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Nanosecond fluorescence from chromatophores of *Rhodopseudomonas sphaeroides* and *Rhodospirillum rubrum*

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Single-photon counting techniques were used to measure the fluorescence decay from *Rhodopseudomonas sphaeroides* and *Rhodospirillum rubrum* chromatophores after excitation with a 25-ps, 600-nm laser pulse. Electron transfer was blocked beyond the initial radical-pair state (P^F) by chemical reduction of the quinone that serves as the next electron acceptor. Under these conditions, the fluorescence decays with multiphasic kinetics and at least three exponential decay components are required to describe the delayed fluorescence. Weak magnetic fields cause a small increase in the decay time of the longest component. The components of the delayed fluorescence are similar to those found previously with isolated reaction centers. We interpret the multi-exponential decay in terms of two small (0.01–0.02 eV) relaxations in the free energy of P^F, as suggested previously for reaction centers. From the initial amplitudes of the delayed fluorescence, it is possible to calculate the standard free-energy difference between the earliest resolved form of P^F and the excited singlet state of the antenna complexes in *R. rubrum* strains S1 and G9. The free-energy gap is found to be about 0.10 eV. It also is possible to calculate the standard free-energy difference between P^F and the excited singlet state of the reaction center bacteriochlorophyll dimer (P*). Values of 0.17 to 0.19 eV were found in both *R. rubrum* strains and also in *Rps. sphaeroides* strain 2.4.1. This free-energy gap agrees well with the standard free-energy difference between P^F and P* determined previously for reaction centers isolated from *Rps. sphaeroides* strain R26. The temperature dependence of the delayed fluorescence amplitudes between 180 K and 295 K is qualitatively different in isolated reaction centers and chromatophores. However, the temperature dependence of the calculated standard free-energy difference between P* and P^F is similar in reaction centers and chromatophores of *Rps. sphaeroides*. The different temperature dependence of the fluorescence amplitudes in reaction centers and chromatophores arises because the free-energy difference between P* and the excited antenna is dominated by the entropy change associated with delocalization of the excitation in the antenna. We conclude that the state P^F is similar in isolated reaction centers and in the intact photosynthetic membrane. Chromatophores from *Rps. sphaeroides* strain R-26 exhibit an anomalous fluorescence component that could reflect heterogeneity in their antenna.

Introduction

When chromatophores from the photosynthetic bacteria *Rhodopseudomonas sphaeroides* or *Rhodospirillum rubrum* absorb light, an antenna bacteriochlorophyll complex is raised to an excited state. Energy transfer then occurs within a large pool of complexes, until the excitation is trapped by a reaction center. The time rquired for trapping varies from strain to strain between about 50 and 300 ps [1–7]. Trapping occurs by electron

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Abbreviations: BChl, bacteriochlorophyll; RC, reaction center.

transfer from the excited singlet state of the reaction center (P*) to an initial electron acceptor (I), forming the radical pair state P^F (or P^+I^-) with a time constant of about 4 ps [8–16]. P is a bacteriochlorophyll (BChl) dimer; I involves an anionic radical of a bacteriopheophytin, which interacts with a nearby BChl [17–19]. An electron is subsequently transferred to a quinone (Q) in about 200 ps, forming the state P^+Q^-. It is possible to reduce Q chemically, inhibiting this transfer. Under these conditions P^F lives for about 10 ns, decaying by several alternate pathways [19–22]. Charge recombination during the lifetime of P^F can regenerate P*, which equilibrates with the antenna pigments and gives rise to a long-lived 'delayed' or variable fluorescence [19,22–29].

In isolated raction centers, which have no antenna, the delayed fluorescence is due to emission from P*. This delayed fluorescence has been characterized extensively with isolated *Rps. sphaeroides* reaction centers (strain R26) [22,23,28], and has been analyzed in terms of three exponential decay components of about 1, 4 and 10 ns. These components have been interpreted as reflecting relaxations in the free energy of P^F with time [23]. Previous work has indicated that the decay of the emission from chromatophores is also multi-exponential [1,29]. In the present study we have examined the fluorescence from chromatophores in more detail, with the aim of determining whether P^F undergoes relaxations similar to those seen with isolated reaction centers.

From the initial amplitude of the delayed fluorescence, it is possible to calculate the standard free-energy difference between P^F and P*. In isolated reaction centers, the value is between 0.12 and 0.17 eV, depending on the conditions of the sample [22,23]. In this report we determine the free energy difference in chromatophores from three different strains of photosynthetic bacteria, in the presence and absence of magnetic fields and as a function of temperature between 180 and 300 K. In addition, for two of these strains we determine the free-energy difference between A* (the excited singlet state of the antenna) and P^F in a similar manner.

The four strains of bacteria that we examined differ in the structures of their antenna systems. *R. rubrum* S1 and G9 both contain predominantly, if not exclusively, a single type of antenna complex (B875) that probably consists of two closely interacting molecules of BChl on a dimer of polypeptides [6,30,31]. Strain S1 is the wild-type strain; G9 is a carotenoidless mutant. *Rps. sphaeroides* 2.4.1 is a wild-type that has two types of antenna complexes, B875 and B800–850. The latter probably consists of three molecules of BChl on a polypeptide dimer [6,31,32]. Excitations probably move from the B800–850 complexes to the B875 antenna, and then to the reaction centers [33,34]. *Rps. sphaeroides* R-26.1, a carotenoidless mutant, has an altered antenna that appears to be less effective than that of strain 2.4.1 in funneling excitations to the reaction centers [34].

Methods

Chromatophores from *R. sphaeroides* strains 2.4.1 and R26 and *R. rubrum* strains S1 and G9 were prepared from late log phase cells as follows. Dense suspensions of cells in 10 mM Tris-HCl/100 mM NaCl/1 mM phenylmethylsulfonylfluoride (PMSF) (pH 8) were passed twice through a French pressure cell at 138 MPa in the presence of a small quantity of DNAase. The resulting solution was centrifuged for 20 min at about $9000 \times g$ to remove any unbroken cells. Chromatophores were pelleted by centrifuging 90 min at $200\,000 \times g$, resuspended in 10 mM Tris-HCl/100 mM NaCl/1 mM PMSF (pH 8), then brought to 30–40% glycerol and stored at 255 K. Chromatophores stored in this way did not change spectrally for at least several months.

Samples were prepared for single-photon counting measurements by dilution with 50 mM Tris-HCl (pH 8) and glycerol to give a final glycerol concentration of 75% (w/w). Measurements of the fluorescence emission intensity at 920 nm were made as described previously [23], with high concentrations of chromatophores ($\text{absorbance}_{600} \approx 0.5$) to obtain maximal count rates. Emission spectra were measured with much lower concentrations (a maximum absorbance of 0.2 in the long-wavelength band) to avoid self-absorption effects. No qualitative differences in the time-course of the delayed fluorescence were observed between these two concentrations, though a detailed kinetic

analysis was not possible at the lower concentration.

