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DELAYED FLUORESCENCE FROM *RHODOPSEUDOMONAS SPHAEROIDES* REACTION CENTERS

ENTHALPY AND FREE ENERGY CHANGES ACCOMPANYING ELECTRON TRANSFER FROM *P*-870 TO QUINONES

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Delayed fluorescence from isolated reaction centers of *Rhodopseudomonas sphaeroides* was measured to study the energetics of electron transfer from the bacteriochlorophyll complex (*P*-870, or P) to the primary and secondary quinones (Q_A and Q_B). The analysis was based on the assumption that electron transfer between P and Q reaches equilibrium quickly after flash excitation, and stays in equilibrium during the lifetime of the P^+Q^- radical pair. Delayed fluorescence of 1Q reaction centers (reaction centers that contain only Q_A) has a lifetime of about 0.1 s, which corresponds to the decay of $P^+Q_A^-$. 2Q reaction centers (which contain both Q_A and Q_B) have a much weaker delayed fluorescence, with a lifetime that corresponds to that of $P^+Q_B^-$ (about 1 s). In the presence of *o*-phenanthroline, the delayed fluorescence of 2Q reaction centers becomes similar in intensity and decay kinetics to that of 1Q reaction centers. From comparisons of the intensities of the delayed fluorescence from $P^+Q_A^-$ and $P^+Q_B^-$, the standard free energy difference between $P^+Q_A^-$ and $P^+Q_B^-$ is calculated to be 78 ± 8 meV. From a comparison of the intensity of the delayed fluorescence with that of prompt fluorescence, we calculate that $P^+Q_A^-$ is 0.86 ± 0.02 eV below the excited singlet state of P in free energy, or about 0.52 eV above the ground state PQ_A. The temperature dependence of the delayed fluorescence indicates that $P^+Q_A^-$ is about 0.75 eV below the excited singlet state in enthalpy, or about 0.63 eV above the ground state.

Introduction

The reaction centers of photosynthetic bacteria contain a photochemically reactive bacteriochlorophyll complex (P), a complex of initial electron acceptors (I), and two quinones [1,2]. When P is raised to the excited singlet state (P*) by the absorption of light, it transfers an electron to I, generating a radical-pair state (P^+I^-, or P^F) [1–3]. I^- reduces one of the quinones (Q_A), and Q_A^- in turn passes an electron to the second quinone (Q_B) in about 100 μs [3–5]. To understand these early steps of photosynthesis, one would like to know the thermodynamic parameters of each of the transient states of the system. The thermodynamic parameters control the kinetics [3] and direction of electron transfer at each step, and ultimately determine the amount of work that the photosynthetic apparatus can do [6,7].

The standard partial molecular free energies of the states $P^+Q_A^-$ and $P^+Q_B^-$ have been estimated from the midpoint redox potentials (E_m) of P and the quinones. In both chromatophores and isolated reaction centers of *Rhodopseudomonas sphaeroides*, P has an E_m of about +450 mV [8]. In isolated reaction centers of the same species, Q_A has an apparent E_m of about −50 mV, essentially independent of pH [9,10]. Combining these values gives an estimate of 0.50 eV for the standard free energy of $P^+Q_A^-$ relative to the ground state, PQ_A. In chromatophores, however, the

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apparent E_m of Q_A decreases linearly with increasing pH up to about pH 9, probably because the uptake of a proton is coupled to the reduction [1,8,11]. During the normal operation of the photosynthetic apparatus, proton uptake is not fast enough to occur before Q_A^- transfers its electron to Q_B [8,11]. The effective E_m of Q_A is therefore considered to be about −180 mV, which is the value measured at high pH [11]. This gives a free energy of about 0.63 eV for $P^+Q_A^-$ relative to PQ_A for chromatophores.

The apparent E_m of Q_B is about +70 mV in chromatophores of *Rps. viridis* at pH 8, and −15 mV at pH 10 [12,13]. In *Chromatium vinosum* chromatophores, E_m values of +80 and −90 mV have been measured at pH 7.7, depending on the redox mediators used for the titration [14]. Both values become more negative with increasing pH. In isolated reaction centers, studies of the decay kinetics of $P^+Q_A^-$ and $P^+Q_B^-$ suggest that the standard free energy of $P^+Q_B^-$ is about 70 mV below that of $P^+Q_A^-$, or about +0.43 eV above PQ_B [15,17].

