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Charge recombination from the $P^+Q_A^-$ state in reaction centers from *Rhodopseudomonas viridis*

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The rate of decay of the flash-oxidized primary electron donor, P^+, from the state $P^+Q_A^-$ was studied in reaction centers from *Rhodopseudomonas viridis*, containing only the primary menaquinone electron acceptor (Q_A). At 295 K, in 100 mM NaCl and in the presence of *o*-phenanthroline, the rate of recombination was 470 ± 15 s^{-1} at pH 7 and 570 ± 20 s^{-1} at pH 9. The rate at ambient temperatures varied somewhat with viscosity, pH and ionic strength. Between 310 K and 275 K, the temperature dependences of the rate, at pH 7 and pH 9, were linear in an Arrhenius plot, with apparent activation energies of 0.20 eV and 0.16 eV, respectively. At lower temperatures, however, the dependences deviated from this behavior. In 60% glycerol (pH 7) the recombination rate was 370 ± 10 s^{-1} at 295 K. As the temperature was lowered, the rate decreased but leveled off to a value of 105 ± 5 s^{-1} at 170 K and was independent of temperature from 170 K to 100 K. In 60% ethylene glycol, the temperature dependence was similar, but the rate fell to a minimum of 75 s^{-1} at 170 K and then increased slightly at lower temperatures; it finally became temperature independent, with a value of about 100 s^{-1}, at 110 K. The overall temperature dependence is consistent with charge recombination by two competing pathways: a direct electron-tunneling process which dominates at low temperature (less than 250 K), and a thermally activated process via a higher energy state, M, which decays rapidly to the ground state. The indirect route dominates at high temperature (above 250 K). Taking into account the contribution from the low-temperature pathway, the activation energy (enthalpy) for the activated process, in aqueous buffer, was determined to be 0.25 eV (at pH 7) and 0.19 eV (at pH 9). A likely candidate for M is P^+I^- (P^F), where I is the intermediate bacteriopheophytin electron acceptor, and energetic arguments are presented in favor of this assignment. If a rate of decay of P^+I^- to the ground state, derived from the experimental value, was used in the description of the thermally activated $P^+Q_A^-$ recombination process, the free-energy gap separating M and $P^+Q_A^-$ could be estimated to be 0.27–0.28 eV, placing it about 0.95 eV above the ground state and 0.30 eV below the excited singlet state, P*. This may imply additional relaxation compared to P^+I^- in the pico- to nanosecond time range of the forward pathway. The lack of an observable magnetic-field effect on the recombination process through P^+I^- can be explained by the expected rapid cycling between P^+I^- and $P^+Q_A^-$, with the latter providing an efficient

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Abbreviations: Mops, 4-morpholinepropanesulfonic acid; BChl, bacteriochlorophyll; BPheo, bacteriopheophytin; MK-8, menaquinone-8; Q-10, ubiquinone-10; PES, phenazineethosulfate; PMS, phenazinemethosulfate.

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spin-rephasing state. The pH dependence of the recombination kinetics could be accounted for if the influence of protonation on the $I^-Q_A \leftrightarrow IQ_A^-$ equilibrium was taken into account. Further implications of the identity of M with P^+I^- are discussed.

Introduction

The primary events in bacterial photosynthesis occur in the photochemical reaction center, a bacteriochlorophyll (BChl) protein complex in the cell membrane. Light activation results in the photooxidation of the primary donor, P, a BChl dimer or special pair, and the transfer of an electron to the primary quinone, Q_A, via a transient intermediate, I (a bacteriopheophytin (BPheo) possibly interacting with a BChl). In the absence of subsequent stabilizing reactions, the charge-separation state $P^+Q_A^-$ recombines in approx. 1–100 ms. In *Rhodobacter* (formerly *Rhodopseudomonas*) *sphaeroides*, the recombination reaction has been quite extensively studied, especially in isolated reaction centers [1–5]. In particular, it has been shown by several workers to be essentially temperature independent and has been interpreted as being controlled by a quantum mechanical, nuclear tunneling process (for a review, see Ref. 6).

For the species, *Rhodopseudomonas viridis*, the temperature dependence data are sparse and less coherent. The reaction centers of this species are similar to those of the more common, BChl *a* containing, purple bacteria. Carithers and Parson [7] reported a room-temperature recombination rate of about 10^3 s^{-1} in *Rps. viridis* chromatophores, and concluded that there was little temperature dependence in the range of 296 to 273 K. We have measured a similar rate (630 s^{-1} at 298 K and pH 9) in isolated reaction centers [8]. However, at lower temperatures, lower recombination rates of 106 s^{-1} at 233 K and 76 s^{-1} at 78 K in *Rps. viridis* chromatophores have been measured by a delayed fluorescence method [9]. This suggests a substantial change in the rate with temperature variation. In view of this uncertainty in the older literature, and the recent interest in this species arising from the X-ray structural work on the reaction-center complex [10,11], we were prompted to examine the temperature dependence of the recombination kinetics for $P^+Q_A^-$ in isolated, *Rps. viridis* reaction centers. We report here that the back reaction is strongly dependent on temperature in a manner suggesting the existence of two recombination pathways – an activated route, dominant at high temperature, and a temperature-independent pathway, dominant at low temperature.

Materials and Methods

Reaction centers from *Rps. viridis* were isolated as detailed by Prince et al. [12]. Reaction centers from *Rb. sphaeroides* were prepared as described by Wraight [13], except that the reaction centers were eluted from the DEAE cellulose (or Sephacel, Pharmacia) column in 0.15 M NaCl, rather than 0.35 M NaCl as erroneously stated in the earlier work. Except where noted, all samples of *Rps. viridis* reaction centers were unreconstituted after isolation and therefore contained little secondary quinone (Q_B, ubiquinone) activity [14]. *O*-Phenanthroline (4 mM) was also routinely present. Further ubiquinone extraction was performed, as necessary, by the method of Okamura et al. [15] to yield *Rb. sphaeroides* reaction centers with less than 0.1 ubiquinone per reaction center (Q-less reaction centers) or *Rps. viridis* reaction centers with a single menaquinone (Q_A only) [8]. Menaquinone-8 (MK-8) was isolated from *Escherichia coli* and used to substitute for ubiquinone-10 (Q-10) in *Rb. sphaeroides* reaction centers as previously described [8]. Anthraquinone, from Kodak (Rochester, NY), was purified by sublimation prior to addition to Q-less *Rb. sphaeroides* reaction centers.

The experimental apparatus, a kinetic spectrophotometer for optical measurements in the ambient temperature range, was as previously described [8,13]. A cold-finger, liquid nitrogen, optical dewar with a cell of pathlength 3.5 mm was also used. P^+ decay was monitored at 450 nm and the decay kinetics were fit to a single exponential. The bound cytochromes of the *Rps. viridis* reaction center were chemically oxidized by the addition of 0.5 mM ferricyanide, which was then

removed, to a concentration of less than 1 μM, by repeated dilution and ultrafiltration using an XM-100 filter (Amicon) [14].