The concentration of reaction centers in the chromatophore samples was determined by measuring the 605-nm absorbance change due to P^+ formation after a 30-ns saturating flash at 860 nm. The difference between the extinction coefficients of P and P^+ at 605 nm was taken to be 20 $mM^{-1} \cdot cm^{-1}$ [35]. The total BChl concentration in the chromatophore samples was determined by methanol extraction using an extinction coefficient of 59.2 $mM^{-1} \cdot cm^{-1}$ at 770 nm [36]. From the reaction center concentration, the total BChl concentration and the number of BChls per antenna complex, one can calculate the number of antenna pigment complexes per reaction center, N_A. N_A was calculated only for *R. rubrum* strians S1 and G9; each antenna complex was assumed to contain two closely interacting BChl molecules.

The excitation light intensity was maintained at about 0.16 $\mu W \cdot cm^{-2}$ ($4 \cdot 10^{11}$ photons per s per cm^2) for samples at moderate potential (Q unreduced), unless otherwise specified. This intensity was calculated to give a 1% steady-state level of the state P^+Q^-, assuming a 1 s lifetime for this radical pair. After reduction of the quinone, the light intensity was increased about 10-fold until the count rate was approx. 4000 counts/s, 0.5% of the firing rate of the excitation laser. The laser intensity used for reduced samples can be higher than that for unreduced samples because P^F, the state formed under these conditions, decays very rapidly compared to P^+Q^- [19–22].

The single-photon counting apparatus and the methods used for measuring and analyzing the fluorescence decay kinetics and obtaining time-resolved fluorescence spectra have been described previously [23]. The dye laser used for excitation was tuned to 600 nm; the pulse duration was about 25 ps. The machine response time (measured as the full-width-at-half-maximum of the fluorescence signal from unreduced reaction centers) was about 0.8 ns. As in previous studies of reaction centers [22,23], fluorescence from an unreduced chromatophore sample was used to generate the excitation profile for deconvolution of the fluorescence from the same sample after reduction of Q. All fluorescence data are expressed relative to the fluorescence from the unreduced sample at room temperature. Thus, the initial amplitudes of the exponential decay components (B_i) are given in ns^{-1} and are scaled such that $B_i\tau_i$ (where τ_i is the decay-time constant) gives the total yield of fluorescence from component i, relative to the integrated fluorescence yield from the unreduced sample at 295 K. The dimensionless parameter α gives the yield of 'prompt' fluorescence from the reduced sample, relative to the total fluorescence yield from the unreduced sample. For temperatures below 295 K, all amplitude data were corrected for the change in fluorescence detection efficiency due to shifting of the emission spectrum. These corrections were typically about 30% at 180 K and less at higher temperatures.

Unreduced chromatophore samples were used to obtain the excitation profiles for deconvolution because we were intested in the decay kinetics of fluorescence due to back reactions that regenerate P* or A* from P^F. This decay is convoluted not only with the machine response time, but also with the time for equilibration of P^F and P* with the antenna. By using the signal from the unreduced sample for the excitation profile this equilibration time is automatically deconvoluted from the delayed fluorescence decay components. (The trapping time in *R. rubrum* strain S1 chromatophores apparently increases from about 60 ps to about 80 ps upon reduction of Q_A [1,2]. However, such an increase would not significantly affect the time-course of the 0.8-ns excitation function.) The quantum yield of fluorescence (Φ_f) from each unreduced chromatophore sample was determined by direct comparison to the yield from unreduced reaction centers ($4.0 \cdot 10^{-4}$) [8]. The yield was corrected to account for the fraction of the total emission spectrum that was detected.

In principle, delayed fluorescence due to the transient presence of the state P^F in the unreduced chromatophore samples might invalidate the use of the fluorescence from the unreduced sample as an excitation profile. In the analysis of reaction center fluorescence [23], correcting for this effect changed the calculated free-energy gap between P* and P^F by about 6 meV at 295 K, and less at lower temperatures. For chromatophores the effect would be smaller, since the ratio of the delayed to prompt fluorescence is less than the ratio

of delayed to prompt fluorescence from reaction centers. We therefore made no correction for this effect.

Absorption spectra were measured with a GCA-McPherson spectrophotometer equipped with a home-built cryostat.

Results and Discussion

Delayed fluorescence from chromatophores at room temperature

Table I presents measurements made on chromatophores from four strains of bacteria at 295 K. Four pairs of measurements (with and without a 600-g magnetic field) were made on chromatophores from *Rps. sphaeroides* strain 2.4.1; fewer measurements were made on the other three. R26 reaction center measurements reported previously [23] are also shown for comparison. The raw data obtained with chromatophores were qualitatively similar to those obtained with isolated reaction centers in both form and noise level, and therefore no time-course is presented here. As with reaction centers, a 'prompt' component (with a relative yield α) and at least three exponential decay components were needed in order to describe the fluorescence from reduced chromatophores adequately. The relative increase in the total fluorescence yield from chromatophores upon reduction of Q ($\alpha + \Sigma B_i \tau_i$) is lower that seen in reaction centers. In part, this is due to the yield of the prompt component of the fluorescence (α) from reduced chromatophores, which is generally lower than that of reduced reaction centers. The initial amplitudes of the delayed fluorescence (B_i) are generally either similar to or somewhat less than the initial amplitudes seen for reaction centers in aqueous buffer (no glycerol). An exception to this is the fastest component of the delayed fluorescence from *Rps. sphaeroides* R26 chromatophores. Not only does it have an anomalously large initial amplitude ($B_1 \approx 0.35$ ns^{-1}), but the decay time of this component is very short (0.4 ns) in comparison to the τ_1 of about 1 ns for aqueous reaction centers or the other chromatophore samples. More general agreement is seen in the two longer decay times (τ_2 and τ_3). τ_3 in all samples is about the same as the lifetime of the absorbance changes associated with the state P^F in either chromatophores [37,38] or reaction centers isolated from *Rps. sphaeroides* strain R26 [9,20,22,39].

