

Determination of the primary charge separation rate in isolated photosystem II reaction centers with 500-fs time resolution

(electron transfer/ultrafast spectroscopy/photosynthesis/*Spinacia oleracea*)

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ABSTRACT We have measured directly the rate of formation of the oxidized chlorophyll *a* electron donor (P680⁺) and the reduced electron acceptor pheophytin *a*⁻ (Pheo^{a-}) following excitation of isolated spinach photosystem II reaction centers at 4°C. The reaction-center complex consists of D₁, D₂, and cytochrome *b*-559 proteins and was prepared by a procedure that stabilizes the protein complex. Transient absorption difference spectra were measured from 440 to 850 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 505 and 540 nm due to formation of Pheo^{a-}. The appearance of the 820-nm band is monoexponential with $\tau = 3.0 \pm 0.6$ ps. The time constant for decay of ¹P680, the lowest excited singlet state of P680, monitored at 650 nm, is $\tau = 2.6 \pm 0.6$ ps and agrees with that of the appearance of P680⁺ within experimental error. Treatment of the photosystem II reaction centers with sodium dithionite and methyl viologen followed by exposure to laser excitation, conditions known to result in accumulation of Pheo^{a-}, results in formation of a transient absorption spectrum due to ¹P680. We find no evidence for an electron acceptor that precedes the formation of Pheo^{a-}.

The primary processes of photosynthesis commence with light absorption by antenna pigment-protein complexes (1), followed by exciton migration among the pigments (2). Trapping of the excitons by chlorophyll *a* (Chl*a*) or bacteriochlorophyll molecules in the reaction center (RC) is followed by primary charge separation, which converts the excitation energy into chemical potential (3). In isolated RC preparations from purple photosynthetic bacteria, primary charge separation occurs in about 3 ps (4–8). However, the situation is more complex with cyanobacteria and plants because they contain two photosystems. Photosystem I (PSI) oxidizes plastoquinone and reduces nicotinamide-adenine dinucleotide phosphate (NADP⁺), while photosystem II (PSII) oxidizes water to molecular oxygen and reduces plastoquinone. The primary electron donor of PSI consists of one or two Chl*a* molecules and is called P700 because its long-wavelength absorption maximum is at 700 nm; the primary electron donor of PSII is called P680 for similar reasons (3). Using 6-ps and 1.5-ps time resolution, respectively, Fenton *et al.* (9) and Wasielewski *et al.* (10) have shown that PSI preparations enriched in P700 (1 P700 per 40 Chl molecules) undergo primary charge separation in about 14 ps. Nuijs *et al.* (11, 12) have suggested that electron transfer from P680 to pheophytin *a* (Pheo*a*) in PSII preparations enriched in P680 (1 P680 per 80 Chl) occurs in <35 ps. Schatz *et al.* (13), using a preparation containing 1 P680 per 80 Chl from *Synechococcus*, have estimated that the electron transfer from

Pheo^{a-} to a subsequent quinone electron acceptor takes place in 510 ps.

Until now it has proven very difficult to measure the primary charge separation rate in PSII RCs because isolation of a purified, stable RC preparation was not possible. Nanba and Satoh (14) succeeded in isolating a complex that contained only D₁, D₂, and cytochrome *b*-559 proteins (see also refs. 15 and 16). Since this preparation bound four or five Chl*a* molecules, two Pheo*a* molecules, and a single β -carotene molecule, and since Pheo*a* in the preparation was reduced following illumination, these authors suggested that the complex was the RC of PSII analogous to the RC of purple photosynthetic bacteria. The bacterial RC also contains four bacteriochlorophyll and two bacteriopheophytin molecules (17–22). However, unlike RC preparations from *Rhodospseudomonas viridis* and *Rhodobacter sphaeroides*, the PSII RC preparation does not contain bound quinone acceptors and has not been crystallized for structural studies. Nevertheless, Takahashi *et al.* (23) and Danielius *et al.* (24) measured a 32-ns lifetime for P680⁺-Pheo^{a-} in this material. Hansson *et al.* (25) reported that the P680⁺-Pheo^{a-} lifetime depends on the number of antenna Chl*a* molecules attached to the PSII RC. Okamura *et al.* (26) showed that recombination of the P680⁺-Pheo^{a-} radical pair in the PSII RC complex leads to formation of the triplet state of P680. This triplet possesses the spin polarization characteristic of a radical-pair precursor. Unfortunately, the PSII RC as isolated by Nanba and Satoh is rather unstable (27) and thus no data on the kinetics of the formation of P680⁺-Pheo^{a-} have appeared as yet.