Redox titration of the I/I^- couple was performed by monitoring the stable formation of I^- (at 545 nm) following a flash. Because of the great speed with which the low-potential cytochrome donates to P^+ in *Rps. viridis* ($\tau \approx 130$ ns [16]), it can partially compete with the decay of P^+I^- when Q_A is reduced ($\tau \approx 15$ ns [17]). Thus, when Q_A is reduced, about 10% of the maximum cytochrome oxidation is still observed, with the concomitant trapping of a similar amount of I^-. This residual activity disappears at much lower potentials, which we ascribe to the reduction of the intermediate acceptor, I.

The pH buffers were potassium pyrophosphate and Mops. Between 310 K and 275 K the pH was monitored and maintained within ±0.05 units of the reported value. Viscosity was adjusted by the addition of glycerol, ethylene glycol or sucrose as binary mixtures with water and values were taken from tabulated data in the literature [18]. Temperature was measured with an iron-constantan thermocouple [19].

Results

Rate of recombination of $P^+Q_A^-$ vs. temperature

The temperature dependence of the $P^+Q_A^-$ decay kinetics, in Q_B-less reaction centers isolated from *Rps. viridis*, was measured in aqueous buffer at pH 7 and pH 9 between 310 and 275 K (Fig. 1). At 295 K, in the presence of 100 mM NaCl and 4 mM *o*-phenanthroline, the rate constant was 470 ± 15 s^{-1} and 570 ± 20 s^{-1}, at pH 7 and pH 9, respectively. At 298 K the values were about 510 s^{-1} and 640 s^{-1}, in good agreement with our earlier data [8]. These temperature data are also consistent with those of Carithers and Parson, obtained using chromatophores (see Fig. 5 of Ref. 7), but not with their conclusions. We conclude, in contrast to those authors, that there is a definite temperature dependence of the rate of recombination. Between 310 K and 275 K (Fig. 1) the rates declined steadily and were reasonably well fit by linear Arrhenius plots with apparent activation enthalpies, calculated from the slopes, of 0.20 ± 0.01 eV at pH 7 and 0.16 ± 0.01 eV at pH 9. In the absence of *o*-phenanthroline, at pH 7, the rates were somewhat lower but the temperature dependence was more marked, with an apparent activation energy of 0.24 eV.

Extended temperature dependences were done in 60% glycerol or 60% ethylene glycol, to allow freezing of the sample down to 100 K (Fig. 2). At pH 7, in 60% glycerol, the recombination rate was 370 ± 10 s^{-1} at 295 K, and leveled off to 105 ± 5 s^{-1} at about 170 K. The rate remained at this value as the temperature was lowered further from 170 K to 100 K. The data for reaction centers in 60% ethylene glycol were similar, except that the rate was lowest (75 s^{-1}) between 200 K and 170 K. At lower temperatures it increased slightly to an apparent plateau value of 100 s^{-1} at 100 K. These data are in good agreement with the rates found by delayed fluorescence measurements on chromatophores from *Rps. viridis* [9]. All the temperature dependence data in this work are summarized in Tables I and II.

The back reaction behavior in *Rps. viridis* is in sharp contrast to the slight, negative temperature dependence observed for *Rb. sphaeroides* reaction centers, either with the native Q-10 or with MK-8 acting as Q_A (Fig. 3). However, when anthraquinone was substituted for Q_A in *Rb. sphaeroides* reaction centers, the rate of recombination at room temperature was markedly accelerated and the temperature dependence was qualitatively similar to that observed here for native *Rps. viridis* reaction centers (compare Figs. 2 and 3). A normal (positive) temperature dependence for *Rb. sphaeroides* reaction centers with anthraquinone as Q_A has been noted previously in the high (ambient) temperature range [20]. Our rates for *Rb. sphaeroides* reaction centers, for all three quinones, are in good agreement with previous measurements, made at more selective temperatures [1–5,15,20–22].

Rate of recombination of $P^+Q_A^-$ vs. viscosity

The slower room temperature rate observed for *Rps. viridis* reaction centers in cryosolvents (e.g., 370 s^{-1} in 60% glycerol, pH 7), compared to aqueous buffer (510 s^{-1}, pH 7), suggested a viscosity dependence for the recombination reaction. A viscosity dependence was confirmed at 293 K (20 °C) (Fig. 4) and was fit with a modified

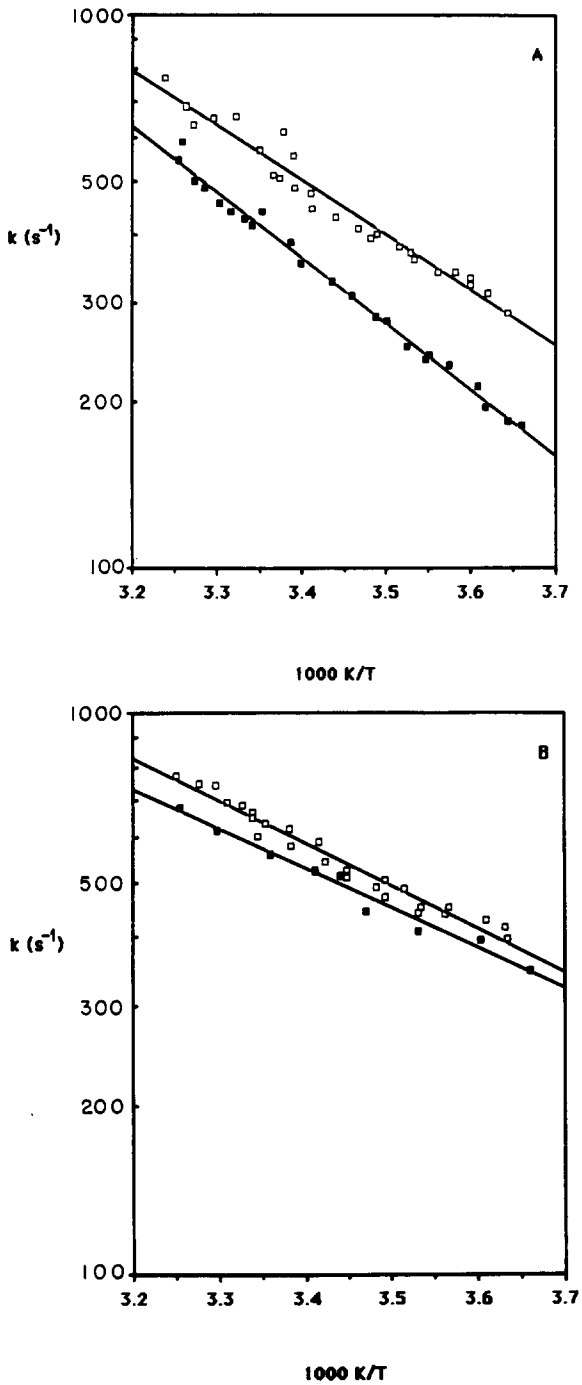


Fig. 1. The temperature dependence, in aqueous buffer, of the rate of charge recombination from the $P^+Q_A^-$ state in reaction centers from *Rps. viridis*. Conditions: 1 μM reaction centers, 0.1% Triton X-100, 100 mM NaCl, 10 mM Mops, 10 mM potassium pyrophosphate and 4 mM *o*-phenanthroline. (A) □, pH 7; ■, pH 7 without *o*-phenanthroline. (B) □, pH 9; ■, pH 9 at a constant viscosity of 3 cP. The lines are least-square fits to the unmodified data (see text).