In the presence of a weak magnetic field, the decay time of both the absorbance changes associated with P^F [22] and the longest component of the delayed fluorescence in reduced reaction centers [23] lengthens by about 10%. This is thought to reflect a decrease in the rate of conver-

TABLE I

INITIAL AMPLITUDES AND DECAY TIMES OF DELAYED FLUORESCENCE AT 295 K

Sample conditions as described in Methods. 600-G magnetic field H: − off, + on. Reduced χ^2 for fitting the photon-counting data to the multiexponential decay expression with deconvolution of the excitation function (average of N runs). N is the number of measurements at 295 K. Data for RCs have been taken from Ref. 23: reduced R26 reaction centers in an aqueous buffer (50 mM Tris/0.05% Triton X-100, pH 8.0)

	H	Total Fluorescence	α	Amplitudes (ns^{-1})			Decay times (ns)			χ^2	N
				B_1	B_2	B_3	τ_1	τ_2	τ_3		
R26 RCs	−	2.20±0.24	1.21±0.12	0.138±0.027	0.096±0.015	0.049±0.007	1.0±0.1	3.8±0.3	10.2±0.3	1.2	4
	+	2.28±0.29	1.22±0.13	0.142±0.022	0.095±0.013	0.046±0.012	1.0±0.1	4.1±0.4	11.6±0.7	1.1	4
2.4.1	−	1.55±0.24	0.97±0.16	0.072±0.018	0.038±0.005	0.029±0.004	1.1±0.1	4.6±0.3	11.2±0.2	1.3	4
Chromatophores	+	1.46±0.11	0.92±0.07	0.065±0.004	0.036±0.002	0.025±0.002	1.1±0.2	4.6±0.5	12.2±0.3	1.2	4
R26	−	1.53	1.15	0.353	0.029	0.014	0.4	3.2	10.8	1.2	1
Chromatophores	+	1.61	1.19	0.348	0.030	0.015	0.4	3.3	12.1	1.2	1
S1	−	1.53	0.75	0.108	0.070	0.046	1.0	3.6	9.1	1.3	2
Chromatophores	+	1.47	0.72	0.132	0.072	0.040	0.9	3.5	9.5	1.2	2
G9	−	1.35	0.79	0.080	0.059	0.029	1.0	3.6	9.3	1.3	1
Chromatophores	+	1.37	0.79	0.079	0.061	0.029	1.0	3.6	9.9	1.3	1

sion between the initially formed singlet radical pair, $^1P^F$, and the triplet form of this state $^3P^+$. The field causes a splitting of the energy levelsof $^3P^F$ so that only one of the three is isoenergetic with $^1P^F$. The only parameter in Table I that changes significantly in the presence of a weak magnetic field is the lifetime of the longest component of delayed fluorescence, τ_3. For *Rps. sphaeroides* 2.4.1 and R26 chromatophores, τ_3 increases by about 10% in the presence of a 600-gauss field. For both *R. rubrum* strains, the increase is about 5%. (The standard deviations given in Table I include day-to-day variations in the sample and the apparatus; variation in the parameters within a single session, such as pairs of measurements with and without a magnetic field, were smaller.) A small increase in the average lifetime of the delayed fluorescence, as well as an increase of 1–2% in the total fluorescence yield, has been observed previously with chromatophores in the presence of a weak magnetic field [29,40–42]. The increase in τ_3 observed in the present work would result in an increase in the total fluorescence yield of about 1–2% depending on the strain.

The multiphasic relaxation of the delayed fluorescence suggests that an increase in the free-energy gap between the excited state (A* or P*) and P^F occurs during the lifetime of the radical pair. The similarity of the results obtained with chromatophores and reaction centers argues, in agreement with earlier conclusions [23], that the relaxation involves a decrease in the free energy of P^F relative to the ground state, and not an increase in the energy of the fluorescing species; the relaxation occurs whether the primary source of the luminescence is P* or A*. In addition, the similarity of the results renders unlikely the possibility that contaminants or artificial heterogeneity resulting from preparation of the reaction centers are responsible for the multiexponential nature of the delayed fluorescence.

Measurements made on *R. rubrum* using a streak camera [1,2] have shown that reduction of the quinone causes a 20–30% increase in the lifetime of the bulk of the subnanosecond fluorescence (from about 60 ps in unreduced chromatophores to about 80 ps in reduced chromatophores). In a model involving fast equilibration of excitations between the antenna and the reaction centers, an effective trapping rate constant would be given by

$$k_{trap} = \frac{k_P K_{A^*P^*}}{K_{A^*P^*} + 1} \quad (1)$$

where $K_{A^*P^*}$ is the equilibrium constant for the raction $A^* \rightleftharpoons P^*$ and k_P is the rate constant for the conversion of P* to P^F. Recent absorbance measurements have shown that k_P decreases by about 30% upon reduction of Q_A in isolated reaction centers [15], which could explain the decrease in k_{trap} upon reduction (Eqn. 1). However, we found that the yield of prompt fluorescence from reduced chromatophores was the same or lower than the total fluorescence from the unreduced chromatophores was the same or lower than the total fluorescence from the unreduced chromophores (Table I). In *R. rubrum* the ratio of these yields (α) was about 0.8. From the streak camera measurements, one would expect α to be about 1.3. We have no convincing explanation for this discrepancy, which is too large to be due simply to inadequate resolution of prompt from delayed fluorescence. However, a significant amount of the prompt fluorescence may occur before equilibration between A* and P*, judging from the emission spectra of the delayed and prompt fluorescence from *Rps. sphaeroides* 2.4.1 chromatophores (see below). This is contrary to the premise underlying Eqn. 1. Studies with higher time resolution will be needed to explore this point in more detail.

Though three exponential components were required to describe the decay of the delayed fluorescence, at least as accurate a description could be obtained using a larger number of exponents. Therefore, the actual number of components in the emission could be greater than three. However, Godik et al. [2] found that the decay of the total emission between 0 and 0.5 ns in reduced, wild-type *R. rubrum* chromatophores could be adequately described by a two-exponential decay. The fast component, presumably prompt fluorescence, decayed with a lifetime of 80 ps. The slow component had a long decay time (greater than 1 ns) and an initial amplitude that was about 5% that of the fast component. If there are earlier components in the delayed fluorescence than τ_1

(1.0 ns), they apparently are very difficult to resolve from the prompt fluorescence.

The temperature dependence of the delayed fluorescence

The greatest difference between the delayed fluorescence from chromatophores and that from reaction centers is in its temperature dependence. With reduced reaction centers, the total fluorescence increases as the temperature is lowered to about 200 K, and decreases below this point [22,23]. With chromatophores from all of the strains except *Rps. sphaeroides* R26, the total fluorescence decreases monotonically with decreasing temperature between 295 and 180 k (Fig. 1A, C and D). The total fluorescence from R26 chromatophores drops initially, but then increases as the temperature is lowered below about 260 K (Fig. 1B). As with isolated reaction centers, the prompt fluorescence yield in S1, G9 and 2.4.1 chromatophores is relatively temperature-independent, dropping by only about 20% between 295 and 180 K (Fig. 1A, C and D). The prompt fluorescence from R26 chromatophores increases between 260 and 200 K but then plateaus as the temperature is decreased further (Fig. 1B).

