Observation of the Reduction and Reoxidation of the Primary Electron Acceptor in Photosystem I†

Gary Hastings, Frank A. M. Kleinherenbrink, Su Lin, Thomas J. McHugh, and Robert E. Blankenship*

Department of Chemistry and Biochemistry and the Center for the Study of Early Events in Photosynthesis, Arizona State University, Tempe, Arizona 85287-1604

Received November 5, 1993; Revised Manuscript Received January 24, 1994®

ABSTRACT: Femtosecond transient absorption spectroscopy has been used to investigate the primary charge separation in a photosystem II deletion mutant from the cyanobacterium *Synechocystis* sp. PCC 6803. These cells contain only the photosystem I reaction center and have a pigment content of ~100 chlorophylls per P700. Utilizing relatively high excitation intensities, the difference spectrum for the reduction of primary electron acceptor [$(A_0^- - A_0)$ difference spectrum] was obtained from experiments performed under both reducing and oxidizing conditions. Both approaches yield very similar results with the $(A_0^- - A_0)$ difference spectrum displaying a maximum bleaching at 687 nm. The shape of the difference spectrum suggests that the primary electron acceptor in photosystem I may be a chlorophyll *a* molecule. The observed rate of primary radical pair formation depends on the overall rate of decay of excitations in the antenna; the radical pair state forms as the antenna decays. The decay of the primary radical pair state is characterized by a 21-ps time constant. Under conditions that avoid annihilation effects, the mean lifetime for excitations in the antenna is 28 ps [Hastings, G., Kleinherenbrink, F. A. M., Lin, S., & Blankenship, R. E. (1994) *Biochemistry* (preceding paper in this issue)]. This indicates that the reduced acceptor decays faster than it forms. Therefore, only a low concentration of the reduced acceptor will accumulate under most conditions.

Light capture and conversion in photosynthetic organisms proceeds by absorption of incident radiation in antenna systems followed by migration of the absorbed energy to a reaction center, where it is trapped via the formation of a series of progressively more stable radical pair intermediates. Study of the primary charge separation in photosystem (PS)[1] II of green plants and purple bacteria has been considerably simplified due to the isolation and characterization of reaction center complexes devoid of accessory antenna pigments. This situation has not been realized in photosystem I (Golbeck & Bryant, 1991), heliobacteria (Trost & Blankenship, 1989), and green sulfur bacteria (Blankenship, 1985), where a relatively large number of pigments are tightly bound to the same polypeptides which also bind the reaction center cofactors.

In heliobacteria (Nuijs et al., 1985b; Lin et al., 1994) and in green sulfur bacteria (Nuijs et al., 1985a) forward electron transfer from the primary to the secondary electron acceptor (defined as secondary electron transfer below) is characterized by a time constant of ~600 ps. In PS II (Nuijs et al., 1986b; Schatz et al., 1987), purple bacteria, and green filamentous bacteria (Kirmaier & Holten, 1987) time constants for secondary electron transfer between 200 and 500 ps are observed. In heliobacteria, green sulfur bacteria, PS II, purple bacteria, and green filamentous bacteria, the reoxidation of the reduced primary electron acceptor was clearly observed in transient absorption experiments because the time constant for decay of antenna excitations is about an order of magnitude smaller than the time constant for secondary electron transfer in these systems.

It has been suggested that, following light excitation, the primary electron acceptor in PS I (A_0) is reduced in 13 ps and reoxidized in about 40 ps (Wasielewski et al., 1987; Fenton et al., 1979). Shuvalov et al. (1986) could not time-resolve the reduction of the primary electron acceptor. However, they indicated that the reduced acceptor was reoxidized with a time constant of 32 ps. A time constant of 32–40 ps for secondary electron transfer in PS I is significantly shorter than that observed for the corresponding process in any other photosynthetic system (see above) and is similar to the overall decay time for antenna excitations in the PS I core antenna (Owens et al., 1988; Holzwarth et al., 1993; see also the preceding paper in this issue). The extremely high rate of secondary electron transfer in PS I may explain why A_0 reoxidation has never been observed in a transient absorption experiment, under normal conditions (P700 neutral), when low excitation intensities are used (Nuijs et al., 1986a; Klug et al., 1989; Holzwarth et al., 1993; see also preceding paper).

The above considerations indicate that it may not be possible to observe absorption changes associated with A_0 directly. It is possible, however, to obtain spectroscopic information concerning the nature, rates of reduction, and rates of reoxidation of the primary electron acceptor, using indirect approaches that require the subtraction of absorption changes associated with a variety of different species ($P700^+$ formation and/or formation and decay of antenna excited states), which may have to be obtained from independent measurements (Mathis et al., 1988; and see below).

By comparison of absorption changes in the presence of both open (P700 neutral) and closed (P700 oxidized) reaction centers, and under reducing conditions, the difference spectrum associated with reduction of the primary electron acceptor in PS I [$(A_0^- - A_0)$ difference spectrum] has been obtained (Nuijs et al., 1986a; Shuvalov et al., 1986). $(A_0^- - A_0)$ difference spectra have also been obtained by comparing absorption

† This work was supported by NSF Grant DMB-9106685.

* Author to whom correspondence should be addressed.

® Abstract published in *Advance ACS Abstracts,* March 1, 1994.

[1] Abbreviations: (B)Chl *a*, (bacterio)chlorophyll *a*; FWHM, full width at half maximum; LHC I, light harvesting complex I; PS I, photosystem I; DAS, decay associated spectrum; P700, primary electron donor in PS I; A_0, primary electron acceptor in photosystem I; A_1, secondary electron acceptor in photosystem I; F_X, tertiary electron acceptor in PS I (iron–sulfur cluster); PMS, phenazine methosulfate.

0006-2960/94/0433-3193$04.50/0

changes in PS I particles in which the secondary electron acceptor has either been deleted or reconstituted (Mathis et al., 1988; Kim et al., 1989). The ($A_0^- - A_0$) difference spectra resemble published (Chl *a*$^-$ – Chl *a*) *in vitro* difference spectra (Fujita et al., 1978), which suggests that the primary electron acceptor in PS I may be a Chl *a* species absorbing near 690 nm (Nuijs et al., 1986a; Shuvalov et al., 1986; Mathis et al., 1988; Kim et al., 1989). The ($A_0^- - A_0$) difference spectra display side bands that are not characteristic of Chl *a* difference spectra, which may indicate that the difference spectra contain contributions from electrochromic effects arising from pigments in the vicinity of the primary electron donor and acceptor (Holzwarth, 1989; Holzwarth et al., 1993).

