Delayed Fluorescence from Fe–S Type Photosynthetic Reaction Centers at Low Redox Potential[†]

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ABSTRACT: Fluorescence kinetics were measured in membranes of a photosystem II-deletion mutant of the cyanobacterium *Synechocystis* sp. PCC 6803 containing photosystem I as the only reaction center and of the anoxygenic photosynthetic bacterium *Heliobacillus mobilis*. The measurements were performed under conditions where forward electron transfer to secondary acceptors was inhibited by the strong reductant sodium dithionite. Delayed fluorescence due to recombination of the primary radical pair $P^+A_0^-$ in both species was found to consist of several kinetic components. The longest-lived component had a lifetime of 35 ns in photosystem I and 18 ns in *H. mobilis*, respectively, which corresponds with the lifetime of the primary radical pair. Delayed fluorescence components with lifetimes of about 4 ns, 1 ns, and 200 ps were also observed in both species and were attributed to relaxations within the radical pair. A standard free energy difference of 0.18 eV was calculated for both species between the relaxed primary radical pair and the excited antenna at room temperature. A value of 0.25 eV was estimated for the free energy difference between the relaxed primary radical pair and the excited primary donor. The temperature dependence of the delayed fluorescence between 25 and 2 °C indicated that more than half of the free energy difference is due to enthalpy. Our measurements indicate an overall similarity between the primary electron transfer process in the Fe–S type (or low potential) reaction centers and the (bacterio)pheophytin–quinone type (or high potential) reaction centers found in purple photosynthetic bacteria and photosystem II.

Photosynthetic reaction centers are generally divided into two groups [see, e.g., Mathis (1990), Nitschke and Rutherford (1991), and Blankenship (1992)]. The so-called high-potential [or (bacterio)pheophytin-quinone type] reaction centers are found in photosystem (PS)[1] II, purple bacteria, and green filamentous bacteria and contain electron carriers with significantly higher redox potentials than those of the low-potential (or Fe–S type) reaction centers of PS I, green sulfur bacteria, and heliobacteria. In the high-potential reaction centers only quinones function as secondary electron acceptors, whereas low-potential reaction centers also contain iron–sulfur centers. Furthermore, the primary electron acceptor in high-potential reaction centers is a (B)Pheo, whereas in low-potential reaction centers it is thought to be Chl *a* (Golbeck & Bryant, 1991) or a related pigment (Van de Meent et al., 1991, 1992). Phylloquinone plays a role in secondary electron transport to the iron–sulfur centers in PS I (Golbeck & Bryant, 1991). The involvement of a quinone in the electron acceptor chain of green sulfur bacteria (Nitschke et al., 1990a) and heliobacteria (Trost et al., 1992; Kleinherenbrink et al., 1993) has not been unambiguously established.

Despite the differences, there appears to be a significant degree of similarity in the overall structure and function of reaction centers from different species, not only within the high- and low-potential groups but also between the two groups (Mathis, 1990; Nitschke & Rutherford, 1991; Hauska, 1988). Recently, it has been established that the reaction centers of heliobacteria (Liebl et al., 1993) and green sulfur bacteria (Büttner et al., 1992) consist of a peptide homodimer rather than a heterodimer, as in PS I and the high potential reaction centers. The consequences of this structure for the electron transfer properties of these reaction centers are not yet clear.

Many studies have been made of primary electron transfer in the reaction centers of purple bacteria, green filamentous bacteria, and PS II. Considerably less is known about the primary reaction center processes in PS I, green sulfur bacteria, and heliobacteria. This is mainly due to the fact that reaction center preparations of these species always contain a considerable amount of antenna (B)Chl, associated with the same polypeptides as the reaction center cofactors. This makes it difficult to obtain transient absorption or fluorescence kinetics of reaction center processes with high signal-to-noise ratios, and requires that the dynamics of excitation migration in the antenna have to be taken into account in the interpretation of the data. Observed antenna excitation lifetimes of 20–80 ps for various PS I preparations (Owens et al., 1987; Hodges & Moya, 1988; Mukerji & Sauer, 1989; Bittersmann & Vermaas, 1991; Trissl et al., 1993; Turconi et al., 1993; Holzwarth et al., 1993) have been interpreted in terms of a charge separation time of 0.5–3 ps in the reaction center (Owens et al., 1987; Hodges & Moya, 1988; Trissl et al., 1993). Recently, a charge separation time of 1.2 ps or less has been estimated for *Heliobacillus mobilis* (Lin et al., 1994), on the basis of observed antenna excitation lifetimes of 20–30 ps (Trost & Blankenship, 1989; Van Noort et al., 1992).

If secondary electron acceptors in the reaction center are either absent or reduced, forward electron transfer from the reduced primary acceptor is inhibited. The primary radical pair decays in ten to several tens of nanoseconds, partly to the ground state and partly to the triplet state of the primary electron donor (Hoff, 1986). In purple bacteria (Van Grondelle, 1978; Schenck et al., 1982; Woodbury & Parson,

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[1] Abbreviations: (B)Pheo, (bacterio)pheophytin; (B)Chl, (bacterio)-chlorophyll; Q, quinone; PS, photosystem; P, primary electron donor; A_0, primary electron acceptor; PMS, phenazine methosulfate.

1984, 1986; Ogrodnik, 1990; Taguchi et al., 1992) and PS II (Schatz et al., 1987; Booth et al., 1990, 1991; Van Mieghem et al., 1991) delayed fluorescence originating from the primary radical pair under these conditions has proven to be a useful probe for the kinetics and energetics of the primary electron transfer process. Multiple exponential components in the kinetics of delayed fluorescence were observed in both types of reaction centers and have been explained in terms of relaxations within the primary radical pair due to protein motion upon electron transfer (Woodbury & Parson, 1984, 1986; Ogrodnik, 1990; Taguchi et al., 1992; Booth et al., 1991). The presence of multiple conformational states (Booth et al., 1991) or the occurrence of electron transfer on the inactive pigment branch (Ogrodnik, 1990) have been proposed as alternative interpretations. The free energy difference between the primary radical pair and the excited primary electron donor may be calculated from the ratio of the amplitudes of prompt and delayed fluorescence. It showed a complicated temperature dependence in both purple bacteria (Woodbury & Parson, 1984, 1986) and PS II (Booth et al., 1990, 1991), with different relative contributions by enthalpy and entropy at different temperatures.

In this paper we present a time-resolved study of the wavelength and temperature dependence of delayed fluorescence from membranes of two species with Fe–S type reaction centers, a PSII-lacking mutant of the cyanobacterium *Synechocystis* sp. PCC 6803 and the anoxygenic bacterium *H. mobilis*, under conditions where primary radical pair recombination occurs. As in purple bacteria and PS II, the kinetics of delayed fluorescence in both species were found to contain several exponential components. The amplitudes of the delayed fluorescence between 25 and 2 °C were used to calculate free energy and enthalpy differences between the primary radical pair and the excited antenna.