Because the initial amplitude of the delayed fluorescence in chromatophores depends on the equilibrium constant between P^F and the excited

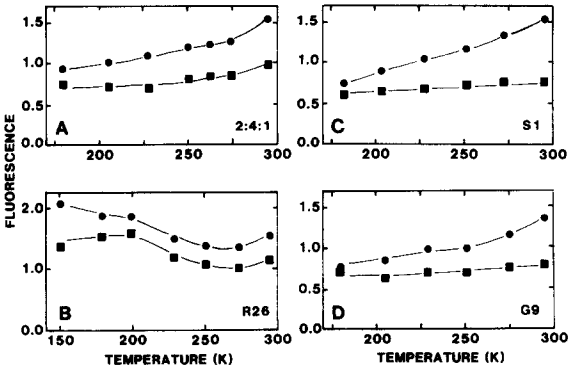

Fig. 1. The temperature dependence of the total fluorescence (circles) and the prompt fluorescence (α, squares) from reduced chromatophores, relative to the total fluorescence from the unreduced samples at 295 K. All measurements shown were in the absence of a magnetic field. Conditions as described in Methods. (A) *Rps. sphaeroides* strain 2.4.1 (B) *Rps. sphaeroides* strain R-26. (C) *Rds. rubrum* strain S1. (D) *R. rubrum* strain G9.

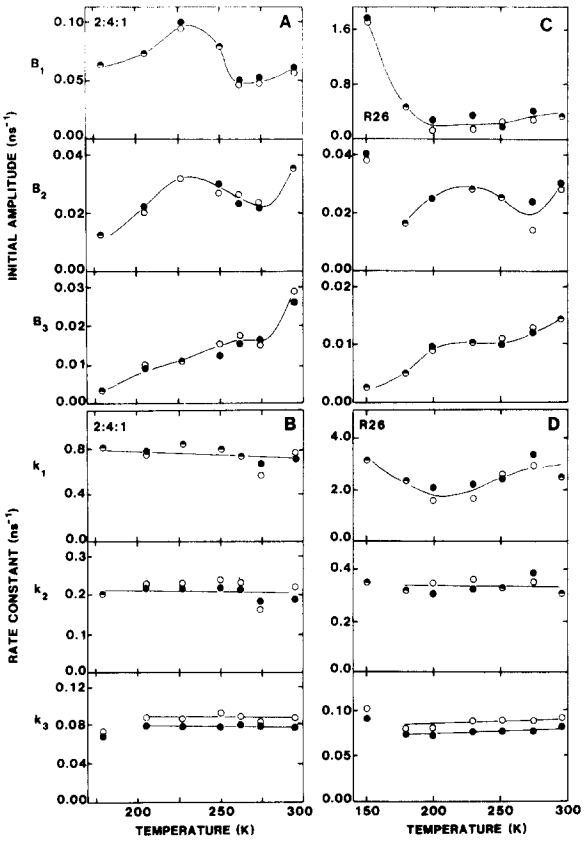

Fig. 2. The temperature dependence of the initial amplitudes (B_i) and decay rate constants (k_i) of the three exponential components of the delayed fluorescence from *Rps. sphaeroides* chromatophores. Conditions as described in Methods. Open symbols are in the absence of a magnetic field; filled symbols are in the presence of a 600-G magnetic field. (A) Initial delayed fluorescence amplitudes for strain 2.4.1. (B) Rate constants for strain 2.4.1. (C) Initial amplitudes for strain R-26. (D) Rate constants for strain R-26. At 150 K in panels C and D, it was difficult to resolve the second component of the delayed fluorescence from the first, because B_1 was much larger than B_2. Therefore, the values of B_2 and k_2 at this temperature may be unreliable. In panels B and D, the values of k_3 at the lowest temperatures shown may also be unreliable, because the initial amplitude of this component (B_3) in both *Rps. sphaeroides* strains becomes very small at low temperatures (see panels A and C).

antenna (A*), one would expect a different temperature dependence for B_1, B_2 and B_3 in chromatophores than that seen in reaction centers, where the initial amplitudes depend on the equilibrium constant between P^F and P^*. This point will be considered in further detail below. The presence of an antenna would not necessarily be

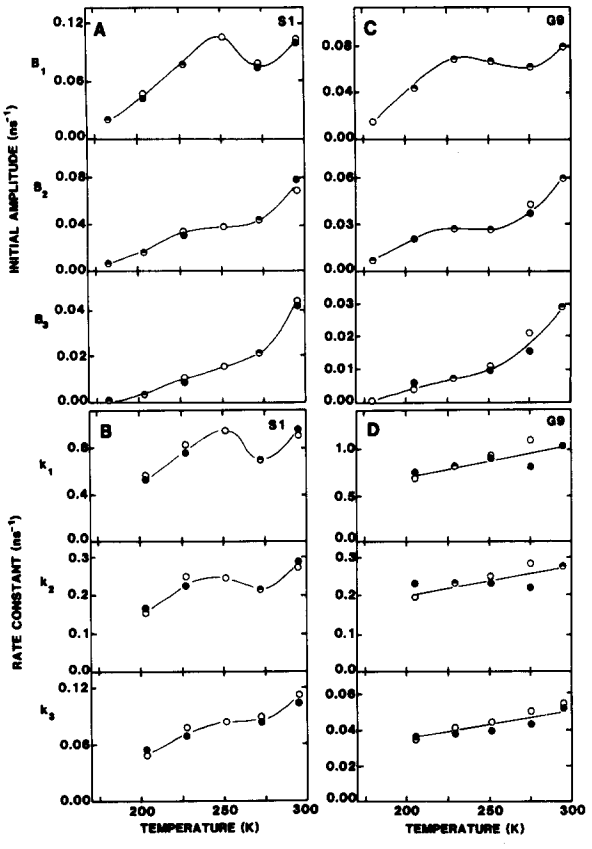

Fig. 3. The temperature dependence of the initial amplitudes (B_i) and rate constants (k_i) of the three exponential components of the delayed fluorescence from *R. rubrum* chromatophores. Conditions as described in Methods. Open symbols are in the absence of a magnetic field; filled symbols are in the presence of a 600-G magnetic field. (A) Initial delayed fluorescence amplitudes for strain S1. (B) Rate constants for strain S1. (C) Initial amplitudes for strain G9. (D) Rate constants for strain G9. In panels B and B the values of k_1, k_2 and k_3 could not be determined reliably at 180 K due to the small amount of delayed fluorescence detected at this temperature (see panels A and C).

expected to alter the temperature dependence of the decay times of the fluorescence components. In reaction centers k_1 and k_2 are nearly temperature independent and k_3 decreases by a factor of about 2 with decreasing temperature [23]. k_1 and k_2 are temperature independent in *Rps. sphaeroides* 2.4.1 chromatophores as is k_2 in chromatophores from strain R26. However, in contrast to the reaction center measurements, k_3 is also nearly constant with temperature in both *Rps. sphaeroides* strains (Fig. 2). In *R. rubrum* G9 and S1 chromatophores the rate constants of all decay components decrease with decreasing temperature (Fig. 3). The reaction center's environment apparently affects the temperature dependence of the relaxation and decay kinetics of the state P^F.