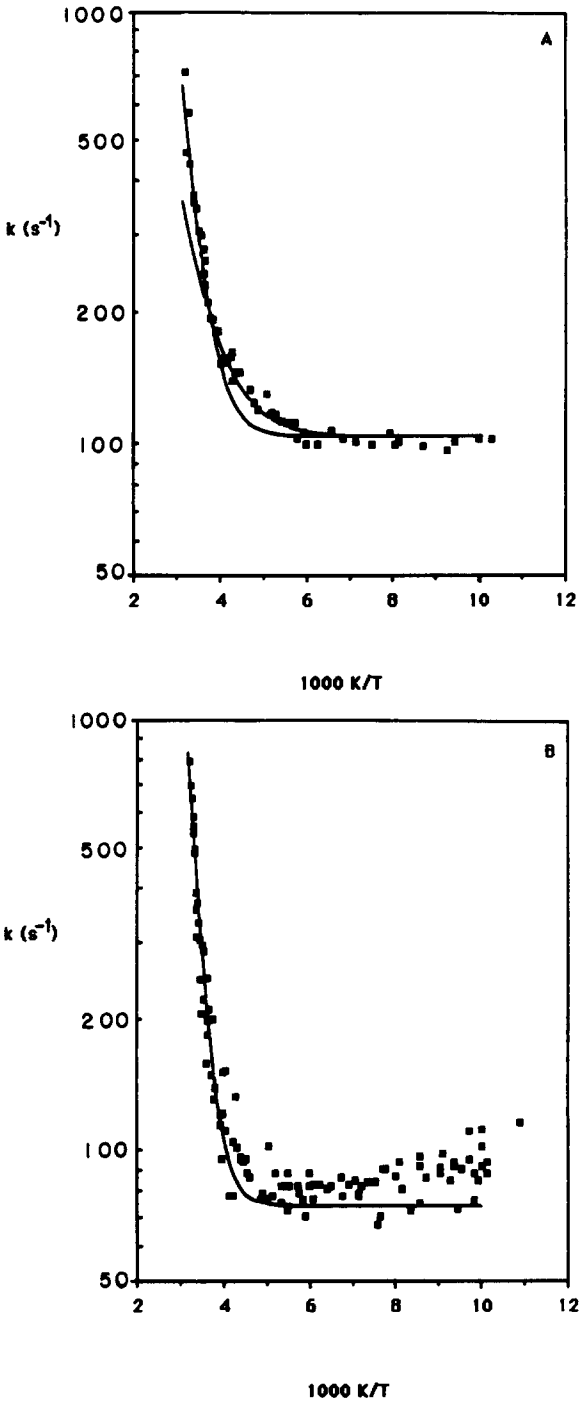


Fig. 2. The temperature dependence, in cryosolvents, of the rate of charge recombination from the $P^+Q_A^-$ state in reaction centers from *Rps. viridis*. Conditions: as in Fig. 1, at pH 7, except 30 μM reaction centers in buffer medium were diluted to 60% glycerol (A) or 60% ethylene glycol (B), to yield a final concentration of 12 μM reaction centers. The pathlength was 3.5 mm. The curves are visual fits, drawn according to Eqn. 2b, with (A): $k_T = 105\ s^{-1}$ and $\Delta H = 0.24$ eV, $k^+ = 3.5 \cdot 10^6\ s^{-1}$ (steeper curve) or $\Delta H = 0.14$ eV, $k^+ = 3.5 \cdot 10^4\ s^{-1}$ (flatter curve); (B): $k_T = 75\ s^{-1}$, $\Delta H = 0.33$ eV, $k^+ = 1.5 \cdot 10^8\ s^{-1}$.

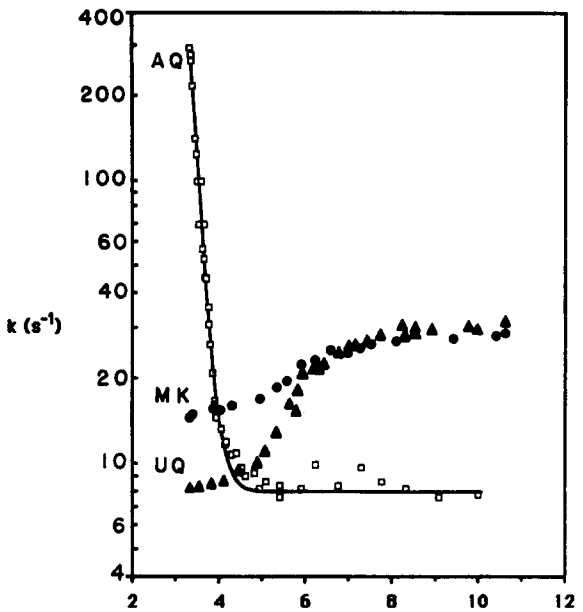


Fig. 3. The temperature dependence of the rate of charge recombination from the $P^+Q_A^-$ state in reaction centers from *Rb. sphaeroides*, strain R-26. Conditions: (□ and ●), 30 μM reaction centers with less than 0.1 Q per reaction center in 10 mM Tris, 30 mM Mops (pH 7), 0.1% LDAO diluted to 60% glycerol (w/w), plus (□) 150 μM anthraquinone or (●) 150 μM menaquinone-8; (▲) the same but for unextracted reaction centers containing approx. 1 ubiquinone per reaction center and no added quinone. The curve for the anthraquinone data is drawn according to Eqn. 2b, with $k_T = 8\ s^{-1}$, $\Delta H = 0.48$ eV, $k^+ = 3.5 \cdot 10^{10}\ s^{-1}$.

