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Radical-pair energetics and decay mechanisms in reaction centers containing anthraquinones, naphthoquinones or benzoquinones in place of ubiquinone

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In reaction centers from *Rhodobacter sphaeroides* (formerly called *Rhodopseudomonas sphaeroides*), light causes an electron-transfer reaction that forms the radical pair state (P^+I^-, or P^F) from the initial excited singlet state (P*) of a bacteriochlorophyll dimer (P). Subsequent electron transfer to a quinone (Q) produces the state P^+Q^-. Back electron transfer can regenerate P* from P^+Q^-, giving rise to 'delayed' fluorescence that decays with approximately the same lifetime as P^+Q^-. The free-energy difference between P^+Q^- and P* can be determined from the initial amplitude of the delayed fluorescence. In the present work, we extracted the native quinone (ubiquinone) from *Rps. sphaeroides* reaction centers, and replaced it by various anthraquinones, naphthoquinones, and benzoquinones. We found a rough correlation between the halfwave reduction potential ($E_{1/2}$) of the quinone used for reconstitution (as measured polarographically in dimethylformamide) and the apparent free energy of the state P^+Q^- relatively to P*. As the $E_{1/2}$ of the quinone becomes more negative, the standard free-energy gap between P^+Q^- and P* decreases. However, the correlation is quantitatively weak. Apparently, the effective midpoint potentials (E_m) of the quinones in situ depend subtly on interactions with the protein environment in the reaction center. Using the value of the E_m for ubiquinone determined in native reaction centers as a reference, and the standard free energies determined for P^+Q^- in reaction centers reconstituted with other quinones, the effective E_m values of 12 different quinones in situ are estimated. In native reaction centers, or in reaction centers reconstituted with quinones that give a standard free-energy gap of more than about 0.8 eV between P^+Q^- and P*, charge recombination from P^+Q^- to the ground state (PQ) occurs almost exclusively by a temperature-insensitive mechanism, presumably electron tunneling. When reaction centers are reconstituted with quinones that give a free-energy gap between P^+Q^- and P* of less than 0.8 eV, part or all of the decay proceeds through a thermally accessible intermediate. There is a linear relationship between the log of the rate constant for the decay of P^+Q^- via the intermediate state and the standard free energy of P^+Q^-. The higher the free energy, the faster the decay. The kinetic and thermodynamic properties of the intermediate appear not to depend strongly on the quinone used for reconstitution, indicating that the intermediate is probably not simply an activated form of P^+Q^-. It has been suggested previously that the intermediate is

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Abbreviation: LDAO, lauryldimethylamine *N*-oxide.

P^F; however, calculations of the decay kinetics of P^+Q^- in kinetic models based on this assumption are inconsistent with the thermodynamic parameters determined from fluorescence measurements. In addition, the triplet state (P^R) normally formed as a decay product of P^F could not be detected. These observations argue that the thermally accessible intermediate is not identical with the P^F state that has been characterized previously, though it could be a strongly relaxed form of this radical pair.

Introduction

In reaction centers of the photosynthetic bacterium *Rhodobacter sphaeroides* (formerly called *Rhodopseudomonas sphaeroides*), absorption of light creates an excited singlet state (P*) of a bacteriochlorophyll dimer, P. Electron transfer from P* to an initial electron-acceptor complex (I) results in the formation of a transient radical pair (P^F, or P^+I^-) in about 4 ps at 295 K [1–9]. A subsequent transfer to a quinone (Q) forms a more stable state, P^+Q^-, in about 200 ps [2,3,5,10]. If electron transfer to a secondary quinone is inhibited, P^+Q^- decays in about 100 ms at 300 K [11–15]. Because P^+Q^- is formed much faster than it decays, P* and P^+Q^- are effectively in equilibrium throughout the lifetime of P^+Q^-. One can calculate the apparent equilibrium constant between P* and P^+Q^- from measurements of the weak, 'delayed' fluorescence of P*. At 303 K and pH 7.8, the standard partial molecular free energy of P^+Q^- is about 0.86 eV below that of P*, or about 0.52 eV above that of the ground state (PQ) in both isolated reaction centers and intact photosynthetic membranes (chromatophores) [16,17]. The latter value agrees well with the value of 0.50 eV calculated from the difference between the midpoint redox potentials of P and Q, as measured by redox titrations of isolated reaction centers (0.45 and −0.05 V) [18–20]. A more negative midpoint-potential value for Q (−0.18 V) has been obtained in redox titrations of chromatophores at high pH [21]. This discrepancy may reflect a difference in the proton uptake associated with reduction of Q in reaction centers and chromatophores [22].

The quinone that is found naturally in *Rps. sphaeroides* reaction centers, ubiquinone, can be removed and replaced by different quinones such as anthraquinone [23–25]. One would expect the free energy gap between P* and P^+Q^- to be smaller in reaction centers substituted with a quinone of lower midpoint potential than ubiquinone. In the present work, we have used measurements of delayed fluorescence to evaluate the equilibrium constant between P* and P^+Q^- in reaction centers that were substituted with several types of quinones. This technique allows one to compare the effective midpoint potentials of the quinones in situ with the potentials measured polarographically in organic solvents.

At room temperature, the decay of P^+Q^- is considerably faster in anthraquinone-substituted reaction centers than it is in 'native' reaction centers containing ubiquinone, and the decay kinetics have a different temperature dependence. In native reaction centers, the decay of P^+Q^- is almost independent of pH and temperature, actually becoming somewhat slower with increasing temperature [26–28]. In anthraquinone-substituted reaction centers, the decay increases in rate with increasing pH or temperature, exhibiting an activation energy of 0.35 to 0.37 eV [29,30]. Apparently, the decay of P^+Q^- in anthraquinone-substituted reaction centers proceeds via a thermally accessible intermediate state, whereas that in native reaction centers involves more direct tunneling of an electron from Q^- to P. Similar results have been obtained with a variety of anthraquinone and naphthoquinone derivatives [25,31]. It has been suggested that the intermediate through which P^+Q^- decays in anthraquinone-substituted reaction centers is the early radical-pair state, P^F [25,29].

The present paper considers the properties of the intermediate state in the decay path of P^+Q^- and concludes that they differ significantly from the properties of P^F, as it is initially created from P*.

Methods

Reaction centers were isolated from *Rps. sphaeroides* R-26 as described [32]. To remove the

ubiquinone, the method of Okamura et al. [23] was used with minor modifications. A column of DEAE-52 sephacryl (Pharmacia) with a total volume of about 10 ml was washed with 20 ml of 10 mM Tris, 0.1% lauryldimethylamine *N*-oxide (pH 8.0) (buffer 1) containing 5 mg/ml bovine serum albumin, followed by 50 ml of buffer 1 with 0.5 M NaCl and then by another 50 ml of buffer 1. The chromatography material was then removed from the column, 0.5–1.0 μmol of reaction centers was mixed with it in a small amount of buffer 1, and the column was repoured on top of a small quantity of sephadex G-200. The temperature was then raised to 26–27°C, and 200 ml 10 mM Tris/4.0% lauryldimethylamine *N*-oxide/10 mM *o*-phenanthroline/1 mM dithiothreitol (pH 8.0) was passed through the column. Next the column was washed with 200 ml of buffer 1 and the reaction centers were eluted with 0.375 M NaCl in buffer 1. After elution, the reaction centers were dialysed against buffer 1 at 4°C and concentrated with an Amicon pressure dialysis apparatus. Approx. 90% of the reaction centers had been depleted of ubiquinone, as judged from measurements of the absorbance changes indicative of the formation of P^+Q^- after excitation with a short flash.

For reconstitution, solutions of the depleted reaction centers (typically 2–5 μM in 10 mM Tris-HCl (pH 8.0) with 0.01% LDAO) were supplemented with various quinones dissolved in methanol, to give about a 10-fold molar excess of quinone over reaction centers. The reaction centers were saturated with quinone, judging from the reappearance of the absorbance changes associated with the formation of P^+Q^- after flash excitation, and the increase in long-lived delayed fluorescence. The dissociation constants for all quinones used in this study are less than 1 μM (Gunner, M.R., Braun, B.S., Bruce, J.M. and Dutton, P.L., unpublished results). The final methanol concentration of the sample never exceeded 1%.