In experiments where the formation and/or decay of A_0^- is reported to have been observed, it is also claimed that P700 is either directly excited (Shuvalov et al., 1986) or the excitation reaches P700 within 1 ps (Wasielewski et al., 1987). These latter results are at variance with the conclusions drawn from a large number of time-resolved fluorescence and absorption measurements (Owens et al., 1987; Klug et al., 1989; Turconi et al., 1993; Holzwarth et al., 1993; see also preceding paper). We do not believe that it is possible to selectively excite P700. However, we show that this is not necessary to resolve absorption changes associated with the formation and/or decay of A_0^-.

At present, the identity as well as the time constants that characterize the rates of reduction and reoxidation of the primary electron acceptor in PS I are not well established (Holzwarth, 1989; Holzwarth et al., 1993; and see above). In this paper we describe the use of subpicosecond transient absorption spectroscopy, utilizing high excitation intensities, to investigate the nature, rate of reduction, and rate of reoxidation of the primary electron acceptor in mutants from the cyanobacterium *Synechocystis* sp. PCC 6803 that contain only the PS I reaction center. We show that under these conditions it is possible to resolve absorption changes associated with the reduction and reoxidation of the primary electron acceptor, and we present ($A_0^- - A_0$) difference spectra obtained using two entirely different approaches.

MATERIALS AND METHODS

Thylakoid preparation. The mutant psbDI/C/DII from the cyanobacterium *Synechocystis* sp. PCC 6803, which contains only the PS I reaction center, has been described previously (Vermaas et al., 1987, 1988). The preparation of the membrane fragments from the whole cells is described in the preceding paper.

Femtosecond Transient Absorption Measurements. All experiments described here were performed at room temperature. Samples were suspended in either of two different buffers. For experiments performed under both neutral and oxidizing conditions, samples were suspended in buffer containing 20 mM Tris-HCl, pH 8.0, approximately 20 mM ascorbate, and 10 μM phenazine methosulfate (PMS). Oxidizing conditions were created using laser illumination rather than chemically, as described below. For experiments performed under reducing conditions, samples were suspended in buffer containing 200 mM glycine, pH 11.5, 20 mM ascorbate, and 20 μM PMS. The sample was suspended in the buffer and degassed. Following this, sodium dithionite was added to a final concentration of 30 mM.

The femtosecond laser system is described in the preceding paper. In some of the experiments described here we also direct a portion (40%) of the intense, 532-nm, 100-ps pulses from a regenerative amplifier along a delay line and use this portion to excite the sample ~2 ns prior to the main excitation pulses. This intense preflash closes a high proportion of the reaction centers (P700 oxidized).

All femtosecond transient absorption experiments described in this paper were performed using high intensity, 590-nm excitation pulses. On the basis of the transient bleaches, we estimate that 4–8 photons were absorbed per reaction center. Under these conditions much of the decay of the excitations occurs via excitation annihilation processes.

Data were collected between 0 and 90 ps in the 640–780-nm spectral region and analyzed globally, assuming multiexponential kinetics. Results obtained from global analysis are presented as decay associated spectra (DAS) as described in the preceding paper.

Microsecond Transient Absorption Measurements. Transient absorption spectra were collected on a microsecond time scale (~20 μs) using a home-built, double-flash spectrometer. The actinic and measuring pulses were supplied by two separate xenon arc lamps (EG&G). The measuring lamp produced pulses of ~2 μs duration (FWHM), while the actinic pulses were ~4 μs in duration. The discharge sequence of the lamps was controlled by a CTM05 timing board (Metrobyte, Inc.). Optical density changes resulting from the actinic flash were monitored using a diode array detector (EG&G Princeton Applied Research) coupled to a model 340S spectrograph (Spex Industries, Inc.) and stored on a personal computer, via an EG&G model 1461 controller interface. Spectra were collected over a 60-nm wavelength interval and were obtained by averaging 100 flashes with ~3 s between each flash. Fluorescence from the sample induced by the actinic lamp was corrected for during data acquisition by subtracting a baseline with the measuring lamp turned off.

RESULTS

To distinguish between absorption changes associated with formation and decay of antenna excited states and absorption changes associated with radical pair formation, and also to distinguish between absorption changes associated with $P700^+$ formation and reduction and reoxidation of the primary electron acceptor, data were collected using three different sets of experimental conditions:

(1) Neutral Conditions. Under these conditions the PS I reaction centers were in the open state (P700 neutral) prior to excitation.

(2) Oxidizing Conditions. Under these conditions the PS I reaction centers were in the closed state (P700 oxidized) prior to excitation. P700 was oxidized by an intense preflash, as described under Materials and Methods.

(3) Reducing Conditions. Highly reducing conditions (see Materials and Methods), combined with illumination, eventually lead to the double reduction of A_1, the secondary electron acceptor in PS I. The doubly reduced quinone acceptor is very stable and blocks secondary electron transfer. Under these conditions the primary radical pair state, $P700^+A_0^-$, decays in 35–50 ns (Sétif & Bottin, 1989; Sétif & Brettel, 1990).

Oxidizing Conditions. Transient absorption data were collected on a 90-ps time scale, under conditions in which the traps were both open and closed. Figure 1 panels A and C show examples of the kinetics at 686 and 700 nm, respectively, when the traps are both open and closed. Figure 1 panels A and C demonstrate that similar excitation intensities were used in both sets of experiments and were such that 4–8 photons were absorbed per reaction center. When such high excitation intensities are used, the main antenna decay is characterized

