Universality of Energy and Electron Transfer Processes in Photosystem I[†]

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ABSTRACT: Femtosecond transient absorption spectroscopy has been used to investigate the photoinduced energy and electron transfer processes in photosystem I (PS I) particles from cyanobacteria, green algae, and higher plants. At room temperature, the kinetics observed in all three species are very similar: Following 590 nm excitation, an equilibration process(es) with a 3.7–7.5 ps lifetime was observed, followed by a 19–24 ps process that is associated with trapping. In all three species long-wavelength pigments (pigments that absorb at longer wavelengths than the primary electron donor) were observed. The difference spectrum associated with reduction of the primary electron acceptor [($A_0^- - A_0$) difference spectrum] was obtained for all three species. The ($A_0^- - A_0$) difference spectra obtained from measurements using detergent-isolated PS I particles from spinach and *Chlamydomonas reinhardtii* are similar but clearly different from the ($A_0^- - A_0$) difference spectrum obtained from measurements using PS I cyanobacterial membrane fragments. In all three species the reduced primary electron acceptor (A_0^-) is reoxidized extremely rapidly, in about 20 ps. The difference spectrum associated with A_0 reduction appears to contain contributions from more than a single chlorophyll pigment.

Photosystem I (PS I)[1] is a polypigment protein complex that catalyzes the transfer of electrons across the thylakoid membrane from plastocyanin to ferredoxin. This light-driven electron transfer results in the formation of reducing equivalents used in carbon assimilation. The PS I reaction center core consists of the PsaA and B membrane-spanning polypeptides, which bind the electron transfer components (P700 through $F_{A,B}$), as well as about 100 accessory chlorophyll *a* (Chl *a*). PsaA and B form a protein heterodimer and are highly conserved in all species from which the *psaA* and *psaB* genes have been sequenced to date (Cantrell & Bryant, 1987; Mühlenhoff et al., 1993). This homology suggests that similarities exist in the organization of the PS I core antenna of the different species, which might then be expected to translate into similarities in the excited-state dynamics in the core antenna of the different species.

A number of authors have considered excitation migration and equilibration among the various spectral forms in the PS I core antenna. At present there is no consensus on either the mechanisms or the lifetimes associated with the energy migration process(es): Du et al. (1993) found a 300 fs component in measurements performed using PS I-50 particles (the PS I particles contain ~50 Chl/P700, hence the term PS I-50) from the green algae *Chlamydomonas reinhardtii*, which they assign to single-step energy transfer between neighboring antenna pigments. They also observed a single, isotropic 5 ps decay which they associated with spectral equilibration. In contrast, multiple depolarization lifetimes were observed in measurements performed using PS I-60 (Causgrove et al., 1988) and PS I-200 (Causgrove et al., 1989) particles from spinach.

We have recently shown that equilibration in the PS I core of the cyanobacterium *Synechocystis* sp. PCC 6803 is characterized by a time constant of 2.7–4.3 ps independent of the excitation wavelength. In PS I particles from the cyanobacterium *Synechococcus* sp., however, the measured decay times associated with equilibration are between 8–12 ps (Holzwarth et al., 1993; Turconi et al., 1993).

Previously, we obtained the ($A_0^- - A_0$) difference spectrum in measurements using PS I cyanobacterial membrane fragments from *Synechocystis* sp. PCC 6803 (Hastings et al., 1994b). The difference spectrum is significantly different from that obtained from measurements using PS I particles from spinach (Shuvalov et al., 1986; Mathis et al., 1988; Kumazaki et al., 1994a,b). Although the ($A_0^- - A_0$) difference spectra for spinach and *Synechocystis* sp. PCC 6803 are significantly different, it appears that A_0^- reoxidation (secondary electron transfer) takes about 20–30 ps in both species. Recently Hecks et al. (1994) performed photovoltage measurements using PS I cyanobacterial membrane fragments from *Synechocystis* sp. PCC 6803 and found a somewhat longer 50 ps time constant.

In summary, there does not appear to be a consensus concerning either the mechanisms or time constants that govern equilibration in the PS I core antenna of different species. In addition, there is little consensus concerning both the difference spectra associated with A_0 reduction and the time constant governing A_0^- reoxidation in the different

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[1] Abbreviations: (B)Chl *a*, (Bacterio)chlorophyll *a*; FWHM, full-width at half-maximum; PS I, photosystem I; LHC I, light-harvesting complex 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; TX-100, Triton X-100; β-DM, β-dodecyl maltoside.

species. Measurements have been performed on a wide variety of biological material (higher plants, green algae, and cyanobacteria, all with varying antenna size), and it is not clear whether the apparent discrepancies in the literature are somehow related to the type of particles used.

To address these issues, and to further our studies on energy and electron transfer processes in PS I, we have performed (sub)picosecond time-resolved absorption measurements on PS I particles from green algae (*C. reinhardtii*), higher plants (spinach PS I-60), and cyanobacteria (*Synechocystis* sp. PCC 6803). We have characterized extensively the energy and electron transfer processes in PS I particles from cyanobacteria (Hastings et al., 1994a,b, 1995), and here we provide a detailed comparison with data obtained using PS I particles from the other two species.

MATERIALS AND METHODS

Preparation of Cyanobacterial PS I Core Particles. Membrane fragments from the mutant psbDI/C/DII of *Synechocystis* sp. PCC 6803, which contains only the PS I reaction center, were prepared as described previously (Hastings et al., 1994a). Purified, detergent-isolated particles were prepared from the membranes as described in Hastings et al. (1995). We have recently modified and simplified the procedure. Briefly, membrane fragments are thawed and suspended for 3 h in buffer containing 20 mM Tris-HCl, pH 8, and 1% β-dodecyl maltoside (β-DM). The suspension was centrifuged at low speed to pellet the membrane debris which was discarded. The supernatant was loaded onto an 8%−32% sucrose gradient containing 0.03% β-DM and 10% glycerol and was then centrifuged at 108 000g for 16 h in a Beckman Ti-45 rotor. Two green bands were found. Only the lower green band was used in the experiments described here, which corresponds to the trimeric form of the PS I core as described previously (Hastings et al., 1995).

Preparation of PS I Core Particles from Green Algae. Detergent-isolated particles from the PS II less, Chl *b*-less mutant of *C. reinhardtii*, strain CC-2696, were prepared using a protocol similar to that of Takahashi et al. (1991). The room temperature absorption spectrum has a Q_y peak at 678 nm, with a full-width at half-maximum (FWHM) very similar to that of the detergent-isolated cyanobacterial preparations. By direct comparison of the millisecond kinetics obtained using the detergent-isolated green algal and cyanobacterial PS I particles and by assuming that there are about 80−98 chlorophyll *a* (Chl *a*)/P700 in the detergent-isolated PS I particles from cyanobacteria (Hastings et al., 1995), we find that the green algal PS I particles contain about 48−63 Chl *a*/P700. Very similar PS I particles from *C. reinhardtii*, which lack Chl *b*, have been used in a number of other studies (Owens et al., 1989; Werst et al., 1992; Du et al., 1993). The H523L mutant of the PsaB protein was prepared as described in Cui et al. (1995).

Preparation of PS I Complexes from Spinach. PS I particles with various antenna sizes can be isolated from spinach. In the measurements described here, we used only PS I particles from spinach that contained ~60 Chl *a*/P700. Firstly, PS I-180 particles were prepared by solubilizing spinach thylakoids in 0.8% (w/v) Triton X-100 (TX-100) as described by Hoshina and Itoh (1987). PS I-100 particles from spinach were prepared both by detergent isolation and heat treatment.