In this paper we report the direct measurement of the kinetics of the primary charge separation in stabilized (27, 28) PSII RCs.

MATERIALS AND METHODS

PSII RC complex was prepared from spinach (*Spinacia oleracea*) PSII appressed membrane fragments (29, 30) by a modification (27, 28) of the original Nanba and Satoh procedure (14). All steps were carried out at 4°C in the dark. PSII membranes (30 mg of Chl at a concentration of 1 mg/ml) were solubilized in 4% (wt/vol) Triton X-100/50 mM Tris-HCl, pH 7.2, for 1 hr with stirring. The material was then centrifuged for 1 hr at 100,000 $\times g$, and the resultant supernatant was loaded onto a 15 \times 1.6-cm column of Fractogel TSK DEAE-650S (EM Science, Gibbstown, NJ, but now sold by Supelco, Bellefonte, PA, under the name TSK-GEL Toyopearl DEAE-650S) preequilibrated with 50 mM Tris-HCl, pH 7.2/30 mM NaCl/0.05% Triton X-100. The column was then washed with the same buffer until the eluent was colorless, and the RC fraction was eluted with a 30–200 mM NaCl gradient in 50 mM Tris-HCl, pH 7.2/0.05% Triton X-100. At

this point the RC fraction was concentrated by precipitation with polyethylene glycol, which also serves to stabilize the material (27, 28). Polyethylene glycol (32.5% wt/vol; $M_r = 3,350$) was added slowly and dispersed with a soft paintbrush. After 90 min of incubation, the suspension was centrifuged at $31,300 \times g$ for 15 min. The pellet was resuspended in 50 mM Tris·HCl (pH 7.2) with no detergent and then centrifuged at $1100 \times g$ for 90 sec to pellet mostly colorless material containing polyethylene glycol aggregates. The supernatant RC material was stored at -80°C until use. Photochemical competence of the RCs was assayed by photoinduced electron transport from diphenylcarbazide to silicomolybdate at 4°C (28) and by observation of the wavelength of the red-most optical absorption band at 674 nm. The photochemical activity of this preparation ranges from 1350 to 2250 μmol of silicomolybdate reduced per mg of Chl per hr. As the RCs degrade, the 674-nm band shifts to 669 nm (27). Measurements made before and after laser excitation showed that the absorption maximum of the sample was always between 672 nm and 674 nm.

RC samples for picosecond spectroscopy (117 μg of Chl per ml) were thawed in dim light and 0.04% Triton X-100 (final concentration) was added immediately to keep the material from aggregating (28). The samples were then transferred to a nitrogen atmosphere and the following were added (final concentration) in the indicated order: glucose (20 mM), catalase (0.039 mg/ml), and glucose oxidase (0.1 mg/ml) (28). After mixing, the samples were transferred anaerobically or aerobically to a sealed cuvette for spectroscopy at 4°C . Under these conditions the samples did not aggregate and remained active for the duration of the experiment.

Subpicosecond time-resolved transient absorption measurements were obtained with the laser apparatus described elsewhere (10). The absorbance of the sample was adjusted to 1.0–1.1 at 674 nm in a 1.5-mm-pathlength cell. In one set of measurements, PSII RCs were treated as described above; in a second set of measurements, the buffer contained 1.5 mM sodium dithionite and 15 μM methyl viologen to reduce Pheoa to Pheoa⁻ under illumination. In the latter case we found that the 10-Hz actinic laser pulses at 610 nm were sufficient to keep Pheoa reduced to Pheoa⁻ in the steady state.