Kramers' equation, as given by Beece et al. [23]:

$$k = \beta + \alpha/\eta^n \qquad (1)$$

with $\alpha = 185 \pm 20\ s^{-1} \cdot cP^n$, $\beta = 430 \pm 10\ s^{-1}$ and $n = 0.5 \pm 0.05$. The viscosity (η, in cP) of an aqueous solution varies by a factor of two in the temperature range of Fig. 1 and the slope might, therefore, overestimate the apparent activation energy. In order to account for this, some of the temperature dependences were repeated with the viscosity held constant, at 3 cP, by changing the solution composition (see Methods). At pH 9, at constant viscosity, the slope and intercept were lower than those obtained from the uncorrected data, in aqueous buffer, by less than 10% and 5%, respectively (Fig. 1B, closed squares). Thus the change of viscosity is not a significant factor for calculations based on the data in Fig. 1. Nevertheless, the viscosity dependence apparent in Fig. 4 does indicate some damping of reaction-center protein vibrational modes coupled to the recombination process. The value of n (0.5) used to fit the data to Eqn. 1, on the other hand, suggests significant isolation of the protein interior from the solvent bath [23].

Rate of recombination of $P^+Q_A^-$ vs. pH and ionic strength

The pH dependence of the recombination reaction at room temperature appears complex (Fig. 5). In the presence of *o*-phenanthroline and 100 mM NaCl, the rate was minimum at pH 7–7.5 (500 s^{-1}), increasing to a possible maximum of about 1000 s^{-1} at pH 10.5–11.0, and to 850 s^{-1} at pH 4. Without *o*-phenanthroline the pH depen-

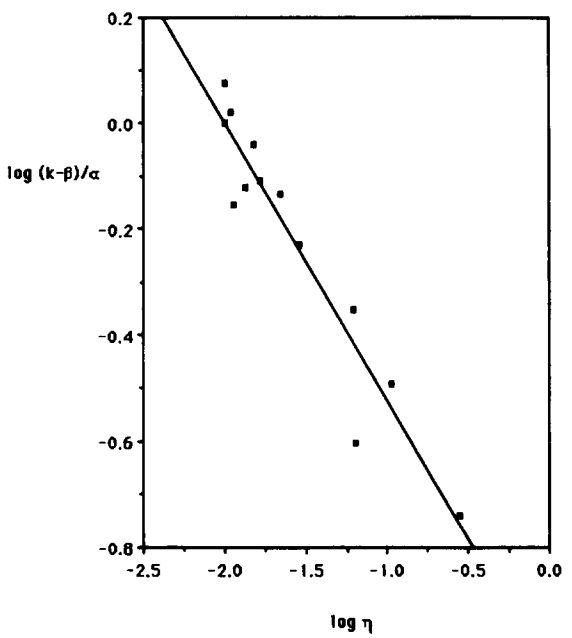


Fig. 4. The viscosity dependence of the rate of charge recombination from the $P^+Q_A^-$ state in reaction centers from *Rps. viridis*. Conditions: 1 μM reaction centers, 0.1% Triton X-100, 100 mM NaCl, 10 mM Tris (pH 9), 4 mM *o*-phenanthroline; temperature is 293 K. The viscosity was modified by the addition of glycerol, sucrose or ethylene glycol. The line is drawn according to the logarithmic transformation of Eqn. 1: $\log(k - \beta)/\alpha = n \log \eta$ with $\alpha = 185\ s^{-1} \cdot cP^n$, and $\beta = 430\ s^{-1}$; the slope, equivalent to n in Eqn. 1, is 0.52.

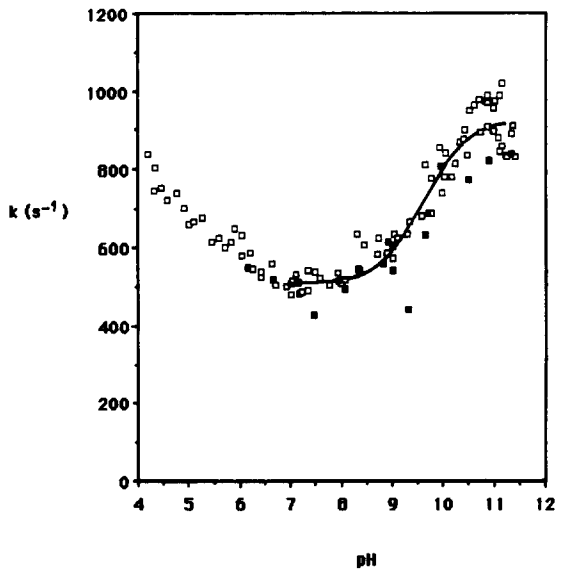


Fig. 5. The pH dependence of the rate of charge recombination from the $P^+ Q_A^-$ state in reaction centers from *Rps. viridis*. 1 μM reaction centers, 0.1% Triton X-100, 100 mM NaCl, in the presence of 4 mM *o*-phenanthroline (□); temperature is 296 K. ■, Rates calculated with Eqn. 2, using the activation parameters shown in Fig. 7. These were determined in separate measurements of the temperature dependence at various pH values, as described in the text. The line is drawn assuming two pK values governing the activation free energy, according to the following expression:

$$k = k_T + \frac{1+10^{pH-pK_I}}{1+10^{pH-pK_{QA}}} k_d \cdot e^{-\Delta G_{H^+}/k_B T}$$

$\Delta G_{H^+} = 0.275$ eV, is the activation free energy at neutral pH where the rate is pH independent; $k_T = 100\ s^{-1}$; $k_d = 2 \cdot 10^7\ s^{-1}$; $pK_I = 9.4$; $pK_{QA} = 9.7$.

dence in 100 mM NaCl was generally similar, except that the minimum value was about 400 s^{-1} (not shown). At low temperature (125 K), there was no significant effect of pH in the range pH 6.4 to pH 9.6 (not shown).

Discussion

The temperature dependence of the kinetics: dual-recombination pathways

The recombination of $P^+ Q_A^-$ in reaction centers from *Rps. viridis* was previously interpreted to proceed via an activationless electron-transfer mechanism [7], analogous to the recombination in *Rb. sphaeroides*. In *Rb. sphaeroides* the process accelerates 3–4-fold with decreasing temperature and becomes temperature independent below about 150 K [1–3,6,21]. From the data presented here for *Rps. viridis* an activationless process is evidently involved at temperatures below 200 K where the electron-transfer rate is essentially temperature independent (Fig. 2). In fact, in certain solvents, e.g., ethylene glycol, below 200 K, the charge-recombination rate increases slightly with decreasing temperature before leveling to a constant value, in much the same manner as seen for *Rb. sphaeroides*.