The values of the standard enthalpies of $P^+Q_A^-$ and $P^+Q_B^-$ are quite unclear. From the temperature dependence of the E_m values of of P, Q_A, and Q_B in *C. vinosum* chromatophores, the enthalpies of both $P^+Q_A^-$ and $P^+Q_B^-$ were calculated to be very close to that of the ground state [14]. Using a capacitor microphone, we recently measured the expansion of the solution due to the release of heat, after flash excitation of *Rps. sphaeroides* reaction centers [18]. The results gave an enthalpy of 0–0.15 eV for $P^+Q_A^-$ relative to PQ_A, and of 0.40 ± 0.02 eV for $P^+Q_B^-$. Contradictory results on the enthalpy of $P^+Q_A^-$ have come from measurements of the temperature dependence of delayed fluorescence from chromatophores of *Rps. viridis* [19]. These measurements indicated that the enthalpy of $P^+Q_A^-$ is about 0.7 eV above that of the ground state. This disagrees seriously with both the calorimetric and potentiometric measurements.

There are several possible explanations for the different results on the enthalpies: The enthalpies could depend on the bacterial species. The measurements of delayed fluorescence in *Rps. viridis* chromatophores could have been influenced by a membrane potential, or by a temperature dependence of the quantum yield of $P^+Q_A^-$. The capacitor microphone measurements could have been distorted by temperature-dependent volume changes due to something other than the release of heat. The E_m values would not reflect interactions between P^+ and Q_A^- or Q_B^-, because they were obtained by titrating the electron donor and acceptor separately [3,7,14].

In the present study, we measured delayed fluorescence from isolated reaction centers of *Rps. sphaeroides*. These cannot have a membrane potential, and their photochemical quantum yield is close to 1.0 and is essentially independent of temperature [20,21]. To consider both $P^+Q_A^-$ and $P^+Q_B^-$, we studied reaction centers that contained both quinones, preparations that contained only Q_A, and samples that had electron transfer between Q_A^- and Q_B^- blocked by the addition of *o*-phenanthroline [15–18, 22].

When reaction centers are excited with a short flash, the electron-transfer reactions between P* and $P^+Q_A^-$ reach equilibrium in about 200 ps [3]. We assume that the equilibrium is maintained throughout the lifetime of $P^+Q_A^-$. The same assumption has generally been made in studies of delayed fluorescence from chromatophores [23]. If P* can fluoresce with a rate constant k_f, the intensity of the delayed fluorescence emitted at any time during the lifetime of $P^+Q_A^-$ will be:

$$\begin{aligned} F_d &= k_f[P^*] = k_f K_A^*[P^+Q_A^-] \\ &= k_f[P^+Q_A^-]\exp(-\Delta G_A^*/kT) \\ &= k_f[P^+Q_A^-]\exp(-\Delta H_A^*/kT + \Delta S_A^*/k) \end{aligned} \quad (1)$$

where K_A^*, ΔG_A^*, ΔH_A^*, and ΔS_A^* are the equilibrium constant and the changes in standard partial molecular free energy, enthalpy, and entropy, respectively, for the process $P^+Q_A^- \rightleftharpoons P^*Q_A$, and k is Boltzmann's constant. Similar considerations apply to delayed fluorescence from $P^+Q_B^-$.

Materials and Methods

Reaction centers of *Rps. sphaeroides* strain R-26 were prepared as described elsewhere (Schenk, C.C., Blankenship, R.E. and Parson, W.W., unpublished data). Preparations containing approximately two ubiquinones per particle (2Q reaction centers) and with approximately one ubiquinone per particle (1Q reaction centers) were made as described previously [16,18].

Samples were placed in a 1 X 1 cm quartz fluorescence cuvette in a thermostatically controlled copper housing. They were excited at 0.08 Hz by 588 nm, 0.5 μs flashes from a rhodamine-6-G dye laser in combination with a Corning 9788 glass filter, a 590 nm interference filter, and neutral density filters. The flash strength was measured with a balistic thermopile. Delayed fluorescence at wavelengths between 900 and 1000 nm was measured at 90° to the excitation beam, with a cooled RCA-7102 (S-1) photomultiplier covered with a Corning 2540 glass filter. An electronically driven shutter protected the photomultiplier from the 'prompt' fluorescence during the excitation flash. The photomultiplier signal was sent to a high-gain preamplifier, followed by a Biomation 802 transient digitizer and a computer for signal averaging. Flash-induced absorbance changes were measured essentially as described previously [24], using the same excitation flashes and sample geometry as were used for the fluorescence measurements. All measurements were made at 303 K, unless otherwise indicated. Additional details are in the figure legends.