MATERIALS AND METHODS

The PS II-deletion mutant of *Synechocystis* sp. PCC 6803 was grown, and membrane fragments, depleted of phycobilisomes, were prepared as described previously (Wittmershaus et al., 1992). *H. mobilis* was grown and membrane fragments were prepared as described by Trost and Blankenship (1989). All samples were diluted in Tris or glycine buffer to have an optical density <0.3 at the Q_y maximum during the measurement.

Steady-state fluorescence spectra were measured with a JASCO FP777 spectrofluorometer. Fluorescence kinetics were measured using a time-correlated single photon counting apparatus (Causgrove et al., 1990). Fluorescence lifetimes were obtained by fitting kinetic traces to the sum of four or five exponentials, convoluted with the response function of the instrument. For some experiments a global analysis was performed of several kinetic traces. Samples were contained in an optical cuvette with 0.15-cm pathlength (1 cm for the steady-state measurements) within a thermally insulated copper sample holder. Temperature was controlled using a refrigerated circulating bath and checked by means of a copper–constantan thermocouple inside the cuvette.

RESULTS

Detection of Delayed Fluorescence under Reducing Conditions. Steady-state fluorescence spectra of membrane fragments of *Synechocystis* sp. PCC 6803 and *H. mobilis* at moderate redox potential (in the presence of ascorbate) are
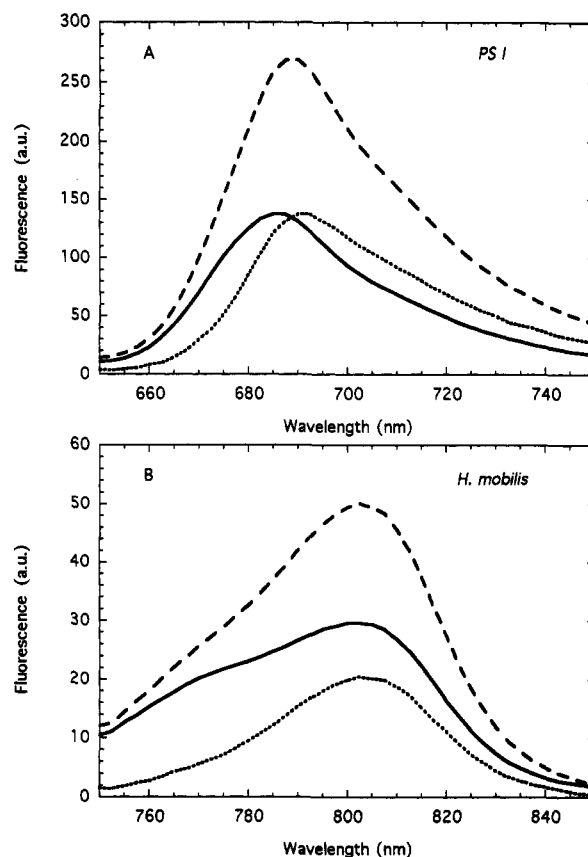

FIGURE 1: (A) Solid line: steady-state fluorescence spectra of membranes of *Synechocystis* sp. PCC 6803 upon excitation at 440 nm at 293 K, in the presence of 200 mM glycine (pH 10.5) and 10 mM ascorbate. Dashed line: same, after addition of dithionite to a final concentration of 50 mM. Dotted line: difference between spectra with and without dithionite. (B) Same as panel A, for membrane fragments of *H. mobilis*, upon excitation at 575 nm.

shown in Figure 1 (solid lines). For *Synechocystis* sp. PCC 6803 (Figure 1A), the spectrum shows a maximum at 686 nm and a shoulder on the long-wavelength side. It is similar to that presented by Wittmershaus et al. (1992) for a comparable preparation. Upon addition of dithionite at pH 10.5, the fluorescence steadily increased and eventually reached a maximum after a few minutes in the excitation beam of the fluorometer (Figure 1A, dashed line). The emission maximum of the spectrum under reducing conditions was found at 689 nm, i.e., slightly red-shifted compared to the spectrum in the absence of dithionite. The spectrum of *H. mobilis* membranes in the absence of dithionite (Figure 1B, solid line) showed a maximum at 803 nm, with a shoulder on the short wavelength side. Upon addition of dithionite, an increase in the long-wavelength emission was observed (dashed line), while the maximum emission remained at the same wavelength.

In order to investigate the origin of the fluorescence increase upon lowering the redox potential in both *Synechocystis* sp. PCC 6803 and *H. mobilis*, time-resolved fluorescence measurements were performed (Figure 2). In *Synechocystis* sp. PCC 6803, the fluorescence decay at 720 nm in the absence of dithionite (Figure 2A, middle curve) was dominated by a component with a lifetime of 25 ps (see Table 1). This decay time may be ascribed to antenna de-excitation due to trapping in the reaction center (Turconi et al., 1993, Holzwarth et al., 1993). It should be noted here that under the conditions of the experiment the lifetime of the oxidized primary donor was several tens of milliseconds (Golbeck & Bryant, 1991), indicating that all reaction centers were in the closed state
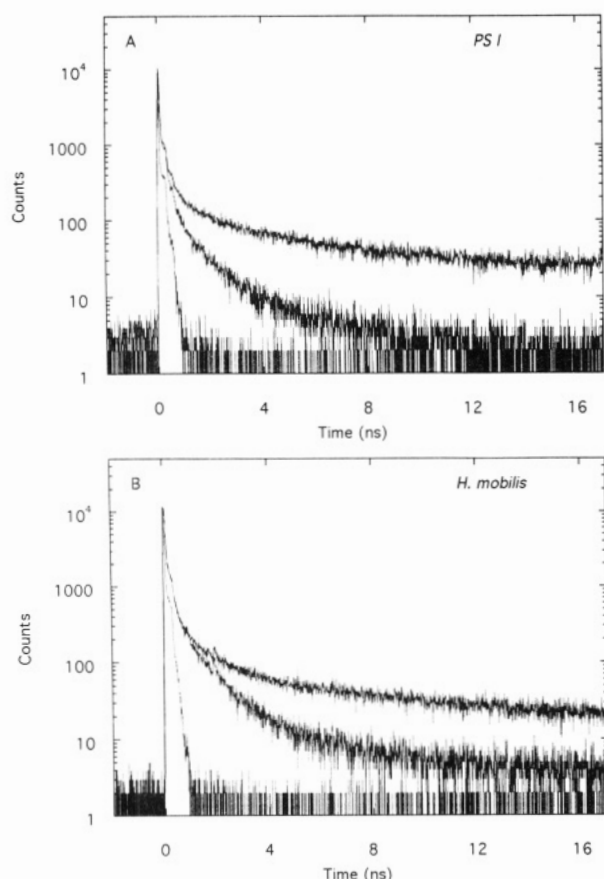
FIGURE 2: (A) Fluorescence kinetics at 720 nm of membranes of *Synechocystis* sp. PCC 6803 upon excitation at 590 nm at 293 K, in the presence of 200 mM glycine (pH 10.5) and 10 mM ascorbate (middle curve) and after addition of dithionite to a final concentration of 50 mM (top curve). The lower curve represents the instrument response function. (B) Same as panel A, for membrane fragments of *H. mobilis* at 820 nm upon excitation at 575 nm, in the presence of 200 mM Tris (pH 9.5), 10 mM ascorbate, and 100 μM PMS.