Detergent-isolated PS I-100 particles from spinach were prepared from PS I-180 according to Mullet et al. (1980) with minor modification as follows. After sucrose density gradient ultracentrifugation, the PS I-100 band was stirred with Bio-Beads SM-2 (1 g of Bio-Beads/10 mg of TX-100) for 3 h to remove the detergent (Holloway, 1973). The mixture was filtered through a nylon mesh (82 μm) and centrifuged at 40 000g for 30 min. The pellet was resuspended in distilled water and stored at −80 °C until use. The resultant complexes contained 100 Chl *a*/P700 and a full complement of electron acceptors (A_0 through $F_{A,B}$), as judged by millisecond absorption spectroscopy. We shall use the terms PS I-100, PS I core, and P700/$F_{A,B}$ complex interchangeably below to describe the PS I complexes that contain ~100 Chl *a*/P700.

Heat-treated PS I-100 particles from spinach were prepared by 60 °C heat treatment of PS I-180, as described by Hoshina et al. (1989, 1990) with minor modification as follows. PS I-180 was incubated at 60 °C for 5 min in a medium containing 50% (v/v) ethylene glycol, 0.04% (w/v) TX-100, 100 mM sorbitol, 10 mM NaCl, and 50 mM tricine-NaOH (pH 7.8) at 1 mg of Chl/mL. The incubation was diluted by a factor of 4 in distilled water and centrifuged at 35 000g for 20 min and then resuspended with 0.8% (w/v) TX-100 at 0.8 mg of Chl/mL. The suspension was incubated at 20 °C for 30 min with stirring and centrifuged at 52 000g for 30 min. The resulting supernatant was loaded onto a linear sucrose density gradient (0.2−1.5 M) containing 0.02% TX-100. The gradients were centrifuged for 15 h in a Hitachi RPS-27 rotor at 24 000 rpm (100 000g max). The lower green band was collected and dialyzed against 15 mM 2-mercaptoethanol for 3 h before centrifuging at 20 000g for 10 min. The precipitate was resuspended in a medium containing 10 mM 2-mercaptoethanol, 20% (v/v) glycerol, and 50 mM tricine-NaOH (pH 7.8) and stored at −80 °C until use. The ethylene glycol heat treatment causes the destruction of small subunits such as PsaC and the peripheral antenna complex LHC I, resulting in the loss of iron−sulfur clusters F_A and F_B and the loss of chlorophylls associated with peripheral antenna complexes (Hoshina et al., 1989, 1990). From millisecond transient absorption measurements we estimate that $F_{A,B}$ is completely removed and that F_X (and probably A_1) is active in about only 20% of the particles (see below).

In both types of isolation procedure described above, we obtained PS I-100 particles with different terminal electron acceptors. PS I-60 particles were prepared by solubilizing the above described PS I-100 particles in 0.8% (w/v) TX-100 containing 2 mM $MgCl_2$, 20 mM sodium ascorbate, 15 mM 2-mercaptoethanol, and 50 mM tricine-NaOH (pH 7.8) at 0.2 mg of Chl/mL. The suspension was loaded on a hydroxylapatite column equilibrated with 10 mM sodium phosphate buffer (pH 7.0). The gel was washed with 0.1% (w/v) TX-100 containing 20 mM sodium ascorbate, 2 mM $MgCl_2$, 15 mM 2-mercaptoethanol, and 50 mM Tris-HCl (pH 7.8) until the eluate became colorless. PS I-60/$F_{A,B}$ complexes were eluted from the column with 0.15 M sodium phosphate buffer (pH 7.0) containing 0.1% TX-100, 5 mM sodium ascorbate, and 15 mM 2-mercaptoethanol. TX-100 was removed using Bio-Beads SM-2 as described above. PS I-60/$F_{A,B}$ complexes were resuspended in 100 mM tricine-NaOH (pH 7.8) containing 100 mM sorbitol and 10 mM NaCl while PS I-60/F_X complexes were resuspended in 50

Table 1: Lifetimes Obtained from Global Analysis of Transient Absorption Data

cyanobacteria[a] *Synechocystis* sp. PCC 6803		green algae *C. reinhardtii* strain CC 2696		higher plant spinach			
				heat-treated		detergent-isolated	
		Chl-*a*/P700					
80–98		48–63		60		60	
		Time scale (ps)					
12	90	12	90[b]	12	90[b]	25	90
		Lifetimes (ps)					
0.2		0.23		0.13		0.16	
3.7	3.7	5.6		5.8		7.5	0.48
37	24	42	22.4	25	21	1000	19
	ND[c]		ND[c]		ND[c]		ND[c]

[a] See also Hastings et al. (1995). [b] Similar lifetimes (and spectra) were obtained from three exponential fits. [c] Nondecaying on the time scale considered here.

mM tricine-NaOH (pH 7.8) containing 10 mM 2-mercaptoethanol and 20% glycerol. Both complexes were stored at −80 °C until use.

In all transient absorption measurements described here the PS I particles were suspended in buffer containing 20 mM Tris-HCl, pH 8, and approximately 20 mM ascorbate and 10 μM PMS. For some experiments using the spinach PS I particles, samples were suspended under highly reducing conditions (as described by Hastings et al., 1994b) in order to reduce the terminal acceptors up to and including A_1. Under these conditions, upon strong illumination, only the primary radical pair state, $P700^+A_0^-$, can form. This state decays in 30−50 ns (Mathis et al., 1988; Sétif & Bottin, 1989; Sétif & Brettel, 1990; Kleinherenbrink et al., 1994).

The laser system is identical to that described previously (Hastings et al., 1994a,b, 1995). All experiments described here were performed at room temperature with the pump and probe beams set at the magic angle. The absorbance of the sample in the spinning cell was ~1−1.2 at the peak of the Q_y absorption band (~678 nm for all four PS I particles). Typically, 25% of the reaction centers were excited by each laser pulse, ensuring that excitation annihilation processes were of minimal significance.

Data were collected as a function of both time and wavelength. The noise on the kinetic traces at each wavelength is similar. At certain wavelengths (usually near 650 nm) isosbestic points could be found (i.e., no signal could be detected). In some cases (Figures 4 and 5) the kinetic trace at the isosbestic point wavelength was subtracted from all the other kinetic traces at other wavelengths. In such cases, backgrounds (measurements with no excitation beam) were also collected and subjected to similar manipulations to ensure that the procedure was appropriate for the data.

RESULTS

Energy Transfer and Trapping in PS I. Femtosecond transient absorption measurements were performed using PS I preparations from cyanobacteria, green algae, and higher plants. Excitation pulses centered at 590 nm were used for all measurements. Data were collected on 12 and 90 ps time scales and globally analyzed as described previously (Hastings et al., 1994a, 1995). Table 1 shows the results obtained from global analysis of data (ΔOD measured simultaneously at 70 wavelengths for 100 different time delays) collected on each time scale.

At the present level of signal to noise, the data are well described by two time constants of 3.7−7.5 and 19−24 ps (neglecting any sub-picosecond components that reflect mainly the instrumental response) in addition to a nondecaying component. Figure 1 shows the decay-associated spectra (DAS) of the 3.7−7.5 ps, 19−24 ps, and nondecaying components obtained from global analysis of data collected following 590 nm excitation of (A) detergent-isolated PS I particles from *Synechocystis* sp. PCC 6803, (B) detergent-isolated PS I particles from *C. reinhardtii*, (C) PS I-60 particles from spinach prepared using heat treatment, and (D) detergent-isolated PS I-60 particles from spinach. Data collected on different time scales have been normalized, as was described previously (Hastings et al., 1995). The DAS in Figure 1A have been presented previously (Hastings et al., 1995) but are reproduced here in a slightly different fashion, both for ease of comparison and for the sake of completeness.