Results

Fig. 1A shows a measurement of the delayed fluorescence from a 1Q reaction center preparation. The time of the excitation flash is indicated by the arrow below the trace, and by a small electrical artifact caused by the laser. The shutter opens about 25 ms after the flash to afford a measurement of the delayed fluorescence. The delayed fluorescence decays exponentially with a (1/*e*) lifetime of about 90 ms (Fig. 2A, open circles). Its decay kinetics are similar to those of $P^+Q_A^-$, as measured by optical absorbance changes following the flash (Fig. 2A, open triangles). A small fraction of the absorbance changes decay with a lifetime of about 1 s, and probably are due to reaction centers that contain Q_B in addition to Q_A. Delayed fluorescence from these is too weak to be distinguished. The assignment of the faster decay phase of the absorbance changes to back-reactions of $P^+Q_A^-$, and the slower phase to back-reactions of $P^+Q_B^-$, has been discussed in detail previously [21,24]. *o*-Phenanthroline, which blocks electron transfer between Q_A^- and Q_B [15–18,22,25], causes a small decrease in the initial amplitudes of both the absorbance changes and the delayed fluorescence, and virtually eliminates the slow decay phase of the absorbance changes (Fig. 2A, closed symbols).

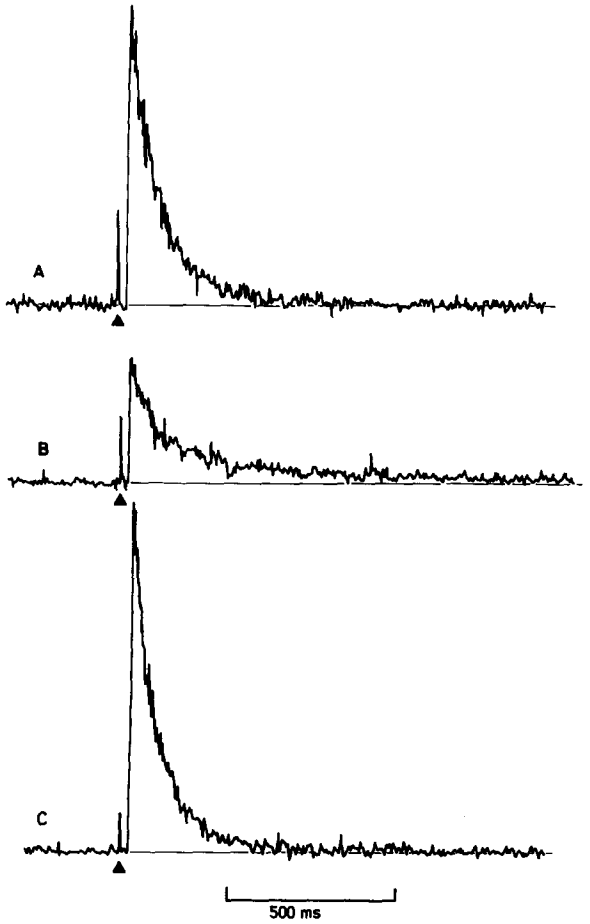

Fig. 1. Time courses of delayed fluorescence of a 1Q reaction center preparation (A) and a 2Q preparation (B), both in the absence of *o*-phenanthroline, and of the 2Q preparation in the presence of 2 mM *o*-phenanthroline (C). Reaction centers (A_{800} = 1.3) were suspended in 10 mM phosphate buffer, pH 7.8, with 100 mM NaCl and 0.1% lauryldimethylamine oxide. Intensity of the flash, 0.01 J. Signals were averaged 64 times. A blank cuvette gave no significant signal. The vertical scale is arbitrary.

Fig. 1B shows the delayed fluorescence from a 2Q reaction center preparation. The amplitude is lower than that from the 1Q preparation, and the decay kinetics are biphasic. Most of the delayed fluorescence decays with a lifetime of about 100 ms (Fig. 2B, open circles), and probably is due to contaminating 1Q reaction centers in the preparation. The

