I, PS II - Photosystem II, RC - reaction center, P680 - primary electron donor in Photosystem II, Chl *a* - chlorophyll *a*, Pheo *a* - pheophytin *a*

Introduction

Determinations of X-ray crystal structures of reaction center proteins (RCs) from purple photosynthetic bacteria have resulted in a new framework for the development of structure–function relationships in photosynthesis (Deisenhofer et al. 1984, Chang et al. 1986, Allen et al. 1987). As a result it is particularly interesting to ask whether the structures and functions of green plant Photosystems I and II (PS I and PS II) are similar to those of bacterial RCs. Until now it has proven very difficult to study the primary photochemistry in both PS I and PS II because isolation of these RCs was always accompanied by large numbers of chlorophyll (Chl) molecules not directly involved in the primary photochemistry. Recently, a complex has been isolated (Nanba and Satoh 1987) that contains only the D_1, D_2, and cytochrome *b*-559 proteins of PS II (see also Satoh et al. 1987), Barber et al. 1987). Since this preparation binds 4 Chl *a* molecules, 2 Pheo a molecules, and a single β-carotene molecule,

a pigment composition similar to that of bacterial RCs (Deisenhofer et al. 1984), and since the D_1 and D_2 proteins possess significant sequence homology to the L and M subunits of bacterial RCs (Erickson et al. 1985), it is likely that the D_1–D_2–cyt *b*-559 complex is the RC of PS II. In addition, a new 4.8 kD protein has been identified recently as intrinsic to the PS II RC (Ikeuchi et al. 1988a, 1988b, Webber et al. 1989). Unlike bacterial RC preparations, the Nanba and Satoh PS II RC preparation does not contain bound quinone acceptors.

The rates of primary charge separation in RCs are very rapid and as such are important to understand within the context of reaction center structure. In isolated RC preparations from *Rhodobacter sphaeroides* and *Rhodopseudomonas viridis*, primary charge separation occurs in 2.8 ps at room temperature (Woodbury et al. 1985, Martin et al. 1986, Breton et al. 1986, Wasielewski and Tiede 1986) and 0.7–1.2 ps at 10 K (Fleming et al. 1988, Breton et al. 1988). Using 500 fs time resolution Wasielewski *et al.* (1988, 1989) have shown recently that the primary charge separation in PS II RCs occurs with a time constant of 3.0 ± 0.6 ps at 277 K. Schatz et al. (1988) estimated a similar time constant from an analysis of fluorescence emission from a larger PS II containing particle. Thus, the analogy between bacterial RCs and PS II is maintained at a functional level. Since the rates of electron transfer in bacterial reaction centers increase at cryogenic temperatures, suggestive of electron tunnelling (DeVault 1984), it is important to determine whether PS II RCs behave similarly. In this paper we report the kinetics of primary charge separation in stabilized (Seibert et al. 1988, McTavish et al. 1989) PSII RCs at 15 K and compare these data with our results obtained earlier at 277 K.

Materials and methods

Photosystem II reaction center complex was prepared from spinach PS II appressed membrane fragments (Dunahay et al. 1984) by a modification Seibert et al. 1988, McTavish et al. 1989) of the original Nanba and Satoh procedure (Nanba and Satoh 1987). After the polyethylene glycol (PEG) aggregates were separated from the PEG-RC material, the latter was concentrated to as high as 900 μg Chl/ml by centrifugation in a Microfuge and stored at −80°C until use. Photochemical competance of the RCs was assayed by photoinduced electron transport from diphenylcarbazide to silicomolybdate (SiMo) at 6°C (McTavish et al. 1989) and by observation of the wavelength of the red-most absorption band at 674 nm. The photochemical activity of this preparation was 5300 μmol SiMo reduced $(\mathrm{mg\,Chl})^{-1}\,\mathrm{hr}^{-1}$. As the RCs degrade, the room temperature 674 nm absorption band shifts to 669 nm (Seibert et al. 1988). At 15 K the red absorption band of the PS II RC resolves into a band at 672 nm with a pronounced shoulder at 682 nm. The absorption spectrum of the RCs at 15 K was measured before and after laser excitation and did not change.

Reaction center samples for picosecond spectroscopy (900 μg Chl/ml in 50 mM Tris-HCl, pH 7.2) were unfrozen in dim green light and 0.04% Triton X-100 (final concentration) was added immediately to keep the material from aggregating (McTavish et al. 1989). The samples were then transferred to a nitrogen atmosphere and the following O_2 scrubbing system was added at the final concentration in the indicated order: 20 mM glucose, 0.039 mg/ml catalase, 0.1 mg ml glucose oxidase (McTavish et al. 1989). The samples were diluted with sufficient Tris-HCl buffer to give a 50 mM final buffer concentration and with glycerol to give a 60/40 v/v glycerol aqueous mixture. After mixing, the samples were transferred anaerobically to an Air Products He flow cryostat, and were immediately cooled to 15 K in the dark.