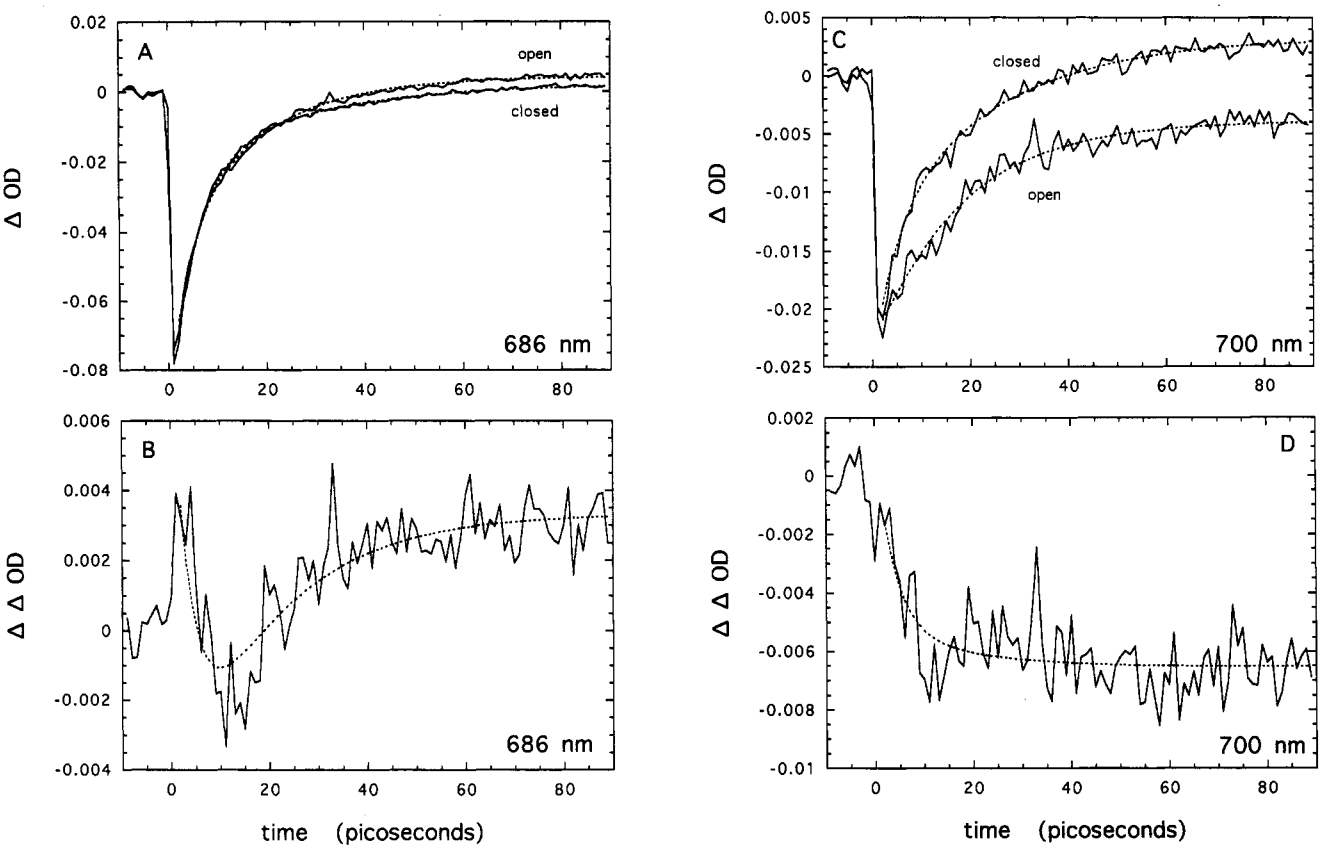

FIGURE 1: Kinetics of the absorption changes observed at (A) 686 nm and (C) 700 nm, in the presence of both open and closed PS I reaction centers, following 590 excitation at room temperature. Panels B and D show the difference (open minus closed) between the kinetics shown in panels A and C, respectively. The dotted lines show the fitted functions obtained from global analysis of the data. In the open trap decays in panels A and C, the fitted function is the sum of two exponential components, with lifetimes of 4 and 16 ps, and a nondecaying component. In the closed trap decays the fitted function is the sum of two exponential components, with lifetimes of 5 and 35 ps, and a nondecaying component. The fitted functions in panels B and D are the sum of two exponential components, with lifetimes of 4 and 21 ps, and a nondecaying component.

by a 4–5-ps time constant in both open and closed traps (see fitted functions in Figure 1A). The absorption changes observed when the traps are closed are due only to processes associated with excitations in the antenna, while the absorption changes observed under neutral conditions (open traps) are due to processes associated with both excitations in the antenna and the formation and decay of the primary radical pair state. A great deal of evidence suggests that the antenna kinetics in PS I are independent of the redox state of P700 (Nuijs et al., 1986a; Shuvalov et al., 1986; Owens et al., 1988; Klug et al., 1989; Turconi et al., 1993; see also the preceding paper). Therefore, subtracting data obtained with the traps closed from data obtained with the traps open should result in data that display absorption changes associated with only the formation and decay of the primary radical pair state. Figure 1 panels B and D show the kinetics at 686 and 700 nm, respectively, when such a subtraction is performed. Figure 2 panels A and B show the difference spectra at a variety of delay times obtained from measurements under conditions in which the traps are open and closed respectively. Figure 2C shows magnified views of the 89-ps spectra in Figure 2A,B. When the traps are closed, a long-lived residual bleaching at ~675 nm is observed. This type of long-lived, blue-shifted, residual bleaching has been observed previously by Klug et al. (1989) and by Nuijs et al. (1986a) and has been interpreted to result from antenna complexes that are uncoupled from the charge separation. Figure 2D shows the resultant spectra, at a variety of delay times, when data obtained with the traps closed (Figure 2B) are subtracted from data obtained with the traps open (Figure 2A). We shall refer below to the data in Figure 2D as neutral minus oxidized. The neutral minus oxidized spectra (some of which are shown in Figure 2D), obtained in 1-ps intervals between 0 and 90 ps, were globally analyzed. Figure 3 shows the decay associated spectra (DAS) obtained from such a global analysis. Two exponentials, with lifetimes of 4 and 21 ps, and a nondecaying component are required to adequately fit the data.

The 4-ps DAS displays peaks at 686 and 696 nm and is positive at all wavelengths above 670 nm. It can be seen from Figures 1B,D that the positive amplitude of the 4-ps component is the result of a grow-in or formation of a bleaching. For this reason we have assigned the 4-ps DAS to formation of the primary radical pair state, $P700^+A_0^-$.