In isolated reaction centers the initial amplitudes of all three components of the delayed fluorescence increase markedly as one lowers the temperature between 250 and 200 K and decrease below 200 K [23]. An indication of this effect is seen in chromatophore delayed fluorescence, though it is not as pronounced. An increase in the initial amplitude of the fastest component (B_1) is observed in G9, S1, and 2.4.1 chromatophores below about 260 K, but it reaches a maximum by 230 to 250 K, somewhat higher then the temperature of the maximum amplitude in reaction centers (Figs. 2 and 3). In chromatophores from both *R. rubrum* strains, the initial amplitude of the middle component (B_2) decreases between 300 and 260 K, then stays level until about 230 K before

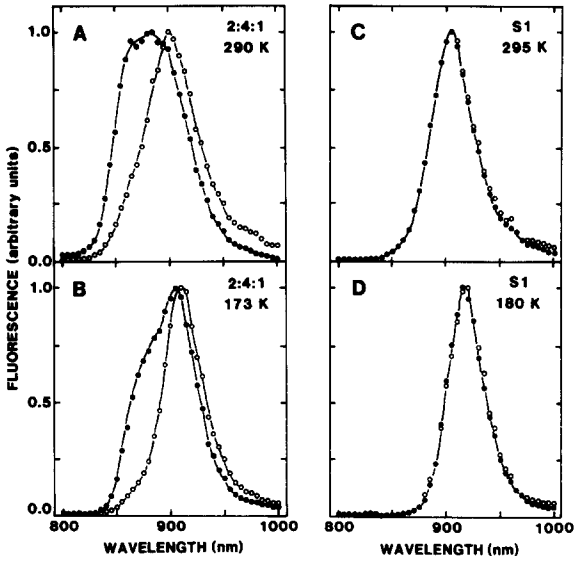

Fig. 4. Spectra of the prompt fluorescence (0–2 ns, filled symbols) and delayed fluorescence (10–35 ns, open symbols) from chromatophores. (A) *Rps. sphaeroides* strain 2.4.1 at 290 K. (B) *Rps. sphaeroides* strain 2.4.1 at 173 K. (C) *R. rubrum* strain S1 at 295 K. (D) *R. rubrum* strain S1 at 180 K. All spectra were normalized to 1 at the wavelength of maximum emission. The ratios of the delayed fluorescence at its peak wavelength to the prompt fluorescence at its peak were about 0.16 and 0.05 at the upper and lower temperatures, respectively, for both 2.4.1 and S1 chromatophores. Conditions as described in Methods.

continuing to decline (Fig. 3). The initial amplitude of the longest component of the delayed fluorescence (B_3) in G9 and S1 chromatophores decreases monotonically with decrease temperature (Fig. 3). In both *Rps. sphaeroides* strains, B_2 and B_3 tend to plateau or increase as the temperature is lowered between 260 and 230 K (Fig. 2).

In *Rps. sphaeroides* R26 chromatophores, the initial amplitude of the fastest component (B_1) generally decreases as the temperature is lowered from 295 K to 200 K and then increases rapidly below 200 K. By 150 K, B_1 in this sample is about a factor of 6 greater than it is at 295 K (Fig. 2). This observation, coupled with the anomalously short lifetime found for this component, and the increase seen in the prompt and total fluorescence as the temperature in lowered (Fig. 1B), suggests that much of the subnanosecond fluorescence may arise from a different source in R26 chromatophores than the other chromatophore samples investigated. The excitation profile obtained using unreduced R26 chromatophores is 1.1 ns (FWHM), about 200 ps wider than the excitation profile from any other chromatophore sample. Possibly, this could reflect a population of antenna complexes that are not well connected to reaction centers. As the temperature is lowered below 200 K, the rate constant for excitation transfer from these poorly connected antenna complexes to the reaction centers may decrease. In support of this hypothesis, the total fluorescence from unreduced R26 chromatophores increases by about a factor of 2.9, and the full-width-at-half-maximum of the fluorescence signal increases from 1.1 to 1.25 ns, as the temperature is lowered from 295 to 150 K. (In the other three chromatophore strains, there is little change in the total fluorescence from the unreduced sample between 295 and 180 K [43].) If the rate constant for excitation transfer in R26 chromatophores decreases substantially at lower temperatures, the time-course of fluorescence from the unreduced sample at 295 K will not be an accurate representation of the time-course of reaction center excitation, which is required for deconvolution in the fitting algorithm, and this could result in fitting artifacts at early times.

Fig. 4 gives examples of the time-resolved fluorescence spectra of *R. rubrum* S1 and *Rps. sphaeroides* 2.4.1 chromatophores at two temperatures. The 'prompt' fluorescence spectra represent photons detected between 0 and 2 ns after the beginning of the initial fluorescence rise and are composed primarily (greater than 80%) of the prompt component of the fluorescence. The 'delayed' fluorescence spectra represent photons detected between 10 and 35 ns after the start of the fluorescence rise and are composed almost entirely of delayed fluorescence (greater than 95%). The *R. rubrum* S1 spectra (Fig. 4C and D) are representative of the three strains that have only one well-resolved component in their steady-state near infrared absorbance and emission spectra above 180 K (*R. rubrum* S1 and G9 and *Rps. sphaeroides* R26). In these, the delayed and prompt fluorescence have essentially identical spectra. This implies that the excited state (or mixture of excited states) that is formed by back electron transfer from P^F is indistinguishable from the state that is formed upon the initial excitation. In *Rps. sphaeroides* 2.4.1 chromatophores (Fig. 4A and B) this is clearly not the case. The prompt fluorescence appears to have substantial contributions from both of the spectrally distinct complexes present in the antenna (B800–850 and B875), while the delayed fluorescence apparently comes primarily from the longer-wavelength absorbing complex (B875). Similar results have been obtained previously by comparing the steady-state fluorescence spectra of 2.4.1 chromatophores at moderate and low redox potentials [43]. Either much of the fluorescence from the short-wavelength component of the antenna (B800–850) occurs before thermal equilibrium is reached in the antenna, or the excited state formed by charge recombination is substantially different from the state that is formed upon initial excitation.