Sub-picosecond time-resolved absorption measurements were obtained with the laser apparatus described elsewhere (Wasielewski et al. 1987). The absorbance of the sample was adjusted to 1.0–1.2 at 672 nm in a 1.5 mm pathlength cell. In one set of measurements, PS II RCs were treated as described above, while in a second set of measurements, the buffer contained 1.5 mM sodium dithionite and 15 μM methyl viologen to reduce Pheo *a* to Pheo a^- under illumination. In the latter case we found that the 10 Hz actinic laser pulses at 610 nm were sufficient to keep Pheo *a* reduced to Pheo a^- in the steady state.

A 2-mm diameter spot on the sample cell was irradiated with the pump and probe beams derived from the transient absorption apparatus (Wasielewski et al. 1987). A 610 nm, 500 fs, 100 μJ pulse

was used to excite the samples, while a 500 fs white light continuum pulse was used to probe the absorbance of the sample. The absorbance of the sample at 610 nm was always < 0.3. Pulse lengths were determined by autocorrelation techniques. Zero-time, i.e. the coincidence of the probe and excitation pulses, was determined by the use of a 1 mm pathlength CS_2 Kerr cell positioned between crossed polarizers. The total instrument response function was 500 fs and the spectral resolution was ± 1 nm. Typically, 256 laser shots were averaged at each time point to obtain the data presented. Exponential time constants for kinetic data were determined either by the method of Provencher (1976) or by Grinvald–Steinberg reconvolution (Grinvald 1976). These fits to the data are depicted as the solid lines in the figures displaying kinetic data.

The ground state absorption spectra of PS II RCs at 15 K were obtained with the dual-beam transient absorption spectrophotometer using the white continuum probe light pulse in the absence of laser excitation of the sample.

Results

The ground state absorption spectrum of the PS II RC at 15 K (Fig. 1) shows that the Q_y absorbances of the Chl *a* and Pheo *a* molecules in the RC are partially resolved. The band maximum occurs at 672 nm with a distinct shoulder at 682 nm. When the untreated sample (Fig. 1A) is compared with the sample in which Pheo *a* is pre-reduced (Fig. 1B), the latter looses significant absorbance intensity at 682 nm suggesting that Pheo *a* absorbs at 682 nm. This observation is consistent with the results of a recent study of these PS II RCs at low temperature (Tetenkin et al. 1989). As will be seen below, P680 also absorbs near 682 nm in this preparation at low temperature, while the remaining chlorophyll pigments probably absorb at 672 nm.

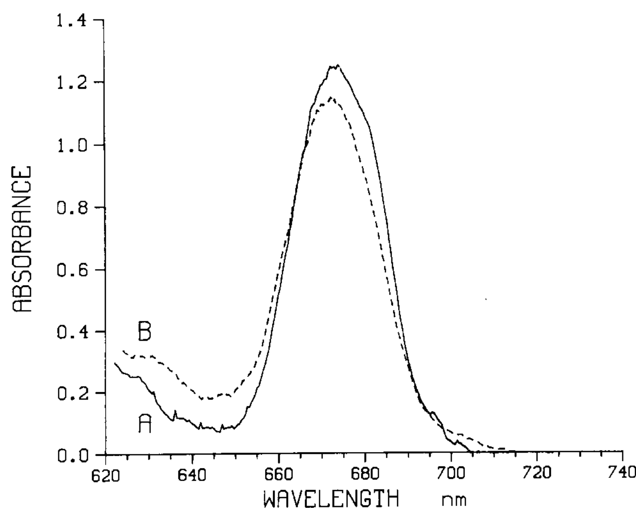

Fig. 1. Ground state absorption spectrum of PS II RCs at 15 K. (A) untreated PS II RCs, (B) PS II RCs treated with 15 μM methyl viologen and 1.5 mM sodium dithionite.

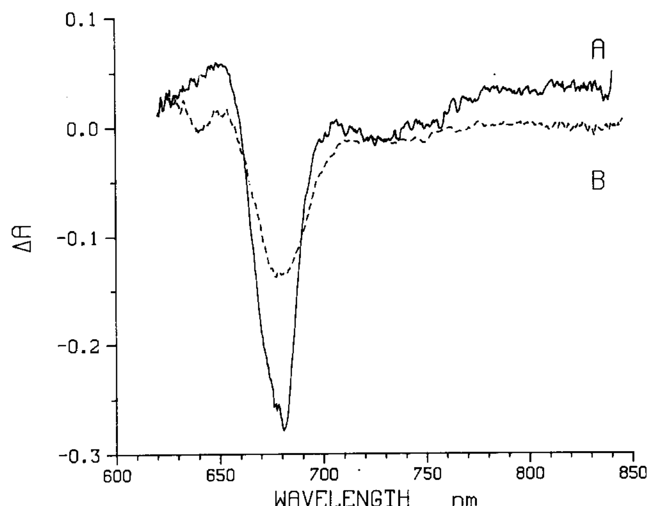

Fig. 2. (A) Solid trace shows the transient absorption spectrum of PS II RCs; (B) Dashed trace shows the transient absorption spectrum of PS II RCs treated wtih 1.5 mM sodium dithionite and 15 μM methyl viologen. Both traces occur 10 ps following a 100 μJ, 500 fs laser flash at 610 nm.