The spectrum of the 21-ps component in Figure 3 shows a negative peak at 686 nm and is flat and positive on both the long and short wavelength sides. The decay in Figure 1B and the spectra at 10 and 89 ps in Figure 2D (essentially before and after the 21-ps process has occurred) indicate that the 21-ps spectrum in Figure 3 is consistent with the ground state recovery of a species which absorbs maximally at 686 nm. Because the spectra in Figure 2D are a result of absorption changes associated only with radical pair formation (see above), it is likely that the 21-ps process is due to secondary electron transfer ($P700^+A_0^- \rightarrow P700^+A_1^-$). The secondary electron acceptor in PS I, termed A_1, is a phylloquinone molecule, which does not absorb in the wavelength region considered here (see Golbeck & Bryant, 1991 for a review). This indicates that the 21-ps spectrum in Figure 3 is due to changes associated only with A_0 reduction (assuming negligible

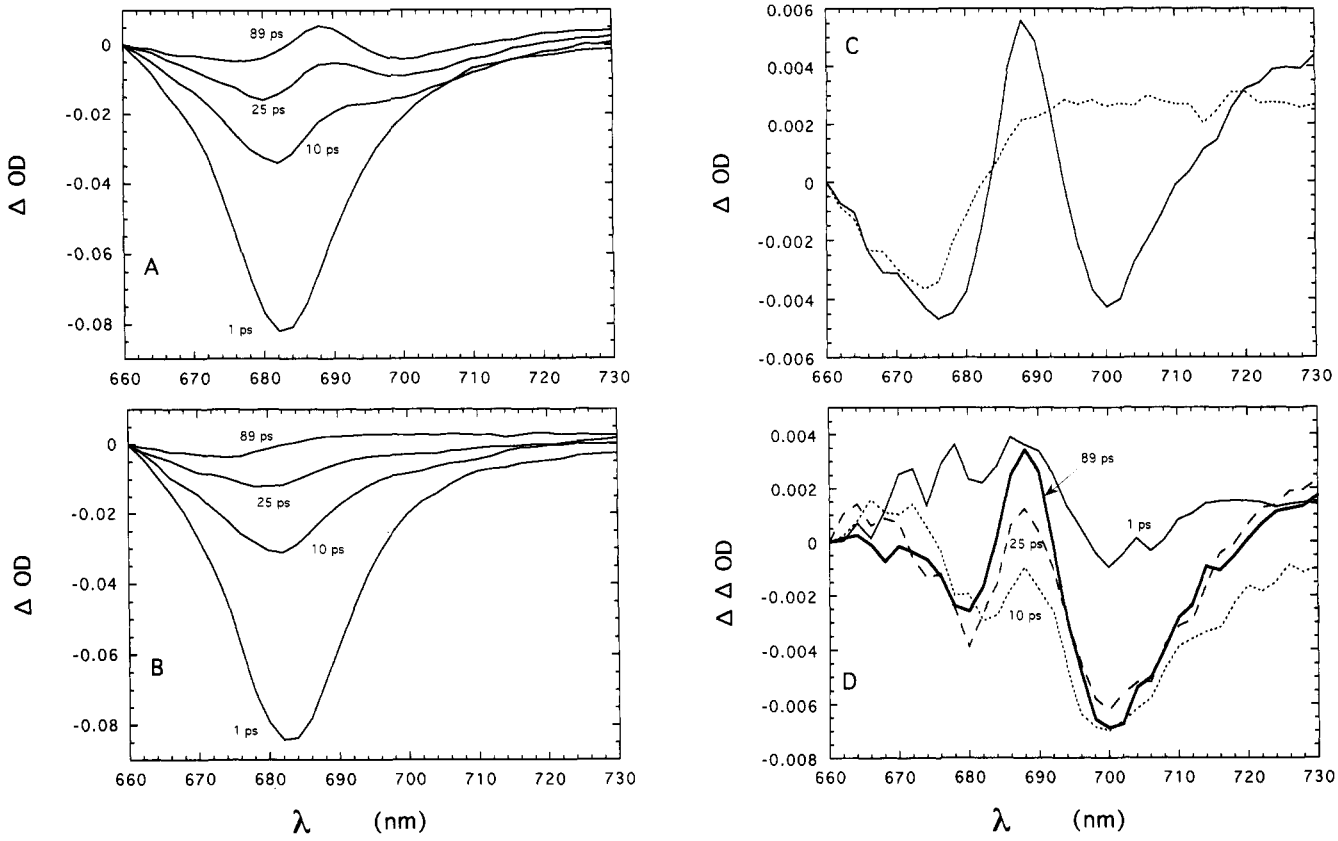

FIGURE 2: Absorbance difference spectra observed following 590-nm excitation of PS I particles from *Synechocystis* sp. PCC 6803 at room temperature. In panel A the reaction centers are open (P700 neutral). In panel B the reaction centers are closed (P700 oxidized). (C) Expanded view of the 89-ps spectra shown in panels A (solid line) and B (dotted line). The difference between the spectra in panels A and B (neutral minus oxidized spectra), at the relevant time delays, are shown in panel D.

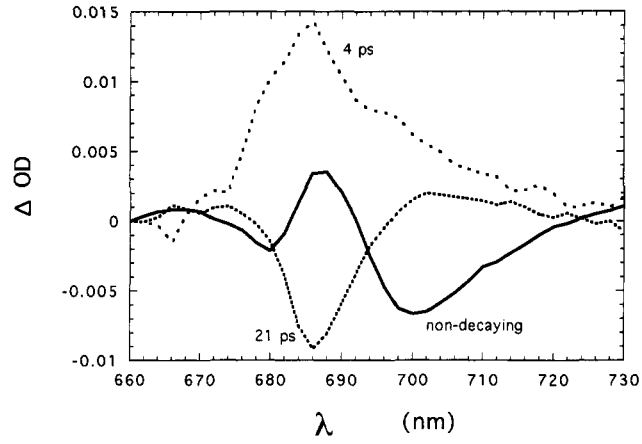

FIGURE 3: Decay associated spectra (DAS) of the 4-ps (dotted line), 21-ps (dashed line), and nondecaying (solid line) components obtained from global analysis of neutral minus oxidized data, some of which are shown in Figure 2D. Data were collected under both neutral and oxidizing conditions on a 90-ps time scale, using high intensity excitation pulses centered at 590 nm.

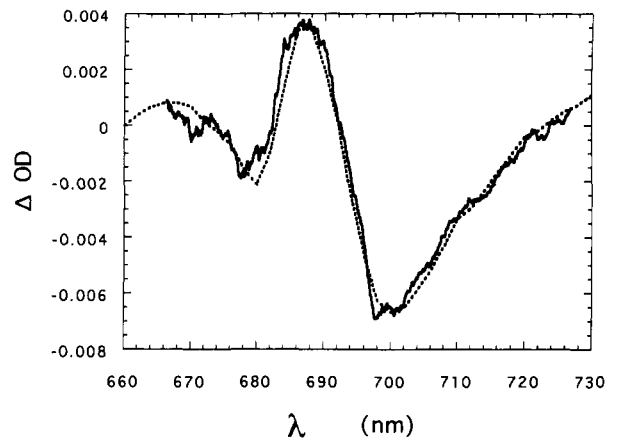

FIGURE 4: Absorption difference spectrum, obtained ~20 μs after excitation of PS I particles from *Synechocystis* sp. PCC 6803 at room temperature (solid line). Also shown is the nondecaying spectrum presented in Figure 3 (dotted line). The microsecond difference spectrum has been normalized at 700 nm. Both spectra are very similar in shape, indicating that the same species is responsible for both spectra.

changes in electrochromism upon electron transfer) and is therefore representative of the ($A_0^- - A_0$) difference spectrum.