In contrast, at ambient temperatures, the recombination rate in *Rps. viridis* reaction centers decreases with decreasing temperature. A single electron-transfer mechanism may, in theory, be preserved for *Rps. viridis* over the entire temperature range if one supposes that, at the high temperatures, thermal activation of vibrational modes assists a tunneling pathway for the return of the electron to P^+ from Q_A^-. Such a description has been used to explain the temperature-dependent oxidation rate of the low-potential cytochrome *c*-552 in *Chromatium vinosum* (for a review, see Ref. 6). However, attempts to fit our data by a single-frequency mode theory [24] resulted in physically unmeaningful parameters, e.g., reorganization energies in excess of 30 eV! Use of more complex and multivariable electron-transfer theories [25] did not seem warranted in the light of a simple and physically reasonable alternative mechanism: the existence of two competing pathways for recombination – an activationless route, dominant at low temperature (less than 250 K), and a thermally activated route (via an energetically elevated intermediate state, M) which is dominant at higher temperatures. Assuming rapid equilibrium between $P^+ Q_A^-$ and the intermediate state, M, the observed decay rate is given by:

$$k = k_T(T) + k_d\, e^{-\Delta G/k_B T} \quad (2a)$$

$$= k_T(T) + k^+\, e^{-\Delta H/k_B T} \quad (2b)$$

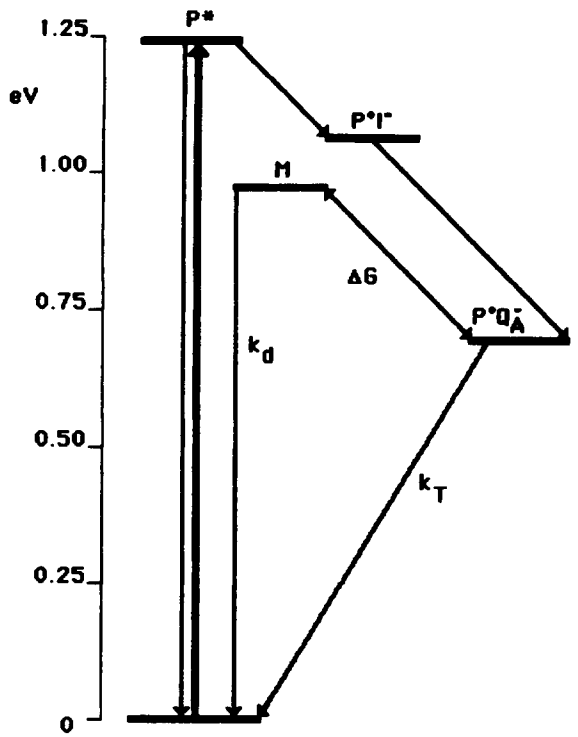
In Eqn. 2a, k_d is the rate of decay of M to the ground state, $k_T(T)$ is the putative tunneling rate at temperature T, k_B is the Boltzman constant and ΔG is the free energy difference between $P^+ Q_A^-$ and M. In Eqn. 2b, k^+ is a composite

preexponential factor, $k_d \cdot \exp(\Delta S/k_B)$, and ΔH is the activation energy or enthalpy. Such a scheme has been proposed previously to account for the recombination kinetics in *Rb. sphaeroides* reaction centers in which the ubiquinone of Q_A function has been replaced by low potential quinones such as anthraquinone [20,21,26,27]. The *Chromatium* cytochrome data has also recently been reinterpreted in terms of two parallel pathways [28]. A schematic diagram of this model for the recombination of $P^+Q_A^-$ in reaction centers from *Rps. viridis* is given in Scheme I.

To calculate thermodynamic parameters from the data of Fig. 1, in terms of Scheme I, we rearrange Eqn. 2:

$$\ln(k - k_T) = \frac{-\Delta H}{k_B}\frac{1}{T} + \frac{\Delta S}{k_B} + \ln k_d \qquad (3)$$

We assume k_T to be temperature independent betwen 310 K and 275 K, and take a reasonable value (i.e., 80–100 s^{-1}) from the low temperature data of Fig. 2. The activation enthalpy, ΔH, is



Scheme I.

TABLE I

ACTIVATION PARAMETERS FOR CHARGE RECOMBINATION IN REACTION CENTERS FROM *RHODOPSEUDOMONAS VIRIDIS*

Sample [a]	k_T (s^{-1})	ΔH (eV)	ΔS [b] (meV per degree)	$-T\Delta S$ [c] (eV)	$\Delta G_{298\,K}$ [d] (eV)	Temp. range (1000/T)
pH 7, 60% glycerol	105	0.25	−0.12	0.035	0.285	3.2–10.4
		(±0.03) [e]	(±0.05)	(±0.01)	(±0.01)	
	105	0.224 [f]	−0.204	0.061	0.285	3.2–3.9
pH 7, 60% ethylene glycol	75	0.31	0.08	−0.025	0.285	3.2–10.8
		(±0.03)	(±0.05)	(±0.01)	(±0.01)	
	75	0.343	0.206	−0.061	0.282	3.2–3.9
pH 7, aq. buf.	100	0.253	−0.080	0.024	0.277	3.25–3.65
pH 9, aq. buf.	100	0.186	−0.285	0.087	0.271	–
pH 9, isovisc.	100	0.178	−0.324	0.096	0.274	–
pH, aq. buf. (*o*-phen)	80	0.302	0.062	−0.018	0.284	–
pH 7.8 chromatophores [g]	75	0.288	0.065	−0.019	0.269	3.4–3.65

[a] Conditions for the reaction center samples are described in the legends to Figs. 1 and 2.

[b] Calculated from Eqn. 3, using $k_d = 2 \cdot 10^7\ s^{-1}$.

[c] $T = 298$ K.

[d] Calculated from ΔH and $-T\Delta S$ in this table.

[e] These limits represent the range of values for which data over the whole temperature range (90–310 K) were reasonably well fit, judged by visual inspection, by two components, as given by Eqn. 2.

[f] The activation parameters in the ambient temperature range (270–310 K) were obtained by subtracting k_T from the data and replotting as $\ln(k - k_T)$ vs. $1/T$, according to Eqn. 3. The errors for these linear plots were given by least-squares analysis, and were all within ±0.01 eV.

[g] Data from Ref. 7; 100 mM potassium phosphate (pH 7.8), 100 μM PMS, 1 mM *o*-phenanthroline.

given directly from a plot of $\ln(k - k_T)$ vs. $1/T$, but ΔS and k_d cannot be determined independently. Subtraction of k_T slightly enhances the linearity of the Arrhenius plots in this temperature range. For $k_T = 100\ s^{-1}$, the data in aqueous buffer at pH 7 yield $\Delta H = 0.25 \pm 0.01$ eV, and an intercept of 16.0 ± 0.25 ($\Delta S/k_B + \ln k_d$). At pH 9, we find $\Delta H = 0.19$ eV and an intercept of 13.5. Data from all sample types are summarized in Table I.

Further analysis is not possible without appropriate values for either ΔS or k_d. These are not available a priori, but assumption of a chemical identity for M allows discussion of these parameters. Following the proposal of Dutton and coworkers [21,22,26,27], a conceivable identity for M is $P^+I^-Q_A$ (termed P^F by Parson et al. [29]), where I is the intermediate electron acceptor which preceeds Q_A in the chain of forward electron flow (for a review, see Ref. 30). k_d would then be a decay rate constant for this state, which has been studied extensively.