(P700 oxidized by the excitation beam). The lifetime of antenna excitations with open reaction centers at moderate redox potential was found to be 28 ps by means of transient absorption measurements (Hastings et al., 1994), confirming that the quenching rates by open and closed reaction centers are very similar (Owens et al., 1988; Turconi et al., 1993). Minor components (<2% of total amplitude; see Table 1) with lifetimes of 251 ps, 1 ns, and 4 ns were needed to obtain a good fit of the fluorescence decay. The lifetimes and spectra (not shown) of the latter two components indicated that they might be due to fluorescence from uncoupled Chl *a* or residual phycobilisomes. The 251-ps component might originate from antenna Chl *a* associated with a small fraction of inactive reaction centers.

Upon addition of dithionite, part of the fluorescence kinetics clearly became considerably slower (Figure 2A, upper curve). A fit with four components now yielded lifetimes of 23 ps, 299 ps, 2.0 ns, and 17.2 ns (Table 1). Illumination of PS I reaction centers at low redox potential has been reported to result in a double reduction of the phylloquinone acceptor A_1 and, subsequently, to charge recombination between P700$^+$ and A_0^- with a time constant of 35–50 ns (Sétif & Bottin, 1989; Sétif & Brettel, 1990). Under these conditions, there is sufficient time between laser flashes to keep the reaction centers in the open state (P700 reduced). The lifetime of the fast 23-ps component indicates that (double) reduction of secondary electron acceptors does not influence the primary charge separation rate in the PS I reaction center in a substantial way.

The slow 17.2-ns component was clearly absent before the addition of dithionite and may be due to recombination fluorescence from the primary radical pair. However, considerably longer lifetimes of 30–50 ns have been observed for the primary radical pair in various PS I preparations (Sétif & Bottin, 1989; Sétif & Brettel, 1990; Mathis et al., 1988; Biggins & Mathis, 1988). Furthermore, instead of the significant 1-ns component in the absence of dithionite (Table 1), a component with a time constant of 2.0 ns was found in the four-component analysis of the kinetics in the presence of dithionite. The 1-ns component was ascribed to some residual phycobilisome or uncoupled Chl *a* fluorescence (see above) and would not be expected to be influenced by the addition of dithionite. We therefore fitted the kinetics in the presence of dithionite with a fixed 1-ns lifetime. A better fit was obtained in a five-component analysis than in a four-component analysis under these conditions (Table 1). The 22-ps lifetime (due to trapping in the reaction center) was still observed in the five-component fit, with additional lifetimes of 218 ps, 5.7 ns, and 92 ns. It should be noted here that the 5.7-ns component has a much larger amplitude than the 4-ns component in the absence of dithionite (Table 1) and may therefore not be ascribed to uncoupled Chl or phycobilisomes. The time constant of 92 ns is not very well determined on the time scale of the experiment of Figure 2. Fits of fluorescence kinetics on a longer time scale (Figure 3A) yielded a longest lifetime of 35 ns. Fixing this lifetime in the analysis of the kinetics on the time scale of Figure 2 did not noticeably alter the quality of the fit but yielded a somewhat shorter time constant of 4.1 ns for the other slow component (Table 1).

Table 1: Fluorescence Decay Kinetics Obtained from the Measurements of Figure 2

	τ_1 (ps)	τ_2 (ps)	τ_3 (ns)	τ_4 (ns)	τ_5 (ns)	A_1[a] (%)	A_2[a] (%)	A_3[a] (%)	A_4[a] (%)	A_5[a] (%)	χ^2	Φ[b] (au)
Synechocystis[d]												
−dithionite	25	251	1.0	4.0		98.1	1.46	0.39	0.03		0.97	33
+dithionite	23	299	2.0	17.2		97.5	1.98	0.40	0.15		1.19	54
	21	173	1.0[c]	11.6		96.7	2.41	0.72	0.21		1.26	56
	22	218	1.0[c]	5.7	92	97.1	2.08	0.55	0.22	0.06	1.18	94
	22	236	1.0[c]	4.1	35[c]	96.4	2.03	0.45	0.23	0.09	1.18	72
H. mobilis[e]												
−dithionite	20	171	0.96	6.2		96.4	2.67	0.87	0.03		1.35	34
+dithionite	24	159	1.1	12.9		96.1	3.01	0.76	0.15		1.12	56
	23	126	0.96[c]	11.8		95.2	3.75	0.89	0.16		1.14	54
	24	139	0.96[c]	7.7	100	95.6	3.38	0.83	0.15	0.03	1.12	76
	24	143	0.96[c]	4.1	18[c]	95.8	3.30	0.79	0.10	0.10	1.12	57

[a] Amplitudes relative to total amplitude $\sum A_i$. [b] Relative fluorescence yield $\sum A_i \tau_i$. [c] Fixed lifetime. [d] Fluorescence excited at 590 nm and detected at 720 nm. [e] Fluorescence excited at 575 nm and detected at 820 nm.
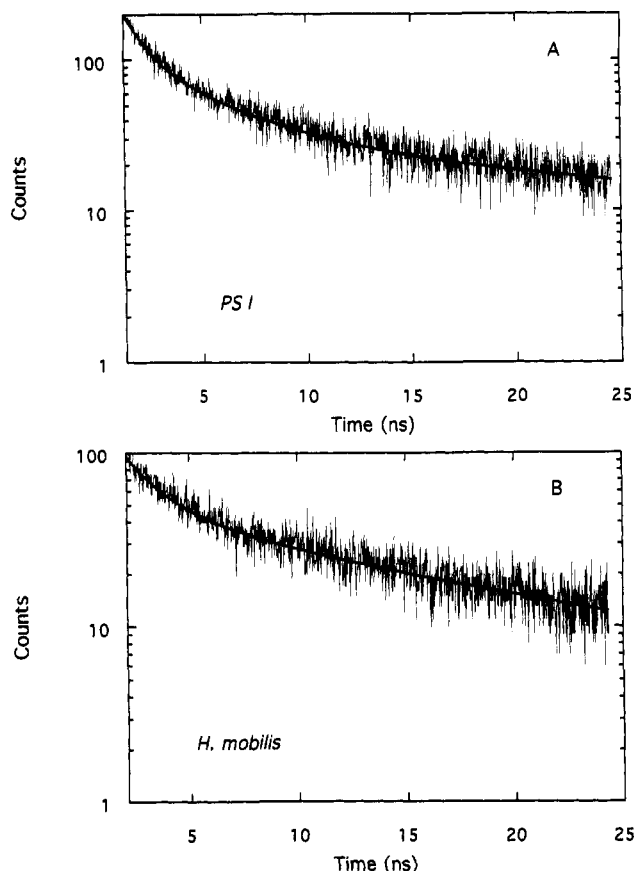
FIGURE 3: (A) Fluorescence kinetics at 720 nm of membranes of *Synechocystis* sp. PCC 6803 upon excitation at 590 nm at 293 K, in the presence of 200 mM glycine (pH 10.5), 10 mM ascorbate, 100 μM PMS, and 50 mM dithionite. The solid line shows the result of a fit with three exponential components between 1 and 25 ns after the laser flash. Time constants were 1 ns (71%), 4.5 ns (23%), and 35 ns (6%). No lifetimes were fixed in the analysis. (B) Same as panel A, for membrane fragments of *H. mobilis* at 820 nm upon excitation at 575 nm, in the presence of 200 mM Tris (pH 9.5), 10 mM ascorbate, 100 μM PMS, and 50 mM dithionite. Time constants for the fit between 2 and 25 ns were 1.3 ns (45%), 4.2 ns (19%), and 18 ns (17%).