The difference spectrum obtained ~0.2 ms after 532 nm laser flash excitation of detergent-isolated PS I particles from *C. reinhardtii* is shown in Figure 1B, while the steady-state light-minus-dark difference spectrum obtained using detergent-isolated PS I-60 particles from spinach is shown in Figure 1D. Both the 0.2 ms spectrum (Figure 1B) and the light-minus-dark difference spectrum (Figure 1D) are similar in shape to the corresponding nondecaying difference spectrum, in good agreement with results obtained previously from measurements performed using detergent-isolated PS I particles from cyanobacteria (Hastings et al., 1995).

We have also performed experiments on the PS I particles from *C. reinhardtii* using 680 nm excitation (data not shown). Following 680 nm excitation, a ~20 ps component and a nondecaying component are also found. The shape of the 20 ps and nondecaying spectra are independent of excitation wavelength (data not shown), as was also found previously for PS I particles from cyanobacteria (Hastings et al., 1995).

An identical set of transient absorption measurements was also performed using a mutant from *C. reinhardtii* in which a histidine residue at position 523 on PsaB is replaced by a leucine residue (Cui et al., 1995). It has been suggested that the histidine provides a ligand to P700; however, we found that the lifetimes and spectra for the mutant are almost identical to those shown in Figure 1B (data not shown).

The kinetics observed using the detergent-isolated PS I-60 particles from spinach can be described by two exponential components with lifetimes of 7.5 and 19 ps, in addition to a nondecaying component, similar to that found for the heat-treated PS I particles. The 19 and 21 ps spectra in Figures

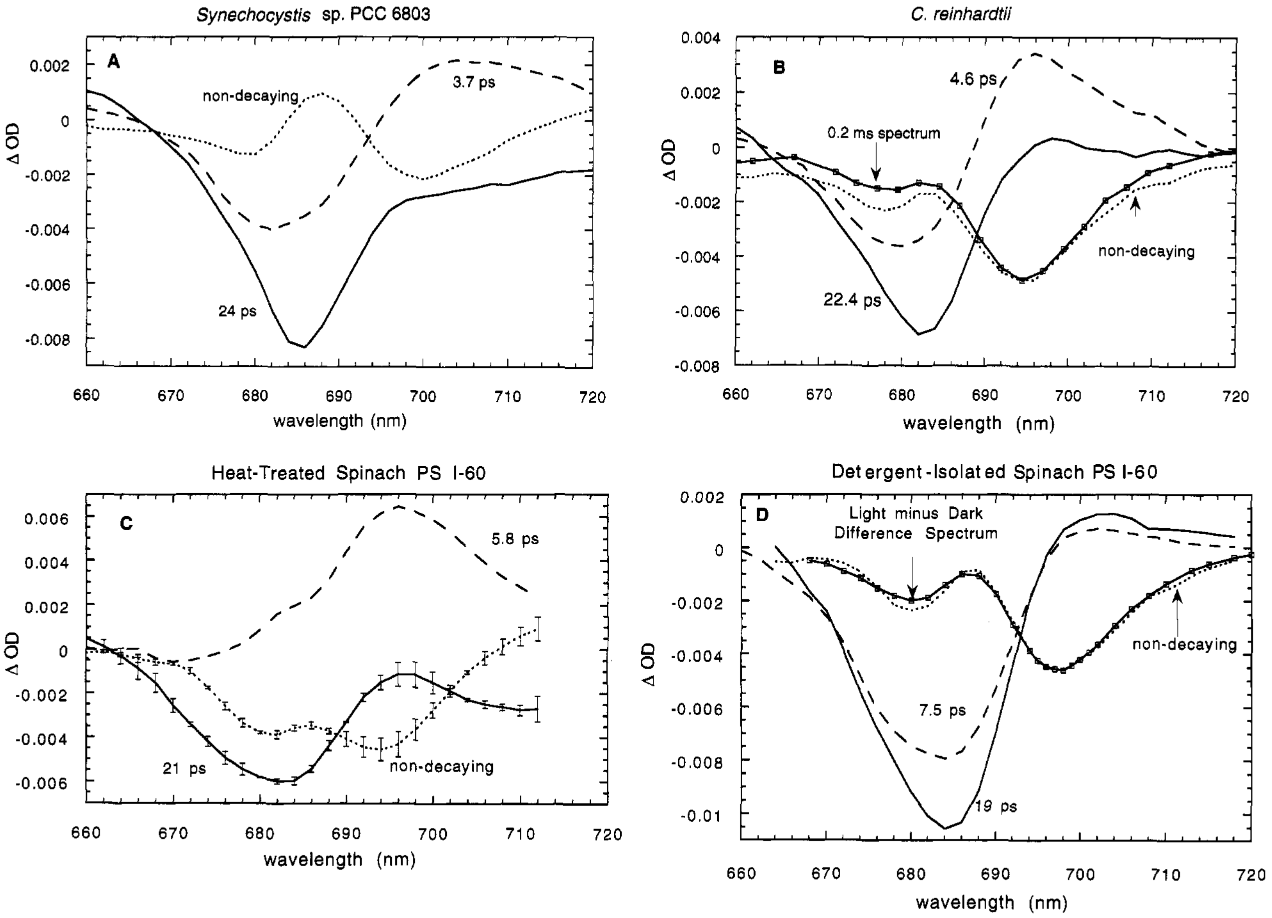

FIGURE 1: Decay associated spectra of the 3.7–7.5 ps (— —), 19–24 ps (—), and nondecaying (- - -) components, obtained from global analysis of transient absorption data collected on two time scales, using (A) detergent-isolated PS I particles from *Synechocystis* sp. PCC 6803, (B) detergent-isolated PS I particles from *C. reinhardtii*, (C) heat-treated PS I-60 particles from spinach, and (D) detergent-isolated PS I-60 particles from spinach. In B, the difference spectrum obtained ~0.2 ms after saturating laser flash excitation of the PS I particles from *C. reinhardtii* is also shown (—□—). In C, the error bars represent the standard error of two independent measurements. In D the steady-state light-minus-dark difference spectrum obtained using PS I-60 particles from spinach is shown (—□—).

1C,D are very similar; however, the 5.8 and 7.5 ps spectra are significantly different (see below for a discussion).

Figure 2A shows the kinetics at 680 and 696 nm obtained following 590 nm excitation of PS I particles from *C. reinhardtii.* Following an initial bleaching, a clear ~5 ps recovery is observed on the shorter-wavelength side with corresponding rise or grow-in of a bleaching on the longer-wavelength side, almost identical to that observed in cyanobacteria (Hastings et al., 1994a, 1995). In spinach the kinetics are not so clear-cut; Figure 2B shows the kinetics observed following 590 nm excitation of the heat-treated PS I particles. A clear ~5 ps rise is observed on the longer-wavelength side; however, the recovery on the short-wavelength side is not so clear. In the detergent-isolated PS I-60 particles from spinach (Figure 2C), very different behavior is observed. The 7.5 ps recovery of absorption changes, following an initial bleaching, is very clear on the shorter-wavelength side. However, the rise on the longer-wavelength side is less distinct. At present we are unable to explain the differences in the 5.8–7.5 ps spectra in Figures 1C,D. Apparently, the heat treatment causes some disruption in the core antenna (we show below that the detergent-isolated particles are relatively intact). In both types of spinach particle it is clear that a 5–7 ps component is necessary, in addition to a longer 20 ps component, to describe the data.