Estimation of the activation parameters from equilibrium redox properties

In the simplest possible situation, the suggested identification of M with $P^+I^-Q_A$ would imply that, if access to P^+I^- is the rate-limiting step, the activation free energy for recombination of $P^+Q_A^-$ should be equivalent to the difference in redox midpoint potentials (ΔE_m) between I/I^- and Q_A/Q_A^-. Electrostatic interactions between P^+ and the reduced acceptors could perturb the redox energy span between I and Q_A, but such an effect would have to be focussed on the P^+/I^- and I^-/Q_A^- interactions, as previous work indicates that the interaction between P^+ and Q_A^- is negligible [5,31,32]. This will be returned to below. In fact, uncertainties in the equilibrium E_m values for I/I^- and Q_A/Q_A^- are at least as serious. A range of values has been reported for the E_m of the I/I^- couple in *Rps. viridis* chromatophores: -600 mV (at pH 11.5) [33], -525 mV (at pH 10.2) [34] and -400 mV (at pH 10.8) [35]. In isolated reaction centers we have found a value of -515 ± 20 mV (at pH 8.8) by measuring flash induced I^- at 545 nm (Fig. 6). For the purposes of this discussion, we will use a value of -520 mV. In chromatophores, the equilibrium E_m of the

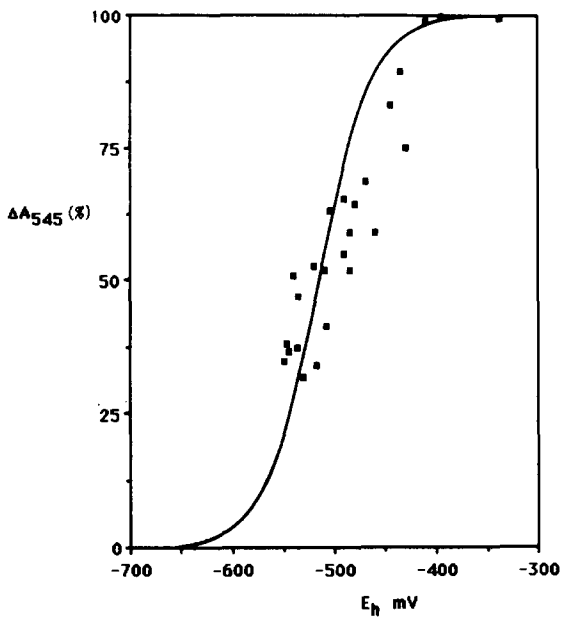


Fig. 6. Equilibrium redox titration of the intermediate acceptor, I, measured as the extent of the flash induced absorption change at 545 nm – see Materials and Methods. 1 μM reaction centers, 4 mM *o*-phenanthroline, 0.1% TX-100, 100 mM NaCl (pH 8.8); temperature, 296 K. 40 μM each of Q-10, methyl viologen, 2-anthraquinone sulfonate, 2-hydroxy-1,4-naphthoquinone, 5-hydroxy-1,4-naphthoquinone, 1,4-naphthoquinone, 1,2-naphthoquinone; 4 μM each PMS and PES. The curve is drawn for a one electron titration, with $E_m = -515$ mV, assuming that complete titration to low potentials would yield $\Delta A = 0$.

Q_A/Q_A^- couple, at pH 7 and in the presence of *o*-phenanthroline, is reported to be 35 mV [35,36]. If, as has been implicitly assumed in the past, the E_m of I/I^- is pH independent, this yields a ΔE_m of 0.55 ± 0.05 V at pH 7.0. Using this value for ΔG (in eV) and a value of 0.25 eV for ΔH (from the fit to the data; see Fig. 1 and Table I), we obtain $\Delta S_{calc} = -1.01$ meV/deg. Comparing this with the intercept of $\ln(k - k_T)$ vs. $1/T$, we find $k_d \approx 10^{12}\ s^{-1}$. This is more than four orders of magnitude faster than the measured decay rate for P^F of $7 \cdot 10^7\ s^{-1}$ [17].

This large discrepancy might be taken to invalidate the identification of M with P^+I^-, but it could also arise from an inappropriate use of equilibrium redox properties for calculating these short lived, dynamic events. However, if we proceed with the presumption of equilibrium values, an interesting, possible resolution arises.

The pH dependence of the activated recombination pathway

In bacteria, the E_m of Q_A/Q_A^-, determined by equilibrium redox titrations, is well established as being pH dependent below the pK for $Q_A^-(H^+)$. The pK is species specific and dependent on conditions of occupancy of the secondary quinone (Q_B) binding site [37,38]. In earlier work, it was suggested that the passage of electrons through Q_A may be much faster than the available protonation rate, leading to Q_A functioning in a quasi-pH independent fashion, with its 'operating' midpoint potential set at the value above the pK ($E_{m,pK}$) [56]. In fact, use of this value ($E_{m,pK}(Q_A/Q_A^-) = -160$ mV, in *Rps. viridis* chromatophores [35]) markedly improves the situation, yielding a ΔE_m of 0.36 V. However, recent work on the acceptor quinones (Q_A and Q_B) in reaction centers from *Rb. sphaeroides* strongly indicates that Q_A^- really is rapidly protonated following a flash, in general agreement with its equilibrium E_m/pH behavior [5,13,31,38–40], and similar behavior is seen for reaction centers from *Rps. viridis* [41].

The equilibrium properties of I/I^- are not clearly established, but the available data (see above) have generally been taken as consistent with a pH-independent E_m. This would imply that at pH values below the pK of $Q_A^-(H^+)$, the equilibrium ΔE_m between I/I^- and Q_A/Q_A^- (i.e., ΔG in Eqn. 2), exhibits a significant pH dependence, approaching -60 mV/pH unit. This would give rise to an equivalent pH dependence for the rate of recombination, i.e., a 10-fold acceleration of the activated pathway per unit increase in pH. In contrast to this expectation, the recombination kinetics are only weakly pH-dependent (Fig. 5). Since the functional behavior of the acceptor quinones supports the use the pH-dependent, equilibrium E_m for Q_A/Q_A^-, this suggests that, if M is P^+I^-, the effective E_m of I/I^- is also pH-dependent, and in a manner that is remarkably similar to that of Q_A/Q_A^-. Thus, ΔE_m would be almost constant. The appropriate E_m for I/I^-, in this case, cannot be the equilibrium parameter, but is a functional value in the presence of oxidized Q_A.