In *H. mobilis* membranes, similar changes in the fluorescence kinetics were observed at 820 nm upon addition of dithionite. Figure 2B, middle curve, shows the fluorescence kinetics in the absence of dithionite, which are dominated by a 20-ps decay component with minor components of 171 ps, 0.96 ns, and 6.2 ns (Trost & Blankenship, 1989). The fastest component is due to trapping of antenna excitations in the reaction center (Trost & Blankenship, 1989; Van Noort et al., 1992), whereas the slower components may be ascribed to uncoupled pigments or pigment complexes. Upon addition of dithionite (Figure 2B, upper curve), the contribution by slower fluorescence components clearly increased, in analogy with the results for *Synechocystis* sp. PCC 6803. A four-component fit of these kinetics yielded time constants of 24 ps, 159 ps, 1.1 ns, and 12.9 ns (Table 1). Under the conditions of the experiment, forward electron transfer to secondary acceptors is inhibited (Kleinherenbrink et al., 1991), and primary radical recombination has been observed with a time constant of 17 ns (Kleinherenbrink et al., 1991). The 24-ps lifetime of the fastest component is similar to that observed at moderate redox potential, again indicating that reduction of the secondary acceptor(s) does not greatly affect the charge separation time in the reaction center.

The longest-lived component may be ascribed to delayed fluorescence but shows a decay time of 12.9 ns, shorter than

the reported lifetime of 17 ns for the primary radical pair (Kleinherenbrink et al., 1991). It thus appears that the kinetics may contain components that are faster than the actual lifetime of the primary radical pair, in analogy with the results with PS I from *Synechocystis* sp. PCC 6803 (see above). A five-component fit with one fixed lifetime of 0.96 ns (uncoupled BChl) yielded long-lived components of 7.7 and 100 ns, but from analysis of kinetics on a longer time scale (Figure 3B) a lifetime of 18 ns was obtained for the longest-lived component. This time constant was fixed in the analysis of the kinetics of Figure 2B, once again resulting in a somewhat shorter time constant of 4.1 ns for the second slow component (Table 1).

In summary, it appears that the increase of the steady-state fluorescence from *Synechocystis* sp. PCC 6803 and *H. mobilis* membranes upon addition of dithionite (Figure 1) may be explained by the appearance of small, but very slow components in the kinetics (compare the relative yields of fluorescence (Φ) in Table 1 under both conditions). We attribute these components to delayed fluorescence due to recombination of the primary radical pair $P700^+A_0^-$ in the reaction center. At least two exponential time constants are needed to accurately fit the delayed fluorescence kinetics on a nanosecond time scale. Multiexponential kinetics of delayed fluorescence have been reported previously for purple bacteria (Woodbury & Parson, 1984, 1986; Ogrodnik, 1990; Taguchi et al., 1992) and PS II (Booth et al., 1991) (see Discussion).

Time-Resolved Fluorescence Spectra. As mentioned in the previous section, the increase in the steady-state fluorescence of *Synechocystis* sp. PCC 6803 membranes upon addition of dithionite mainly occurred at longer wavelengths (Figure 1). This effect was further investigated by global analysis of the fluorescence kinetics under reducing conditions. In Figure 4A the decay associated spectra are shown of the kinetic components that were found in this analysis, with time constants of 17 ps, 197 ps, 1 ns (fixed, see above), 3.9 ns, and 35 ns (fixed, see above).

The components with longer lifetimes have considerably smaller amplitudes (note the multiplication factors in Figure 4) than the main 17-ps component. The 35-ns component may be ascribed solely to delayed fluorescence from the radical pair. This is confirmed by its spectrum, which is identical to that of the increase in the steady-state fluorescence upon addition of dithionite (Figure 1A, dotted line). The spectrum of the 3.9-ns component is slightly blue-shifted, indicating that it may be due partly to delayed fluorescence and partly to uncoupled Chl or residual phycobilisomes. The 1-ns and 197-ps components are blue-shifted even further, but it will be shown below from the temperature dependence that they might also contain a contribution by delayed fluorescence from the primary radical pair.

The 17-ps component is ascribed to de-excitation of the antenna due to trapping in the reaction center. Its lifetime is shorter than that observed in the single fit at 720 nm (see Table 1). Time-resolved fluorescence kinetics on a faster time scale (Hastings et al., 1994) indicated that there is actually an additional component with a time constant of 4–6 ps, which is not resolved on this time scale. The spectrum of this component showed a positive amplitude below 700 nm and a negative amplitude at longer wavelengths. This indicated that energy transfer occurs from short wavelength absorbing Chl *a* to long-wavelength absorbing Chl *a* [see also Turconi et al. (1993) and Holzwarth et al. (1993)]. In the experiments of Figures 2 and 3, the 4–6-ps equilibration could not be resolved due to limited time resolution, indicating that the spectrum
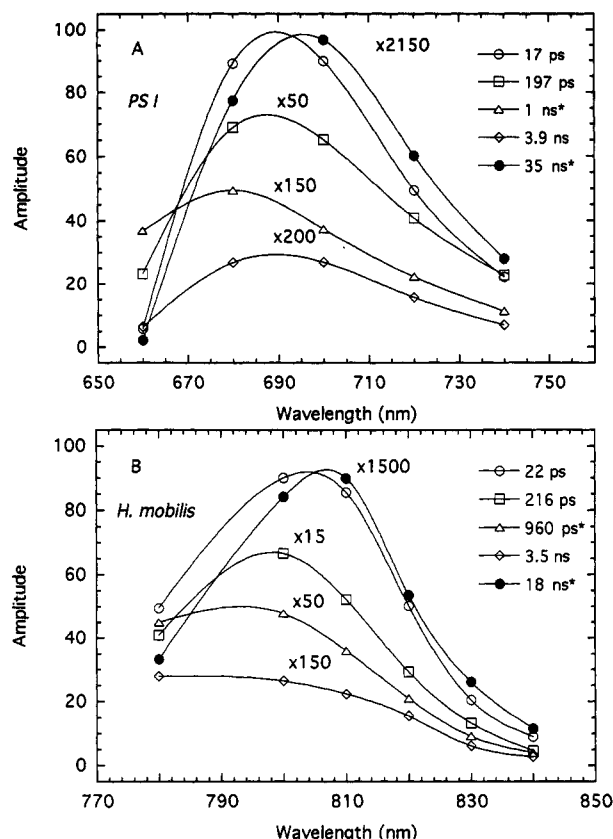
FIGURE 4: (A) Decay-associated spectra obtained in a five-component global analysis of fluorescence kinetics of membranes of *Synechocystis* sp. PCC 6803 under the conditions of Figure 2A in the presence of 100 μM PMS and 50 mM dithionite. (B) Same as panel A, for membrane fragments of *H. mobilis* under the conditions of Figure 2B in the presence of 100 μM PMS and 50 mM dithionite.

of the 17-ps component in Figure 4A contains contributions from both the 6-ps equilibration component and a trapping component of around 25 ps. This results in a decay which has a time constant shorter than the actual decay time due to trapping in the reaction center, with a slightly blue-shifted spectrum.