Reduction and Reoxidation of the Primary Electron Acceptor in PS I. It is well established that in PS I particles with a full complement of electron acceptors (A_0 through $F_{A,B}$) radical pair recombination between the oxidized primary electron donor ($P700^+$) and the reduced terminal electron acceptor ($F_{A,B}^-$) is characterized by a time constant ($t_{1/2}$) of 30–40 ms (Hiyama & Ke, 1972; Ke, 1973; Golbeck & Bryant, 1991). In PS I particles that have been stripped of the terminal acceptors $F_{A,B}$, radical pair recombination between $P700^+$ and the new reduced terminal electron acceptor, F_X^-, is characterized by a time constant of 1.2 ms (Parrett et al., 1990; Golbeck & Bryant, 1991). Therefore, it is possible to monitor the integrity of the electron acceptor chain, and to distinguish between the different terminal electron acceptors in PS I, simply by monitoring radical pair recombination using millisecond time-resolved absorption spectroscopy.

We have performed micro- and millisecond transient absorption measurements on all four PS I particles. Radical pair recombination was monitored at or near 700 nm. For the cyanobacterial and the green algal PS I particles, as well as the detergent-isolated PS I particles from spinach, only recombination kinetics with lifetimes of several tens of milliseconds were observed. This indicates that these PS I particles contain a full complement of electron acceptors (A_0 through $F_{A,B}$).

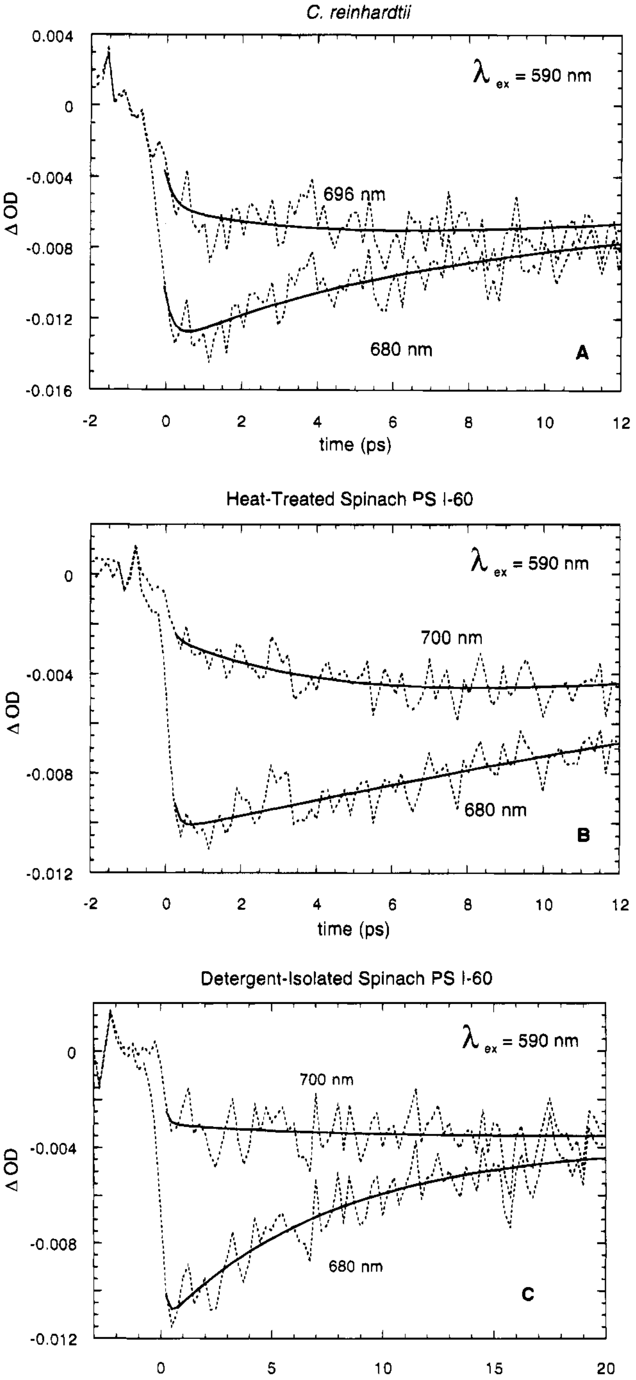

FIGURE 2: Kinetics of the absorption changes observed at two different wavelengths, following 590 excitation of (A) detergent-isolated PS I particles from *C. reinhardtii*, (B) heat-treated PS I-60 particles from spinach, and (C) detergent-isolated PS I-60 particles from spinach. The dotted lines are the experimental data, and the solid lines are the fitted functions. The lifetimes associated with the fitted functions are given in Table 1.

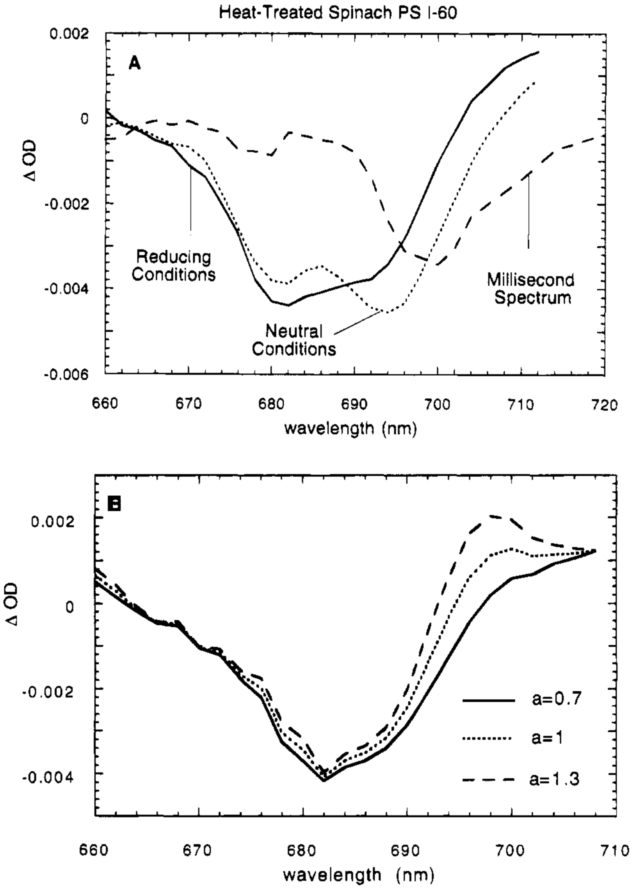

FIGURE 3: (A) Difference spectra obtained from a variety of different measurements using heat-treated PS I particles from spinach. Difference spectrum of the 1.2 ms phase (— —); nondecaying difference spectrum obtained from global analysis of data collected following 590 nm excitation of heat-treated particles [also shown in Figure 1C; (- - -)]; nondecaying difference spectrum obtained from global analysis of data collected following 590 nm excitation of heat-treated particles suspended under highly reducing conditions [(—); see Materials and Methods]. (B) Spectra obtained by subtraction of the 1.2 ms spectrum from the spectrum obtained under reducing conditions. The spectrum of the 1.2 ms phase was first multiplied by three different numerical factors, designated as "*a*", and then the resultant spectrum was subtracted.