It has been suggested previously that the redox-linked protonation site for Q_A in *Rb. sphaeroides* is a protein (amino acid) moiety [13,39,40] and that the same residue may be involved for both Q_A/Q_A^- and Q_B/Q_B^-, with somewhat different pK values of 9.8 and 11.3, respectively [5,13,31,38–40]. Similar conclusions can be drawn for *Rps. viridis* [12,33–36,41]. In the present case, the slight pH dependence of the recombination rate can be accounted for, in terms of an operational ΔE_m for IQ_A^-/I^-Q_A, if the pK of this same protonation site is slightly lower for I^-Q_A (pK_I) than for IQ_A^- (pK_{Q_A}). Below pK_I the effective E_m of I/I^-, with Q_A oxidized, will be pH dependent. This is an unmeasurable state by equilibrium methods. At the very low potentials necessary for equilibrium titrations of I/I^-, Q_A will already be reduced and the protonation state of the site will be determined by the very similar pK of Q_A^-. Thus, the measured E_m of I/I^- would be almost pH independent which is consistent with the, admittedly sparse, equilibrium data. (We note that the postulated electron-transfer equilibrium between IQ_A^- and I^-Q_A is likely to occur much faster than the protolytic events – the forward electron-transfer rate constant is approx. $6 \cdot 10^9$ s^{-1} [17,30]. Thus, the I^-Q_A species formed in the electron-transfer equilibration is short-lived and is locked into the momentary protonation state of IQ_A^-). From the observed pH-dependence of the recombination rate in *Rps. viridis* reaction centers, in 100 mM salt, we can estimate that p$K_I \approx$ pK_{QA} -0.3 and p$K_{QA} \approx 9.7$, in the presence of *o*-phenanthroline (Fig. 5).

This value for pK_{QA} is in reasonable agreement with that determined in chromatophores from the E_m/pH relationship for Q_A in the presence of *o*-phenanthroline (p$K \approx 10.1$) [35]. We are currently investigating the redox and pK properties of Q_A in isolated reaction centers from *Rps. viridis*, by titration of the flash-induced oxidation of cytochrome *c*-553 at low potentials [35–37]. Preliminary data with this assay and by ESR indicate similar behavior as in chromatophores, although the E_m above the pK appears to be somewhat higher in reaction centers (-120 mV vs. -160 mV in chromatophores; Gao, J.-L. and Wraight, C.A., unpublished observations).

According to the view presented here, and developed further below, the pH dependence of the rate of recombination of $P^+Q_A^-$ reflects a weak

pH dependence in the energy gap between $P^+Q_A^-$ and P^+I^-. This can be analyzed in terms of electrostatic effects of protonatable groups which lie near both I and Q_A, within the reaction center protein [31]. The data for reaction centers in 100 mM NaCl (Fig. 5) indicate that a group, which lies nearer to Q_A than I, has a pK between 9 and 10, depending on the location of the electron. Using the same general approach to interpret the whole pH range in *Rps. viridis*, the acceleration at low pH may indicate the influence of another group, with pK values up to 6, that is nearer I than Q_A.

Possible interactions and modifications of redox properties

From the discussion in the previous section, we expect the operational ΔE_m between I/I^- and Q_A/Q_A^- to be only weakly dependent on pH. Ignoring charge interactions between the two centers, ΔE_m should be experimentally obtainable from equilibrium titrations at high pH, where both redox couples (I and Q_A) become pH independent, i.e., above the pK values. Our preliminary data, on reaction centers, for the midpoint potential of Q_A/Q_A^- above the pK of reduced Q_A ($E_{m,pK} = -120$ mV) gives a worst case choice for this parameter. Comparison with our choice of $E_m(I/I^-) = -520$ mV yields $\Delta E_{m,pK}$ of about 400 mV. The difference in pK values for I^- and Q_A^- ($\Delta pK = 0.3$) would increase ΔG by about 20 meV at pH values below both pK values, i.e. up to pH 9.

In contrast with the assumption of no significant interactions, several studies in the literature have suggested that the presence of Q_A^- does affect the energy level of P^+I^-, presumably through a Coulombic interaction between I^- and Q_A^-. This would require a correction to the equilibrium redox potential of I/I^-, which is determined in the presence of Q_A^-. Two groups have used nanosecond-delayed fluorescence to monitor the free-energy level of P^+I^- in Q-reduced and Q-extracted reaction centers from *Rb. sphaeroides* and have reported that they differ by 0.02–0.08 eV [22,42]. Comparison of enthalpy changes, for which there are more data, suggests even larger differences may pertain at longer times. Thus, although determinations of the P^+I^- enthalpy level in Q-reduced reaction centers, on the nanosecond timescale, have generally shown it to lie within 0.05 eV of P* [44–47], in Q-depleted reaction centers, P^+I^-, repopulated on the microsecond timescale from the localised triplet state of the primary donor, ^3P, is about 0.28 eV below P* in enthalpy [43]. The interaction energy between Q_A^- and I^- could, therefore, be as large as 0.23 eV. From the X-ray structure of the *Rps. viridis* reaction center, the center-to-center distance for I and Q_A is 14 Å [11]. An effective dielectric constant of 6–7, in this region of the reaction center, can be calculated from the effect on the recombination kinetics of large electric fields applied across reaction centers in monolayers (Ref. 48; see also Dutton, P.L., personal communication). From this we can calculate an expected Coulombic interaction of about 0.15 eV. This is well within the range of values described above. With these considerations we take here a likely range for ΔG of 0.22–0.32 eV, for which $k_d = (0.2\text{–}10) \cdot 10^7\ s^{-1}$. Although this encompasses the measured decay rate for P^F, the uncertainty in the E_m values and the corrections used is considerable, and a more circumspect view is that the potentiometric data are insufficiently understood to address, definitively, the relative energies and possible identity of M and P^+I^-.

Determination of activation parameters by fixing k_d

Clearly, if we question the suitability or precision of equilibrium redox potential determinations for providing the operating midpoint potential span between I/I^- and Q_A/Q_A^-, and if we do not numerically fix k_d, then the system is underdetermined for analysis of the activated recombination pathway. It is instructive, however, to examine the implications of the assignment of P^+I^- as M by using a rate constant derived from observations on the lifetime of P^+I^- in extracted or reduced reaction centers. The choice of value for k_d is not straightforward, as discussed below, but the range of likely values is not so large as to affect the analysis seriously. As justified in the next section, we shall use $k_d = 2 \cdot 10^7\ s^{-1}$, rather than the net decay constant determined by Holten et al. [17] for P^+I^- in reduced *Rps. viridis* reaction centers ($k = 7 \cdot 10^7\ s^{-1}$).

Applying Eqn. 3, as before, but with $k_d = 2 \cdot 10^7$

s^{-1}, we find from the data of Fig. 1, at pH 7, that the intercept yields $\Delta S = -0.08$ meV per degree; since $\Delta H = 0.25$ eV from the slope, we obtain $\Delta G = 0.28$ eV at 298 K. At pH 9, ΔG is very similar (0.27 eV), although the relative contributions of ΔH (0.19 eV) and ΔS (-0.29 meV per degree) are rather different. At pH 7, similar values for ΔG were obtained from fits to the ambient temperature range for the samples in 60% ethylene glycol and in 60% glycerol, using limiting values for k_T of 75 s^{-1} and 105 s^{-1}, respectively. Consistent values were also obtained by fitting the extended temperature data of these samples to single curves using Eqn. 2b. The data for 60% glycerol showed some departure from a two-component fit between 200 and 250 K, and Fig. 2A shows two fits, covering the distinct temperature regions. The origin of this behavior is not understood at the present time. The steeper curve, which best fits the high temperature region, yields activation parameters similar to those found for the aqueous buffer samples.