For *H. mobilis*, the decay associated spectra obtained from a global analysis of the fluorescence kinetics under reducing conditions are shown in Figure 4B. The 18-ns component may only be ascribed to delayed fluorescence from the primary radical pair and has the same spectrum as that of the steady-state fluorescence increase upon addition of dithionite (Figure 1B, dotted line). The 3.5-ns and 1-ns components are partly ascribed to uncoupled BChl g and partly to delayed fluorescence, similar to what was found for *Synechocystis* sp. PCC 6803 (see above). The spectrum of the 22-ps component, due to trapping of antenna excitations in the reaction center, is slightly blue-shifted compared to the spectrum of the 18-ns delayed fluorescence component. As was described in detail for *Synechocystis* sp. PCC 6803 (see above), this may again be explained by an unresolved energy transfer component from short to long wavelength BChl g. Recent time-resolved absorbance difference measurements indicate that such a spectral equilibration within the antenna of *H. mobilis* takes place within 1–2 ps at room temperature (Van Noort et al., 1992; Lin et al., 1994), i.e., too fast to be resolved in our fluorescence kinetics.

Temperature Dependence of Delayed Fluorescence. The temperature dependence was measured between 25 and 2 °C of the various fluorescence components at 720 and 820 nm in the presence of dithionite for PS I from *Synechocystis* sp.

PCC 6803 and *H. mobilis*, respectively. Single fits with four decay components of kinetic traces measured at different temperatures verified that only the amplitudes and not the time constants obtained for each of the decay components were dependent on temperature (data not shown). We therefore performed a global analysis on the kinetics measured at different temperatures, to obtain a temperature profile of the amplitude factors. Figure 5A shows the temperature dependence of the relative amplitudes of the four decay components that were obtained in this analysis for *Synechocystis* sp. PCC 6803 membranes. An obvious temperature dependence was observed for the 12.6-ns and, to a lesser extent, the 1.4-ns and 247-ps components. Global analysis using five kinetic components showed similar results, with the two slowest components showing the strongest temperature dependence (Figure 5C). A similar analysis of the temperature dependence of fluorescence kinetics under reducing conditions for membrane fragments of *H. mobilis* is shown in Figure 5B,D. Once again, the slower fluorescence components show a clear temperature dependence between 25 and 2 °C.

The temperature dependence of the longest-lived components in Figure 5 may be ascribed to the energy difference between the radical pair $P^+A_0^-$ and the excited primary donor state P^*A_0 in both *Synechocystis* sp. PCC 6803 and *H. mobilis* (see Discussion). A clear decrease with temperature, however, is also observed in the amplitudes of the components with a lifetime of around 1–2 ns in the four-component fits (Figure 4A,B), which might indicate a significant contribution by delayed fluorescence from the primary radical pair to these components. In the five-component analysis, the temperature dependence of the 0.96–1-ns components and, possibly, the components with a lifetime of 150–200 ps indicates that those might also at least partly be due to delayed fluorescence from the radical pair.

DISCUSSION

In this paper we have investigated the weak delayed fluorescence components with lifetimes on the nanosecond time scale that appear in the fluorescence kinetics of membranes of a PS II-deletion mutant of *Synechocystis* sp. PCC 6803 and of *H. mobilis* at low redox potential.

Origin of the Delayed Fluorescence. In the reaction center of PS I, direct chemical reduction can only be achieved for the terminal acceptors F_A and F_B that have redox potentials around -550 mV (Golbeck & Bryant, 1991). The preceding electron acceptor F_X has a redox potential around -705 mV (Golbeck & Bryant, 1991) and can only be completely reduced if low redox potentials are combined with illumination. It has been shown recently that illumination of PS I reaction centers at low redox potential eventually leads to the double reduction of A_1, the phylloquinone molecule that precedes F_X in the electron acceptor chain (Sétif & Bottin, 1989; Sétif & Brettel, 1990). The doubly reduced quinone is very stable and will prevent electron transfer beyond A_0, the primary acceptor. The radical pair $P700^+A_0^-$ shows an exponential decay time of 35–50 ns under these conditions (Sétif & Bottin, 1989; Sétif & Brettel, 1990), which is similar to the observed lifetime after extraction of A_1 (Mathis et al., 1988; Biggins & Mathis, 1988). In our experiments, fluorescence components with lifetimes of 35 ns and shorter were found in membranes of *Synechocystis* sp. PCC 6803 at low redox potential. These lifetimes lead us to conclude that radical pair recombination occurs under our experimental conditions, probably due to double reduction of A_1 as a result of the low redox potential and illumination by the excitation beam. It may be noted
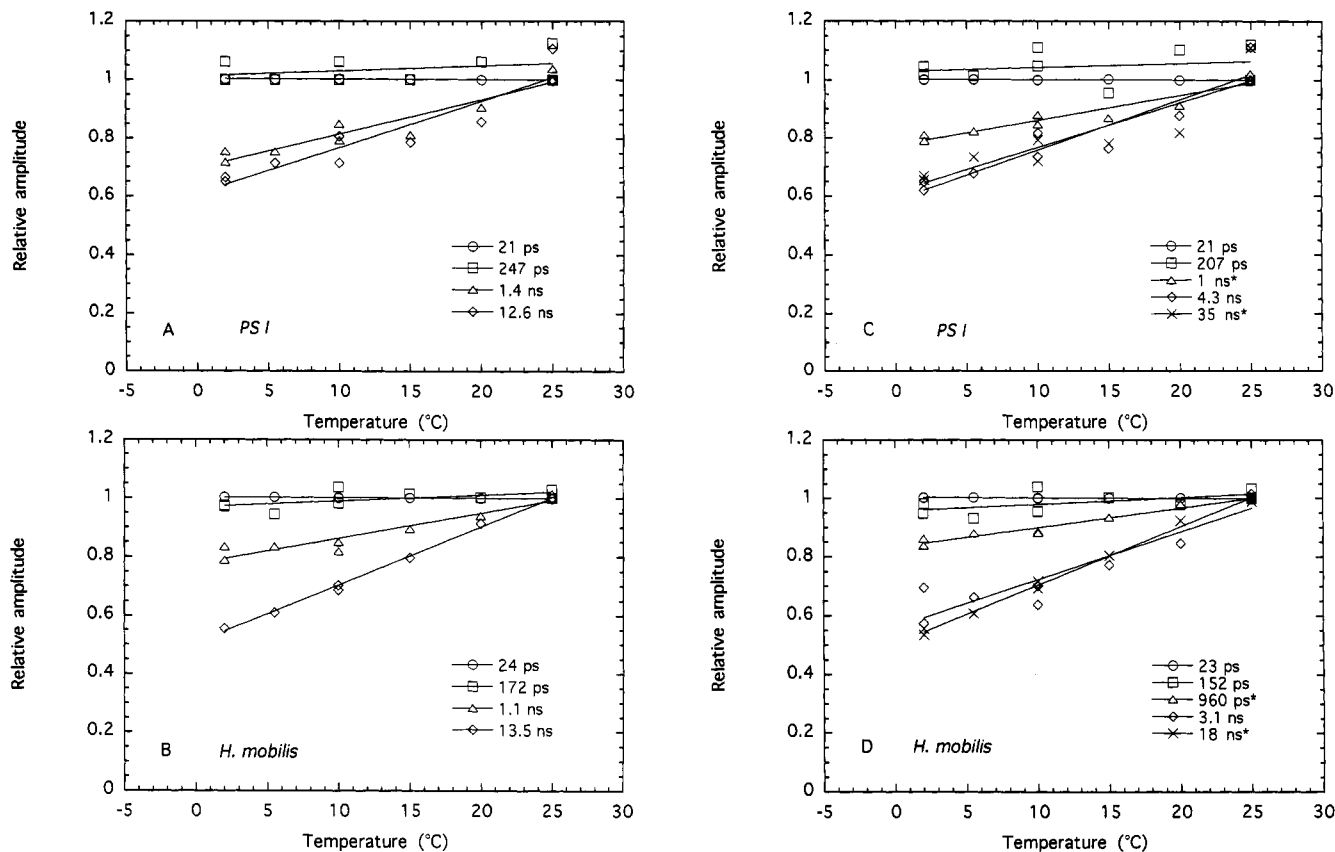
FIGURE 5: (A and B) Amplitudes (relative to the total amplitude) obtained from four-component global analysis of fluorescence kinetics measured under the conditions of Figure 4, panels A and B, respectively, between 25 and 2 °C. Kinetics were measured at 720 nm for *Synechocystis* sp. PCC 6803 and at 820 nm for *H. mobilis* during cooling of the sample and subsequently during warming. The relative amplitude of each component was normalized to that of the first measurement at 25 °C. (C and D) same as panels A and B, but with five exponential components. The markers (*) indicate that the corresponding lifetimes were fixed in the analysis (see text).