A 2-mm-diameter spot on the sample cell was illuminated with the pump and probe beams derived from the transient absorption apparatus (10). 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. 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. Time constants for kinetic data were determined by the method of Provencher (31). These fits to the data are depicted as the solid lines in the figures displaying kinetic data.

RESULTS

The ground-state absorption spectrum of the PSII RC (Fig. 1) shows that the overlap in the red between the absorbances of the Chl a and the Pheoa molecules is quite severe. This is in contrast to the large spectral separation between the absorption of the primary donor and the primary acceptor observed in purple photosynthetic bacteria. The presence of Pheoa and/or cytochrome *b*-559 can be distinguished by the 415-nm Soret band, whereas the small feature near 540 nm is indicative of Pheoa, and the absorbance from 450–500 nm is due to the single β -carotene molecule in this preparation.

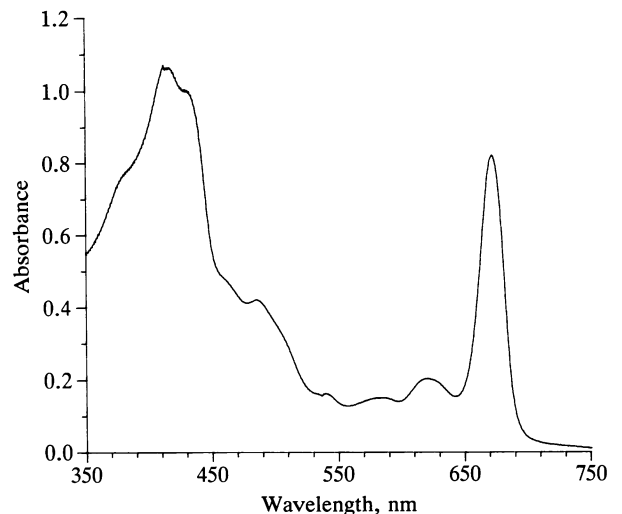


FIG. 1. Ground-state absorption spectrum of PSII RCs used in the transient absorption experiments.

The transient absorption difference spectrum obtained 10 ps following a 500-fs laser flash at 610 nm [Fig. 2, spectrum A (solid trace)] shows a strong bleaching at 674 nm and a positive absorption starting near 740 nm and extending past 850 nm. Since P680 absorbs near 674 nm in detergent-treated PSII membranes (32) and Pheoa absorbs close to 682 nm in PSII RCs (14), the observed red bleach should be a convolution of the bleaches due to P680 and Pheoa. The fact that the observed bleach occurs at 674 nm could be due to a larger change in molar extinction coefficient ($\Delta\epsilon$) for $\text{P680} \rightarrow \text{P680}^+$ compared to $\text{Pheoa} \rightarrow \text{Pheoa}^-$, electrochromic effects, and/or the presence of 0.04% Triton X-100 required to disaggregate the PSII RC. Absorption at 820 nm is characteristic of the formation of P680^+ , whereas Pheoa⁻ is known to have an absorption band centered near 790 nm (33). In the blue-green region of the spectrum, a general absorption increase is seen with a maximum at 485 nm and two minima at 505 nm and 540 nm. These features are known to accompany the formation of Pheoa⁻ (32, 34). The minimum at 540 nm is similar to the 540-nm trough observed in PSII membranes 4 ns after a 3-ns flash (34) but is blue-shifted relative to the corresponding