Free-energy differences between P^F, P and the excited antenna*

In isolated reaction centers, the equilibrium constant between the initial form of P^F and P* is given by [22,23]:

$$K_{P^F P^*} = \frac{F_D(0)\,\Phi_f}{k_f} \qquad (2)$$

where $F_D(0)$ is the initial amplitude of the delayed fluorescence relative to the total fluorescence from

TABLE II

CALCULATED FREE-ENERGY DIFFERENCE

600-G magnetic field H: −, off; +, on. λ_{O-O} is the O–O transition wavelength, determined as the wavelength about which the absorbance and weighted emission spectra are mirror images (see Ref. 36). $k_f K_{P^*,A^*}$ was calculated using method 2, Data for R26-RCs were taken from Ref. 23: reduced R26 reaction centers in an aqueous buffer (50 mM Tris-HCl/0.05% Triton X-100, pH 8.0).

Sample	H	λ_{O-O} (nm)	ϕ_f	k_f (10^{-8} s^{-1})	$k_f K_{P^*,A^*}$ (10^{-9} s^{-1})	$F_D(0)$ (ns^{-1})	$\Delta G_{P^F,A^*}$ (eV)	$\Delta G_{P^F,P^*}$ (eV)		$\Delta G_{P^*,A^*}$ (eV)	
								calculated using method 1	calculated using method 2	calculated from Eqns. 5 and 6	calculated as $\Delta G_{P^F,A^*} - \Delta G_{P^F,P^*}$
R26-RCs	−	891.5	0.0004	0.8	–	0.29 ± 0.04	–	0.168 ± 0.004	–	–	–
	+	891.5	0.0004	0.8	–	0.28 ± 0.05	–	0.169 ± 0.006	–	–	–
2.4.1	−	887	0.0196	–	2.1	0.14 ± 0.03	–	–	0.169 ± 0.003	–	–
Chromatophores	+	887	0.0196	–	2.1	0.13 ± 0.01	–	–	0.171 ± 0.002	–	–
S1	−	893	0.0093	1.2	3.6	0.26	0.102	0.184	0.190	−0.083	−0.088
Chromatophores	+	893	0.0093	1.2	3.6	0.26	0.100	0.182	0.188	−0.083	−0.086
G9	−	883	0.0096	1.0	1.3	0.17	0.106	0.177	0.172	−0.071	−0.066
Chromatophores	+	883	0.0096	1.0	1.3	0.17	0.106	0.177	0.171	−0.071	−0.065

unreduced reaction centers and is given by $B_1 + B_2 + B_3$, k_f is the reciprocal of the natural radiactive lifetime of P* and Φ_f is the quantum yield of fluorescence from unreduced reaction centers. The same expression can be used to calculate the equilibrium constant between the earliest form of P^F and A*, $K_{P^FA^*}$, if A*, P* and P^F are in thermal equilibrium. In this case, k_f is the effective radiative rate constant of the emitters in the chromatophore (A*), Φ_f is the quantum yield of fluorescence from unreduced chromatophores and $F_D(0)$ is the total initial amplitude of delayed fluorescence from reduced chromatophores relative to the total fluorescence from the unreduced sample.

In S1, G9 and 2;4;1 chromatophores, the reported times for trapping of excitation from the antenna by the reaction center are less than or equal to 100 ps [1–7]. This is short enough so that P^F should reach equilibrium with the excited antenna rapidly with respect to even the fastest component (τ_1) of the delayed fluorescence. The same may not be true for R26 chromatophores, which have an apparent prompt fluorescence lifetime of about 300 ps [7]. For this reason, and because of the uncertain origin of the fastest component of the delayed fluorescence, we did not attempt to calculate equilibrium constants or free-energy differences for R26 chromatophores.

It is important to note, in this context, that Eqn. 2 still holds even if the prompt component of the fluorescence from the reduced sample or the total fluorescence from the unreduced sample does not come from a system in thermal equilibrium. The fluorescence from unreduced chromatophores is used simply as a standard. The only requirement is that the yield of this fluorescence, Φ_f, be known.

P^F is initially formed as a radical pair state with singlet character. The singlet and triplet forms of P^F can interconvert on the nanosecond time scale [22,44–46]. Since delayed fluorescence monitors only the population of the singlet form of P^F, one might think that the amplitude of this fluorescence would not accurately reflect the overall concentration of P^F. However, since the amplitude of the delayed fluorescence can be measured at times less than a nanosecond, before a significant amount of singlet-triplet interconversion has occurred, the initial amplitude of the delayed fluorescence should represent the total population of the earliest form of P^F. (It is possible that the kinetics of delayed fluorescence decay are affected by singlet-triplet interconversion. However, it seems unlikely that this process is the primary cause of the multiple exponential components found in the kinetic analysis of the delayed fluorescence, since the decay times of the two fastest components (τ_1 and τ_2) do not appear to change

in the presence of a magnetic field.)

In Eqn. 2, k_f is simple to evaluate only if all of the emitters in the photosynthetic apparatus have the same standard free-energy difference between their ground and excited states (see appendix). In chromatophores this is never strictly true, because some emission will come from P* and some from A*; but for single-component antennas it is approximately true. Both samples of *R. rubrum* chromatophores have only one resolvable component in their near-infrared absorption spectra (data not shown) and fluorescence spectra (Fig. 4C and D), at least above 180 K, and it therefore seems reasonable to assume that all antenna complexes in these chromatophores have approximately the same free-energy difference between their ground and excited states. Almost all of the delayed fluorescence from these chromatophores probably comes from the antenna, because the antenna complexes considerably outnumber P. N_A was found to be 24 ± 3 for strain S1 and 28 ± 3 for G9. This is consistent with the fact that in both *R. rubrum* chromatophore strains the emission spectrum of the delayed fluorescence is indistinguishable from that of the prompt fluorescence (Fig. 4C and D), which probably originates primarily in the antenna. Table II lists k_f values calculated from the absorption and emission spectra of *R. rubrum* S1 and G9 chromatophores (see Appendix). The initial standard free energy differences between A* and P^F for these chromatophores are also given, as calculated from Eqn. 2 and the expression

$$\Delta G_{P^F,A^*} = -kT \ln K_{P^F,A^*} \tag{3}$$

where k is the Boltzmann constant. In both cases, $\Delta G_{P^F,A^*}$ is about 0.10 eV (Table II).