The transient absorption difference spectrum at 15 K obtained 10 ps following a 500 fs laser flash at 610 nm (Fig. 2A) shows a strong bleaching of the entire red band and a positive absorption starting near 760 nm and extending past 840 nm. The bleach at 682 is assigned to the disappearance of the ground states of both P680 and Pheo *a*. Absorption at 820 nm is characteristic of the formation of $P680^+$ (Takahashi et al. 1987), while Pheo a^- is known to have an absorption band centered near 790 nm (van Gorkom et al. 1975, Fujita et al. 1978). Careful examination of the bleach in the 665–685 nm region in Fig. 2A shows that the 672 nm band is also strongly bleached at 10 ps following

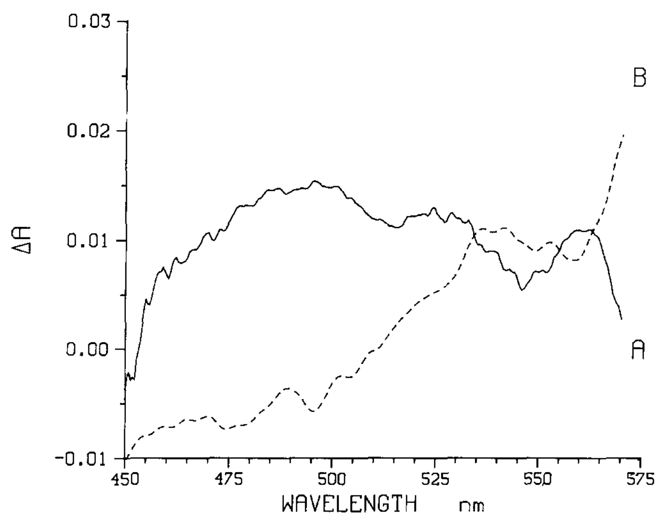

Fig. 3. (A) Solid trace shows the transient absorption spectrum of untreated PS II RCs; (B) Dashed trace shows the transient absorption spectrum of PS II RCs treated with 1.5 mM sodium dithionite and 15 μM methyl viologen. Both traces occur 10 ps following a 100 μJ, 500 fs laser flash at 610 nm.

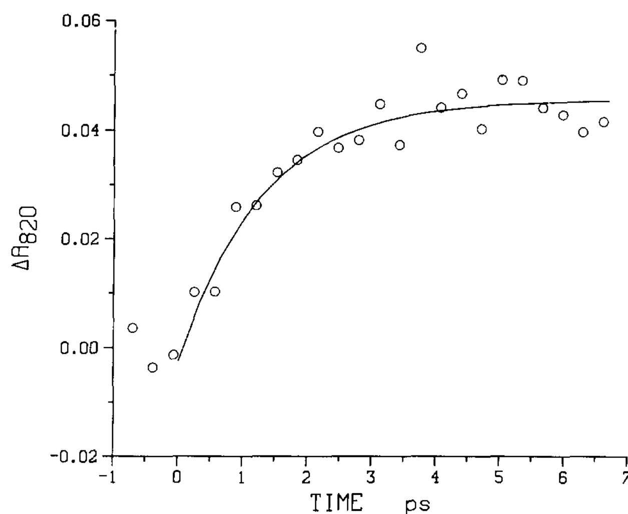

Fig. 4. Transient absorption changes at 820 nm for untreated PS II RCs following a 100 μJ, 500 fs laser flash at 610 nm.

the laser flash. The recovery of this bleach displays interesting wavelength dependent kinetics that will be discussed below.

Since the formation of the lowest excited singlet state of Chl *a* also results in a small absorbance increase in the near-infrared (Wasielewski et al. 1989), our measurements on PS II RCs with Pheo *a* pre-reduced serve as controls regarding our assignment of the absorption changes that occur in untreated PS II RCs to the formation of $P680^+$–Pheo a^-. Figure 2B shows the transient difference spectrum at 15 K obtained 10 ps following a 500 fs laser flash at 610 nm in the PS II RC sample in which Pheo *a* is pre-reduced. Comparing the spectrum in Fig. 2A with that in Fig. 2B, the near-infrared features present in Fig. 2A are nearly absent in Fig. 2B. In addition, the 672–682 nm bleach in Fig. 2A is replaced by a weaker bleach centered at 682 nm in Fig. 2B.