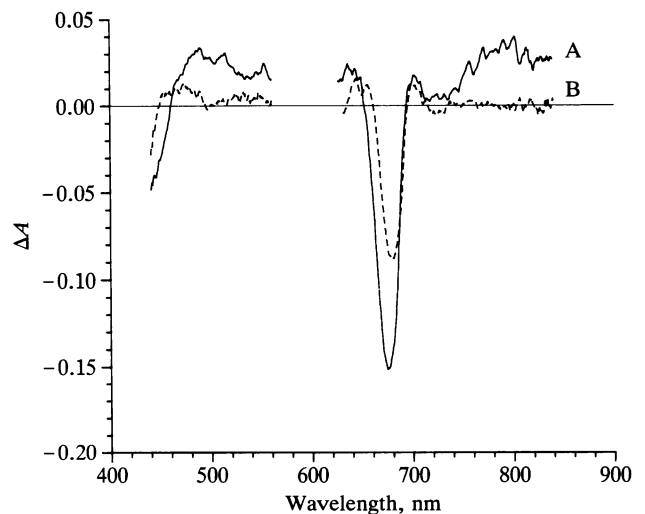


FIG. 2. Spectrum A (solid trace), transient absorption spectrum of PSII RCs; spectrum B (dashed trace), transient absorption spectrum of PSII RCs treated with 1.5 mM sodium dithionite and 15 μM methyl viologen. Both traces occur 10 ps following a 100- μJ , 500-fs laser flash at 610 nm. Filters needed to reject residual 610-nm light following continuum-probe light generation block the 560- to 620-nm wavelength range.

543-nm (at 4 ns) and 546-nm (at 25 ns) minima observed by Danielius *et al.* (24) after 35-ps flashes. The formation of Chla^+ results in positive absorption changes between 460 nm and 580 nm.

The signal-to-noise ratio of the data at 540 nm is about 4. If this feature is due to the formation of Pheoa^- , the magnitude of ΔA at 800 nm should be consistent with that observed at 540 nm. For both P680^+ and Pheoa^- , $\Delta \epsilon$ is about $10^4 \text{ M}^{-1}\cdot\text{cm}^{-1}$ at 800 nm (3, 33), so that the total $\Delta \epsilon = 2 \times 10^4 \text{ M}^{-1}\cdot\text{cm}^{-1}$. The $\Delta \epsilon$ for Pheoa^- at 540 nm is $4 \times 10^3 \text{ M}^{-1}\cdot\text{cm}^{-1}$ (33). Thus, the absorption change at 540 nm should be about one-fifth that at 800 nm. Examination of spectrum A (solid trace) in Fig. 2 shows that the magnitude of the ΔA trough at 540 nm is 0.007, while the magnitude of the ΔA peak at 800 nm is 0.035. Therefore these absorbance changes are self-consistent and agree with the analysis presented above.

Spectrum B (dashed trace) in Fig. 2 is the transient difference spectrum obtained 10 ps following a 500-fs laser flash at 610 nm in the PSII RC sample treated with sodium dithionite and methyl viologen. Comparing this spectrum with spectrum A, we see that the near-infrared features are nearly absent in spectrum B. In the blue-green spectral region, the absorption increase near 485 nm in spectrum A is greatly reduced in spectrum B. In addition, the 674-nm bleach in spectrum A is replaced by a 680-nm bleach in spectrum B. The absorption changes of the features near 505 nm and 540 nm in spectrum A are nearly absent in spectrum B.

The kinetics of selected transient absorption changes are depicted in Figs. 3–6. The only unambiguous spectral region in which the formation of $\text{P680}^+\text{-Pheoa}^-$ is not convolved with the appearance and decay of the lowest excited singlet state ($^1\text{P680}$) of P680 (and of the other pigments) is in the near-infrared. A monophasic exponential increase at 820 nm is observed with a best fit of 3.0 ± 0.6 ps (Fig. 3). The kinetics for the appearance of this band do not vary within experimental error from 750 nm to 850 nm.

The appearance of the strong absorption decrease at 674 nm (Fig. 4) reflects the formation of both the excited states of all the Chla and Pheoa pigments in the RC as well as the formation of $\text{P680}^+\text{-Pheoa}^-$. The fit to the kinetic data in Fig. 4 is biphasic. An initial bleach occurs with the instrument function $\tau_1 = 0.5 \pm 0.4$ ps ($54 \pm 10\%$), presumably excited state formation, followed by a further bleach that occurs with $\tau_2 = 3.3 \pm 0.4$ ps ($46 \pm 10\%$), presumably formation of $\text{P680}^+\text{-Pheoa}^-$. The time constant τ_2 is within experimental error of τ for formation of the 820-nm band. The amplitudes of these kinetic components are consistent within experimental error