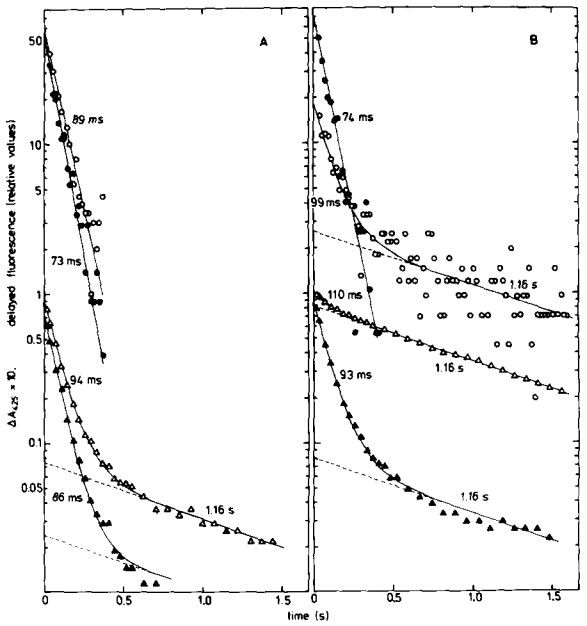

Fig. 2. Time course of delayed fluorescence (○ and ●) and absorbance change at 425 nm (△ and ▲), plotted on a semi-logarithmic scale. (A) 1Q reaction center preparation, (B) 2Q reaction center preparation. ● and ▲, 2 mM *o*-phenanthroline. Solid lines show theoretical single or double exponential decay time courses which give the least-squares fit. The numbers near the lines are the lifetimes of the two decay phases. The analyses included additional data in the time range between 0.5 and 1.5 s that are omitted from the figure for clarity. To obtain the lifetime of the slow components, we analyzed only the absorption changes with 2Q reaction center preparations. The value obtained (1.16 s) was used for the analysis of the other time courses. The experimental conditions were as in Fig. 1. Signals were averaged 64 times for delayed fluorescence and 16 times for absorbance changes.

optical absorbance changes indicate that about 18% of the reaction centers decay with the short lifetime that is characteristic of $P^+Q_A^-$ (Fig. 2B, open triangles). The largest part of the absorbance changes have the slower decay kinetics that are characteristic of $P^+Q_B^-$. A component of the delayed fluorescence with similar decay kinetics is weak, but detectable (Figs. 1B and 2B, open circles). The addition of *o*-phenanthroline greatly decreases the slow components of both the absorbance changes (Fig. 2B, closed triangles) and the delayed fluorescence (Figs. 1C and 2B, closed circles), and increases the amplitudes of the fast components.

The decay of the absorbance changes measured with 2Q reaction centers in the absence of *o*-phenanthroline was analyzed by fitting the data to a double-exponential decay expression, using a nonlinear least-squares procedure [26]. This afforded values for the initial amplitude and the lifetime of each of the two components. To analyze the delayed fluorescence from the 2Q reaction centers, we again assumed a double-exponential decay expression, but with the lifetime of the slow component fixed at the value (1.16 s) obtained from the analysis of the absorbance changes. The amplitude of the slow component of the delayed fluorescence was too small to be determined reliably without this reduction in the number of free parameters. The same restriction was used for analysis of the absorbance changes in the 1Q reaction centers and in the 2Q reaction centers treated with *o*-phenanthroline. For the delayed fluorescence from 1Q reaction centers, and from both types of reaction centers in the presence of *o*-phenanthroline, we simply used a single exponential expression. The lifetimes obtained by the curve fitting are indicated in Fig. 2, and the initial amplitudes of the various components are summarized in Table I. The table also includes the results of similar experiments with a second preparation of reaction centers. The error limits indicated in Table I are the uncertainties of the curve fitting only, and do not include sampling errors due to variations in the state of the reaction centers or the experimental apparatus.

The curve fitting generally gave slightly shorter decay times for the fast component of the delayed fluorescence than it did for the fast component of the absorbance change. In eight measurements of the delayed fluorescence under various conditions, the fast decay component had a mean lifetime of 0.081 ± 0.009 s. Parallel measurements of the absorbance changes gave a mean lifetime of 0.096 ± 0.012 s. The difference is probably not significant, and could result from a tendency of the fitting procedure to mix more of the slow component in with the fast when the amplitude of the slow component is large.

The numbers in the last two columns of Table I give the ratio of $F_d(0)$, the initial amplitude of the delayed fluorescence, to $\Delta A(0)$, the initial optical absorbance change for the corresponding decay component. For the fast component, $F_d(0)/\Delta A(0)$ should be proportional to the product $k_f K_A^*$ in Eqn. 1. The

TABLE I

INITIAL AMPLITUDES OF DELAYED FLUORESCENCE AND ABSORBANCE CHANGES FOR REACTION CENTERS WITH AND WITHOUT *o*-PHENANTHROLINE

The fast phase of the delayed fluorescence and the absorbance change is the component with a lifetime of about 0.1 s, and the slow phase the component with a lifetime of about 1 s. The experimental conditions were as in Fig. 1. The meaning of the uncertainties in the values is described in the text. n.d., not determined.