For fluorescence measurements, the reaction centers were placed in a quartz cuvette, which was held in a thermostatted aluminum block with windows at right angles for excitation and detection. The sample temperature was monitored with a copper-constantan thermocouple or with a thermometer. Except where indicated, all measurements were made at 303 ± 0.5 K. The excitation source for the fluorescence was a 500-ns, 600-nm pulse from a flashlamp-pumped, rhodamine-590 dye laser (Phasar DL2100A) that fired at approx. 0.1 Hz. The flash was passed through a broadband, red-absorbing filter (Schott KG3) to block any flashlamp light at the detection wavelength and through calibrated neutral density filters to adjust the intensity. Fluorescence usually was detected with an S1 photomultiplier (RCA 7102 or Hamamatsu R632) through a 20-nm band-width interference filter with a maximum transmittance at 920 nm. For detection of very weak fluorescence signals, the 920-nm filter was replaced with a red cut-off filter (Schott RG-830). Prompt fluorescence was always detected through the 920-nm interference filter to remove a small amount of 800-nm fluorescence that is not seen in the delayed fluorescence spectrum [33,34]. Fluorescence amplitudes measured through different filters were normalized using the delayed fluorescence from reaction centers reconstituted with a quinone that gave a relatively strong signal. During measurements of delayed fluorescence, the prompt fluorescence was blocked by either a rapidly rotating disk with two apertures [33] or a shutter that was driven by a solenoid [16], depending on the time scale of the measurement. The firing of the laser was synchronized with the rotation of the disk to within ±25 μs, or with the opening of the shutter to within about ±2 ms.

In most experiments, the laser intensity was monitored with a photodiode and automatically recorded on each flash through a transient digitizer (Tektronix 7912) interfaced to a computer. The delayed fluorescence signals were recorded by a second transient digitizer (Biomation 802), averaged in a home-made signal average for 25–300 flashes (depending on the signal size), and then transferred to the same computer. All fluorescence signals increased linearly with excitation energy at the laser intensities used.

The delayed fluorescence signals were analyzed by a nonlinear least-squares fitting of the data to one or two exponentials [34]. (Two-exponential fits were needed only for the data from reaction centers substituted with 2-methylanthraquinone and 2-ethylanthraquinone, to remove a small 700-μs electronic artifact. The initial amplitude of the

700-μs component was less than 20% that of the delayed fluorescence. The time constant of this component was not allowed to vary during the curve fitting.) Only data taken after the shutter had opened completely were fit. The total dead time between the laser flash and the beginning of the fit was about 1.5 ms in the case of anthraquinone, about 250 μs with the substituted anthraquinones and 2–5 ms with the substituted naphthoquinones. The initial amplitudes reported represent extrapolations back to the time of the flash.

Prompt fluorescence from native reaction centers was measured by holding the shutter open and reducing the laser intensity with calibrated neutral density filters until the signal was on the same scale as the delayed fluorescence signal from the quinone-substituted sample at the same photomultiplier voltage [16]. The trace thus obtained was integrated.

Single-photon counting measurements of the nanosecond fluorescence from reaction centers lacking quinone were made as described previously [34].

Measurements of the decay kinetics of P^+Q^- and P^R were performed essentially as described previously [35]. A weak, 30-ns, 860-nm laser pulse from a ruby laser-pumped dye laser with Eastman Kodak IR144 as the laser dye was used for excitation of the sample. The quantum yield of P^+Q^- in reaction centers reconstituted with different quinones was determined by measuring ΔA_{600} or $\Delta A_{605-540}$ following excitation with a 30-ns, 860-nm laser flash or with a 10 μs flash from a xenon flashlamp. Absorbance changes due to the small fraction of the reaction centers that still contained ubiquinone were measured before the addition of the exogenous quinone, and were subtracted. The absorbance changes were compared with the signal from native reaction centers, or from 2,3-dimethylnaphthoquinone-substituted reaction centers, which have a quantum yield equivalent to that of native reaction centers (Gunner, M.R., and Dutton, P.L., unpublished results).

The oxidation-reduction halfwave potentials ($E_{1/2}$) for the Q/Q^- couples were measured in *N,N'*-dimethylformamide (Baker Analytical Grade) containing 50 mM tetrabutylammonium tetrafluoroborate (Aldrich, crystallized from 4:1 water/methanol) as electrolyte and dried over 0.4 nm molecular sieves. Cyclic voltammetry was performed with a glassy carbon electrode using Princeton Applied Research 173 or 264A potentiostats and a saturated calomel reference electrode [36,37]. Under the conditions used here, ferrocene had $E_{1/2} = +524$ mV.

Results and Discussion

The free-energy and enthalpy differences between P^+Q^- and P, and the effective E_m of Q in situ*

We examined the delayed fluorescence from

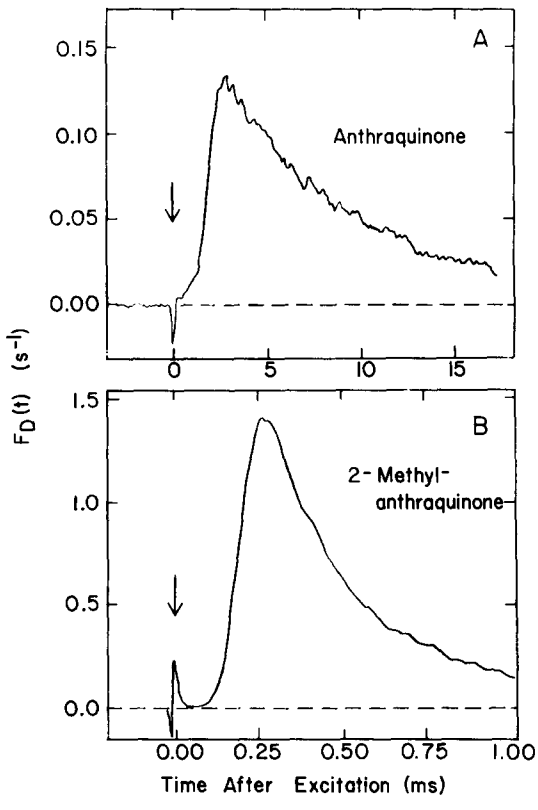


Fig. 1. Time-course of delayed fluorescence. (A) Anthraquinone-substituted reaction centers. The trace represents an average of 101 flashes. The arrow indicates the time of the flash. The temperature was 295.6 K; other conditions as described in the text. (B) 2-Methylanthraquinone-substituted reaction centers. Conditions as in (A), except that the trace represents an average of 237 flashes. The amplitude of the delayed fluorescence, $F(t)$, is expressed relative to the integrated amplitude of the (prompt) fluorescence from native reaction centers. Note that $F_D(t)$ has units of s^{-1}. Multiplying $F_D(0)$ by the decay time constant of the delayed fluorescence gives the ratio of the total yields of delayed and prompt fluorescence.

TABLE I

DECAY KINETICS AND RELATIVE FREE ENERGY OF P^+Q^- IN NATIVE REACTION CENTERS AND IN QUINONE-DEPLETED REACTION CENTERS AFTER RECONSTITUTION WITH VARIOUS QUINONES

The observed decay time τ_Q of P^+Q^- was measured either from the decay of the absorbance changes associated with the formation of P^+ or the decay of the delayed fluorescence. γ is the initial amplitude of the absorbance change associated with the formation of P^+, relative to the initial amplitude of the P^+ signal from ubiquinone-containing reaction centers. γ includes only the component of the absorbance change that decayed with the time constant given in the third column. The uncertainties of γ are approx. ± 0.2. ϕ_Q is the quantum yield of P^+Q^- formation after a single flash, measured as described in Methods. The uncertainties of ϕ_Q are approx. ± 0.1. DMF, dimethylformamide; NQ, naphthoquinone; AQ, anthraquinone.