that it took several minutes after the addition of dithionite to reach the maximum level of delayed fluorescence (Figure 1), which emphasizes the effect of illumination. Furthermore, the redox-mediator PMS was present in most experiments, which should also facilitate double reduction of A_1 (Sétif & Brettel, 1990).

The electron acceptor chain in the reaction center of heliobacteria is thought to be broadly similar to that of PS I (Nitschke & Rutherford, 1991; Nitschke et al., 1990b; Trost et al., 1992; Liebl et al., 1993, also see, the introduction). The F_A and F_B iron–sulfur centers can, for the most part, be chemically reduced by dithionite at high pH (Nitschke et al., 1990b). The sequence similarity between the heliobacterial and PS I reaction center polypeptides in the F_X binding region provides strong evidence for the presence of a similar iron–sulfur center in heliobacteria (Liebl et al., 1993). Recently, spectral evidence for F_X has been obtained in *H. mobilis* (F. A. M. Kleinherenbrink, H. C. Chiou, R. Lobrutto, and R. E. Blankenship, unpublished results), but no information about its redox potential is available. Titration experiments of absorbance changes on a millisecond time scale have been interpreted in terms of the double reduction of a quinone acceptor similar to A_1 of PS I (Trost et al., 1992), but the involvement of a quinone in the electron transfer chain has also been challenged (Kleinherenbrink et al., 1993). Recombination of the primary radical pair with a time constant of 17 ns has been observed in the presence of dithionite at high pH (Kleinherenbrink et al., 1991), but the redox state of the secondary electron acceptors remained unclear under these conditions. However, the similarity between this time constant for primary radical pair recombination and the lifetime of the

longest-lived fluorescence component in *H. mobilis* membranes found by us (18 ns) strongly indicates that it is due to the same process.

We may conclude that the delayed fluorescence observed in membranes of *Synechocystis* sp. PCC 6803 and *H. mobilis* originates from recombination of the radical pair $P^+A_0^-$ in both species, as a result of inhibited electron transfer to secondary acceptors. The slowest decay times of 35 ns and 18 ns, respectively, correspond with the lifetimes of the radical pair. The cause of the observed faster components of delayed fluorescence will be discussed below.

Kinetics of Delayed Fluorescence. In previous studies the kinetics of delayed fluorescence from the radical pair in PS I were described with a single exponential decay time of 50 ns (Itoh & Iwaki, 1988) or even 100 ns (Sonneveld et al., 1981). The time resolution in both studies, however, was at least several nanoseconds, and faster decay components may have been missed. We used several kinetic components to accurately fit the measured kinetics of delayed fluorescence, both in *Synechocystis* sp. PCC 6803 and *H. mobilis* (Table 1). The longest-lived components that appeared upon addition of dithionite had a time constant 35 ns in *Synechocystis* sp. PCC 6803 and 18 ns in *H. mobilis*, respectively, i.e., very similar to the observed lifetimes of the primary radical pair under these conditions (see above). These components originate from the radical pair $P^+A_0^-$, which is in equilibrium with the excited primary donor P^* and therefore with the excited antenna pigments, from which the fluorescence is emitted. The energy difference between the radical pair and the excited antenna is the cause of the strong temperature

dependence of the amplitudes of these components shown in Figure 5 (see below).

The interpretation of the faster components is less straightforward and somewhat clouded by the presence of fluorescence components with similar time constants due to uncoupled (B)-Chl or phycobilisomes. It is clear, however, that the amplitude of a component with a lifetime of 3–7 ns in both species had much larger amplitude after addition of dithionite (Table 1), indicating that it is mainly due to delayed fluorescence from the primary radical pair. This conclusion is supported by its spectrum (Figure 4) and by the strong temperature dependence of its relative amplitude (Figure 5). A third component with a lifetime of about 1 ns did not increase significantly in either species upon addition of dithionite (Table 1) but may be ascribed at least partly to delayed fluorescence also because of its temperature dependence (Figure 5). A fourth component with a lifetime of 100–300 ps showed an increase upon addition of dithionite and possibly also a slight temperature dependence.

The multiple exponential components in the fluorescence kinetics could in principle be explained by different populations of reaction centers showing different recombination times of the radical pair. However, transient absorption measurements of primary radical pair recombination in several different PS I preparations show only a single exponential component with a lifetime of 35–50 ns (Sétif & Bottin, 1989; Sétif & Brettel, 1990; Mathis et al., 1988; Biggins & Mathis, 1988), and measurements with 35-ps time resolution on a spinach PS I preparation under reducing conditions showed essentially no decay of P700$^+$ or A_0^- within the first few nanoseconds (Nuijs et al., 1986). In membranes of *Heliobacterium chlorum* under reducing conditions, radical pair recombination with a single time constant of 17 ns was observed in a transient absorption measurement with a time resolution of several nanoseconds (Kleinherenbrink et al., 1991). Furthermore, recent measurements with picosecond time resolution showed virtually no decay of A_0^- within the first 2 ns (Lin et al., 1994). The above observations make it unlikely that the observed multiple components of delayed fluorescence are due to actual differences in the decay times of the primary radical pair, either in *Synechocystis* sp. PCC 6803 or *H. mobilis*.