Because all antenna processes have occurred well within 90 ps (see preceding paper), and since secondary electron transfer to A_1 (which does not absorb in this wavelength region) occurs in 21 ps, it follows that a nondecaying spectrum in Figure 3 should be due only to P700 oxidation. To verify that the nondecaying spectrum in Figure 3 is due only to P700 oxidation, we have also performed transient absorption measurements on a microsecond time scale. Figure 4 compares the difference spectrum obtained on a microsecond time scale with the nondecaying spectrum presented in Figure 3. The spectra are identical within experimental error, which provides strong evidence that the nondecaying spectrum in Figure 3 is due only to P700 oxidation.

Reducing Conditions. Data were collected under both neutral and reducing conditions. The excitation intensities used were the same under both sets of experimental conditions and were also similar to the excitation intensities used in the experiments described above. Data were collected on a 90-ps time scale under both neutral and reducing conditions and were globally analyzed. Figure 5A compares the nondecaying spectra, obtained from global analysis of data obtained under

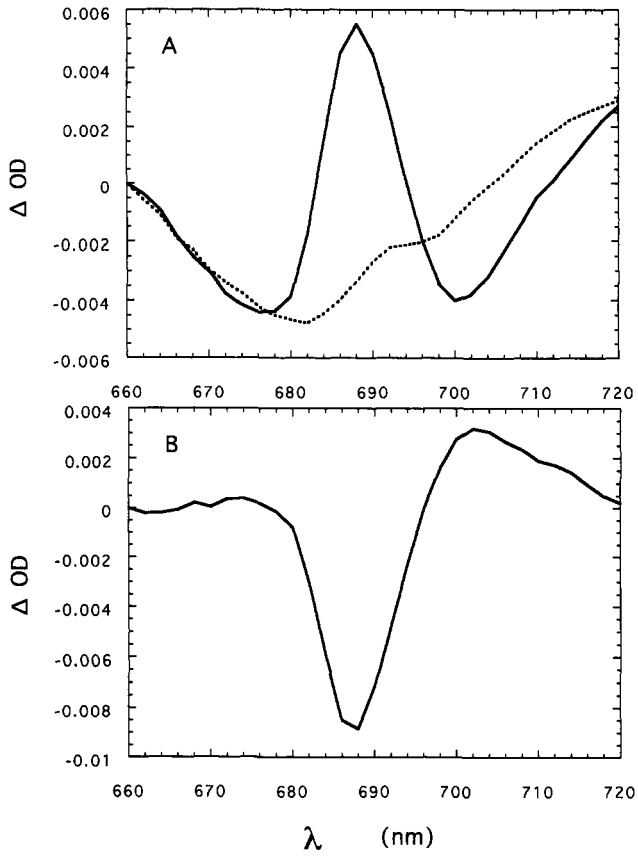

FIGURE 5: (A) Spectra of the nondecaying components obtained from global analysis of data collected following 590-nm excitation of PS I particles from *Synechocystis* sp. PCC 6803, suspended under reducing conditions (dotted line) and neutral conditions (solid line). Data under both sets of experimental conditions were obtained using the same excitation intensities, on a 90-ps time scale. (B) Result of subtracting the nondecaying spectrum obtained under neutral conditions from the nondecaying spectrum obtained under reducing conditions.

both neutral and reducing conditions. The nondecaying spectrum, obtained from global analysis of data obtained under neutral conditions, has peaks at 700 and 676 nm which are of similar magnitude, while the nondecaying spectrum obtained under reducing conditions has peaks at 698 and 680 nm. Under reducing conditions, the nondecaying spectrum is due to $P700^+A_0^-$ formation [the lifetime of the radical pair state, $P700^+A_0^-$, under reducing conditions is about 50 ns (Sétif & Bottin, 1989)], while the spectrum obtained under neutral conditions is due to $P700^+$ formation only (see above). The long-lived, residual bleaching at ~675 nm can clearly be seen in the data obtained under neutral conditions [compare the nondecaying spectrum in Figure 4 and the nondecaying spectrum (neutral conditions) in Figure 5A]. This residual bleaching should also be present in data obtained under reducing conditions. Because the data obtained under both sets of conditions are subtracted, this residual bleaching is automatically eliminated. Subtraction of the nondecaying spectrum obtained under reducing conditions from the nondecaying spectrum obtained under neutral conditions should therefore result in a difference spectrum that is due only to the reduction of A_0. Figure 5B shows the result of such a subtraction. The spectrum in Figure 5B peaks at 688 nm and is positive on both the long and short wavelength sides, similar to that of the 21-ps spectrum in Figure 3. This similarity is highlighted in Figure 6, where the $(A_0^- - A_0)$ difference spectra obtained using the two different approaches are compared.

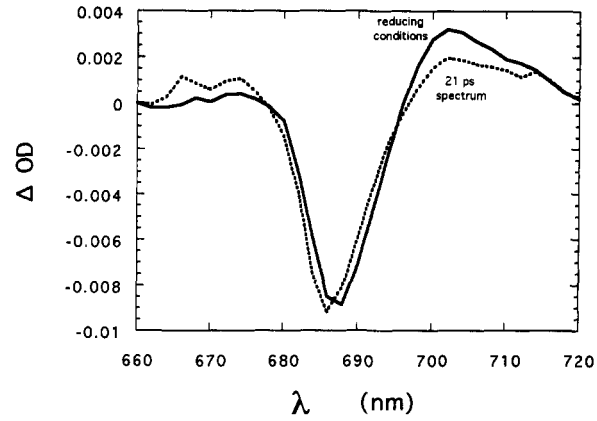

FIGURE 6: Comparison of the $(A_0^- - A_0)$ difference spectra, obtained using two different approaches: DAS of the 21-ps component shown in Figure 3 (dotted line) and the subtraction shown in Figure 5B (solid line).