	Preparation	*o*-Phenanthroline (mM)	$\Delta A(0)$ (425 nm) ($\times 10^2$)		$F_d(0)$ (relative values)		$F_d(0)/\Delta A(0)$	
			Fast phase	Slow phase	Fast phase	Slow phase	Fast phase	Slow phase
Expt. 1	2Q reaction	–	1.8 ± 0.04	8.19 ± 0.04	16.2 ± 0.3	2.7 ± 0.2	876 ± 25	33 ± 3
	centers	1	8.62 ± 0.06	0.80 ± 0.02	78.0 ± 0.5	<1.5	905 ± 9	n.d.
	1Q reaction	–	8.53 ± 0.04	0.76 ± 0.01	60.8 ± 0.8	<1.5	713 ± 10	n.d.
	centers	2	7.08 ± 0.05	0.24 ± 0.02	55.5 ± 0.5	<1.5	784 ± 9	n.d.
Expt. 2	2Q reaction	–	3.16 ± 0.04	6.75 ± 0.04	26.3 ± 0.4	3.4 ± 0.3	832 ± 17	50 ± 5
	centers	4	7.77 ± 0.04	0.46 ± 0.01	62.9 ± 0.6	<1.5	809 ± 9	n.d.
	1Q reaction	–	9.08 ± 0.03	2.02 ± 0.03	75.1 ± 0.6	<1.5	827 ± 7	n.d.
	centers	4	8.81 ± 0.02	0.36 ± 0.01	59.0 ± 0.7	<1.5	670 ± 8	n.d.

values of $F_d(0)/\Delta A(0)$ measured in the presence of *o*-phenanthroline do not differ significantly from those measured in the absence of the inhibitor. The values for the fast component in the 2Q reaction center preparations also are probably not significantly different from those measured in the 1Q preparations. For the slow decay component, $F_d(0)/\Delta A(0)$ should be proportional to $k_f K_B^*$, where K_B^* is an equilibrium constant similar to K_A^*, but for the process $P^+Q_B^- \rightleftharpoons P^*Q_B$. A comparison of the values for the two phases allows one to estimate $K_A^B = K_A^*/K_B^*$, the equilibrium constant for the process $P^+Q_A^-Q_B \rightleftharpoons P^+Q_AQ_B^-$. Using only the data obtained with the 2Q reaction centers (Table I, rows 1 and 5) gives $K_A^B = 22 \pm 7$. A more reliable estimate can be obtained by using all of the data for the fast phase in the absence of *o*-phenanthroline (rows 1, 3, 5 and 7). This gives 20 ± 6 for K_A^B, or -78 ± 8 meV for the standard free energy change of the reaction. The uncertainties stated here include errors due to sampling, as well as to the curve fitting. Including the data obtained for the fast phase in the presence of *o*-phenanthroline would not affect the estimate of K_A^B significantly.

To determine the absolute value of K_A^*, we compared the intensity of the delayed fluorescence with that of the prompt fluorescence. From Eqn. 1, the toal amount of delayed fluorescence from 1Q reaction centers, or from 2Q reaction centers in the presence of *o*-phenanthroline is:

$$\int_0^\infty F_d(t)\,dt = k_f K_A^* \int_0^\infty [P^+Q_A^-]_0 \exp(-k_d t)\,dt$$

$$= k_f K_A^* [P^+Q_A^-]_0 / k_d \qquad (2)$$

where $F_d(t)$ is the intensity of the delayed fluorescence at time t, k_d is the decay rate constant, and $[P^+Q_A^-]_0$ is the concentration of $P^+Q_A^-$ initially produced by the flash. The ratio of delayed fluorescence to prompt fluorescence is thus:

$$\frac{\int F_d(t)\,dt}{\int F_p(t)\,dt} = \frac{(k_f/k_d)\,K_A^*[P^+Q_A^-]_0}{\phi_f \sigma \int I(t)\,dt} = \frac{k_f K_A^* \phi_A}{k_d \phi_f} \qquad (3)$$

Here $I(t)$ and $F_p(t)$ represent the instantaneous intensities of the excitation light and the prompt fluorescence, respectively; ϕ_f is the quantum yield of prompt fluorescence ($4.0 \pm 1.5 \cdot 10^{-4}$ [27]); σ is the absorption cross-section of the reaction centers at the excitation wavelength; and ϕ_A is the quantum yield of $P^+Q_A^-$ (essentially 1.0 with weak excitation [20]). k_f can be calculated from the Strickler-Berg relationship [28,29]; it is approx. $8 \cdot 10^7\ s^{-1}$ if one takes the