A more likely cause for the multi-exponential kinetics of delayed fluorescence are time-dependent changes in the equilibrium between the radical pair $P^+A_0^-$ and the excited antenna. It may be noted here that the equilibrium constant between the primary radical pair and the excited antenna, taken from the longest-lived component in Table 1, is about 1000. If we take a typical value of 1 ns for the antenna excitation lifetime in the absence of photochemistry, the observed lifetimes of 35 and 18 ns for the primary radical pair in PS I and *H. mobilis*, respectively, would indicate that less than 4% of the primary radical pair decay actually occurs to the ground state through the P* pathway (see Figure 6). The majority of the radical pairs decay by recombination directly back to the ground state or through the triplet state. Therefore, the proposed time-dependent changes in the equilibrium between the primary radical pair and the excited antenna are not expected to significantly change the actual lifetime of the primary radical pair in these species.

Multiple exponential components in delayed fluorescence from the primary radical pair have been observed before in reaction centers of purple bacteria (Woodbury & Parson, 1984, 1986; Ogrodnik, 1990; Taguchi et al., 1992) and PS II (Booth et al., 1991). The slowest decay time obtained in purple bacteria was 10–15 ns, corresponding with the single exponential lifetime of the radical pair found in transient absorption
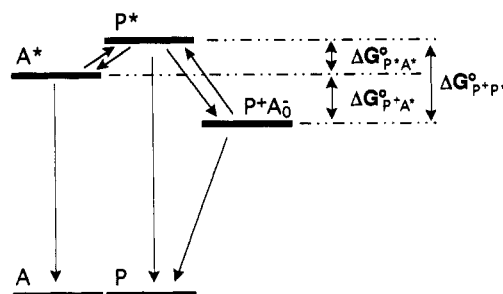

FIGURE 6: Schematic representation of the equilibrium between the primary radical pair $P^+A_0^-$, the excited primary donor P^*, and the excited antenna A^*. For simplicity, recombination of the primary radical pair to the ground state and to the triplet state of P has been depicted as a single process. See the text for the definition of the standard free energy differences ΔG°.

measurements (Schenck et al., 1982). Faster time constants of about 3 ns, 1 ns, and even 100 ps (J. M. Peloquin and N. W. Woodbury, personal communication) in the delayed fluorescence have therefore been interpreted by Woodbury and co-workers as reflecting relaxations within the radical pair, resulting in changes in the equilibrium with the excited primary donor. The slowest component of 10–15 ns would then be the true lifetime of the primary radical pair, originating from the relaxed state. The relaxations in the energy of the radical pair were proposed to be due to reorganization of the protein matrix upon charge separation, providing stabilization of the charge-separated state.

Booth et al. (1991) observed multiexponential kinetics in both delayed fluorescence and transient absorption measurements of PS II reaction centers. It may be noted that the excited state is much more favored in the equilibrium with the primary radical pair in PS II (Booth et al., 1990, 1991) than it is in purple bacteria (Woodbury & Parson, 1984, 1986), PS, I or heliobacteria (our results). This means that an actual multiexponential decay of the primary radical pair in PS II would not be incompatible with the concept of time-dependent relaxations.

The multiexponential kinetics of delayed fluorescence presented here for PS I from *Synechocystis* sp. PCC 6803 and for *H. mobilis* are generally similar to those obtained for purple bacteria and PS II. This indicates that there may be an overall similarity between the primary electron transfer process in (B)Pheo-Q type (high-potential) reaction centers and Fe–S type (low-potential) reaction centers. Since transient absorption data appear to yield only single exponential lifetimes for the primary radical pair in PS I and *H. mobilis*, the multiexponential kinetics for these species are explained more easily in terms of relaxations within the radical pair than in terms of actual differences in its lifetime. It has been proposed that the different kinetic components in the delayed fluorescence from the primary radical pair in purple bacteria (Woodbury & Parson, 1984, 1986) and PS II (Booth et al., 1991) might represent a continuous distribution rather than a discrete number of decays. The variability with time scale and fitting conditions of the obtained time constants (Table 1 and text) indicates that the same conclusion might apply to PS I and heliobacteria.

Free Energy Difference between the Radical Pair and the Excited State. Delayed fluorescence from the primary radical pair can be a useful probe of the energetics of the primary charge separation process. The ratio of the amplitudes of the prompt and delayed fluorescence components may be used to calculate the equilibrium between the charge separated and

the excited states, K_{eq}. In membrane preparations, prompt and delayed fluorescence are emitted mainly by antenna pigments, and K_{eq} will therefore represent the equilibrium between the radical pair and the excited antenna. The standard free energy difference, $\Delta G°_{P^+A^*}$ (see Figure 6), between the radical pair and the excited antenna may be calculated through

$$\Delta G°_{P^+A^*} = -k_B T \ln K_{eq} \qquad (1)$$

in which k_B is Boltzmann's constant and T is the temperature. From the amplitudes of the longest-lived delayed fluorescence component (35 and 18 ns) and the initial prompt component (22 and 24 ps) in Table 1, we can calculate a standard free energy difference of 0.18 eV for both *Synechocystis* sp. PCC 6803 and *H. mobilis* at room temperature. Note that this value applies to the relaxed radical pair state, whereas the other kinetic components of delayed fluorescence would originate from unrelaxed states. After correcting for a contribution by uncoupled pigments (i.e., the amplitude of the 4–6-ns components in Table 1 in the absence of dithionite), we may calculate from the sum of the amplitudes $A_4 + A_5$ that the 4.1-ns fluorescence components that we detected originates from an unrelaxed state with roughly 0.01–0.02 eV higher free energy than the relaxed state.

Because energy transfer between the primary electron donor and the antenna is fast compared to the lifetime of the primary radical pair, we may assume the excited primary donor P* to be in equilibrium with the excited antenna. The free energy difference between the excited primary donor and antenna, $\Delta G°_{P^*A^*}$ (Figure 6), is given by (Woodbury & Parson, 1986)

$$\Delta G°_{P^*A^*} = -k_B T \ln N + E_A - E_P \qquad (2)$$

in which T is the absolute temperature, N is the number of antenna pigments per primary donor, and E_A and E_P are the energies of the 0–0 transitions of the antenna and primary donor pigments, respectively.