The transient absorption spectrum at 15 K for untreated PS II RCs in the blue-green spectral region at 10 ps following a 500 fs laser flash at 610 nm is shown in Fig. 3A. A general increase in absorption is found throughout the 450–570 nm region with a maximuma at 490 nm and two minima at 515 nm and 546 nm. These features are known to accompany the formation of Pheo a^- (van Gorkom et al. 1975, Fujita et al. 1978). The formation of Chl a^+ also results in positive absorption changes between 450 nm and 570 nm (Takahashi et al. 1987, Davis et al. 1979). If the 546 nm transient absorption change is due to the formation of Pheo a^-, the magnitude of ΔA at 800 nm should be consistent with that observed at 546 nm based on the change in extinction coefficient for the reaction P680–Pheo $a \rightarrow P680^+$–Pheo a^-. For both $P680^+$ and Pheo a^-, $\Delta\varepsilon$ is about $10^4\,M^{-1}cm^{-1}$ at 800 nm (Parson and Ke 1982, Fujita et al. 1978), so that the total $\Delta\varepsilon = 2 \times 10^4\,M^{-1}cm^{-1}$. The $\Delta\varepsilon$ for Pheo a^- at 546 nm is $4 \times 10^3\,M^{-1}cm^{-1}$. Thus, the absorption change at 546 nm should be about 5 times smaller than that at 800 nm. Examination of Figs. 2A and 3A shows that the magnitude of the absorbance change trough at 546 nm relative to the positive background absorbance change is 0.0065, while the magnitude of the absorbance change peak at 800 nm is 0.035. The ratio $\Delta A_{800}/\Delta A_{546} = 5.4$ and therefore is consistent with the analysis presented above.

Figure 3B shows the transient difference spectrum at 15 K of pre-reduced PS II RCs at 10 ps following the 0.5 ps, 610 nm laser flash. Both the absorption maximum at 490 nm and the absorption troughs near 515 nm and 546 nm that were present for the untreated PS II RCs in Fig. 3A are nearly absent for the pre-reduced PS II RCs in Fig. 3B. Thus, in both the blue-green and near-infrared regions of the spectrum reduction of Pheo *a* to

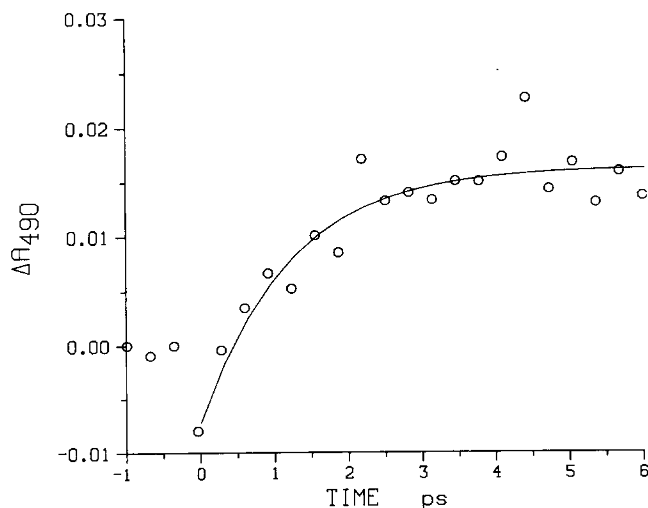

Fig. 5. Transient absorption changes at 490 nm for untreated PS II RCs following a 100 μJ, 500 fs laser flash at 610 nm.

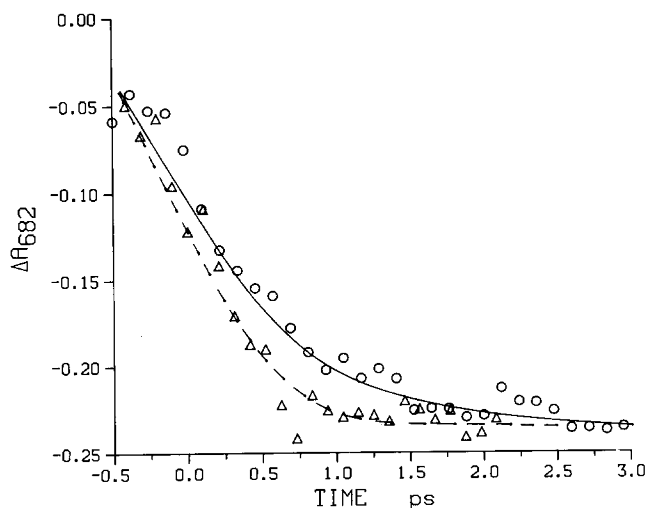

Fig. 6. Transient absorption changes at 682 nm for PS II RCs following a 100 μJ, 500 fs laser flash at 610 nm. (A) untreated RCs, ○ and ——. (B) pre-reduced RCs, △ and ----.

Pheo a^- results in spectral changes that are consistent with blocking P680$^+$-Pheo a^- formation.