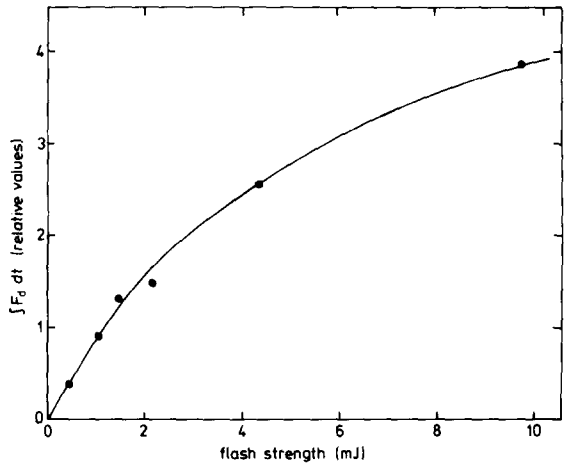

Fig. 3. Dependence of the delayed fluorescence of 2Q reaction centers on the intensity of the flash. Integrals of the delayed fluorescence were taken from a 100-times average for the point at the lowest intensity and from 36-times averages for the others. The buffer solution was as in Fig. 1. 2 mM *o*-phenanthroline. $A_{800} = 1.2$.

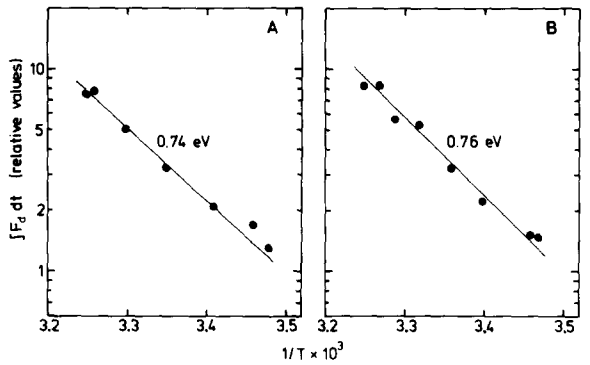

Fig. 4. Van't Hoff plot of the intensity of delayed fluorescence. (A) 2Q reaction center preparation with 2 mM *o*-phenanthroline; (B) 1Q reaction center preparation without *o*-phenanthroline. Integrals of the delayed fluorescence were taken from 36-times averages. The buffer solution was as in Fig. 1. A_{800}, 1.5 for A and 1.4 for B. Intensity of the flash, 0.01 J.

refractive index to be 1.5, and assumes that P and P* have similar partition coefficients, defined with respect to the lowest vibrational levels of the two states.

In Fig. 3, the integrated intensity of the delayed fluorescence from 2Q reaction centers in the presence of *o*-phenanthroline is plotted against the intensity of the excitation flash. In practice, we integrated the signal from 31 to 161 ms after the flash, and calculated the total integral using the value of k_d obtained from measurements with high flash intensities. The delayed fluorescence is proportional to flash intensity, as long as the intensity is less than about 1.5 mJ. We therefore compared the delayed fluorescence obtained with a 0.44 mJ flash to the prompt fluorescence obtained with a much weaker flash ($1.34 \cdot 10^{-8}$ J). (The shutter in front of the photomultiplier was simply held open for the prompt fluorescence measurements.) The prompt fluorescence was essentially the same in the presence and absence of *o*-phenanthroline. After correction for the different flash intensities, the ratio of the delayed fluorescence to the prompt fluorescence was $(7.3 \pm 3.4) \cdot 10^{-5}$. Using the values of ϕ_F, ϕ_A, and k_f quoted above, and our measured value of 12.6 s^{-1} for k_d, Eqn 3 gives $K_A^* = (4.4 \pm 2.6) \cdot 10^{-15}$. This corresponds to a value of 0.86 ± 0.02 eV for ΔG_A^* at 303 K. These values are the means and standard deviations of three independent measurements. If one takes the standard free energy of P* to be about 1.38 eV (the 0-0 transition energy) above the ground state, PQ_A, the standard free energy of P^+Q^- must be 0.52 ± 0.02 eV with respect to PQ_A.

$[P^+Q_A^-]_0$, k_f, and k_d are all essentially independent of temperature over the range 15–35°C. Eqns. 1 and 2 therefore give:

$$\ln \int_0^\infty F_d(t)\,dt = -\Delta H_A^*/kT + \text{constant} \qquad (4)$$

Plots of $\ln[\int_0^\infty F_d(t)dt]$ vs. $(1/T)$ are shown in Fig. 4 for a 2Q reaction center preparation with *o*-phenanthroline (panel A) and for a 1Q reaction center in the absence of *o*-phenanthroline (panel B). The slopes of the plots are similar for the two samples, and give a value of 0.75 ± 0.014 eV for ΔH_A^*. Taking the standard enthalpy of P* as 1.38 eV, this means that $P^+Q_A^-$ lies about 0.63 eV above PQ_A in enthalpy.