It is possible to calculate the initial standard free-energy difference between P* and P^F in chromatophores, again assuming thermal equilibration between these states and A*, by using the expression

$$K_{P^F,P^*} = \frac{K_{P^F,A^*}}{K_{P^*,A^*}} = \frac{F_D(0)\Phi_f}{k_f K_{P^*,A^*}} \tag{4}$$

which is readily derived from Eqn. 2. This calculation can be done in two ways. First, one can calculate the number of antenna complexes per reaction center (N_A, see Methods) and then determine the equilibrium constant between P* and A* from the expression:

$$K_{P^*,A^*} \approx N_A \exp\frac{E_P - E_A}{kT} \tag{5}$$

or

$$\Delta G_{P^*,A^*} \approx -kT \ln(N_A) + E_A - E_P \tag{6}$$

Here E_P and E_A are the O–O transition energies between the excited and ground states of P and A, which can be obtained from the absorption and emission spectra (Table II). Using this method the free-energy difference between P* and A* ($\Delta G_{P^*,A^*}$) is calculated to be -0.07 to -0.08 eV for both chromatophore samples (Table II). Substituting K_{P^*,A^*} from Eqn. 5 into Eqn. 4 and converting K_{P^F,P^*} into an initial standard free-energy difference, $\Delta G_{P^F,P^*}$ is calculated to be about 0.18 eV for both S1 and G9 chromatophores at 295 K (Table II), in agreement with the value of 0.17 eV reported in reduced, isolated reaction centers in an aqueous buffer with 0.05% Triton X-100 [23].

The second method utilizes the treatment of Arata and Nishimura [47] and Ross [48] to determine the product ($k_f K_{P^*,A^*}$) from the absorption and emission spectra of the chromatophores (see Appendix). This then can be used in Eqn. 4 to determine K_{P^F,P^*}. This technique has the advantage that multicomponent antenna systems can be treated (see Appendix). Thus the standard free-energy difference between P* and P^F can be calculated not only in *R. rubrum* S1 and G9 chromatophores but in *Rps. sphaeroides* 2.4.1 chromatophores as well. At 295 K, the free-energy differences between P^F and P* in 2.4.1, S1 and G9 chromatophores calculated in this way range between 0.17 and 0.19 eV (Table II), again in reasonable agreement with the free-energy difference determined for reaction centers in aqueous Tris/Triton buffer [23].

A summary of standard free-energy differences now known between A* and the state $P^+Q_B^-$ at 295 K in *Rps. sphaeroides* is shown schemically in Fig. 5. The free-energy difference shown between P^F and P* was determined, as described above, for reduced chromatophore samples. In principle the

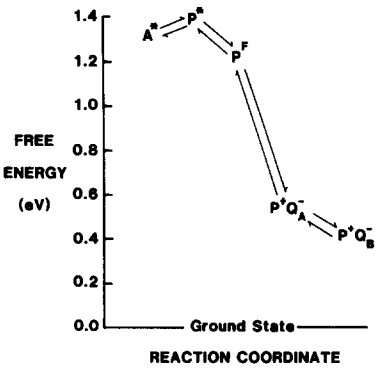

Fig. 5. Scheme showing the relative initial standard free energies of photochemical intermediates in chromatophores of *Rps. sphaeroides* at 295 K, as determined from delayed fluorescence measurements. The free energy of P* is taken to be above that of the ground state by the zero–zero transition transition energy, E_P. The free energy of A* includes the entropy of delocalization of the excitation in the antenna. Data for this scheme were taken from this work and from Refs. 47 and 50.

presence of the reduced quinone in the reaction center could make electron transfer between P* and P^F less favorable. We have considered this possibility in isolated reaction centers by analyzing the delayed fluorescence from samples lacking quinone (Woodbury, N.W. and Parson, W.W., unpublished results). Though an unambiguous determination of the initial free energy between P^F and P* was difficult, we determined a maximum free-energy difference of 0.19 eV. Apparently, the presence of the reduced quinone does not greatly affect the standard free-energy difference between p^F and P*.

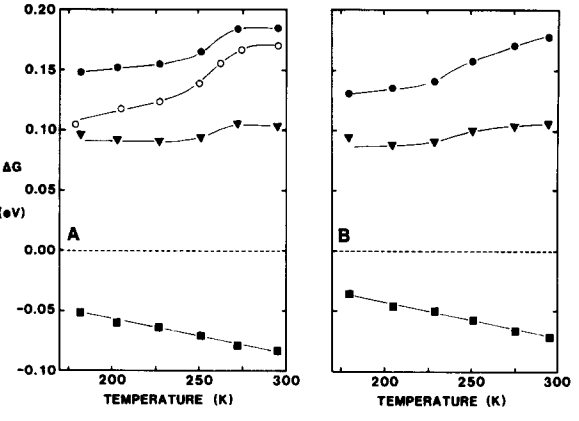

Fig. 6. The temperature dependence of the initial standard free-energy difference between P^F and P* (circles), P^F and A* (triangles), and P* and A* (squares). The free-energy differences shown are calculated from the data in Figs. 2 and 3 as described in the text. Essentially the same free-energy differences for *R. rubrum* strains G9 and S1 were obtained irrespective of which method was used for calculating the free-energy difference between P^F and P* (see Results and Discussion). (A) Filled symbols are *R. rubrum* strain S1; open symbols are *Rps. sphaeroides* strain 2.4.1. (B) *R. rubrum* strain G9.

Fig. 6 shows the temperature dependence of the initial standard free-energy difference between P* and P^F in 2.4.1, S1 and G9 chromatophores. All strains show a similar temperature dependence. The free-energy difference decreases with decreasing temperature in a nonlinear fashion, dropping very little between 295 and 275 K, then dropping more abruptly between 275 and 225 K, and finally declining more gently at lower temperatures. A similar temperature dependence was seen for the standard free-energy difference between P^F and P* in reaction centers [23]. The similarity is particularly strong between reaction centers (isolated from *Rps. sphaeroides* strain R-26) and chromatophores from *Rps. sphaeroides* strain 2.4.1. Fig. 6 also shows the temperature dependence of the free-energy gaps between P^F and A* and between P* and A*. The nearly linear temperature dependence of the free-energy difference between P* and A* is required by the entropic term, $-kT \ln N_A$, in Eqn. 5; the slight deviation from linearity is due to the temperature dependence of E_P and E_A.

The multi-exponential decay of the delayed fluorescence from chromatophores can be interpreted in terms of relaxations in the energy of P^F, as was done previously for isolated reaction centers [23]. The relaxations appear to occur in two main steps of 0.01 to 0.02 eV each and involve a total free-energy change of 0.02 to 0.03 eV at 295 K. Similar results were obtained with reaction centers [23].