The comparison of the transient spectra of both untreated and pre-reduced PS II RCs given above suggests that the kinetics of P680$^+$-Pheo a^- formation is not strongly convolved with the appearance and decay of the lowest excited singlet state of P680 (and of the other pigments) at wavelengths > 790 nm and near 490 nm. A monophasic exponential increase at 820 nm is observed with a best fit of 1.4 ± 0.2 ps (Fig. 4). The kinetics for the appearance of this band do not vary within experimental error from 790 nm to 840 nm. The transient absorption change kinetics observed at 490 nm are shown in Fig. 5. A small ($\Delta A = -0.0075$) negative absorption change appears within the 500 fs instrument function and is followed by a positive absorption change that can be fit to a single exponential time constant $\tau = 1.4 \pm 0.2$ ps. The time constant for the appearance of the positive absorption change at 490 nm is the same as the time constant determined at 820 nm. Since the magnitude of the small trough at 546 nm is only 0.0065 ΔA, the low signal-to-noise ratio of the data precludes a determination of the kinetics for the appearance of the 546 nm feature. Thus, a 1.4 ps time constant is found consistently for the appearance of the transient absorption features assigned to P680$^+$-Pheo a^- in PS II RCs at 15 K.

The appearance of the strong absorption decrease at 682 nm in untreated RCs (Fig. 6A) reflects the formation of both the excited states of P680 and Pheo a in the RC as well as the formation of P680$^+$-Pheo a^-, while in pre-reduced RCs this bleach reflects only excited state formation (Fig. 6B). The magnitudes of the 682 nm bleaches in both untreated and prereduced RCs were normalized at 3 ps for comparison in Fig. 6. The fit to the kinetic data in Fig. 6A is biphasic. An initial bleach occurs with the instrument function, $\tau_1 = 0.5 \pm 0.4$ ps (50 ± 20%), presumably formation of ^{1*}P680, followed by a further bleach that occurs with $\tau_2 = 1.4 \pm 0.4$ ps (50 ± 20%), presumably formation of P680$^+$-Pheo a^-. Time constant τ_2 is the same as τ for the formation of the 820 nm band. The amplitudes of these kinetic components are consistent within experimental error with the magnitudes of the bleaches at 682 nm in Figs. 2A and 2B, respectively. The bleach at 682 nm in pre-reduced RCs (Fig. 6B) occurs with a single exponential time constant of 0.5 ± 0.4 ps and presumably reflects only excited state formation within the RC pigments.

In untreated RCs charge separation competes very efficiently with ^{1*}P680 excited state decay. Thus, the rate constant for the decay of ^{1*}P680 should be nearly identical to that for the formation of P680$^+$-Pheo a^-. The lifetime of ^{1*}P680 can be obtained directly by measuring the decay of its

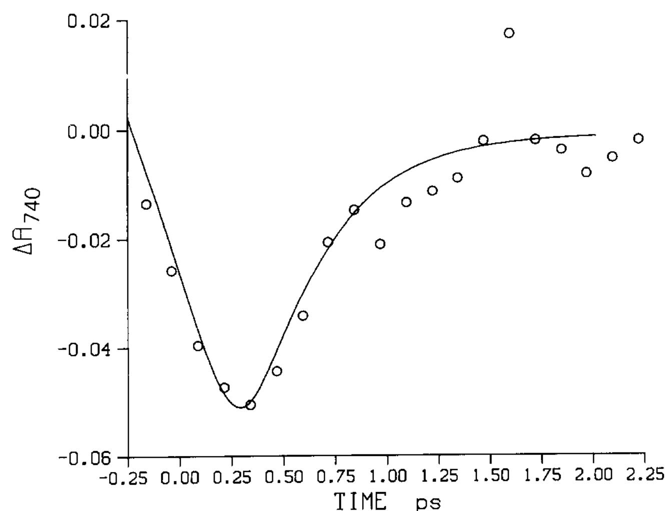

Fig. 7. Formation and decay of ^{1*}P680 stimulated emission at 740 nm following a 100 μJ, 500 fs, 610 nm laser flash.

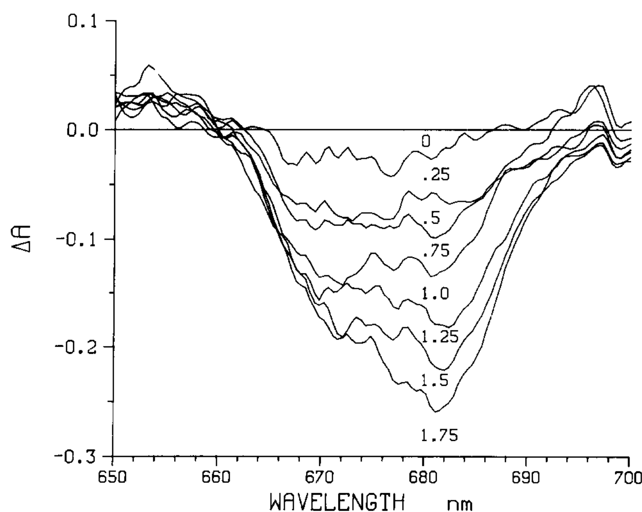

Fig. 8. Transient absorption spectra for untreated PS II RCs at 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, and 1.75 ps following a 100 μJ, 500 fs laser flash at 610 nm.

stimulated emission (Breton et al. 1986). Since the Stokes shift between the stimulated emission from ^{1*}P680 and the absorption of P680 is small, we monitored weak stimulated emission arising from the Q(0, 1) vibronic band of ^{1*}P680 at 740 nm. The appearance of the apparent bleach in the transient absorption spectrum due to stimulated emission and its subsequent decay are shown in Fig. 7. The appearance of the bleach is instrument limited followed by a single exponential decay with $\tau = 1.2 \pm 0.4$ ps. The decay of the stimulated emission signal, which yields the lifetime of ^{1*}P680, is in good agreement with the 1.4 ± 0.2 ps time constant measured for the formation of $P680^{+}$–Pheo a^{-}.