Following flash excitation of the heat-treated PS I-60 particles from spinach, however, we observe no ~30 ms phase. We do observe a 1.2 ms phase, but its amplitude is considerably reduced relative to the amplitude of the 30 ms phase observed for the cyanobacterial or green algal PS I particles. It may be that the heat-treated spinach particles are inactive; however, the picosecond data in Figure 1C argue against this. An alternative explanation is that some/most of F_X is also stripped (or has somehow been inactivated) from the PS I core during the isolation procedure. Under these conditions, radical pair recombination between P700$^+$ and A_1^- occurs directly to the ground state in about 10 μs (Warren et al., 1993). We are unable to clearly resolve absorption changes in this time range. By comparing the amplitude of the bleaching due to P700 oxidation in the cyanobacterial or green algal PS I preparations (both of which are fully active) with that for heat-treated spinach PS I-60, in addition to comparison of the absorption spectra and taking into account the different chlorophyll contents of the different particles, we estimate that in the spinach PS I-60 particles prepared using heat treatment, at most 20% of the centers have functional F_X. Although only about 20% of the reaction centers display a 1.2 ms phase, we have managed to determine the spectrum of this phase (Figure 3A). We find a very similar spectrum from steady-state light-minus-dark measurements (data not shown). The spectrum of the 1.2 ms phase represents the (P700$^+$ − P700) difference spectrum, since F_X does not absorb in this wavelength region (Golbeck & Cornelius, 1986; Golbeck & Bryant, 1991). The spectrum of the 1.2 ms phase is normalized for ease of comparison and is clearly distinct from the nondecaying spectrum in Figure 1C, which is reproduced again in Figure 3A for closer comparison (spectrum labeled "Neutral Conditions").

Previously, we associated the nondecaying spectrum obtained from global analysis of picosecond data with P700 oxidation only (Hastings et al., 1994a,b, 1995). This was done by showing that the nondecaying spectrum is similar to the millisecond spectrum (see Figures 1B,D). The nondecaying spectrum in Figure 1C is not similar to the millisecond spectrum (Figure 3A) and is therefore due to P700 oxidation *and* absorption changes associated with another pigment that absorbs in the 660–720 nm spectral region.

In PS I, it is now well established that A_1 is a phylloquinone (Hauska, 1988; Biggins & Mathis; 1988, Golbeck & Bryant, 1991; Itoh & Iwaki, 1992; Iwaki & Itoh, 1994) and does not absorb in the 660–720 nm spectral region. It appears, therefore, that A_1 is probably also stripped or nonfunctional in the centers that lack F_X activity (see below), in agreement with Warren et al., (1993) who found that removal of F_X, in the presence of $<1\%$ TX-100, led to the irreversible destruction of A_1. Since both A_1 and F_X are not functional, the nondecaying spectrum in Figure 1C is most likely due to $P700^+A_0^-$, which is known to decay in about 30–50 ns (Mathis et al., 1988; Sétif & Bottin, 1989; Kleinherenbrink et al., 1994), in addition to a small additional bleaching ($\sim$20%) from P700 oxidation in particles with active terminal acceptors. A_0 reduction clearly gives rise to significant bleaching in the 680–690 nm spectral region, consistent with A_0 being a chlorophyll species (Hastings et al., 1994b). To obtain the (A_0^- − A_0) difference spectrum for the heat-treated PS I particles from spinach we have performed experiments under highly reducing conditions [see Hastings et al. (1994b) for details]. Under these conditions the nondecaying spectrum obtained from global analysis is due only to $P700^+A_0^-$ (Hastings et al., 1994b). This nondecaying spectrum obtained under reducing conditions is also shown in Figure 3A. It is possible to extract the (A_0^- − A_0) difference spectrum by subtracting the ($P700^+$ − P700) difference spectrum (1.2 ms phase) from the $P700^+A_0^-$ spectrum (spectrum obtained under reducing conditions); however, the appropriate normalization for the ($P700^+$ − P700) difference spectrum is unclear. Figure 3B shows the difference spectra obtained by subtracting the millisecond spectrum (and different multiples, designated by the factor "*a*") from the spectrum obtained under reducing conditions. Below 690 nm, the shape of the spectra is not strongly dependent on the multiplicative factor. We shall discuss the "a = 0.7" spectrum in detail below. The spectrum shows a peak at 682 nm (which does not depend on the normalization) and is positive below about 665 and above 695 nm, with a shoulder near 670 nm.

We note also that if we subtract the nondecaying spectrum from measurements performed under reducing conditions from the nondecaying spectrum from measurements under neutral conditions (assuming an appropriate normalization) we can obtain a spectrum that is identical to the millisecond spectrum (data not shown).

To further verify the validity of the (A_0^- − A_0) spectrum found for the heat-treated PS I particles, we have also determined the (A_0^- − A_0) spectrum using the detergent-isolated PS I-60 particles from spinach. The detergent-isolated particles contain a full complement of electron acceptors, as was verified by millisecond transient absorption measurements. Therefore, the (A_0^- − A_0) difference spectrum in these particles can be obtained using an intense preflash, as was described previously for cyanobacterial membrane fragments, which also contain a full complement of electron acceptors (Hastings et al., 1994b).

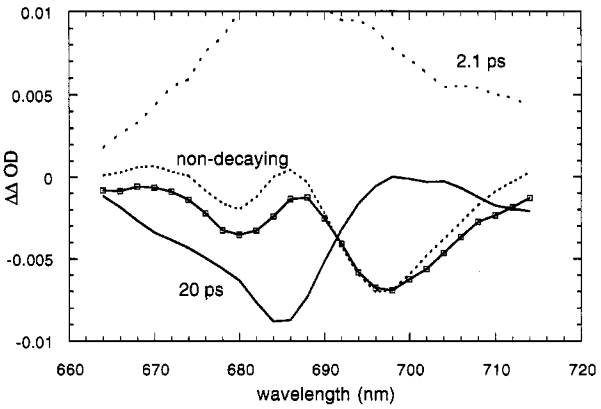

FIGURE 4: (A) Decay-associated spectra of the 2.1 ps (- - -), 20 ps (—), and nondecaying, (---) components obtained from global analysis of "neutral-minus-oxidized" data of the detergent-isolated PS I-60 particles from spinach. Also shown is the nondecaying difference spectrum from Figure 1D (—□—). The nondecaying spectra have been normalized at 698 nm. The spectra are similar in shape indicating that the same species is responsible for all three spectra.

We performed two sets of identical transient absorption measurements. One set of experiments was performed with the preflash exciting the sample $\sim$2 ns prior to the main, 590 nm excitation pulses. The other experiment was performed without the preflash. Subtracting the data collected with the preflash from the data collected without the preflash yields the radical pair kinetics (Hastings et al., 1994b; we shall refer to the data obtained following the above outlined subtraction as "neutral-minus-oxidized" data). Results from global analysis of the neutral-minus-oxidized data are presented in Figure 4. The data can be described by two exponential components with lifetimes of 2.1 and 20 ps, in addition to a nondecaying component. Almost identical results were obtained in the corresponding measurements on cyanobacterial PS I membrane fragments (Hastings et al., 1994b). Also shown in Figure 4 for comparison is the nondecaying difference spectrum obtained using low excitation intensities (reproduced from Figure 1D). The overall similarity in the nondecaying spectra in Figure 4 suggest that they represent the same state [namely, the ($P700^+$ − P700) difference spectrum]. This result is also identical to that obtained previously from corresponding measurements on cyanobacterial PS I membrane fragments (Hastings et al., 1994b).