DISCUSSION

Primary and Secondary Electron Transfer. We have shown that the nondecaying spectrum in Figure 3 is due only to P700 oxidation. This interpretation is based on the observation that the shape of the spectrum is invariant between the picosecond and microsecond time scales (Figure 4). There is some overall similarity in shape with previously published ($P700^+$ – P700) difference spectra (Schaffernicht & Junge, 1981, 1982); however, the positive peak at 687 nm has only previously been observed at low temperature (Vermeglio et al., 1976; Sétif et al., 1984; Parrett et al., 1989). The absorption changes observed near 687 nm in previous ($P700^+$ – P700) difference spectra vary widely, depending strongly on the type of particle used, and are thought to be related to electrochromic shifts of absorption bands of antenna pigments in close proximity to the positively charged primary electron donor (Schaffernicht & Junge, 1981, 1982).

In experiments performed with the traps closed, we observe a long-lived, residual bleaching which peaks at ~675 nm (Figure 2C). This residual bleaching may be due to excitation of uncoupled chlorophyll or particles in which charge separation does not occur, or it may be due to excitation of residual phycobilisomes that were not removed during the isolation procedure. This residual bleaching should be present in data obtained using PS I particles in which the traps are both open and closed. Because we are primarily interested in the neutral minus oxidized data, this residual bleaching is of little concern, as it is subtracted out. The fact that the same $P700^+$ difference spectrum is observed on both the picosecond and microsecond time scales indicates that this is indeed the case and that the subtraction procedures employed here are appropriate for our data. The 89-ps difference spectrum, observed when the traps are closed (Figure 2C), does not display any absorption changes that can be associated with $P700^+$ (Figure 4). This indicates that the intense preflash results in oxidation of P700 in virtually all of the reaction centers.

Since the 4-ps DAS, obtained from neutral minus oxidized data is the result of a grow-in or formation of a bleaching (see Figures 1B,D and 2D), and also because the neutral minus oxidized data are associated with changes which are a result of only radical pair formation, we have assigned the 4-ps DAS to formation of the primary radical pair state, $P700^+A_0^-$.

Because the excitation pulses predominately excite antenna pigments, and the antenna kinetics are independent of the redox state of P700, the absorption changes at very early time delays in the neutral minus oxidized data should be approx-

imately zero. The 1-ps spectrum in Figure 2D and the decay in Figure 1B indicate that this is not quite the case. This may be due to slight variations in excitation intensities between the two sets of experiments. The data subtraction procedure has a pronounced effect on the 4-ps spectrum because at very early time delays the bleaching at 686 is ~0.08 (see Figure 1A). The changes we are interested in are about an order of magnitude smaller (compare the DAS in Figure 3 with the 1-ps spectra in Figures 2A,B). We are subtracting two large signals to obtain a small resultant signal. Figure 1A–D shows that there is clearly a 4-ps phase that has a different amplitude in the presence of open and closed traps. Therefore, there is clearly a 4-ps process that is associated with radical pair formation. It is likely, however, that the spectrum we present for the 4-ps process in Figure 3 is slightly distorted, especially in the 680 nm spectral region.

We have performed a large number of global simulations in which the initial starting parameters were varied, and it is clear from these simulations that both the lifetime and shape of the 21-ps spectrum is not affected significantly by the faster 4-ps component. The fact that the spectrum of the nondecaying component in Figure 3 can be assigned only to P700 oxidation, and that we observe the same difference spectrum for the primary electron acceptor using two different approaches, supports the appropriateness of the data subtraction procedures. The fact that we observe the same (A_0^- – A_0) difference spectrum, using two entirely different approaches, also provides strong evidence that the antenna kinetics in PS I are independent of the redox state of P700, as has been found previously (Nuijs et al., 1986a; Shuvalov et al., 1986; Owens et al., 1988; Klug et al., 1989; Turconi et al., 1993; see also the preceding paper).

Although the spectrum of the 4-ps component in Figure 3 is not well resolved in the 680-nm spectral region, there is some similarity in shape between the 4-ps spectrum in Figure 3 (inverted) and the nondecaying spectrum obtained under reducing conditions in Figure 5A (neglecting the long-lived residual bleaching). The nondecaying spectrum obtained under reducing conditions is assigned to primary radical pair state, $P^+A_0^-$, which again suggests that this is the origin of the 4-ps process.

We have separately globally analyzed the data obtained with the traps both open and closed (Figures 2A,B). We find in both cases that the main component in the overall decay of excitations in the antenna is characterized by a 4–5-ps time constant (see fitted functions in Figures 1A,C). This suggests that the observed rate of radical pair formation is limited by the decay of excitations in the antenna, which is in turn dominated by the excitation annihilation process. This was investigated further by performing experiments with lower excitation intensities, which results in a mean antenna lifetime of 16 ps (data not shown). Under these conditions we cannot resolve any processes with lifetimes of a few picoseconds. We can still resolve a ~20-ps lifetime; however, the shape of the spectrum is distorted (data not shown), presumably due to the incomplete resolution of a number of components all with similar lifetimes. The fact that we do not observe a 4-ps component when lower excitation intensities are used is further evidence that the observed rate of formation of the primary radical pair state is limited by the overall rate of decay of the excitations in the antenna.

Using low excitation intensities, we have shown that the mean antenna lifetime is 28 ps (see preceding paper). It is also shown above that the 21-ps spectrum in Figure 3 is due to reoxidation of A_0^-. If low excitation intensities are used, the above observations would indicate that the primary electron acceptor is reduced in 28 ps (A_0^- forms as the excitations in the antenna decay) and then reoxidized in 21 ps. Assuming a simple, irreversible, sequential reaction, and the observed 28- and 21-ps time constants, it can be calculated that the maximum transient population of A_0^- is about 30%. This small intermediate population of reduced acceptor, together with the similarity in time constants, is likely to make detection of this intermediate difficult and explains why the reduction and the reoxidation of the primary electron acceptor in PS I has not previously been resolved in transient absorption experiments, under normal conditions (P700 neutral), utilizing low excitation intensities (Klug et al., 1989; Holzwarth et al., 1993; see also the preceding paper).

We show above that the observed time constant characterizing primary radical pair formation is 4 ps and depends on the decay time of excitations in the antenna. If the intrinsic time constant for primary radical pair formation is greater than 4-ps, we would not expect to observe a 4-ps time constant in the neutral minus oxidized data, because charge separation would be the limiting process and act as a bottleneck. Our assignments therefore suggest that the intrinsic time constant characterizing radical pair formation in the PS I particles used here must be less than 4 ps. This is in contrast to the 13-ps time constant proposed by Wasielewski et al. (1987) but agrees with the upper limit of 1.6 ps that was calculated in the preceding paper.