The data presented in Figs. 8–10 focus on the kinetics of the bleach and recovery of the Q_y bands of the pigments in the PS II RC. Figure 8 shows the spectral changes that occur immediately following the laser pulse in untreated PS II RCs. The spectra are shown at 0.25 ps intervals. The formation of the bleach at 672 follows the 500 fs instrument function, while the formation of the 682 nm bleach reflects both excited state and $P680^{+}$–Pheo a^{-} formation as noted in Fig. 6. The magnitude of the bleach is somewhat larger for the 682 nm band than for the 672 nm band. However, in the ground state spectrum of untreated RCs (Fig. 1A) the 682 nm absorbance is slightly smaller than that at 672 nm. The additional apparent bleach of the 682 nm band is due to stimulated emission occurring at this wavelength. Since the Stokes shift for fluorescence emission from PS II RCs is small, the stimulated emission appears as an additional bleach that is added to the absorption bleach.

As mentioned above, the recovery of the transient absorption bleach at 665–685 nm in the PS II RCs exhibits wavelength-dependent kinetics. Figure 9 shows the recovery of the bleach in untreated PS II RCs at 5 nm intervals between 665 and 680 nm. This bleach recovers with monoexponential time constants of $\tau = 25 \pm 4$, 43 ± 4, 101 ± 15, and > 200 ps at 665, 670, 675, and 680 nm, respectively. Figure 10 shows similar data for this spectral region in PS II RCs that have been pre-reduced. In this case at $\tau = 10 \pm 3$, 16 ± 5, 20 ± 5, and > 30 ps at 665, 670, 675, and 680 nm, respectively. The fact that the 672 nm bleach recovers quickly regardless of the oxidation state of Pheo *a* suggests that this recovery is not associated with the primary charge separation or recombination process.

Discussion

Both the ground state and transient absorption data suggest that both P680 and the photoactive Pheo *a* in the PS II RC absorb near 682 nm. As such

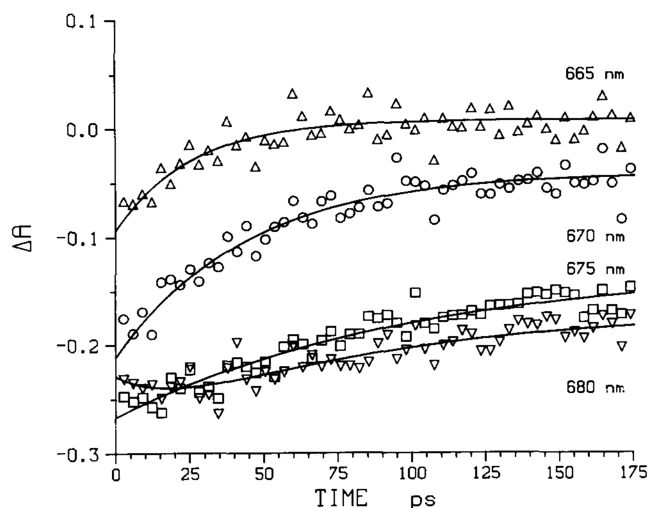

Fig. 9. Transient absorption changes of untreated PS II RCs at 665, 670, 675, and 680 nm following a 100 μJ, 500 fs laser flash at 610 nm.

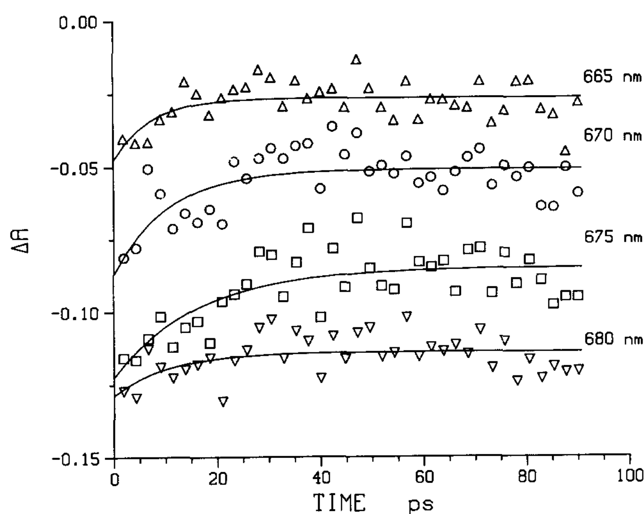

Fig. 10. Transient absorption changes of pre-reduced PS II RCs at 665, 670, 675, and 680 nm following a 100 μJ, 500 fs laser flash at 610 nm.