The PS I particles from *C. reinhardtii* have a full complement of electron acceptors, and we have also performed "preflash" experiments on these particles. Figure 5 shows the DAS obtained from global analysis of the neutral-minus-oxidized data. Again the data can be described by two exponential components with lifetimes of 0.5 and 23 ps, in addition to a nondecaying component. Figure 5 also compares the nondecaying difference spectrum from Figure 1B with the nondecaying difference spectrum obtained from neutral-minus-oxidized data. Again there is some similarity. The noise in these preflash experiments is more pronounced since the OD in the rotating cuvette was about 0.5 (compared

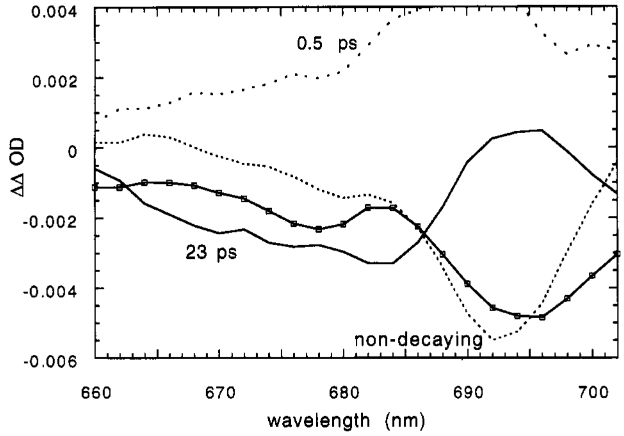

FIGURE 5: (A) Decay-associated spectra of the 0.5 ps (- - -), 23 ps (—); and nondecaying (---) components obtained from global analysis of "neutral-minus-oxidized" data (see text) of the detergent-isolated PS I particles from *C. reinhardtii*. Also shown is the nondecaying difference spectrum from Figure 1B (—□—). The spectra have been scaled for ease of comparison. The spectra are similar in shape, indicating that the same species is responsible for all three spectra.

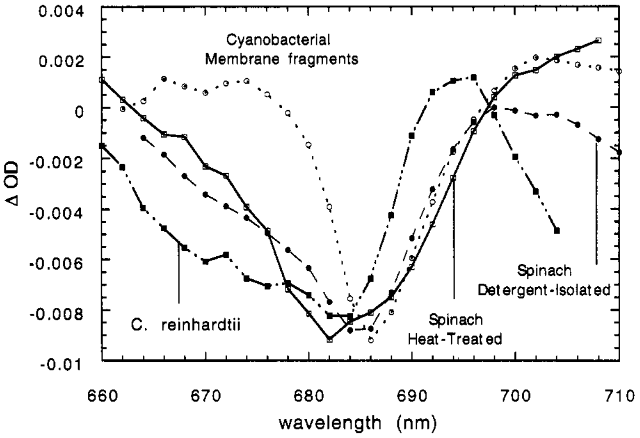

FIGURE 6: Comparison of the ($A_0^- - A_0$) difference spectra obtained using heat-treated PS I-60 particles from spinach (—□—) (see also the $a = 0.7$ spectrum shown in Figure 3B); detergent-isolated PS I-60 particles from spinach (—●—) (see also the 20 ps spectrum in Figure 4), and detergent-isolated PS I particles from *C. reinhardtii* (—■- -) (see also the 23 ps spectrum in Figure 5). The ($A_0^- - A_0$) difference spectrum obtained using PS I cyanobacterial membrane fragments is also shown (—O—) [see also Figure 6 in Hastings et al. (1994b)]. The spectra have been scaled so that the amplitudes are similar in the 680−690 nm spectral region.

to 1−1.2 in the other experiments). The similarity suggests that the nondecaying spectra in Figure 5 are both due to P700 oxidation.

As described previously (Hastings et al., 1994b), the 20−23 ps spectra in Figures 4 and 5 are the ($A_0^- - A_0$) difference spectra for the two detergent-isolated PS I particles. The ($A_0^- - A_0$) difference spectrum for the heat-treated PS I-60 particles from spinach (Figure 3B, $a = 0.7$) is compared in Figure 6 to the ($A_0^- - A_0$) difference spectra for the detergent-treated PS I-60 particles from spinach and the detergent-treated PS I particles from *C. reinhardtii*. The spectra for the two different spinach PS I particles are very similar, suggesting little disruption of the A_0 acceptor site due to the relatively harsh isolation protocols. For comparison we also show the ($A_0^- - A_0$) difference spectrum obtained previously from "preflash" experiments on cyanobacterial membrane fragments [taken from Figure 6 of Hastings et al. (1994b)].

DISCUSSION

Excited-State Dynamics in the PS I Core Antenna. The shape of the 3.7 ps spectrum in Figure 1A indicates that it is associated with energy transfer from shorter- to longer-wavelength-absorbing pigments. The broad positive lobe of the 3.7 ps spectrum extends well beyond 700 nm, suggesting the presence of pigments with absorption maxima near or below that of P700. The DAS associated with the 3.7 ps process is strongly excitation wavelength dependent and is due to the relaxation of the initially excited distribution of pigments to the Boltzmann distribution (Hastings et al., 1995). The 24 ps spectrum in Figure 1A is associated with trapping and contains contributions from absorption changes associated with both the decay of excited singlet states of antenna pigments and also the absorption changes associated with radical pair formation. The nondecaying spectrum in Figure 1A is associated with absorption changes due only to P700 oxidation (Hastings et al., 1995).

Following identical experimental and analysis procedures for detergent-isolated PS I particles from *C. reinhardtii* we find very similar results. The data can be described using an almost identical set of time constants (see Table 1). Furthermore, the shape of the 4.6 ps component again indicates that it is associated with an energy transfer process(es), from shorter- to longer-wavelength-absorbing pigments. The 4.6 ps spectrum has a positive peak at 696 nm and is positive well beyond 700 nm. The primary electron donor in *C. reinhardtii* has an absorption difference peak at 694 nm (nondecaying and 0.2 ms spectra in Figure 1B), indicating that long-wavelength pigments (pigments with absorption maxima near or below that of the primary electron donor) are also present in PS I particles from this species. The similarity in the shape of the 3.7 and 4.6 ps spectra in Figures 1A,B indicates that the same process is observed in both cyanobacterial and green algal PS I particles. Although the overall lifetimes governing the energy transfer process in the cyanobacterial and green algal PS I particles are similar, the spectra of the 3.7 and 4.6 ps components in Figures 1A,B indicate that the pigment distribution in the two PS I particles is slightly different. The positive lobe of the 3.7 ps spectrum in Figure 1A is broader and more red shifted compared to the positive lobe of the 4.6 ps spectrum in Figure 1B. This indicates that the content or concentration of longer-wavelength-absorbing pigments is greater in the cyanobacterial preparations.

Woolf et al. (1994) studied the same cyanobacterial PS I particles as used here and estimated that there are 8−11 pigments in the PS I core that absorb at 703−708 nm. In contrast, using similar PS I particles, Gobets et al. (1994) estimated that there are only one or two long-wavelength pigments in the PS I core, which absorbs at 708 nm at 4 K. The reason for the differences is not entirely clear but is likely to be related to the different methods of calculation. Werst et al. (1992) estimated that there are one or two long-wavelength pigments in PS I-50 particles from *C. reinhardtii*, but it is not clear at present how or if this can be related to our data. In our own studies on PS I particles from cyanobacteria we indicated that there were a relatively large number of pigments absorbing at 690−700 nm (Hastings et al., 1995). On the basis of a simple three-pool model it can be estimated that there are about 10 pigments that absorb at 690–700 nm. It may be that it is this group of pigments that is greater in number in cyanobacteria, compared to in green algae, and causes the observed differences in the spectra associated with equilibration in the two species.

In the heat-treated and detergent-isolated PS I particles from spinach we associate the 5.8 and 7.5 ps component with energy transfer processes partly because of the shape of the spectra and partly because we observe energy transfer components with similar lifetimes in the other two species. In recent time-resolved fluorescence measurements employing detergent-isolated PS I-65 particles from spinach a 6 ps lifetime was obtained (Turconi et al., 1994). The 6 ps lifetime was also assigned to energy transfer processes (Turconi et al., 1994). The 5.8 and 7.5 ps spectra in Figures 1C,D are very different. We do not fully understand what type of disruption could cause the different energy transfer kinetics in the core antenna of the different spinach PS I particles. The disruption probably occurs in the core antenna of the heat-treated particles (see below); however, this disruption probably does not extend to the early electron acceptors (see below).