No.	Quinone	τ_Q (ms)		γ	ϕ_Q	$F_D(0)$ (s^{-1})	K_{Q^*}	ΔG_{Q^*} (eV)	$E_{1/2}$ in DMF (V)	E_m in situ (V)
		from ΔA	from F_D							
1	duroquinone	530	> 250	0.8	0.9	$2.10 \cdot 10^{-4}$	$1.4 \cdot 10^{-15}$	0.89	−0.75	−0.02
2	6,7-dichloro NQ	209	245	0.6	0.7	$1.37 \cdot 10^{-4}$	$1.2 \cdot 10^{-15}$	0.90	−0.42	−0.01
3	2-methylthio NQ	160	100	0.9	0.9	$2.60 \cdot 10^{-4}$	$1.5 \cdot 10^{-15}$	0.89	−0.62	−0.02
4	ubiquinone	112	96	1.0	1.0	$9.01 \cdot 10^{-4}$	$4.8 \cdot 10^{-15}$	0.86	−0.60	(−0.050)
5	2,3-dimethyl NQ	120	86	0.9	1.0	$1.78 \cdot 10^{-3}$	$1.1 \cdot 10^{-14}$	0.84	−0.75	−0.07
6	5-methyl NQ	91	57	0.6	0.9	$1.03 \cdot 10^{-3}$	$9.2 \cdot 10^{-15}$	0.84	−0.64	−0.07
7	2,3,5-trimethyl NQ	71	46	0.8	1.0	$2.14 \cdot 10^{-2}$	$1.4 \cdot 10^{-13}$	0.77	−0.84	−0.14
8	5-methoxy NQ	35	36	0.6	0.9	$5.81 \cdot 10^{-2}$	$5.2 \cdot 10^{-13}$	0.74	−0.68	−0.17
9	1-chloro AQ	26	29	0.6	1.0	$4.55 \cdot 10^{-2}$	$4.0 \cdot 10^{-13}$	0.75	−0.75	−0.17
10	AQ	5.0	5.3	0.7	0.9	$3.30 \cdot 10^{-1}$	$2.5 \cdot 10^{-12}$	0.70	−0.83	−0.21
11	1-amino-5-chloro AQ	2.2	2.2	0.6	0.8	1.40	$1.2 \cdot 10^{-11}$	0.66	−0.87	−0.26
12	2-ethyl AQ	0.26	0.18	0.8	0.8	3.54	$2.4 \cdot 10^{-11}$	0.62	−0.85	−0.29
13	2-methyl AQ	0.30	0.19	0.8	0.8	4.66	$3.1 \cdot 10^{-11}$	0.61	−0.85	−0.30
14	1-amino AQ	0.052	< 0.10	–	–	–	–		−0.94	–
15	2,3-dimethyl AQ	0.040	< 0.10	0.5	0.45	–	–		−0.89	–

'native' reaction centers and from reaction centers substituted with seven different anthraquinone derivatives, six different naphthoquinone derivatives, and tetramethylbenzoquinone (duroquinone). Fig. 1 shows typical measurements for reaction centers containing anthraquinone (Fig. 1A) and 2-methylanthraquinone (Fig. 1B). The fluorescence decays exponentially with a time constant of 5.3 ms in the former case, and 0.18 ms in the latter. The decay time constants of the fluorescence from reaction centers containing these and other quinones are collected in Table I. The delayed fluorescence from reaction centers reconstituted with 2,3-dimethylanthraquinone and 1-aminoanthraquinone had decay times of less than 100 μs and could not be measured accurately because of the dead-time of the phosphoroscope's shutter.

Table I also gives the time constants for the decay of the absorbance changes associated with P^+Q^-. Unlike the delayed fluorescence, the absorbance changes in the substituted reaction centers decayed with biphasic or multiphasic kinetics. A biphasic decay was expected, because about 10% of the reaction centers still contained ubiquinone (see Methods). Additional complexity in the decay could result from heterogeneity in the orientation or position of the quinone in the reconstituted reaction centers. The time constant listed in Table I is for the major component of the absorbance signal. The factor γ that is listed gives the initial amplitude of this kinetic component, relative to the initial amplitude of the P^+Q^- signal in native, ubiquinone-containing reaction centers. Since the quantum yield of P^+Q^- in native reaction centers is essentially 1.0 [38], γ represents a product of the fraction of the reaction centers in which P^+Q^- decayed with the indicated time constant and the quantum yield (ϕ_Q) with which P^+Q^- is generated in these reaction centers. Values for the quantum yields themselves also are given in Table I.

In most cases, there is a good agreement be-

tween the decay kinetics of the delayed fluorescence and the major component of the absorbance changes. It therefore seems reasonable to assume that the fluorescence results from back reactions that regenerate P* from P^+Q^-. In several cases, the delayed fluorescence appears to decay more rapidly than the absorbance changes, but this could reflect heterogeneity in the quinone binding. As discussed below, the delayed fluorescence signals will be weighted in favor of reaction centers in which the free-energy difference between P* and P^+Q^- is smallest, and P^+Q^- will decay most rapidly in this fraction of the population.

With samples 1–13 (Table I), the initial amplitude of the delayed fluorescence was calculated by extrapolation back to the time of the flash. Table I gives the initial amplitude ($F_D(0)$), expressed relative to the integrated (prompt) fluorescence from native reaction centers containing ubiquinone in its functional (unreduced) state. In general, the initial amplitude of the delayed fluorescence increases as the halfwave redox potential of the quinone is made more negative, implying that the concentration of P* in equilibrium with P^+Q^- increases as P^+Q^- moves closer to P* in free energy. This point can be analyzed more quantitatively as follows.

The equilibrium constant for the back electron transfer reaction $P^+Q^- \rightleftarrows P^*$ is given by [16,17,32,34]:

$$K_{Q^*} = \frac{F_D(0)\,\phi_f}{\gamma k_f} \qquad (1)$$

where ϕ_f is the absolute yield of fluorescence from native reaction centers ($4.0 \cdot 10^{-4}$, see Ref. 39), k_f is the reciprocal of the natural radiative life-time of P* ($7.5 \cdot 10^7\ s^{-1}$, see Refs. 32 and 34), and the other terms are as defined above. Using Eqn. 1 and the expression $\Delta G_{Q^*} = -kT \ln K_{Q^*}$, the standard partial molecular free energy difference (ΔG_{Q^*}) for the reaction $P^+Q^- \rightleftarrows P^*$ can be calculated (Table I).

Taking the value for the midpoint potential (E_m) of ubiquinone determined by redox titrations and fluorescence measurements on native reaction centers (−0.050 V) [16–20] as a reference, one can use the following equation to calculate the apparent E_m values of the other quinones in situ:

$$E_m = -0.05\ V + \frac{\Delta G_{Q^*} - 0.86\ eV}{e} \qquad (2)$$

The term 0.86 eV is the measured standard free-energy difference (ΔG_{Q^*}) between P* and P^+Q^- for native reaction centers (Ref. 16 and Table I). The results of these calculations are shown in the last column of Table I and are indicated on the right-hand ordinate in Fig. 2. The in situ E_m values range from −0.01 V (for 6,7-dichloronaphthoquinone) to −0.30 V (for 2-methylanthraquinone).

The polarographic half-wave potentials ($E_{1/2}$) for the fifteen different quinones were measured in dimethylformamide, and are included in Table I. The $E_{1/2}$ values cannot be converted directly to E_m values for aqueous solution or for the quinone-binding site in the reaction center, in part

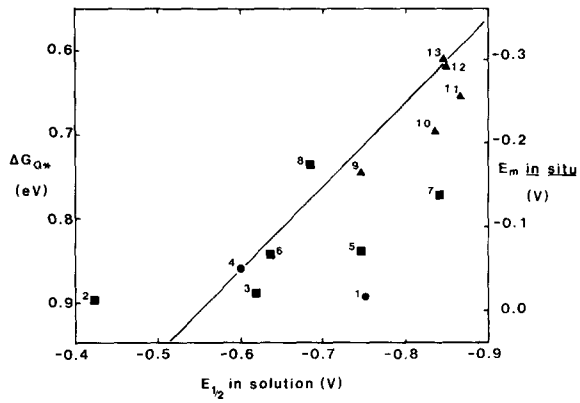


Fig. 2. Calculated standard free-energy difference between P* and P^+Q^- (ΔG_{Q^*}) in reaction centers substituted with various quinones, as a function of the polarographic half-wave potential of Q/Q^- in dimethylformamide ($E_{1/2}$). The numbers by the data points identify the quinones as listed in Table I. ΔG_{Q^*} was calculated from the initial amplitude of the delayed fluorescence as explained in the text. The right-hand ordinate gives the apparent E_m values for the quinones in situ. The uncertainties in these values are typically ±0.01 V. The line through the point for ubiquinone (sample 4) has a slope of 1 and represents the values of the in situ E_m that might be expected on the basis of the $E_{1/2}$ values, when ubiquinone is taken as a reference. For the majority of the quinones that were examined, the apparent E_m values lie below this line (at less negative potentials). However, lines with slopes differing from 1 are sometimes obtained in comparisons of $E_{1/2}$ values measured in different solvents [40]. ●, benzoquinones; ■, naphtho quinones; ▲, anthraquinones.

because they depend on the nature of the solvent (see Ref. 37). However, $E_{1/2}$ values for a series of quinones measured in two different solvents are expected to be linearly related, providing that the redox couple under consideration is unchanged (i.e., is always Q/Q^-). Jaworski et al. [40] found that, while the measured half-wave potentials of the Q/Q^- couple for a series of quinones varied with the solvent by more than 200 mV, these potentials varied in concert, and the rank ordering of the values remained unchanged. We have found similar effects when measuring in dimethylsulfoxide and chloroform, although in these experiments the differences in $E_{1/2}$ measured with respect to ferrocene varied by less than 100 mV. In no case was the rank order of the potentials altered. A similar rank ordering might also be expected if the semiquinone were allowed to protonate, for both pK values [41] and halfwave potentials [37] correlate well with the Hammett substituent constant σ.