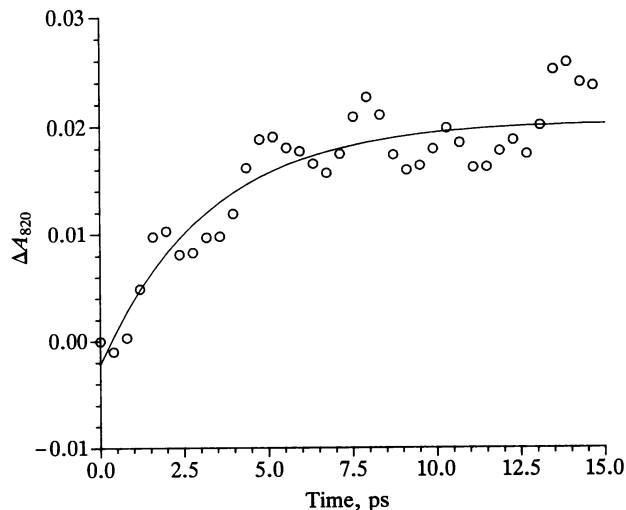


FIG. 3. Transient absorption changes at 820 nm for PSII RCs after a 100- μJ , 500-fs laser flash at 610 nm.

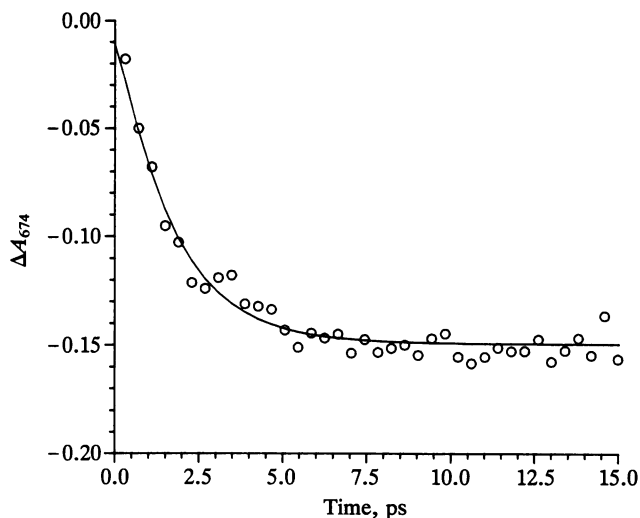


FIG. 4. Transient absorption changes at 674 nm for PSII RCs after a 100- μJ , 500-fs laser flash at 610 nm.

with the magnitudes of the bleaches at 674 nm and 680 nm in Fig. 2, spectra A and B, respectively.

Since the magnitude of the small trough at 540 nm is only 0.007 ΔA on a background absorbance of 0.025 ΔA , the 4:1 signal-to-noise ratio of the data is insufficient to determine the kinetics for the appearance of the 540-nm feature. The absorption band at 485 nm can be assigned to absorption changes due to the formation of $\text{P680}^+\text{-Pheoa}^-$ (33, 35). A small negative absorption change at 485 nm that occurs with the 500-fs instrument function is probably due to $^1\text{P680}$ formation and is followed by a positive absorption change due to $\text{P680}^+\text{-Pheoa}^-$ formation (33, 35) that can be fit with a single exponential time constant $\tau = 3.6 \pm 0.8$ ps (Fig. 5). The time constant for the appearance of the positive absorption change at 485 nm is within experimental error of the time constant for the formation of $\text{P680}^+\text{-Pheoa}^-$ determined at 820 nm. Thus, a 3-ps time constant for formation of $\text{P680}^+\text{-Pheoa}^-$ is found consistently at several key wavelengths throughout the transient absorption spectrum of the PSII RC.