For the detergent-isolated PS I particles from spinach, from measurements performed on a 90 ps time scale, we cannot resolve the 7.5 ps process (Table 1). The similarity in the shape of the 19 and 7.5 ps spectra (Figure 1D) and the fairly similar lifetimes make resolution of both components difficult with the present signal to noise ratio.

We think that the core antenna of the detergent-isolated PS I particles from spinach is relatively intact for a number of reasons. Firstly, we have performed steady-state fluorescence measurements at 77 K using the detergent-isolated PS I particles from spinach and found that the emission displays peaks at 680 and 725 nm. The ratio of the two peaks is ~1, suggesting a relatively intact preparation (Nechushtai et al., 1986; Turconi et al., 1994). Secondly, the millisecond spectra show that the samples are highly active. Thirdly our data agrees remarkably well with the recent time-resolved fluorescence measurements of Turconi et al. (1994), who used PS I-65 particles from spinach prepared by incubation in the relatively mild detergent octyl glucoside: For our detergent-isolated PS I-60 particles from spinach, we find two lifetimes of 7.5 and 19 ps, similar to Turconi et al. (1994), who found lifetimes of 6 and 18 ps. The 7.5 ps spectrum in Figure 1D has a large negative amplitude on the short-wavelength side with only a small positive lobe on the longer-wavelength side, also in remarkably good agreement with the shape of the 6 ps spectrum in Figure 4 of Turconi et al. (1994). These observations suggest that the detergent-isolated PS I-60 particles from spinach are relatively intact. The differences found for the 5.8 ps spectrum of the heat-treated particles may somehow be associated with disruption of the core antenna pigments in these particles.

In all four PS I particles studied, we found a component with lifetime of 19–24 ps. The shapes of the 19–24 ps spectra are similar for all four particles. All spectra display a broad negative peak at 682–686 nm (the exact peak position depends on the species). Near 700 nm and beyond, the 19–24 ps spectra can be positive or negative, depending on the pigment stoichiometry of the particle studied. For example, the 24 ps spectrum for the cyanobacterial PS I particles has relatively large negative amplitude near and beyond 700 nm, whereas the corresponding spectra for the green algal and higher plant PS I particles have significantly smaller amplitude near 700 nm. These observations reflect the fact that the cyanobacterial PS I particles have a higher concentration of pigments that absorb near and beyond 700 nm, relative to the other two species, in agreement with the energy transfer spectra. The positive amplitude at wavelengths near 700 nm in the 22.4 ps spectrum in Figure 1B suggests that the spectrum is dominated by the decay of excited-state absorption of shorter-wavelength-absorbing pigments and the rise or grow-in of a bleaching due to P700 oxidation. In line with our previous work (Hastings et al., 1994a, 1995), we associate the 19–24 ps spectra in Figure 1A–D with overall decay of excitations due to trapping at the reaction center. For the detergent-isolated PS I particles from *C. reinhardtii*, we have also performed measurements using 680 nm excitation and found that the spectrum (and lifetime) associated with trapping and radical pair formation are independent of excitation wavelength, as was also found previously in measurements using detergent-isolated PS I particles from cyanobacteria (Hastings et al., 1995), further confirming this assignment.

In Figure 1A,B,D, the nondecaying spectra represents the ($P700^+ - P700$) difference spectra, because the millisecond flash-induced (or steady-state light-minus-dark) difference spectra are very similar in shape (Hastings et al., 1994b, 1995). The shape of the nondecaying spectra in Figure 1 varies significantly, depending on the species. Schaffernicht and Junge (1981, 1982) showed that the main difference in the ($P700^+ - P700$) spectra from the different species was in the magnitude of the electrochromic response of antenna pigments near P700. We showed above that the pigment stoichiometry appears to be species dependent, so the electrochromic response of antenna pigments near P700 may also be species dependent and hence can explain the differences in the nondecaying spectra in Figure 1A,B,D.

Excluding the species dependent shifts in the spectra, there appears to be some degree of universality in the room temperature kinetics. In all three species studied, equilibration between the different spectral forms in the core antenna occurs in 3.7–7.5 ps. Trapping occurs from this equilibrated state in about 19–24 ps, resulting in the formation of a radical pair state. The nondecaying difference spectra in Figure 1 are associated with the formation of this radical pair state, which in the case of the detergent-isolated particles is due only to P700 oxidation. We show below that the nondecaying spectrum in Figure 1C is also due only to formation of radical pair states.

Previously, we showed that there was a large difference in the area under the absorption difference spectra associated with the spectrally equilibrated state and the radical pair state (Hastings et al., 1995; see also Figure 1A). One possible explanation is that some of the pigments in the core antenna are excitonically coupled.

In the measurements of Hastings et al., (1995) only trimeric PS I particles from cyanobacteria were used and efficient energy transfer between the individual units in the trimer could not be ruled out. Energy transfer between the trimeric units does not occur at 77 K (van der Lee et al., 1993; Gobets et al., 1994) but may occur at room temperature. PS I particles from *C. reinhardtii* or spinach have not been reported to form trimers; however, large differences in the area under the 19–24 ps spectra and nondecaying spectra are still observed (Figure 1). The 19–24 ps spectra contain contributions from absorption changes associated with P700 oxidation; therefore, the area under the 19–24 ps spectra plus the area under the nondecaying spectrum give a measure of the area under the absorption difference spectrum associated with the equilibrated distribution of excitations. It is this area that should be compared to the area under the ($P700^+ - P700$) spectra (nondecaying spectra in Figure 1). The ratio in the area under the absorption difference spectrum at equilibrium, compared to the area under the nondecaying difference spectrum, can be estimated from the data in Figure 1 and is about 4.6 in Figure 1A, 2.3 in Figure 1B, 2.1 in Figure 1C (the nondecaying difference spectrum in Figure 1C is due to at least two pigments: see below), and 3 in Figure 1D. It is clear that there is a relatively large difference in the area under the absorption difference spectra associated with the equilibrated excited state and the radical pair state. This difference in the area under the spectra appears to be a universal property of the pigments in the PS I core antenna from the different species, as may be expected in view of the high degree of amino acid sequence homology of PsaA and PsaB from many different species (Cantrell & Bryant, 1987; Mühlenhoff et al., 1993).

Recently, Kumazaki et al. (1994a,b) performed measurements using parallel polarized pump and probe pulses to investigate absorption changes in PS I particles from spinach containing 12–30 Chl/P700. In PS I-12 particles, using an excitation intensity of about 0.8 photons per particle, Kumazaki et al. (1994b) found that the area under the 500 fs absorption difference spectrum (equilibrated distribution of excitations) is at least 1.5 times greater than the area under the 230 ps spectrum (the 230 ps spectrum is due to at least two pigments, $P700^+$ and A_0^-). Similar observations were made for PS I-30 and PS I-16 particles from spinach (Kumazaki et al., 1994a).

Spectrum of the Primary Electron Acceptor in Photosystem I (A_0 and A_0'). The ($A_0^- - A_0$) difference spectra for detergent-isolated and heat-treated PS I-60 particles from spinach are shown in Figure 6. Both spectra display a broad bleaching at 682–686 nm and a shoulder near 670 nm. There appear to be differences on the longer-wavelength side; however, this is probably noise associated with the two types of measurement. The overall similarity suggests little disruption of the A_0 binding site following the two different isolation procedures, which does not appear to be the case for the antenna pigments. The ($A_0^- - A_0$) difference spectra for the two spinach PS I particles are remarkably similar in shape to that found by Kumazaki et al. (1994a,b) and Shuvalov et al. (1986) but are considerably blue shifted compared to the spectrum presented by Mathis et al., (1988).