As shown in Fig. 2, the free-energy differences (ΔG_{Q^*}) and the E_m values calculated from the delayed fluorescence are correlated only weakly with the $E_{1/2}$ values of the isolated quinones. Apparently, the E_m values in situ are sensitive to interactions of the quinones with the protein. Previous redox titrations of chromatophores have shown that the E_m of the Q/Q^- couple in ubiquinone-containing reaction centers is the same as that in menaquinone-reaction centers, when the pH is above the pK of the semiquinone [18]. The $E_{1/2}$ values for these two quinones are quite different in dimethylformamide [42].

The effective E_m of a quinone in situ depends on both the intrinsic mid-point potential of the quinone in the reaction center and the free energy of proton uptake in the state P^+Q^-. A change in the E_m thus could reflect a change in the pK of the semiquinone or a nearby amino acid sidechain. A change in pK apparently does not occur in the case of anthraquinone, however. The measured pK for proton uptake is the same in reaction centers reconstituted with anthraquinone as it is in native reaction centers [26].

Fig. 3 shows the initial amplitude of the delayed fluorescence from anthraquinone-substituted reaction centers as a function of temperature. Anthraquinone was chosen for detailed study

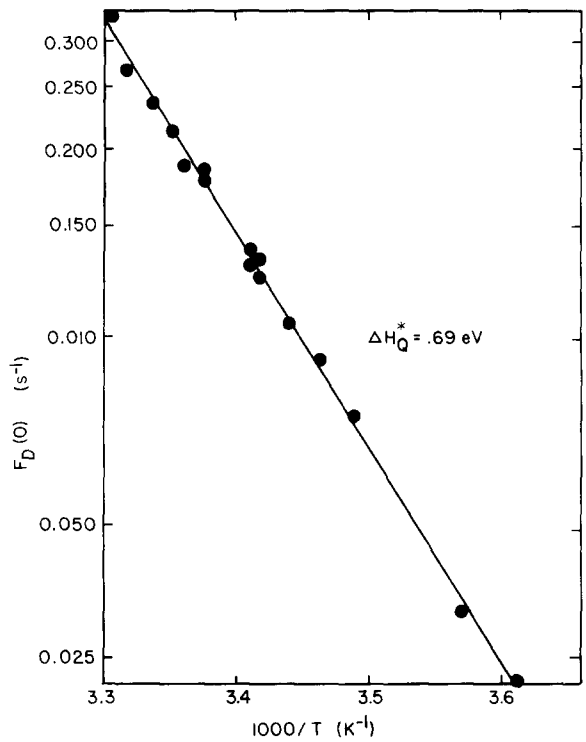


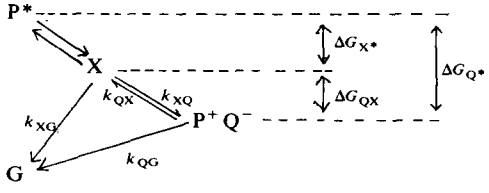
Fig. 3. Van 't Hoff plot of the initial amplitude of the delayed fluorescence ($F_D(0)$) from anthraquinone-substituted reaction centers. Conditions as in Fig. 1A except that the temperature was varied.

because it gives delayed fluorescence with a relatively long lifetime that is easily measured by using a mechanical shutter to block the prompt fluorescence. The line shown is a fit of the data to the Van 't Hoff expression $\ln F_D(0) = \ln K_{Q^*} + A = -\Delta H_{Q^*}/kT + B$, where ΔH_{Q^*} is the standard enthalpy difference between P* and P^+Q^- and A and B are constants. From the slope of the plot, P^+Q^- is calculated to lie 0.69 eV below P* in enthalpy. This is approximately equal to the calculated free-energy difference of 0.70 eV (Table I). Thus, in anthraquinone-substituted reaction centers essentially all of the free-energy change between P* and P^+Q^- appears to be due to an enthalpy decrease. A similar (though somewhat less extensive) series of measurements was performed with 2,3,5-trimethylnaphthoquinone-substituted reaction centers (data not shown). Again, the calculated enthalpy value (0.79 eV) was essentially identical to the total free energy difference

between P* and P^+Q^- in these reaction centers (0.77 ev). The results obtained with these two quinones differ from those obtained with native reaction centers, where the formation of P^+Q^- appears to involve a significant entropy change (at 295 K, $\Delta G_{Q^*} = 0.86$ eV and $\Delta H_Q = 0.75$ eV; see Ref. 16).

The thermally accessible intermediate in the decay of P^+Q^-

The return of an electron from ubiquinone to P^+ occurs by a relatively slow reaction that is essentially independent of temperature [27,28]. Gunner et al. [25] have suggested that the faster decay of P^+Q^- seen when ubiquinone is replaced by a quinone with a strongly negative E_m proceeds through a thermally accessible intermediate (X). This is shown in the following scheme (Scheme I), in which 'G' represents the ground state (PQ). The free-energy differences between P*, P^+Q^-, and the putative intermediate state X are indicated on the right side of the scheme.



Scheme I

In this scheme, the overall rate constant for the decay of P^+Q^- (k_Q) is given by

$$k_Q = k_{QX}(1-\phi_{XQ}) + k_{QG} \quad (3)$$

where ϕ_{XQ} is the probability that X returns to P^+Q^- instead of decaying to the ground state. ϕ_{XQ} can be related to the rate constants k_{XG} and k_{XQ}:

$$\phi_{XQ} = \frac{k_{XQ}}{k_{XQ} + k_{XG}}$$

or

$$k_{XQ} = \frac{k_{XG}\phi_{XQ}}{1-\phi_{XQ}} \quad (4)$$

The rate constants in turn are related by a set of equilibrium constants:

$$\frac{k_{QX}}{k_{XQ}} = K_{QX} = \frac{K_{Q^*}}{K_{X^*}} \quad (5)$$

where K_{Q^*} is the equilibrium constant for the process $P^+Q^- \rightleftarrows P^*$ (see Table I) and K_{X^*} is an equilibrium constant for $X \rightleftarrows P^*$. (This does not imply that X is necessarily an intermediate in the initial forward electron transfer between P* and P^+Q^-.) Combining Eqns. 3–5 gives

$$k_Q = \frac{K_{Q^*}}{K_{X^*}} k_{XG}\phi_{XQ} + k_{QG} \quad (6)$$

or

$$\log(k_Q - k_{QG}) = \frac{-\Delta G_{Q^*}}{2.3kT} + \frac{\Delta G_{X^*}}{2.3kT} + \log k_{XG} + \log \phi_{XQ} \quad (7)$$

If the free-energy gap between P^+Q^- and X is small enough, and if k_{XG} is large, the decay through X will predominate over the direct reaction from P^+Q^- to P, so the term k_{QG} in Eqn. 7 will be neglibile compared to k_Q.