Discussion

The comparison of the integrated intensities of prompt and delayed fluorescence indicates that the free energy of $P^+Q_A^-$ is 0.86 ± 0.02 eV below that of

the excited state (P^*Q_A), and 0.52 ± 0.02 eV above that of the ground state (PQ_A). The latter value agrees well with the value of 0.50 eV calculated from the E_m values of P and Q_A in isolated reaction centers [8–10], but the agreement could be partly fortuitous. Coulombic interactions between P^+ and Q_A^- would contribute to the free energy gap estimated from the delayed fluorescence, but not the the estimate obtained from the E_m values [3,14].

The error bars on our estimate of the free energy of $P^+Q_A^-$ do not include an uncertainty in the radiative rate constant k_f, which is one of the parameters in Eqn. 3. The actual value of k_f differs from that calculated from the Strickler-Berg equation [28] by the factor Z/Z^*, where Z and Z^* are the partition functions of PQ_A and P^*Q_A, respectively, defined with respect to the lowest vibrational levels of the two states [29]. The magnitude of Z/Z^* probably depends mainly on the changes in molecular vibrational frequencies that occur when P is excited. Ross [29] has evaluated the ratio of the partition functions for the ground and excited states of 11 aromatic molecules, and has found ratios ranging from 0.2 to 3, with values between 0.3 and 1 predominating. If $Z/Z^* = 0.3$, ΔG_A^* would differ from the 0.86 eV that we calculated, by $kT\ln(0.3)$ or about −0.03 eV. The correction could be larger than this, because the excitation could affect the frequencies of intermolecular vibrations involving both of the bacteriochlorophyll molecules that make up P [30]. However, no correction is needed in the calculation of the free energy gap between $P^+Q_A^-$ and the ground state, PQ_A. That calculation involves setting the standard partial molecular free energy of P^*Q_A equal to the 0-0 transition energy. The standard energy of P^*Q_A differs from the 0-0 transition energy by $kT\ln(Z/Z^*)$, so that the correactions cancel.

From the initial intensities of the fast and slow components of the delayed fluorescence from 2Q reaction centers, the equilibrium constant for electron transfer from Q_A^- to Q_B (K_A^B) appears to be 20 ± 6. This means that the effective E_m of Q_B is 78 ± 8 mV more positive than that of Q_A. This is consistent with the value of 70 mV estimated from the decay kinetics of $P^+Q_A^-$ and $P^+Q_B^-$ [15–17].

The estimate of K_A^B based on the decay kinetics depends on the assumptions that $P^+Q_A^-$ can decay to the PQ_A ground state directly by reverse electron transfer, but that the decay of $P^+Q_B^-$ proceeds exclusively via $P^+Q_A^-$. On these assumptions, the ratio of the decay rate constants for 1Q and 2Q reaction centers is $(1 + K_A^B)$. In our experiments, the ratio of the rate constants was 12, which gives $K_A^B \simeq 11$. Because some of the decay of $P^+Q_B^-$ might proceed by a route that does not involve $P^+Q_A^-$, this approach gives only a lower limit to K_A^B [16]. Blankenship and Parson [16] have suggested that $P^+Q_B^-$ in fact decays mainly by a route that bypasses $P^+Q_A^-$. The basis for this was the observation that the decay of $P^+Q_B^-$ is not inhibited by *o*-phenanthroline. However, Wraight and Stein [15] and Vermeglio et al. [31] have argued that *o*-phenanthroline does not bind to reaction centers that are in the state $P^+Q_B^-$. They showed that it does not inhibit the transfer of a second electron to Q_B^-, when reaction centers that are in the state $PQ_AQ_B^-$ are excited by a second flash. The agreement between the estimates of K_A^B obtained from the delayed fluorescence and from the decay kinetics supports the view of Wraight and Stein [15] that most of the decay of $P^+Q_B^-$ proceeds via $P^+Q_A^-$. Other estimates of K_A^B have been obtained by measuring the photochemical activity of the reaction center on a second excitation flash, several milliseconds or more after the first [14,15]. With isolated reaction centers, this approach gives a similar value for K_A^B of about 15 [15].

An important reservation concerning our estimate of K_A^B is that the properties of $P^+Q_A^-$ were studied only in reaction centers that either lacked Q_B or had electron transfer blocked between the two quinones. It is possible that the free energy of $P^+Q_A^-$ depends on the presence of Q_B. In this regard, one must remember that the fast component of the delayed fluorescence that we measured in 2Q reaction center preparations probably was due to the small fraction of the centers that lacked Q_B. Delayed fluorescence has not yet been measured during the 100 μs lifetime of $P^+Q_A^-$ in reaction centers that contain both quinones. The same reservation applies to the estimates of K_A^B based on the decay kinetics.