In the preceding paper we show that a strictly diffusion-limited kinetic model cannot describe excitation transfer in the PS I core antenna and that a trap-limited or pseudo-trap-limited model is appropriate. When we use high excitation intensities, we observe singlet–singlet annihilation processes; however, the radical pair state still forms in greater than 70% of the reaction centers. These observations may appear to indicate that P700* is in equilibrium with the surrounding pigments. However, without first accounting for the increased concentration of excitons in the antenna (due to the high excitation intensities), it is not possible to unambiguously distinguish between the two trapping-limited cases. The data presented here and in the preceding paper [and also by Holzwarth et al. 1993)] are not sufficient to unambiguously distinguish between the two different trapping-limited cases. To distinguish between the special-trap-limited and trap-limited cases, it is necessary to have some knowledge of when P700 receives the excitation energy.

Difference Spectra Associated with the Reduction of A_0. We show above that the 21-ps spectrum in Figure 3 is due to secondary electron transfer and that it represents the (A_0^- – A_0) difference spectrum. We also show that the difference between the two nondecaying spectra in Figure 5A is representative of the (A_0^- – A_0) difference spectrum. The (A_0^- – A_0) difference spectra, obtained using the two approaches, are shown in Figure 6. The two difference spectra are essentially the same, with a maximum bleaching at 686–688 nm. There may be a slight wavelength shift between the two spectra in Figure 6. If there is a difference, it is extremely small (<2 nm) and is at the limits of our wavelength resolution.

It has been suggested that the (A_0^- – A_0) difference spectra that have been obtained previously from experiments performed under reducing conditions could be complicated by contributions from electrochromic effects associated with chlorin species situated near the reduced terminal acceptors (Holzwarth, 1989; Holzwarth et al., 1993). The overall shape of the spectra in Figure 6 are not consistent with an electrochromic shift. Furthermore, we obtain similar spectra

in experiments performed under either oxidizing or reducing conditions, which would suggest that this electrochromic effect would have to be very small or the same in both cases and due only to the positive charge on P700.

The overall shape of the spectra in Figure 6 show some similarity to the difference spectrum obtained by Fujita et al. (1978) for the reduction of Chl *a* in vitro (except for a ~20-nm wavelength shift). This indicates that the primary electron acceptor may be a Chl *a* species, as has been suggested previously (Shuvalov et al., 1986; Nuijs et al., 1986a; Mathis et al., 1988; Kim et al., 1989).

Based on a difference extinction coefficient of 64 mM^{-1} cm^{-1} for the ($P700^+$ – P700) difference spectrum (see nondecaying spectrum in Figure 3) at 700 nm (Hiyama & Ke, 1972), a difference extinction coefficient of 82 mM^{-1} cm^{-1} is obtained from the (A_0^- – A_0) difference spectrum (21-ps spectrum in Figure 3) at 686 nm. This is slightly higher than that obtained in previous studies of PS I (Nuijs et al., 1986a) and *in vitro* studies of Chl *a* anions (Fujita et al., 1978).

Comparison with Previous Studies. As mentioned above, Shuvalov et al. (1986) interpreted a 32-ps kinetic phase as reoxidation of the primary electron acceptor in PS I. It is interesting to note that Shuvalov et al. (1986) used 710-nm excitation and claimed to directly excite P700. We do not believe we can directly excite P700 in our particles. However, using 590-nm excitation, we obtain a time constant for the decay of A_0^- similar to that reported by Shuvalov et al. (1986).

Wasielewski et al. (1987) monitored the absorption changes in open and closed PS I particles from spinach containing 30–40 Chl/P700. They report that P700* forms within 1.5 ps by energy transfer from antenna pigments and that A_0^- forms in about 13 ps. This conclusion is at variance with a number of fluorescence and transient absorption studies (Owens et al., 1987, 1988; Klug et al., 1989; Turconi et al., 1993; Holzwarth et al., 1993; see also preceding paper) as well as with the conclusions drawn in this paper.

Kim et al. (1989) used picosecond transient absorption measurements to investigate the kinetics in PS I particles from spinach containing 7–10 Chl/P700. They compared the absorption changes in particles in which A_1 had been either removed or reconstituted by adding artificial quinone acceptors. From this comparison they derived a spectrum for the primary electron acceptor which displayed a bleaching at 686 nm with a slight shoulder near 670 nm.

(A_0^- – A_0) difference spectra have been obtained from experiments performed under reducing conditions (Shuvalov et al., 1986; Nuijs et al., 1986a). The spectra display a bleaching at ~692 nm and a prominent shoulder near 670 nm, which we do not observe.

Secondary Electron Transfer in Other Species. Photosynthetic reaction centers are usually divided into two categories [see e.g., Blankenship (1992)]. High potential reaction centers, which contain quinones as the terminal electron acceptors, are found in PS II, purple bacteria, and green filamentous bacteria. In this type of reaction center, the electron transfer cofactors are bound to distinct polypeptides which do not bind accessory antenna pigments. Low potential reaction centers, which contain iron–sulfur clusters as the terminal electron acceptors, are found in PS I, heliobacteria, and green sulfur bacteria. In iron–sulfur-type reaction centers a relatively large number of accessory antenna pigments are also bound to the same polypeptides that bind the electron transfer cofactors.

The observed time constant for secondary electron transfer, in the PS I particles used here, is 21 ps. In PS II (Nuijs et al., 1986b; Schatz et al., 1987), purple bacteria, and green filamentous bacteria (Kirmaier & Holten, 1987) the observed time constant for secondary electron transfer is 200–500 ps (for the purposes of this discussion it is assumed that the bacteriopheophytin on the L branch constitutes the primary electron acceptor in purple bacteria). The large differences in rates of secondary electron transfer might be due to differences in the structure of the two types of reaction center. However, reaction centers from heliobacteria (Nitschke et al., 1990b; Liebl et al., 1993) and green sulfur bacteria (Büttner et al., 1988; Hauska, 1988) are thought to be similar in terms of both structure and function to PS I reaction centers. In heliobacteria (Nuijs et al., 1985b; Lin et al., 1994) and green sulfur bacteria (Nuijs et al., 1985a) the observed time constant for secondary electron transfer is about 600 ps. This even larger difference in the time constants for secondary electron transfer between heliobacteria or green sulfur bacteria and PS I is unresolved at present.