In Fig. 4, the measured values of log k_Q are plotted as a function of ΔG_{Q^*} for the first 13 quinones in Table I. There is a linear relationship between log k_Q and ΔG_{Q^*} when ΔG_{Q^*} is less than about 0.75 eV. Referring to Eqn. 7, this suggests that ΔG_{X^*} and log k_{XG} do not depend strongly on the nature of the quinone, which implies that X is probably not simply an activated form of P^+Q^-. The nature of X will be discussed in more detail below. Log ϕ_{XQ} presumably does depend on the quinone, but this term will be negligible if $\phi_{XQ} \approx 1.0$. At values of ΔG_{Q^*} above about 0.75 eV, the linear relationship breaks down, presumably because the decay via X becomes slower than direct charge recombination between P^+ and Q^- (k_{QG} in scheme I). The six quinones for which G_{Q^*} exceeds 0.80 eV give decay rate constants that range from 1.9 s^{-1} to 18 s^{-1}. P^+Q^- probably decays mainly by the direct route (k_{QG}) in all of these cases, because the observed rate constants are more than 10-times the extrapolated rate constant for decay via the thermally accessible intermediate (Fig. 4, dashed line). The scatter in the values of the decay rate constants at large ΔG_{Q^*} might be expected,

considering the strong dependence of electron tunnelling on distance and orientation. As noted above in connection with the effective E_m values, different quinones may fit differently into the quinone-binding site.

With 2,3,5-trimethylnaphthoquinone, 1-chloroanthraquinone, and 5-methoxynaphthoquinone, which have intermediate values of ΔG_{Q^*}, the decay of P^+Q^- probably proceeds partially via the thermal intermediate and partially by the direct pathway. If the rate constants of the direct reaction (k_{QG}) were known for these quinones, subtracting them from the observed k_Q would allow a more extensive analysis of how the thermally activated decay depends on ΔG_{Q^*}. Since k_{QG} evidently depends on the nature of Q, such a correction of k_Q cannot be made rigorously. The downward vertical bars for points 7, 8 and 9 in Fig. 4 were obtained by subtracting the largest rate constant obtained with the six quinones that have $\Delta G_{Q^*} >$ 0.80 eV (18 s^{-1}). This seems likely to give a lower limit for the decay via the thermal intermediate. The filled symbols for points 7–9 show the uncorrected values of k_Q, and are therefore upper limits. The correction for k_{QG} is negligible for points 10–13.

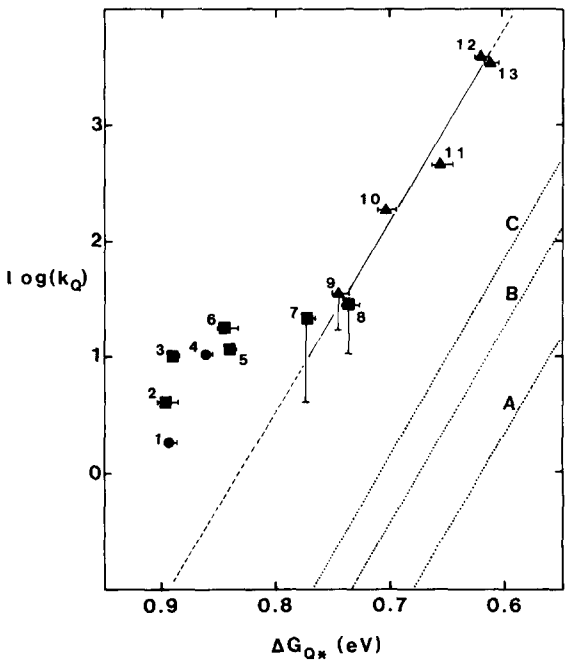


Fig. 4. Log of the observed rate constant for the decay of P^+Q^-, as a function of the standard free energy difference between P^+Q^- and P*, ΔG_{Q^*}. Sample numbers correspond to those in Table I. For samples 1, 12 and 33, k_Q was taken from the decay of the absorbance changes associated with P^+Q^- because the delayed fluorescence was too weak for the decay time to be determined accurately (sample 1), or because a small 700-μs electronic artifact complicated the fitting of the delayed fluorescence (samples 12 and 13). For all other samples k_Q was taken from the delayed fluorescence measurements. The horizontal bars shown indicate an estimate of the uncertainty in the calculation of ΔG_{Q^*}. (The measurement of γ is probably the largest source of uncertainty.) The vertical bars shown for samples 7, 8 and 9 are described in the text. Values and equations used to calculate the dotted lines are as follows: (A) Eqn. 7 with $\Delta G_{X^*} = 0.17$ eV, $k_{XG} = 5 \cdot 10^7$ s^{-1}, $\phi_{XQ} = 0.9$ and $k_{QG} = 0$; (B) same as (A), except $\Delta G_{X^*} = 0.22$ eV; (C) Eqn. 12 with $k_{1D} = 5 \cdot 10^7$ s^{-1}, $\Delta G_{F^*} = 0.22$ eV, $\phi_Q = 0.90$, $z = 0.5$ and $k_{QG} = 0$. ●, benzoquinones; ■, naphthoquinones; ▲, anthraquinones.

Earlier studies has suggested that there was an approximately linear relationship between log k_Q and the $E_{1/2}$ of the quinone, as determined in dimethylformamide [25]. However, the quantitative correlation between log k_Q and $E_{1/2}$ was poor. For example, 2,3-dimethylnaphthoquinone has an $E_{1/2}$ similar to that of anthraquinone in DMF, and 2,3,5-trimethylnaphthoquinone has an $E_{1/2}$ similar to that of 2-methyl-anthraquinone, yet the two naphthoquinones decay at least partially by direct charge recombination, whereas the two anthraquinones appear to decay almost exclusively via the thermal intermediate. However, when log k_Q is compared to ΔG_{Q^*} (Fig. 4) or to the apparent E_m of the quinone in situ, these inconsistencies disappear. Apparently, k_Q provides a reliable index for the E_m in situ when P^+Q^- decays primarily via the thermally accessible state. The three dotted lines in Fig. 4 will be discussed below.

The temperature dependence of the decay kinetics of the delayed fluorescence from anthraquinone-substituted reaction centers is shown in Fig. 5. The line shown is a fit of the data above 283 K to the Arrhenius expression $\ln k_Q = -\Delta H/kT + \ln A$, where ΔH is the activation enthalpy for the decay of P^+Q^- via X, and A is a constant. The Arrhenius plot is nearly linear, but shows a small deviation from linearity near 275 K. At this temperature, there is evidently another route for the decay of P^+Q^-. This presumably reflects the increasing contribution of the direct, temperature independent decay route as the tem-

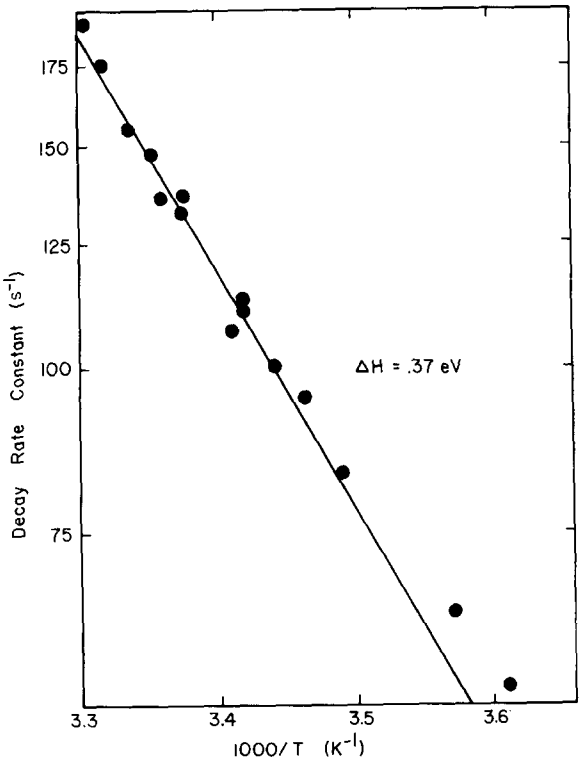


Fig. 5. Arrhenius plot of the decay rate constant of the delayed fluorescence from anthraquinone-substituted reaction centers. Conditions as in Fig. 1A except that the temperature was varied. The line is a fit to the Arrhenius expression, neglecting the two data points at the lowest temperatures. The enthalpy change indicated is the apparent activation energy for the back reaction forming PQ from P^+Q^-.

perature is decreased. For comparison we measured the decay kinetics of P^+ absorbance changes as a function of temperature between 275 and 295 K (data not shown). At temperatures above 283 K, approx. 90% of the absorbance change decayed with a time constant that matched that of the delayed fluorescence. The remainder had a lifetime of about 100 ms, and presumably reflected reaction centers that still contained ubiquinone instead of anthraquinone. Analysis of the kinetics becomes difficult below 283 K because the decay of P^+Q^- in the anthraquinone-substituted reaction centers slows down, whereas that in native reaction centers speeds up with decreasing temperature [27–30].