P680 is a shallow trap for excitation energy relative to the other Chl *a* and Pheo *a* pigments in the PS II RC. The assignment of the remaining pigments to the 672 and 682 nm bands remains ambiguous. For example, it is not clear whether the remaining photochemically inactive Pheo *a* also absorbs at 682 nm. Small environmental influences of the RC protein can result in the 10 nm bandshifts that are observed between the various pigments. The situation is even worse near room temperature where the Q_y bands of all the pigments coalesce to a single narrow absorption at 674 nm. Weak hydrogen-bonding interactions between the protein and the 9-keto carbonyl group of both Chl *a* and Pheo *a* are all that is necessary to red-shift their absorption maxima by as much as 10 nm (Shipman et al. 1976).

When Pheo *a* in PS II RCs is pre-reduced (Figs. 2B and 3B), flash excitation of the RCs results in a transient absorption spectrum that differs significantly from that obtained for untreated RCs (Figs. 2A and 3A). Since both $P680^+$ and Pheo a^- absorb in the near-infrared region of the spectrum, the presence of the near-infrared absorbance in untreated RCs and its loss in the pre-reduced RCs is consistent with the assignment of these bands to the formation of $P680^+$-Pheo a^- in the untreated RCs. Since the data show that the absorbance changes in the pre-reduced RCs, Figs. 2B and 3B, are not due to the formation of $P680^+$-Pheo a^-, these changes may be due either to formation of ^{1*}P680, excited states of the other Chl *a* and Pheo *a* pigments, or to reduction of an electron acceptor preceding Pheo *a*. If the observed spectrum is due to the formation of another intermediate acceptor preceding the formation of Pheo a^-, the most likely candidate for this acceptor would be another Chl *a* in the PS II RC that is not part of P680. However, it is well known that the optical absorption spectrum of Chl a^- is very similar to that of Pheo a^- in the near-infrared with a broad band centered at 780 nm (Fujita et al. 1978). Thus, the absence of such a band in Fig. 2B makes it unlikely that Chl a^- is an intermediate acceptor in PS II RCs at times > 500 fs. This result is the same as that found for these PS II RCs at 277 K (Wasielewski et al. 1989).

The transient absorption changes shown in Fig. 2B are most likely due to the formation of pigment excited states. The transient absorption spectrum of Chl *a in vitro* (Wasielewski et al. 1989) shows that the transient absorbance of ^{1*}Chl *a* is relatively weak in the near-infrared spectral region. This is consistent with the absorption changes in the near-infrared spectral region in Fig. 2B, which are substantially diminished relative to those for $P680^+$-Pheo a^- shown in Fig. 2A. If these changes were due to the formation of ^{1*}Pheo *a*, the Q_x bands of Pheo *a* between 500 and 550 nm would be bleached.

Figure 3B shows that this is not the case. If the transient absorption spectrum in Fig. 2B is due to formation of ^{1*}Chl a among the accessory Chl a molecules, it is likely that the bleach at 672 nm would persist at times > 50 ps. Yet, the data in Fig. 10 show that the 672 nm band recovers significantly in < 25 ps. Thus, it is likely that the spectrum displayed in Fig. 2B is due primarily to the formation of ^{1*}P680.

The results presented in Fig. 7 suggest that the lifetime of ^{1*}P680 is 1.2 ± 0.4 ps. Another estimate of the ^{1*}P680 lifetime can be obtained from photochemical hole-burning experiments (Jankowiak and Small 1987). The width of a hole burnt in the inhomogeneously broadened optical absorption at 682 nm can be related to the ^{1*}P680 lifetime through the uncertainty principle. Photochemical hole-burning experiments using stabilized PS II RCs, yield transient zero-phonon hole widths of 5–6 cm^{-1} at 4.2 K, which correspond to an excited state lifetime for ^{1*}P680 of 1.8–2.1 ps (Jankowiak et al. 1989). This data agrees reasonably well with the 1.2 ± 0.4 ps lifetime of ^{1*}P680, which we obtain from the decay of the stimulated emission from ^{1*}P680 at 15 K.

The rate of charge separation in PS II RCs increases by approximately a factor of 2 when the temperature decreases from 277 K to 15 K. This increase in rate is indicative of a slightly negative activation energy for the electron transfer process. Bixon and Jortner (1986) have proposed a model of electron transfer reaction in RCs which suggests that apparent negative activation energies can be expected for an activationless electron transfer. In their model the electron transfer is assume to proceed non-adiabatically, thus the usual golden-rule expression can be used to calculate the rate constant, k, for electron transfer:

$$k = (2\pi/\hbar)|V|^2 \mathrm{FCWD} \quad (1)$$

where V is the electronic coupling matrix element and FCWD is the Franck–Condon weighted density of states. The Bixon–Jortner treatment assumes that low frequency vibrational modes of the protein are important in promoting the electron transfer. If a single active mode, ω, is assumed to dominate:

$$k = k_0 \left[\frac{\exp(\hbar\omega/kT) - 1}{\exp(\hbar\omega/kT) + 1}\right]^{1/2} \quad (2)$$

where

$$k_0 = 2\pi|V|^2/\hbar\omega(2\pi\Delta G/\hbar\omega)^{1/2} \quad (3)$$

and ΔG is the energy gap between ^{1*}P680 and $P680^{+}$-Pheo a^{-}.