In examining the kinetics of the absorption changes across the transient absorption difference spectrum of $\text{P680}^+\text{-Pheoa}^-$, we found that 650 nm is a near-isosbestic point for the difference between the absorbances of $\text{P680}^+\text{-Pheoa}^-$ and P680-Pheoa . The absorption at 650 nm rises sharply within the time resolution of the laser pulse and decays with

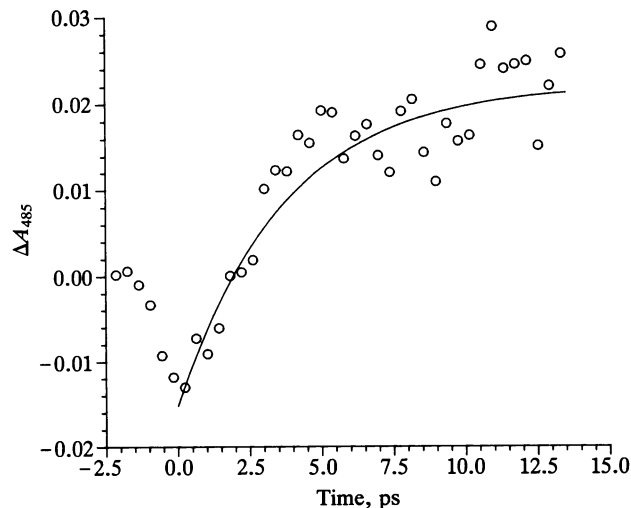


FIG. 5. Transient absorption changes at 485 nm for PSII RCs after a 100- μJ , 500-fs laser flash at 610 nm.

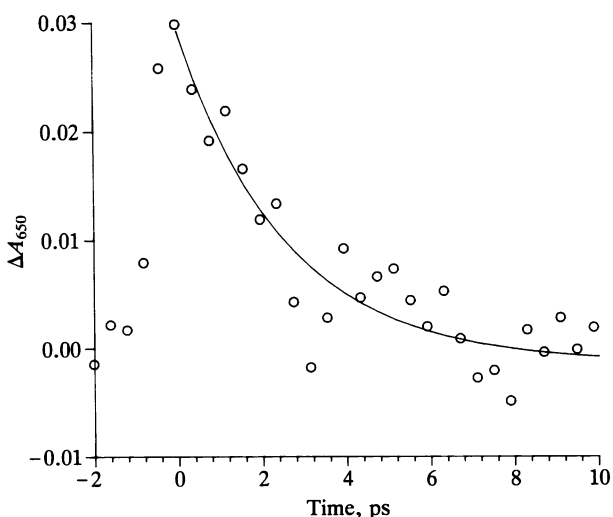


FIG. 6. Transient absorption changes at 650 nm for PSII RCs after a 100- μ J, 500-fs laser flash at 610 nm.

$\tau = 2.6 \pm 0.6$ ps (Fig. 6). This absorption change is ascribed to $^1\text{P680}$. The decay time for this state agrees with the value for the formation of $\text{P680}^+-\text{Pheoa}^-$ obtained at 820 nm.

At no wavelength in the transient absorption spectrum of the PSII RC was there found any spectral or kinetic evidence for an electron acceptor that precedes Pheoa^- .

DISCUSSION

When Pheoa in PSII RCs is prereduced, flash excitation of the RCs results in a transient absorption spectrum (Fig. 2, spectrum B) that differs significantly from that obtained for untreated RCs (spectrum A). Since both P680^+ and Pheoa^- absorb in the near-infrared region of the spectrum, the presence of the near-infrared absorbance in untreated RCs and its loss in the prereduced RCs are consistent with the assignment of these bands to the formation of $\text{P680}^+-\text{Pheoa}^-$ in the untreated RCs.

Since the data show that the absorbance changes in the prereduced RCs are not due to the formation of $\text{P680}^+-\text{Pheoa}^-$, these changes may be due either to formation of $^1\text{P680}$ or to reduction of an electron acceptor prior to Pheoa . If we hypothesize that the observed spectrum is due to the formation of another intermediate acceptor preceding the formation of Pheoa^- , the most likely candidate for this acceptor would be another Chla in the PSII RC that is not part of P680 . However, it is well known that the optical absorption spectrum of Chla^- is very similar to that of Pheoa^- in the near-infrared, with a broad band centered at 780 nm (33). Thus, the absence of such a band in spectrum B makes it unlikely that Chla^- is an intermediate acceptor in PSII RCs at times >500 fs.