A phylloquinone molecule is thought to be the secondary electron acceptor in PS I (Golbeck & Bryant, 1991). The role of the quinones in heliobacteria (Trost et al., 1992; Kleinherenbrink et al., 1993) and green sulfur bacteria (Nitschke et al., 1990a) remains uncertain. If the quinones in heliobacteria and green sulfur bacteria do not function as electron carriers, it may be that the secondary electron acceptor in heliobacteria and green sulfur bacteria is an iron–sulfur cluster, analogous to F_X in PS I. The longer time constants for secondary electron transfer in heliobacteria and green sulfur bacteria, compared to PS I, might then be explained in terms of a greater distance between the primary and secondary electron acceptors. Rigorous explanations of the differences in rate constants that exist between the heliobacteria, green sulfur bacteria, and PS I will probably have to await high-resolution crystal structures for all three systems.

ACKNOWLEDGMENT

We thank Wim Vermaas and Cathy Madsen for providing the PS II deletion mutant and giving advice on growth procedures. This is publication 182 from the Arizona State University Center for the Study of Early Events in Photosynthesis.

REFERENCES

Blankenship, R. E. (1985) *Photosynth. Res. 6*, 317–333.
Blankenship, R. E. (1992) *Photosynth. Res. 33*, 91–111.
Büttner, M., Xie, D. L., Nelson, H., Pinther, W., Hauska, G., & Nelson, N. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 8135–8139.
Fenton, J. M., Pellin, M. J., Govindjee, & Kaufmann, K. J. (1979) *FEBS Lett. 100*, 1–4.
Fujita, I., Davis, M. S., & Fajer, J. D. (1978) *J. Am. Chem. Soc. 100*, 6280–6282.
Golbeck, G. H., & Bryant, D. A. (1991) *Curr. Top. Bioenerg. 16*, 83–175.
Hauska, G. (1988) *Trends Biochem. Sci. 13*, 415–416.
Hiyama, T., & Ke, B. (1972) *Biochim. Biophys. Acta 267*, 160–171.
Holzwarth, A. R. (1989) *Q. Rev. Biophys. 22*, 269–295.
Holzwarth, A. R., Schatz, G. H., Brock, H., & Bittersman E. (1993) *Biophys. J. 64*, 1813–1826.
Kim, D., Yoshihara, K., & Ikegami, I. (1989) *Plant Cell Physiol. 30*, 679–684.
Kirmaier, C., & Holten, D. (1987) *Photosynth. Res. 13*, 225–260.
Kleinherenbrink, F. A. M., Ikegami, I., Hiraishi, A., Otte, S. C. M., & Amesz, J. (1993) *Biochim. Biophys. Acta 1142*, 69–73.

Klug, D. R., Giorgi, L. B., Crystal, B., Barber, J., & Porter, G. (1989) *Photosynth. Res. 22*, 277–284.
Liebl, U., Mockensturm-Wilson, M., Trost, J. T., Brune, D. C., Blankenship, R. E. & Vermaas, W. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 7124–7128.
Lin., S, Chiou, H.-C., Kleinherenbrink, F. A. M., & Blankenship, R. E. (1994) *Biophys. J. 66*, 437–445.
Mathis, P., Ikegami, I., & Sétif, P. (1988) *Photosynth. Res. 16*, 203–210.
Nitschke, Feiler, U., & Rutherford, A. W. (1990a) *Biochemistry 29*, 3834–3842.
Nitschke, W., Sétif, P., Liebl, U., Feiler, U., & Rutherford, A. W. (1990b) *Biochemistry 29*, 11079–11088.
Nuijs, A. M., Vasmel, H., Joppe, H. L. P., Duysens, L. N. M., & Amesz, J. (1985a) *Biochim. Biophys. Acta 807*, 24–34.
Nuijs, A. M., van Dorssen, R. J., Duysens, L. N. M., & Amesz, J. (1985b) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6865–6868.
Nuijs, A. M., Shuvalov, V. A., van Gorkom, H. J., Plijter, J. J., & Duysens, L. N. M. (1986a) *Biochim. Biophys. Acta 850*, 310–318.
Nuijs, A. M., van Gorkom, H. J., Plijter, J. J., & Duysens, L. N. M. (1986b) *Biochim. Biophys. Acta 848*, 167–175.
Otte, C. M., Kleinherenbrink, F. A. M., & Amesz, J. (1993) *Biochim. Biophys. Acta 1143*, 84–90.
Owens, T. G., Webb, S. P., Mets, L., Alberte, R. S., & Fleming, G. R. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 1532–1536.
Owens, T. G., Webb, S. P., Alberte, R. S. Mets, L., & Fleming, G. R. (1988) *Biophys. J. 53*, 733–745.
Parrett, K. G., Mehari, T., Warren, P. G., & Golbeck, J. H. (1989) *Biochim. Biophys. Acta 973*, 324–332.
Schaffernicht, H., & Junge, W. (1981) *Photochem. Photobiol. 34*, 223–232.
Schaffernicht, H., & Junge, W. (1982) *Photochem. Photobiol. 36*, 111–115.
Schatz, G. H., Brock, H., & Holzwarth, A. R. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8414–8418.
Sétif, P., & Bottin, H. (1989) *Biochemistry 28*, 2689–2697.
Sétif, P., & Brettel, K. (1990) *Biochim. Biophys. Acta 1020*, 232–238.
Sétif, P., Mathis, P., & Vänngärd, T. (1984) *Biochim. Biophys. Acta 767*, 404–414.
Shuvalov, V. A., Nuijs, A. M., van Gorkom, H. J., Smit, H. W. J., & Duysens, L. N. M. (1986) *Biochim. Biophys. Acta 850*, 319–323.
Turconi, S., Schweitzer, G., & Holzwarth, A. R. (1993) *Photochem. Photobiol. 57*, 113–119.
Trost, J. T., & Blankenship, R. E. (1989) *Biochemistry 28*, 9898–9904.
Trost, J. T., Brune, D. C., & Blankenship, R. E. (1992) *Photosynth. Res. 32*, 11–22.
Vermaas, W. F. J., Williams, J. G. K., & Arntzen, C. J. (1987) *Z. Naturforsch 42c*, 762–768.
Vermaas, W. F. J., Ikeuchi, M., & Inoue, Y. (1988) *Photosynth. Res. 17*, 97–113.
Vermeglio, A. J., Breton, J., & Mathis P. (1976) *J. Supramol. Struct. 5*, 109–117.
Wasielewski, M. R., Fenton, J. M., & Govindjee (1987) *Photosynth. Res. 12*, 181–190.