Above 283 K, the temperature dependence of the delayed fluorescence decay time in anthraquinone-substituted reaction centers gives an apparent activation enthalpy, ΔH, of 0.37 eV (Fig. 5), which agrees well with the value measured spectrophotometrically [29,30]. In Scheme I, ΔH can be equated to the enthalpy difference between P^+Q^- and X, provided that $(k_{XG}\phi_{XQ})$ is relatively independent of temperature (Eqns. 6 and 7). Note that ΔH is much smaller than the calculated enthalpy gap of 0.69 eV between P^+Q^- and P* (Fig. 3). The thermally accessible intermediate state that participates in the decay of P^+Q^- in anthraquinone-substituted reaction centers thus appears to lie about 0.32 eV below P* in enthalpy.

Is the thermally accessible intermediate P^F?

Gunner et al. originally suggested that the thermally accessible decay path of P^+Q^- in anthraquinone-substituted reaction centers involved back electron transfer to the early radical-pair state, P^F [25,44]. The standard free-energy difference for the reaction $P^F \rightleftarrows P^*$ has been calculated from measurements of the delayed fluorescence from reaction centers in which electron transfer between I^- and Q is blocked by reducing the quinone chemically. It is about 0.17 eV at 295 K [32,34]. If P^F and X are equivalent, one should be able to set ΔG_{X^*} equal to 0.17 eV in Eqn. 7. As a first approximation, one also might replace the rate constant k_{XG} by the measured rate constant for the decay of P^F in reaction centers that are depleted of quinone (about $5 \cdot 10^7\ s^{-1}$; Refs. 32 and 43). (The decay rate constant is slightly greater in reaction centers that contain Q^- [32,47]. One would not expect it to depend strongly on the nature of the quinone if Q is present in the unreduced state.) ϕ_{XQ} can be set equal to the measured quantum yield of P^+Q^-, which is about 0.9 for most of the quinones that we studied (Table I). The dotted line labeled A in Fig. 4 shows log k_Q as calculated with Eqn. 7 using these values of ΔG_{X^*}, $k_{XG}\phi_{XQ}$ and with $k_{QG} = 0$. The calculated values of k_Q are smaller than the experimental values by a factor of about 2500.

The free-energy difference between P* and P^F reported previously [34] was determined with reaction centers in which the quinone had been chemically reduced. This free-energy difference could be less than it is in reaction centers with unreduced quinones or in reaction centers that lack

TABLE II

NANOSECOND FLUORESCENCE FROM REDUCED AND QUINONE-DEPLETED REACTION CENTERS

The total 'prompt' fluorescence, A, is given relative to that from unreduced native reaction centers. Prompt fluorescence includes any emission which occurs too rapidly for the single-photon counting apparatus to resolve. B_1, B_2 and B_3 are the initial amplitudes of the three kinetic components of the delayed fluorescence. The total fluorescence from component i is given by $B_i\tau_i$. τ_1, τ_2 and τ_3 are the time constants for the decay of the three kinetic components of the delayed fluorescence. χ^2 is the reduced χ^2 obtained after the nonlinear-least-squares fit which resulted in the parameters A, B_i and τ_i. ΔG_{F^*} is the initial standard free-energy difference between P^F and P^*. Data for reduced reaction centers are from Ref. 34.

	Magnetic field	A	Amplitudes (ns^{-1})			Time constants (ns)			χ^2	ΔG_{F^*} (eV)	
			B_1	B_2	B_3	τ_1	τ_2	τ_3			
Quinone-depleted reaction centers	−	1.10	0.45	0.091	0.022	0.91	3.1	12.5	1.8	0.148 [a]	0.188 [b]
	+	1.12	0.43	0.090	0.021	0.94	3.3	15.0	2.0	0.149 [a]	0.189 [b]
Reduced reaction centers	−	1.21	0.14	0.10	0.049	0.98	3.8	10.2	1.2	0.168 [a]	
	+	1.22	0.14	0.10	0.046	1.02	4.1	11.6	1.1	0.169 [a]	

[a] Calculated from the total initial amplitude of delayed fluorescence ($B_1 + B_2 + B_3$).
[b] Calculated from the initial amplitudes of the two slowest components of delayed fluorescence ($B_2 + B_3$).

quinone, because the negatively charged quinone in reduced reaction centers could make electron transfer from P to I less favorable. Potentially, such an effect could explain the discrepancy between the measured and calculated kinetic parameters shown in Fig. 4.

To evaluate this possibility, we measured the nanosecond delayed fluorescence from reaction centers lacking quinones. The results are shown in Table II, along with previously reported measurements [34] of the fluorescence from reaction centers with the quinone reduced. In both cases, the fluorescence decays with multiphasic kinetics. The initial amplitude of the early component of the delayed fluorescence is larger in reaction centers lacking quinone than it is in reduced reaction centers: the amplitude of the middle component is about the same in both samples; and the amplitude of the slow component is lower in the reaction centers lacking quinone. The total initial amplitude of delayed fluorescence in reaction centers lacking quinone is greater than that in reduced reaction centers. This suggests that the free energy gap between P^F and P^* is smaller in reaction centers lacking quinone than in reduced reaction centers, opposite what would be required to explain the discrepancy noted above. However, it is possible that the increased initial amplitude of the early component of the delayed fluorescence in reaction centers lacking quinone is due to fluorescence from contaminating pigments in the sample. Such contaminants could result from the treatment that we used to remove the quinone (see Methods). This possibility is strengthened by the observation that decreasing the temperature to 200 K causes the early component's initial amplitude to decrease somewhat (data not shown). With reduced reaction centers, the initial amplitudes of all three delayed fluorescence components increase when the temperature is lowered from 295 K to 200 K and then decrease at lower temperatures [34]. The initial amplitudes and decay time constants of the middle and long components of the delayed fluorescence from reaction centers lacking quinones have a temperature dependence similar to that seen in reduced reaction centers (data not shown).

If we ignore the fast component entirely, we can calculate a minimum value for the total initial amplitude of the delayed fluorescence from the two slower components. Using this gives a maximum value of 0.188 eV for the initial drop in free energy between P^* and P^F. This compares with the value of 0.17 eV used above. Although the

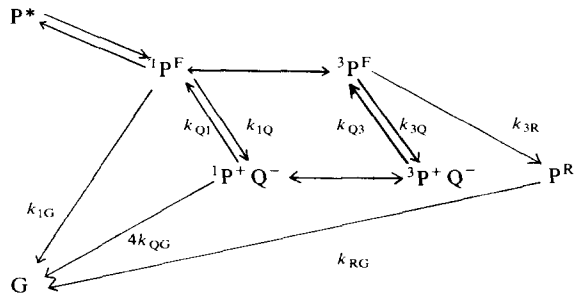
free-energy difference between P* and P^F may be somewhat larger in reaction centers lacking quinone than in reduced reaction centers, the effect is much too small to remove the discrepancy shown in Fig. 4.

An estimate of the enthalpy difference between P* and P^F can be obtained by measuring the temperature dependence of the initial amplitude of the delayed fluorescence from reaction centers in which electron transfer is blocked between P^F and P^+Q^- [32,34]. The initial amplitude of the delayed fluorescence from P^F has a complex temperature dependence between 200 and 295 K, but at lower temperature fits a linear Van 't Hoff plot with an apparent ΔH of 0.015 eV. Again, this is substantially less than the value of 0.32 eV obtained above for the enthalpy difference between P* and X.

These discrepancies could mean that the decay of P^+Q^- in the substituted reaction centers proceeds not via P^F, but via another state that lies farther below P* in free energy and enthalpy. Another possibility is that the form of P^F that is regenerated from P^+Q^- is significantly different from the form that is created initially from P*. For example, a decrease in the free energy of the system could result from nuclear movements that follow the formation of P^+Q^-. The multiphasic decay kinetics of delayed fluorescence from blocked reaction centers [34,45] suggests that such relaxations occur on the time scale of 0.5–5 ns after the formation of P^F, although these relaxations appear to involve a total free energy change of only about 0.05 eV. Increasing ΔG_{X*} by this amount, to 0.22 eV, and keeping the other parameters constant still leaves a large discrepancy between the observed and calculated values of k_Q (Fig. 4, dotted line B). It is possible, however, that reaction centers undergo further relaxations on the micro- or millisecond time scale after the formation of P^+Q^- [46].