The values of $F_d(0)/\Delta A$ measured in the presence of *o*-phenanthroline were not significantly different from those measured for the fast phase with either 1Q or 2Q reaction centers in the absence of the inhibitor (Table I). Apparently, *o*-phenanthroline does not greatly affect the standard free energy of $P^+Q_A^-$ relative to P^*Q_A. This agrees with the observation [10]

that *o*-phenanthroline does not alter the E_m of Q_A in isolated reaction centers, but disagrees with the calculation of Wraight and Stein [15] that the binding constant for *o*-phenanthroline is at least 2.5-times smaller when Q_A is reduced than it is when the quinone is not reduced. If the binding constants were this different, one would expect that *o*-phenanthroline would increase the value of K_A^* by at least a factor of 2.5. The significance of this discrepancy is not clear. Wraight and Stein [15] did not measure the binding constants for *o*-phenanthroline directly, but rather calculated them from the effects of the inhibitor on photochemical activity under various conditions.

From the temperature dependence of the delayed fluorescence, we conclude that $P^+Q_A^-$ lies about 0.75 eV below P^*Q_A in enthalpy, or about 0.63 eV above PQ_A (Fig. 4). The latter value is comparable to the 0.7 eV obtained by delayed fluorescence measurements on *Rps. viridis* chromatophores [19], but the comparison may not be very meaningful. First, it is a comparison between chromatophores and isolated reaction centers. As mentioned above, extraction of reaction centers from the membrane alters the pH dependence of the E_m of Q_A. Also, the E_m values of P and Q_A are both somewhat lower in *Rps. viridis* than they are in *Rps. sphaeroides* [8]. Q_A is ubiquinone in *Rps. sphaeroides* [2], but menaquinone in *Rps. viridis* [32].

The calculation of the enthalpy gap between $P^+Q_A^-$ and PQ_A involves setting the standard enthalpy of P^*Q_A equal to the 0-0 transition energy. This neglects the term $kT^2\partial \ln (Z/Z^*)\partial T$ which is probably relatively small (less than 0.03 eV). The standard enthalpy difference between P^*Q_A and $P^+Q_A^-$ is obtained directly from Fig. 4, and is not sensitive to the uncertainty concerning Z/Z^*.

Our results on the enthalpy of $P^+Q_A^-$ disagree seriously with our previous conclusions based on calorimetric measurements [18]. The calorimetric measurements indicated that the standard enthalpy of $P^+Q_A^-$ was within 0.15 eV of that of PQ_A. A possible explanation for the discrepancy is that the capacitor microphone calorimeter that we used was sensitive to volume changes due to processes other than the release of heat. We determined the amount of heat released by measuring the temperature dependence of the volume changes caused by a flash. The method depends on the fact that the thermal expansion of water is a strong function of temperature. But the formation of $P^+Q_A^-$ also involves relatively large volume changes due to intrinsic differences between the volumes of the reactant (PQ_A) and the product ($P^+Q_A^-$). These probably are caused by ordering (electrostriction) of molecules in the vicinity of the electrical charges on P^+ and Q_A^-. We assumed that these volume changes were independent of temperature, but this might not be the case.

The enthalpy and free energy gaps between P* and P^+I^- (state P^F) have been calculated from measurements of the delayed fluorescence of reaction centers in which electron transfer was blocked between I^- and Q_A [33–35]. In isolated reaction center from *Rps. sphaeroides* at 290 K, the free energy of P^+I^- appears to be about 0.25 eV below that of P* (Schenk, C.C., Blankenship, R.E. and Parson, W.W., unpublished data). The enthalpy of P^+I^- is very close to that of P*. P^+I^- appears to be about 0.03 eV above P* in enthalpy in the 250–300 K temperature region, and about 0.03 below P* in the 100–200 K region. By combining these values with the values that we have obtained for the enthalpy and free energy gaps between P* and $P^+Q_A^-$, one can estimate the gaps between P^+I^- and $P^+Q_A^-$. At temperatures near 300 K, P^+I^- is calculated to be about 0.78 above $P^+Q_A^-$ in enthalpy, and about 0.61 eV above it in free energy. Electron transfer from I^- to Q_A thus involves a substantial drop in both enthalpy and entropy ($T\Delta S$ = 0.61 – 0.78 = –0.17 eV).

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