Using the measured rate constants of $3.3 \times 10^{11}\,s^{-1}$ and $7.1 \times 10^{11}\,s^{-1}$ for the charge separation in PS II RCs at 277 K and 15 K, respectively, eqn. (2) yields $\hbar\omega = 100\,cm^{-1}$. This value is similar to that obtained for the primary charge separation in *Rb. sphaeroides* (Fleming et al. 1988). Since the free energy of the charge separation is not known accurately, it is difficult to use the value of $\hbar\omega$ to calculate the electronic coupling matrix element V using eqn. (3). Nevertheless, Klimov (1979) has estimated that the midpoint potential for reduction of Pheo a to Pheo a^{-} is −610 mV, whereas that for oxidation of P680 to $P680^{+}$ is estimated to be around + 1.1 V. Thus, if the energy of ^{1*}P680 is about 1.83 eV and that of $P680^{+}$-Pheo a^{-} is about 1.6 eV above the ground state, then a crude estimate of the free energy of the reaction ^{1*}P680–Pheo a → $P680^{+}$-Pheo a^{-} is about 0.2 eV. This value is similar to the 0.25 eV estimated for *Rhodobacter sphaeroides*. Using an energy gap of 0.2 eV in eqn. (3), $V = 29\,cm^{-1}$. This large electronic coupling is consistent with the ultrafast charge separation rate that is observed in the PS II RCs. The increase in rate constant upon cooling can be attributed either to a larger Franck–Condon factor or to an increase in electronic coupling or both. Cooling the protein may cause the donor–acceptor distance to decrease, thereby increasing the electronic coupling.

The wavelength dependent kinetics for the recovery of the bleach of the 672 nm band in both untreated and pre-reduced PS II RCs suggests that a second photophysical process occurs in these RCs, which is not coupled to the primary charge separation. Since the 672 nm band of the PS II RCs remains strongly convolved with the 682 nm band at 15 K, we may assume that the kinetic data shown at 665 nm in both Figs. 9 and 10 more cleanly reflect the kinetics of this secondary process. The data show that the bleach at 665 nm recovers nearly completely with a 10–25 ps single exponential time constant. Interestingly, Mimuro et al. (1988) have observed a similar wavelength dependence of the emission spectra obtained at 77 K in PS II RCs prepared by Satoh. They observe an instrument-limited 50 ps fluorescence lifetime at 670 nm, which

increases to about 200 ps at 690 nm. These observations suggest that the recovery of the 672 nm bleach is due to an energy transfer process. One speculation as to the nature of this process involves the photochemically inactive Pheo *a* in the PS II RC. If this second Pheo *a* within the PS II RC also absorbs near 680 nm, it is possible that excitation of the 670 nm absorbing Chl *a* may result in energy transfer to this Pheo *a*. It is also possible that the energy acceptor pigment could be one of the other Chl molecules not involved in P680. The data cannot make this distinction. A third possibility is that energy is transferred to a Chl *a* that resides in a "damaged" i.e. partially denatured site of the protein. We consider this possibility less likely because wavelength dependent absorption changes similar to those in Figs. 9 and 10 *are not* observed at 277 K.

Since the depth of the P680 energy trap is shallow relative to the other Chl *a* and Pheo *a* pigments in the PS II RC, it is possible that at low temperatures excitation energy can be partitioned competitively between P680 and the photochemically inactive Pheo *a* (or accessory Chl *a*). At 277 K the initial bleach of the 674 nm band at 277 K shows no recovery at times less than 200 ps. It is likely that small changes in protein structure occur upon cooling which result in a change in the degree of partitioning of excitation energy between P680 and a secondary trap, perhaps the photochemically inactive Pheo *a*. It is not particularly reasonable that at room temperature excitation energy would be wasted in the PS II RC by significant partitioning into a photochemically inactive trap.

Wavelength dependent absorption changes have been observed previously in bacterial RCs. Kirmaier et al. (1985, 1986a, 1986b) have shown that the bacteriopheophytin anion to quinone electron transfer displays wavelength dependent kinetics. In addition, Breton et al. (1986) have observed fast transients due to excited state processes involving the intermediate bacteriochlorophyll *a* in bacterial reaction centers, even when electron transfer within the RC is blocked by oxidation of the primary donor. These observations were attributed mainly to changes in protein conformation. More data on the secondary photophysical process in PS II RCs are needed before a firm assignment as to its origin can be made. We are currently studying the temperature and excitation wavelength dependence of this phenomenon in order to provide some of this additional information.

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