Expansion of Scheme I to include triplet states

One shortcoming of the simple model in Scheme I is that it does not take into account the effects of electron spin rephasing in P^F and P^+Q^-. When P^F is formed from the excited singlet state P*, it must initially be entirely singlet in character. (The spins of the unpaired electrons on P^+ and I^- must be antiparallel.) This form of P^F($^1P^F$) can decay by at least three pathways. It can transfer an electron to the quinone, forming P^+Q^-; it can decay by direct charge recombination to the ground state P; or it can undergo spin rephasing to form a radical pair with triplet character ($^3P^F$), which then undergoes charge recombination to produce a long-lived triplet state, P^R (Scheme II) [32,47–49]. The decay of $^3P^F$ to P^R appears to occur rapidly, relative to the spin rephasing that mixes $^1P^F$ and $^3P^F$ [49–54]. There may also be an additional route for the decay of $^3P^F$ that does not involve P^R [32,43].



Scheme II

If photochemical electron transfer to Q is blocked, the decay of $^1P^F$ to P occurs more rapidly than spin rephasing, so that the quantum yield of P^R is only 10–20% at 295 K [32,47–54]. In contrast, the lifetime of P^+Q^- is long compared to the spin-lattice relaxation times of P^+ and Q^-. The spin states of Q^- relax particularly rapidly by interactions with the nearby non-heme Fe atom [55]. The spin-lattice relaxation time is about 4 μs at 4 K, and is probably much shorter than this at 300 K. Complete spin randomization therefore should occur rapidly, relative to the decay of P^+Q^-. After spin equilibration, about 1/4 of P^+Q^- will be in the singlet state, $^1[P^+Q^-]$, and 3/4 in a triplet state, $^3[P^+Q^-]$. (This spin equilibration occurs faster than electron transfer between Q and the next electron acceptor (Q_B), and thus provides about 0.04 eV (kT ln 4) of stabilization after the initial formation of $^1[P^+Q^-]$.) If P^+Q^- then undergoes back reactions that regenerate P^F, approx. 3/4 of the back reactions would form $^3P^F$, which should rapidly either reform $^3[P^+Q^-]$ or decay to P^R. The other 1/4 of the

back reactions would generate $^1P^F$, which will decay partly back to $^1[P^+Q^-]$, partly to P, and partly to P^R via $^3P^F$.

The overall rate constant for the decay of P^+Q^- in Scheme II is

$$k_Q = 0.25\, k_{Q1}(1-\phi_{1Q}) + 0.75\, k_{Q3}(1-\phi_{3Q}) + k_{QG} \qquad (8)$$

where ϕ_{1Q} and ϕ_{3Q} are the probabilities that $^1P^F$ and $^3P^F$, respectively, regenerate P^+Q^-. (The macroscopic rate constant k_{QG} is multiplied by 4 in Scheme II, but not in Eqn. 8, in accord with the assumption that 1/4 of the P^+Q^- is in the singlet state.) To a reasonable approximation, we can set $k_{Q3} = k_{Q1} = k_{QF}$ and $k_{3Q} = k_{1Q} = k_{FQ}$. (The rate of electron transfer from I^- to Q or from Q^- back to I should not be highly sensitive to the spin state of P^+, because the exchange interactions of P^+ with I^- or Q^- are relatively weak [56,57].) The rate constants k_{QF} and k_{FQ} can be related by an equilibrium constant K_{QF}:

$$k_{QF} = K_{QF} k_{FQ} \qquad (9)$$

The yield ϕ_{1Q} should be approximately equal to the measured quantum yield of P^+Q^-, ϕ_Q. This is related to k_{FQ} by

$$\phi_Q \approx \frac{k_{FQ}}{k_{FQ}+k_{1D}} \text{ or } k_{FQ} \approx \frac{k_{1D}\phi_Q}{1-\phi_Q} \qquad (10)$$

where k_{1D} is the overall rate constant for the decay of $^1P^F$ by routes other than the formation of P^+Q^-. As before, we assume that k_{1D} is approximately $5 \cdot 10^7\ s^{-1}$, the measured rate constant for the decay of P^F in reaction centers that lack Q. The yield ϕ_{3Q} is smaller than ϕ_{1Q}, because k_{3R} is about 10-times greater than k_{1G} [57]. Let $\phi_{3Q} = z\phi_{1Q}$ with $0 < z < 1$. With these substitutions in Eqn. 8, we have:

$$k_Q \approx K_{QF} k_{1D} \phi_Q \frac{1-(0.25+0.75\,z)\phi_Q}{1-\phi_Q} + k_{QG} \qquad (11)$$

$$\log(k_Q - k_{QG}) \approx \frac{-\Delta G_{Q^*}}{2.3kT} + \frac{\Delta G_{F^*}}{2.3kT} + \log k_{1D} + \log\left\{\phi_Q \frac{1-(0.25+0.75z)\phi_Q}{1-\phi_Q}\right\} \qquad (12)$$

If $\phi_{1Q} = \phi_Q = 0.90$, Eqn. 10 gives $k_{FQ} = 9\ k_{1D} \approx 5 \cdot 10^8\ s^{-1}$. Taking $k_{3R} \approx 10 k_{1D}$ [55] gives $z \approx 0.5$. With these values of ϕ_{1Q} and z, about 94% of the decay of P^+Q^- would proceed via $^3P^F$, and about 6% via $^1P^F$. If ϕ_{1Q} is increased to 0.99, z becomes approx. 0.9, and about 97% of the decay proceeds via $^3P^F$.

Line C in Fig. 4 was calculated using Eqn. 12 with $k_{1D} = 5 \cdot 10^7\ s^{-1}$ and $\Delta G_{F^*} = 0.22$ eV (as for line B), and with $\phi_Q = 0.90$, $z = 0.5$, and $k_{QG} = 0$. The last term in Eqn. 12 makes a contribution of 0.60 to log k_Q, but the calculated values of k_Q are still significantly smaller than the observed values.

The discrepancy between the observed and calculated k_Q could be decreased by using a larger value for ϕ_Q. If $\phi_Q = 0.99$ (approximately the yield found in native reaction centers) and $z = 0.9$, the last term in Eqn. 9 would contribute 0.92 to log k_Q. However, the calculated values of k_Q would still be smaller than the observed values by about a factor of 40. In addition, a ϕ_Q of 0.99 would require a forward rate constant k_{FQ} of $5 \cdot 10^9\ s^{-1}$, which is about 5-times greater than the rate constant that has been measured in anthraquinone-containing reaction centers [58].

The treatment that we have used for Scheme II assumes that the equilibration of $^1[P^+Q^-]$ and $^3[P^+Q^-]$ is fast, relative to the back reactions that regenerate P^F. This assumption could break down if k_{QF} becomes large, because each time $^1[P^+Q^-]$ returns to $^1P^F$ the spin rephasing must start over. However, if $\phi_Q \approx 0.9$, the back reactions must occur only about 10-times as rapidly as the observed decay of P^+Q^-, or at about $5 \cdot 10^4\ s^{-1}$ for 2-methylanthraquinone and more slowly for the other quinones in Fig. 4. This is too slow to compete with the spin rephasing of $^1[P^+Q^-]$ and $^3[P^+Q^-]$.

Is P^R formed during the decay of P^+Q^-?

According to Scheme II, many of the back reactions that regenerate P^F from P^+Q^- should result in the formation of P^R. Any P^R that is formed during the decay of P^+Q^- will augment the P^R formed initially in competition with the first relaxation of P^F to P^+Q^-. This would lengthen the apparent lifetime of the triplet state and make the overall decay multiexponential. The decay of p^R to the ground state P in reaction centers lacking quinone has a time constant of 20–30 μs. Because of this short lifetime, one would

not expect to observe the formation of P^R in significant amounts after flash excitation of anthraquinone-substituted reaction centers, in which P^+Q^- decays with a time constant of about 8 ms (Table I). The steady-state concentration of P^R would be, at most, 0.5% that of P^+Q^-. However, in reaction centers that have been reconstituted with 2,3-dimethylanthraquinone, P^+Q^- decays in about 40 μs. Here, one would expect P^R to be created in a detectable amount if P^+Q^- decays primarily through P^F, provided that the decay of P^R in the substituted reaction centers is not substantially faster than the P^R decay in reaction centers lacking quinone (see below). We have attempted to observe this effect.