The ($A_0^- - A_0$) difference spectra obtained from measurements using spinach PS I-60 are significantly different in shape from the corresponding spectra obtained using PS I cyanobacterial membrane fragments (Figure 6). The ~670 nm shoulder observed in the ($A_0^- - A_0$) difference spectra from spinach suggests the presence of an additional pigment in the vicinity of P700 and A_0. Below we shall refer to this hypothetical, additional pigment as A_0'. The presence of an additional pigment near P700 and A_0 is a useful idea because it provides a coherent explanation to some apparently disparate observations: Firstly, the presence of an additional pigment near P700 and A_0 has been suggested on the basis of the recently reported PS I crystal structure (Krauss et al., 1993). However, it should be pointed out that it is not yet possible to assign the kinetically and spectroscopically identified pigment A_0 unambiguously to a particular pigment in the structure. For this reason we have chosen the nomenclature A_0' for the putative additional pigment, which does not imply a particular order of acceptors. Secondly, the shoulder at 670 nm in the spinach ($A_0^- - A_0$) difference spectra can be explained. The shoulder may arise if A_0' acts as a real electron acceptor or if there is an electrochromic shift of the A_0' absorption band. Thirdly, in heliobacterial reaction centers (heliobacteria also contain an iron–sulfur type reaction center and are widely considered as a bacterial analog to PS I; Liebl et al., 1993) two bands at 670 and 780 nm are found to be associated with the primary electron acceptor (Lin et al., 1994). The kinetics at these two wavelengths are best explained by the presence of an additional BChl *g* molecule near P798 and A_0 which can function as an electron acceptor (A_0'). Electrons could transfer between A_0 and A_0', and an equilibrium distribution of radical pair states is established.

The ($A_0^- - A_0$) difference spectrum obtained using cyanobacterial membranes displays no sideband (Figure 6); however, the peak difference extinction coefficient calculated previously from the difference spectrum was ~82 mM^{-1} cm^{-1} (Hastings et al., 1994b), significantly higher than that found for Chl *a* anions *in vitro* (Fujita et al., 1978). This may suggest that the ($A_0^- - A_0$) difference spectrum for cyanobacterial membranes (Figure 6) is also associated with more than one pigment. In the cyanobacterial case, however, both A_0 and A_0' may absorb near 686 nm, explaining why no sideband is observed in the cyanobacterial ($A_0^- - A_0$) difference spectrum. The shoulder at ~670 nm in the spinach ($A_0^- - A_0$) difference spectra may indicate that the absorption bands of A_0 and A_0' are less overlapped compared to the bands in the cyanobacterial case. In the spinach ($A_0^- - A_0$) difference spectra, one may expect a slightly smaller peak difference extinction coefficient compared to that found for cyanobacteria. Assuming a difference extinction coefficient of 64 mM^{-1} cm^{-1} at 696 nm for the nondecaying spectrum, a peak difference extinction coefficient of 77 mM^{-1} cm^{-1} can be calculated from the spinach ($A_0^- - A_0$) difference spectrum in Figure 4 (20 ps spectrum). This is not significantly smaller than that found from measurements using cyanobacteria. The above calculations, however, rely on the fact that the ($P700^+ - P700$) difference spectrum for both species has the same peak difference extinction coefficient (64 mM^{-1} cm^{-1}). This may not be a valid assumption (Sonoike & Katoh, 1989).

Notwithstanding the above, we suggest the presence of a pigment between P700 and A_0 in cyanobacteria also. The additional acceptor absorbs at 686 nm in cyanobacteria, compared to 670 nm in spinach or 780 nm in heliobacteria. In PS I particles from *C. reinhardtii*, A_0 and A_0' may be even more widely separated spectrally, explaining the even broader difference spectrum (Figure 6). This may also explain the smaller peak extinction coefficient of ~40 mM^{-1} cm^{-1}, which can be calculated from the 23 ps spectrum, assuming a difference extinction coefficient of 64 mM^{-1} cm^{-1} at 694 nm for the neutral-minus-oxidized nondecaying spectrum in Figure 5.

The above discussion indicates that an additional pigment, termed A_0', is required to describe the ($A_0^- - A_0$) difference spectra in PS I particles from all three species. In PS I it is unclear whether A_0' functions as a real intermediary electron acceptor, as was postulated in heliobacteria (Lin et al., 1994). In heliobacteria, secondary electron transfer occurs in 600 ps and an equilibrium of radical pair states may easily be established. In PS I, the secondary electron transfer step is extremely rapid (see below) and it is unclear whether an equilibrium distribution of radical pair states has time to be established. If an equilibrium distribution cannot be established, then the shoulders in the ($A_0^- - A_0$) difference spectra would need to be associated with electrochromic effects and A_0' need not act as a real electron transfer component. It may, however, be involved in promoting the secondary electron transfer step with such rapidity.

Reoxidation of A_0. From studies using PS I cyanobacterial membrane fragments, it was found that A_0^- is reoxidized in 21 ps (Hastings et al., 1994b). Using an identical experimental approach, we show that A_0^- in spinach is reoxidized in 20 ps (Figure 4), in good agreement with other studies (Shuvalov et al., 1986; Kumazaki et al., 1994a,b). Furthermore, we also show that A_0^- in *C. reinhardtii* is reoxidized in 23 ps (Figure 5). In all three PS I species studied, A_0^- is reoxidized at least as fast as it forms, giving a possible explanation as to why this acceptor has never been observed in transient absorption measurements in which low excitation intensities are used [Figure 1; see also Klug et al. (1989), Holzwarth et al. (1993), and Hastings et al. (1994a, 1995)]: The intermediate transient concentration of reduced acceptor never exceeds about 20%, making observation difficult.

Recently Hecks et al. (1994) also studied PS I cyanobacterial membrane fragments from *Synechocystis* sp. PCC 6803 using a time-resolved photovoltage technique. They found that trapping is characterized by a 22 ps time constant while secondary electron transfer is characterized by a 50 ps time constant, significantly longer than our observations as well as other previously published transient absorption experiments (Klug et al., 1989; Holzwarth et al., 1993; Hastings et al., 1994a, 1995; Kumazaki et al., 1994a,b). If the actual time constant for A_0^- reoxidation were 50 ps, as proposed by Hecks et al., then the maximum transient population of A_0^- would be about 52%, occurring ~32 ps after excitation, assuming a simple irreversible, sequential reaction scheme. All available optical evidence indicates that there is much less buildup of A_0^-, consistent with the faster time constant reported here. The reasons for the discrepancy between the optical and photovoltage results are not yet entirely clear, although the photovoltage measurements may possibly be sensitive to dielectric relaxation processes following electron transfer that are not observable optically.

In summary, the ($A_0^- - A_0$) difference spectrum for all PS I particles studied appear to require the presence of at least two pigments which we term A_0 and A_0'. A_0' absorbs at ~686 nm in cyanobacterial PS I particles, ~670 nm in PS I particles from spinach, and is even more blue shifted in PS I particles from green algae. The presence of an accessory pigment may help in the mediation of the secondary electron transfer in PS I with such extreme rapidity.

Finally, we found that the energy transfer kinetics and difference spectra of the H523L mutant of PsaB from *C. reinhardtii* are essentially identical to those observed in the wild type. While it appears likely that His 523 is a chlorophyll-binding residue, current evidence suggests that it is probably not a direct ligand to P700 (Cui et al., 1995). The results reported here also suggest that it is also probably not a residue that coordinates a pigment that plays a pivotal role in the energy transfer process or the earliest electron transfer steps. It may be that no single pigment is essential for the integrity of the energy transfer network, although it seems likely that the electron transfer pathway will be sensitive to significant changes to residues that ligate pigments, such as is found in reaction centers from purple photosynthetic bacteria (Woodbury & Allen, 1995).

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