The remaining hypothesis, the one that the data favor, is that the transient absorption changes in spectrum B are due to the formation of $^1\text{P680}$. The transient absorption spectrum of Chla *in vitro* (Fig. 7) shows that the transient absorbance of $^1\text{Chla}$ is relatively weak in the near-infrared spectral region. This is consistent with the absorption changes in the near-infrared region in spectrum B, which are substantially diminished relative to those for $\text{P680}^+-\text{Pheoa}^-$ shown in spectrum A (Fig. 2).

Since we were unable to obtain the lifetime of $^1\text{P680}$ by observing its stimulated emission (4–6), we examined the transient absorption spectrum depicted in spectrum A for wavelengths that are isosbestic points for the differential absorbance between $\text{P680}^+-\text{Pheoa}^-$ and $\text{P680}-\text{Pheoa}$. The transient absorption kinetics at 650 nm show that there is a

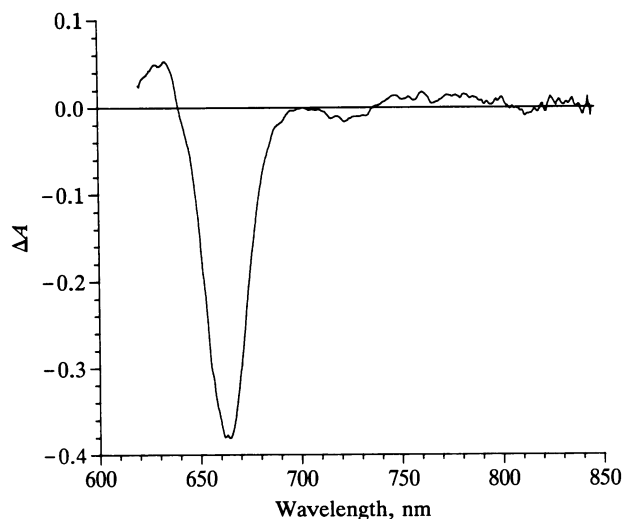


FIG. 7. Transient absorption spectrum of Chla in diethyl ether at 10 ps after a 100- μ J, 500-fs laser flash at 610 nm.

process that occurs within the time resolution of the laser and decays with a monophasic time constant of 2.6 ± 0.6 ps (Fig. 6). We interpret these changes as due to the formation and decay of $^1\text{P680}$. The fast rise of this transient further implies that any energy transfer occurring between the Chla and Pheoa pigments in the RC and between these pigments and P680 is faster than the 500-fs instrument response.

These results are also consistent with the assignment of the transient absorption changes in spectrum B to $^1\text{P680}$. The absorption changes in spectrum B occur within the 500-fs instrument response time. Energy transfer from the other pigments in the PSII RC to $^1\text{P680}$ is complete within this time. In untreated RCs, charge separation competes very efficiently with decay of the $^1\text{P680}$ excited state. The result is that the decay time for $^1\text{P680}$ very closely tracks the time for formation of $\text{P680}^+-\text{Pheoa}^-$, 2.6 ± 0.6 ps vs. 3.0 ± 0.6 ps, respectively. However, when Pheoa is prereduced, electron transfer is eliminated as a rapid quenching mechanism for the excited states, and $^1\text{P680}$ decays with a time constant more typical of $^1\text{Chla}$, which is a few nanoseconds.

An estimate of the lifetime of excited states can be obtained from photochemical hole-burning experiments (36). The width of a hole burnt in the inhomogeneously broadened optical absorption at 680 nm [the 674-nm absorption band of the PSII RC splits and part of it shifts to 680 nm at low temperatures (37)] can be related to the $^1\text{P680}$ lifetime through the uncertainty principle. Photochemical hole-burning experiments, using a sample from the same batch of stabilized PSII RCs reported in this paper, yielded transient zero-phonon hole widths of $5\text{--}6\text{ cm}^{-1}$ at 4.2 K, which correspond to an excited-state lifetime for $^1\text{P680}$ of 1.8–2.1 ps (37). These data are consistent with the 2.6 ± 0.6 -ps lifetime of $^1\text{P680}$ that we obtain from the transient absorption measurements at 4°C.

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