Fig. 6A shows the decay of the absorbance change associated with P^+ in 2,3-dimethylanthraquinone-substituted reaction centers. These measurements were made at 600 nm, where absorbance changes due to P^R are negligible [35]. A nonlinear least squares fit of the data to a single exponential plus a constant gives a decay time of 39 μs. The constant term is needed to accommodate the small amount of native (ubiquinone-containing) reaction centers remaining in the sample. (The 100-ms decay of P^+Q^- in the native reaction centers is slow enough to be unimportant.) The quantum yield of P^+Q^- was measured in 2,3-dimethylanthraquinone-substituted reaction centers, by comparing the initial absorbance change at 600 nm with that exhibited by native reaction centers. Weak excitation flashes at 860 nm were used. The quantum yield was 0.45 (Table I).

The decay kinetics of P^R were measured at 680 nm in quinone-depleted reaction centers before and after addition of 2,3-dimethylanthraquinone (Fig. 6B). (Absorbance changes due to P^+ are negligible at this wavelength. The P^+ signal at 680 nm in native reaction centers was less than 8% of the P^R signal in 2,3-dimethylanthraquinone-substituted reaction centers.) When the reaction centers were reconstituted with 2,3-dimethylanthraquinone, the initial amplitude of the P^R signal dropped to 33% of that from reaction centers lacking quinone (Fig. 6B) *. After normalization

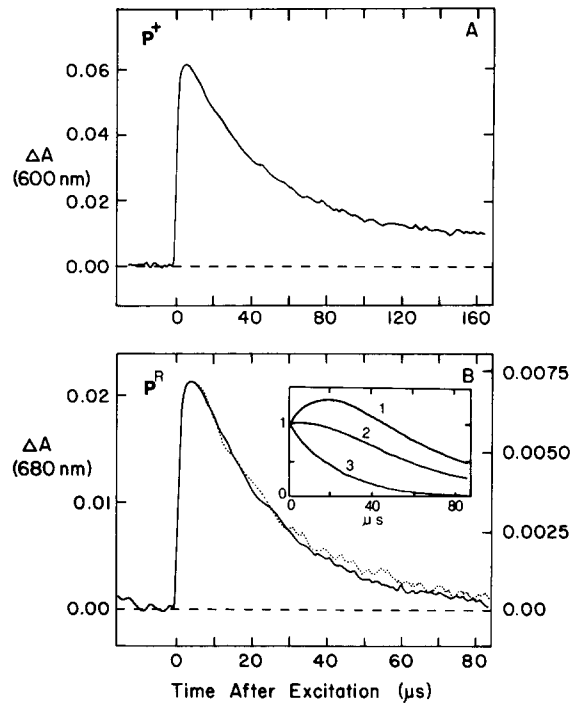


Fig. 6. (A) Time-course of P^+Q^- decay in 2,3-dimethylanthraquinone-substituted reaction centers, monitored by the absorbance change at 600 nm. 2,3-Dimethylanthraquinone (approx. 30 μM) was added to quinone-depleted reaction centers (about 2.7 μM) in 50 mM Tris (pH 8). The temperature was approx. 293 K. This trace represents an average of three flashes. (B) The time-course of P^R decay, monitored by the absorbance change at 680 nm. Sample and conditions as in (A). The solid line represents an average of 30 flashes using quinone-depleted reaction centers and corresponds to the left hand ordinate. The dotted line represents an average of 79 flashes using the same sample of reaction centers reconstituted with 2,3-dimethylanthraquinone and corresponds to the right hand ordinate. The two traces were normalized by their average amplitudes between 6 and 11 μs after excitation. Inset, theoretical curves showing the relative concentrations of P^R as a function of time ($[P^R](t)/[P^R](0)$) if 90% (curve 1), 50% (curve 2) or none (curve 3) of P^+Q^- decays via P^R. These curves were generated as described in ref. 59. They assume the model shown in scheme II and a delta function excitation pulse.

* Because saturating excitation flashes were used, this amplitude does not accurately reflect the relative quantum yield of P^R. A saturating flash allows recycling of reaction centers that decay to the ground state via charge recombination in P^F, resulting in optical pumping of the longer-lived state P^R, while reaction centers that form P^+Q^- become unavailable for recycling. When the flash intensity was reduced by a factor of 10, the initial quantum yield of P^R after reconstitution increased to about 50% of that before reconstitution. This is in good agreement with the expected 55%, calculated as one minus the quantum yield of P^+Q^- in 2,3-dimethylanthraquinone-substituted reaction centers (45%).

between 6 and 11 μs, the P^R signals measured before and after addition of the quinone are almost superimposable (Fig. 6B). Least-squares fits of the data to a single exponential between 6 and 75 μs after excitation give decay times of 23 μs for reaction centers lacking quinone and 25 μs for 2,3-dimethylanthraquinone-substituted reaction centers. Qualitatively, there is no evidence that P^R is formed in significant amounts during the decay of P^+Q^-. For comparison, the inset in Fig. 6B shows the kinetic traces expected for the decay of P^R if 90%, 50% or none of the P^+Q^- decay results in P^R formation.

If P^+Q^- decays partly via P^R, the P^R signal should include a component with a time constant of 39 μs, the time constant measured for the decay of P^+Q^- (Fig. 6A). We fit the data of Fig. 6B to a double-exponential expression including a component with this time constant, and estimated the fraction of the decay that proceeds through P^R from the amplitudes of the two components [59]. The result was that less than 5% of the decay of P^+Q^- proceeds via P^R.

A major assumption in this analysis is that reconstituting the reaction centers with 2,3-dimethylanthraquinone does not greatly alter either k_{3R} or k_{RG} in Scheme II. If the presence of the quinone accelerated the decay of P^R, it could prevent detection of P^R during the decay of P^+Q^-. One might expect the quinone to influence the decay kinetics of P^R, because the triplet state appears to decay largely through P^F, where spin rephasing and the paths to the ground state, to P^+Q^-, and back to P^R are all interrelated (Ref. 60, see also Goldstein, R. and Boxer, S., personal communication). Although the measured decay kinetics are essentially the same before and after the addition of the quinone (Fig. 6B), it is possible that the P^R that is seen represents reaction centers that have bound the quinone in a nonfunctional configuration. Thus, we cannot state definitively that no P^R is formed during the decay of P^+Q^-, but only that no P^R was detected.

Conclusions

It seems clear that there are at least two mechanisms for the decay of P^+Q^- in quinone-substituted reaction centers, and that the path used depends on the effective midpoint potential of the quinone in situ. In reaction centers containing quinones with E_m values near that of ubiquinone, the decay of P^+Q^- apparently proceeds via direct charge recombination. When quinones with substantially more negative midpoint potentials are used, decay proceeds via a thermally accessible intermediate (X in Scheme I). The experiments described here suggest that the intermediate state is not identical with the state P^F that has been described on the nanosecond time scale, nor is it a state, such as an activated form of P^+Q^-, that depends strongly on the nature of the quinone.

Perhaps the simplest interpretation of these observations is that X is a highly relaxed form of P^F. The increase in total fluorescence upon reduction of Q in *Rhodopseudomonas viridis* reaction centers [61] is similar to that in *Rhodobacter sphaeroides* [32,34], suggesting that the initial free energy difference between P* and P^F is approximately the same in the two organisms (about 0.17 eV). However, the difference between the steady state midpoint potentials of P and the initial electron acceptor (I), as determined by redox titrations of *R. viridis* reaction centers, is about 0.90 V [62]. Combining this number with the 0–0 transition energy of P* (1.24 eV in *R. viridis*) gives a free-energy difference of about 0.34 V between P* and P^F. This suggests that the reaction center may undergo a relaxation that decreases the free energy of P^F by about 0.17 eV sometime after the initial charge separation. Other evidence for the occurrence of relaxations of P^F and P^+Q^- in *R. sphaeroides* reaction centers has been presented recently [34,45,46]. If the free energy of the relaxed form of P^F is below that of P^R, one would not expect P^R to be formed in measurable amounts during the decay of P^+Q^-.

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