The antennae of both PS I from *Synechocystis* sp. PCC 6803 and *H. mobilis* are known to be spectrally heterogeneous. Because energy transfer between individual pigments is extremely fast, this results in a concentration of excitations on the longer-wavelength antenna pigments within the first few picoseconds after a laser flash [Hastings et al., 1994 and Lin et al., 1994; see also Turconi et al. (1993), Holzwarth et al. (1993), and Van Noort et al. (1992)]. Subsequently, the equilibrated antenna excitations will decay to form the charge-separated state. The fast equilibration process within the antenna is not resolved in our measurements (see Results), and the fast, prompt fluorescence component will mainly represent the decay of equilibrated antenna excitations. Delayed fluorescence will also be emitted from the equilibrated antenna (note that the spectra of the prompt and delayed fluorescence components in Figure 4 are similar). Free energy differences calculated by means of eqs 1 and 2 will therefore involve the equilibrated antenna. Such an equilibrated system may be described by the hypothetical, but equivalent, situation in which an effective number of pigments per reaction center, N_{eff}, are isoenergetic with the longest wavelength antenna pigment (Trissl, 1993). We may therefore use this effective antenna size in eq 2.

Calculation of the value of N_{eff} requires knowledge about the spectral composition of the antenna (Trissl, 1993; Trissl et al., 1993). Information about the spectral composition of the PS I core antenna of *Synechocystis* sp. PCC 6803 was recently obained by means of fluorescence polarization measurements (Woolf et al., 1994). It was estimated that there are about six long wavelength chlorophylls which absorb at 703 nm at room temperature, with the bulk of antenna pigments (there are about 120 Chls per P700) absorbing at 680 nm. These numbers may be used to calculate an effective antenna size of 17 for PS I from *Synechocystis* sp. PCC 6803. A somewhat smaller value of N_{eff} = 9.5 has been proposed for PS I from peas (Trissl, 1993). An effective antenna size of 12 can be calculated for *H. mobilis*, on the basis of a spectral deconvolution of the antenna absorption spectrum, with the longest-wavelength pigments absorbing at 808 nm (Smit et al., 1989).

The difference between the values of E_A and E_P of the antenna and the primary electron donor, respectively, may in principle be estimated from the maxima of their respective absorption spectra and their Stokes shifts. Because an effective antenna size is used in the calculations (see above), the absorption maximum of the longest-wavelength absorbing antenna pigments should be used to calculate E_A (703 nm for PS I from *Synechocystis* sp. PCC 6803 and 808 nm for *H. mobilis*). We will assume Stokes shifts of 6 nm for antenna Chl *a* (Schatz et al., 1987), 10 nm for antenna BChl *g*, 12 nm for P700 (Trissl, 1993), and 20 nm for P798.

Using the above numbers in eq 2, we find free energy differences of 73 meV between P* and the (equilibrated) antenna in both *Synechocystis* sp. PCC 6803 and *H. mobilis*. We may thus estimate a standard free energy difference $\Delta G°_{P^+P^*}$ (Figure 6) of 0.25 eV between the primary radical pair P700⁺A₀⁻ and the excited primary donor P700* at room temperature. The same value is obtained for the standard free energy difference between the primary radical pair P798⁺A₀⁻ and the excited primary donor P798* in *H. mobilis*. This is in reasonable agreement with the value of 0.28 eV for PS I estimated by a different method (Vos & Van Gorkom, 1988). Similar values of around 0.25 eV have been reported for the free energy difference between the relaxed primary radical pair and the excited primary donor in purple bacteria at room temperature [see Ogrodnik (1990)]. Interestingly, a significantly smaller value of 0.11–0.14 eV was obtained for the PS II reaction center (Booth et al., 1990, 1991).

Contribution of Enthalpy to the Free Energy Difference. The standard free energy difference given by eq 1 consists of an enthalpy and an entropy term:

$$\Delta G° = \Delta H° + T\Delta S° \qquad (3)$$

in which $\Delta H°$ and $\Delta S°$ are the enthalpy and entropy contributions, respectively. From eqs 1 and 3 it may be derived that

$$\Delta H° = -k_B \, d \ln(K_{eq})/d \, (1/T) \qquad (4)$$

This means that the enthalpy contribution to the free energy difference can be determined from the temperature dependence of the ratio of prompt and delayed fluorescence.

Analysis of the longest-lived delayed fluorescence components from Figure 5 yielded values of 0.13 and 0.19 eV for the enthalpy differences between the relaxed radical pair and the antenna in *Synechocystis* sp. PCC 6803 and *H. mobilis*, respectively (Figure 7). It thus appears that, at least around room temperature, the contribution of enthalpy to the total free energy difference is quite considerable in these species. This is similar to what has been observed for purple bacteria at room temperature (Woodbury & Parson, 1984, 1986; Ogrodnik, 1990). At lower temperatures a complicated behavior of the enthalpic and entropic contributions was observed, both in purple bacteria (Woodbury & Parson, 1984,
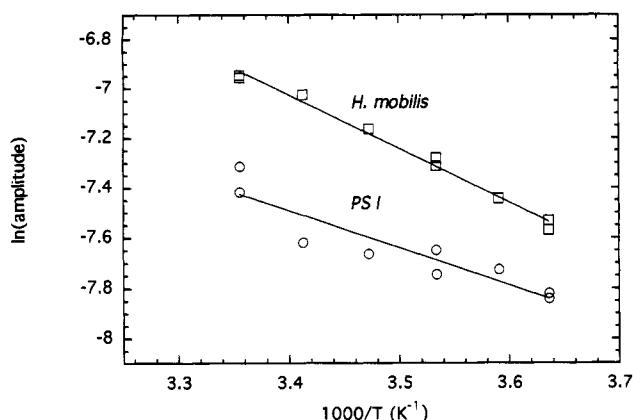
FIGURE 7: Determination of the enthalpy difference between the relaxed primary radical pair and the excited antenna, using eq 4. (O) Relative amplitude of the 35-ns component obtained for PS I from *Synechocystis* sp. PCC 6803 (Figure 5C). (□) Relative amplitude of the 18-ns component obtained for *H. mobilis* (Figure 5D).

1986) and in PS II reaction centers (Booth et al., 1990, 1991). In these species, the free energy difference between the primary radical pair and the excited state only showed a linear decrease with temperature below about 200 K, with a very small contribution by enthalpy. An enthalpy difference of only 25 meV was determined from the temperature dependence of delayed fluorescence between 270 and 100 K in a PS I preparation from spinach (Sonneveld et al., 1981). This finding indicates that the low temperature behavior of the free energy difference in PS I may also be similar to that of purple bacteria and PS II.

CONCLUSION

The kinetics of delayed fluorescence due to recombination of the primary radical pair in chemically reduced membranes from a PS II-deletion mutant of *Synechocystis* sp. PCC 6803 and *H. mobilis* showed multiple components. The slowest of these components had a time constant of 35 and 18 ns, respectively, which reflected the actual lifetime of the primary radical pair in these species. Further components with lifetimes of about 4 ns and possibly 1 ns and 200 ps were attributed to relaxations within the radical pair. Standard free energy differences of 0.18 eV between the relaxed primary radical pair and the equilibrated antenna were determined in both species at room temperature, from which free energy differences of 0.25 eV were estimated between the radical pair and the excited primary donor. Our results for PS I from *Synechocystis* sp. PCC 6803 and for *H. mobilis* are generally similar to results obtained for reaction centers from purple bacteria and PS II.

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