In summary, the nanosecond fluorescence from chromatophores is qualitatively similar to that from isolated reaction centers. At least three exponential components are required to describe the decay kinetics of the delayed fluorescence, with the longest-lived components having the lowest initial amplitude. The standard free-energy difference calculated between P^F and P* from the initial delayed fluorescence amplitudes in chro-

matophores is consistent with the range of values determined in isolated reaction centers, and this difference evidently increases with time, giving rise to the multiexponential fluorescence decay kinetics. The initial amplitudes of the delayed fluorescence behave somewhat differently as a function of temperature in reaction centers and chromatophores, due to the entropy of equilibration between P* and the surrounding antenna in the photosynthetic membrane. However, the calculated free-energy difference between P* and P^F is about the same in reaction centers and chromatophores from *Rps. sphaeroides* between 180 K and 300 K. Apparently the state P^F is very similar in whole photosynthetic membranes and in isolated reaction centers.

Appendix

Following the treatment of Ross [48], Arata and Nishimura [47] have derived an expression for what they term the radiative rate constant of the photosynthetic unit. Briefly, assuming thermal equilibration among vibrational states,

$$\kappa_{fi}(\nu)\,d\nu = 8\pi\left(\frac{n\nu}{c}\right)^2 \cdot \exp\frac{\mu_{Ai} - h\nu}{kT} \cdot \sigma_i(\nu)\,d\nu \qquad \text{(A-1)}$$

where $\kappa_{fi}(\nu)\,d\nu$ is the radiative rate constant of the ith antenna complex at frequency ν, n is the index of refraction, c is the speed of light, k is Boltzmann's constant, h is Planck's constant, μ_{Ai} is the standard partial molecular free-energy difference between the ground and excited states of the ith antenna complex and $\sigma_i(\nu)$ is the absorption cross section of that complex. This is Eqn. A-4 in reference 47. If equilibrium is established among all antenna complexes in the photosynthetic unit, then the total rate constant for radiation at frequency ν, $\kappa_f(\nu)\,d\nu$, is

$$\kappa_f(\nu)\,d\nu = \frac{\sum_i \kappa_{fi}(\nu)\,d\nu\, N_{Ai} \exp\frac{-\mu_{Ai}}{kT}}{\sum_i N_{Ai} \exp\frac{-\mu_{Ai}}{kT}} \qquad \text{(A-2)}$$

where N_{Ai} is the number of antenna complexes of the ith type per reaction center. By substituting Eqn. A-1 into Eqn. A-2, multiplying both sides of the resulting equation by $e^{\mu_P/kT}$, and simplifying one finds:

$$\kappa_f(\nu)\,d\nu \sum_i N_{Ai} \exp -\frac{\mu_{Ai} - \mu_P}{kT}$$

$$= 8\pi\left(\frac{n\nu}{c}\right)^2 \cdot \exp\frac{\mu_P - h\nu}{kT} \cdot \sigma(\nu)\,d\nu \qquad \text{(A-3)}$$

where μ_P is the standard molecular free-energy difference between the ground and excited states of P, and $\sigma(\nu)$ is $\sum_i \sigma_{Ai}(\nu) N_{Ai}$, the cross section of the photosynthetic unit (one reaction center and its associated antenna complexes). This is identical to Eqn. A-7 in Ref. 47, except for the factor of $\sum_i N_{Ai} \exp(-(\mu_A - \mu_P)/kT)$ which is just the equilibrium constant K_{P^*,A^*} between P* and the excited antenna. Making this substitution, realizing that the emission spectrum is proportional to $\kappa_f(\nu)\,d\nu$, evaluating the proportionality constant at the O–O transition wavelength of P and integrating over all frequencies (see Ref. 47 for a detailed procedure) one obtains:

$$K_{P^*,A^*} k_f = K_{P^*A^*} \int \kappa_f(\nu)\,d\nu$$

$$= \frac{Z_P}{Z_{P^*}} 8\pi\left(\frac{n\nu_0}{c}\right)^2 \frac{\sigma(\nu_0)}{F(\nu_0)} \int F(\nu)\,d\nu \qquad \text{(A-4)}$$

where Z_P/Z_{P^*} is the ratio of the vibrational partition coefficients for the ground and excited states of P, ν_0 is the O–O transition frequency and $F(\nu)$ is the fluorescence at frequency ν in arbitrary units. The right hand side of Eqn. A-4 is the same as the definition of the radiative rate constant for the photosynthetic unit in Ref. 47. Thus, this constant is really the product of k_f, an overall radiative rate constant for the fluorescent pigments in the chromatophore, and K_{P^*,A^*}, the effective equilibrium constant between P* and A*. It is easy to calculate this product from the fluorescence spectra given the O–O transition frequency of P [23] and the absorption cross section of the photosynthetic unit at that frequency, assuming that the ratio of the partition coefficients of the ground and excited states of P (Z_P/Z_{P^*}) is unity. $\sigma(\nu_0)$ can be calculated from the reaction-center concentration of the chromatophore sample (see Methods) and the sample's total absorbance at ν_0. This treatment assumes thermal equilibrium among all the excited states of the

photosynthetic apparatus, so the fluorescence spectrum used must be the spectrum of the delayed fluorescence, obtained after thermal equilibrium is achieved.

If all of the emission comes from components with the same standard partial molecular free-energy difference between their ground and excited states, k_f can be calculated directly for the individual antenna complexes from either the Strickler-Berg relationship, which is based on the absorbance spectrum [49]:

$$k_f = \frac{Z_A}{Z_{A^*}} 8\pi \left(\frac{n}{c}\right)^2 \int \frac{(2\nu_0 - \nu)^3}{\nu} \sigma(\nu)\, d\nu \qquad \text{(A-5)}$$

or from another expression developed by Ross, which uses the fluorescence spectrum [48]:

$$k_f = \frac{Z_A}{Z_{A^*}} 8\pi \left(\frac{n\nu_0}{c}\right)^2 \frac{\sigma(\nu_0)}{F(\nu_0)} \int F(\nu)\, d\nu \qquad \text{(A-6)}$$

Essentially identical results were obtained using either Eqn. A-6 or Eqn. A-5. K_{P^*,A^*} can be calculated from the expression:

$$K_{P^*,A^*} = \frac{Z_P}{Z_{P^*}} \frac{Z_{A^*}}{Z_A} N_A \exp - \frac{E_A - E_P}{kT} \qquad \text{(A-7)}$$

where Z_A and Z_{A^*} are the partition coefficients of the ground and excited states of the antenna. Eqn. A-7 becomes Eqn. 5 in Results and Discussion when the product $(Z_P/Z_{P^*})(Z_{A^*}/Z_A)$ is assumed to be unity. The equilibrium constant K_{P^F,P^*} thus can be calculated from Eqn. 4 using the product $k_f K_{P^*A^*}$ determined either from Eqns. A-6, A-7 and A-5 or Eqn. A-4.

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