In both *Rb. sphaeroides* [32] and *Rps. viridis* [7], the free energy of $P^+Q_A^-$, calculated from the difference in midpoint potentials of P/P^+ and Q_A/Q_A^- (above the pK of Q_A^-), is in reasonable agreement with measurements of delayed fluorescence, supporting the lack of significant interaction between these two redox centers. For *Rps. viridis* summation of the redox free energy of $P^+Q_A^-$ (0.68 eV [7,35,41]) with the activation free energy for the recombination process (0.28 eV), determined here, yields a value for the energy level of M (0.96 eV) which is not inappropriate for P^F – approx. 0.3 eV below P*. The excited-state energy level (1.24 eV) was taken from the 0-0 vibrational substate of the first excited singlet-to-ground state transition. The peak absorption wavelength of P (965 nm) is independent of temperature down to about 200 K, and moves to longer wavelengths at lower temperatures (not shown). There is limited data available on the energy levels of P^F and the other transient states, in *Rps. viridis* but a recent study on time-resolved, nanosecond delayed fluorescence from reduced *Rps. viridis* reaction centers, has indicated that P^F is about 0.25 eV below P* in free energy [49].

Table I summarizes our data for several different types of reaction-center sample analyzed according to Eqns. 2 and 3, as well as a re-analysis of the data of Carithers and Parson [7] for chromatophores. The agreement between the various ΔG values is striking and contrasts somewhat with the range of values for ΔH and ΔS. The determinations of ΔH and ΔS, however, are not independent and errors or variations in one tend to be offset by changes in the other. The choice of k_d directly affects the values of ΔS and ΔG, but the contribution is still small for the range of k_d values under consideration: for $k_d = (2\text{–}7) \cdot 10^7$ s^{-1}, $\Delta\Delta G = 0.032$ eV. The choice of k_T, to be subtracted before the Arrhenius analysis, has little effect on ΔG but does influence the distribution between ΔH and ΔS.

Table II compares our data for anthraquinone-substituted reaction centers from *Rb. sphaeroides* with two earlier studies, re-analysed according to Eqn. 3. There is reasonable agreement between the ΔG values, but the range of ΔH and ΔS values is considerable for these three, very different sample preparations – one in glycerol, one in buffer and one in phospholipid. A previous study revealed a surprising sensitivity of these parameters to buffer and detergent conditions [47], and our finding of a significant viscosity dependence in *Rps. viridis* is also consistent with these results. It is noteworthy that the more viscous environments – glycerol and phospholipid – reveal the greater contributions from ΔH.

The effect of spin rephasing on the choice of values for k_d

The decay rate, k_d, involved in our description of the $P^+Q_A^-$ recombination process, should be related to the lifetime of P^+I^- observed following flash activation of blocked reaction centers, but is not expected to be equal to it, since there are multiple decay channels for P^+I^- (see Scheme II). Furthermore, in reaction centers from *Rb. sphaeroides* the net decay rate for P^+I^- is different when Q_A is reduced ($k = 9 \cdot 10^7$ s^{-1}) or extracted ($k = 7 \cdot 10^7$ s^{-1}) [22,45,51,53]. The more relevant value for the charge recombination path from $P^+Q_A^-$ is with Q_A extracted, i.e., uncharged and diamagnetic, as for oxidized Q_A, but, in *Rps. viridis*, the decay of P^+I^- has only been measured in reduced reaction centers, for which $k = 7 \cdot 10^7$ s^{-1} [17].

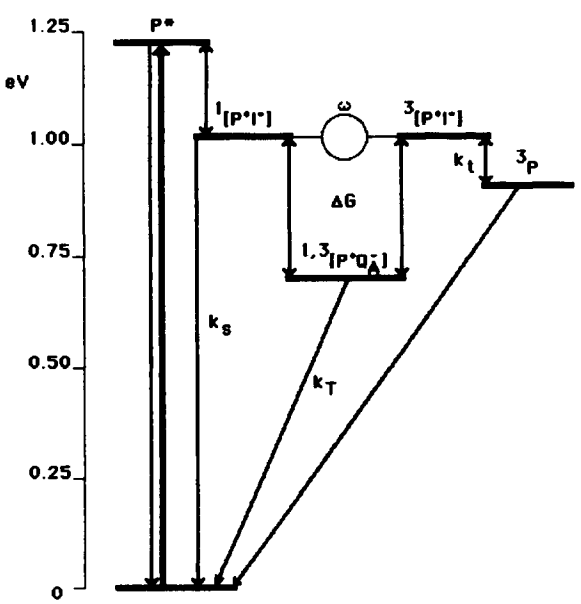
TABLE II

ACTIVATION PARAMETERS FOR CHARGE RECOMBINATION IN ANTHRAQUINONE-SUBSTITUTED REACTION CENTERS FROM *RHODOBACTER SPHAEROIDES*

Sample	k_T (s^{-1})	ΔH (eV)	ΔS [a] (meV per degree)	$-T\Delta S$ [b] (eV)	$\Delta G_{298\,K}$ [c] (eV)	Temp. range (1000/T)
pH 7, 60% glycerol [d]	8	0.47 (±0.02) [e]	0.60 (±0.05)	−0.18 (±0.01)	0.29 (±0.01)	3.3–10.6
– [d]	8	0.484 [f]	0.662	−0.197	0.287	3.3–3.8
pH 8, aq. buf. [g]	8	0.371	0.218	−0.065	0.306	3.3–3.5
pH 7, liposomes [h]	8	0.431	0.359	−0.107	0.324	3.35–3.65
– [h]	0 [i]	0.340	0.035	−0.010	0.350	–

[a] Calculated using $k_d = 2 \cdot 10^7\ s^{-1}$.
[b] $T = 298$ K.
[c] Calculated from ΔH and $-T\Delta S$ in this table.
[d] This work; conditions as described in the legend to Fig. 3.
[e] See note e, Table I.
[f] See note f, Table I.
[g] Data from Ref. 22; 10 mM Tris (pH 8), 0.01% LDAO.
[h] Data from Ref. 20; partially purified soy bean lipids in 100 mM KCl, 10 mM imidazole (pH 7).
[i] As analysed in Ref. 20.

Consideration of the multiplicities of the P^+I^- and $P^+Q_A^-$ states also introduces corrections to the choice of k_d. Following light activation, the radical pair is formed in the singlet state, $^1[P^+I^-]$, and forward electron-transfer normally occurs from this state. Spin rephasing can occur, however, to yield the triplet radical pair state, $^3[P^+I^-]$.



Scheme II.

The rate of electron transfer to Q_A is probably independent of the spin state of P^+I^-, but the triplet species is also rapidly connected to the localized, or 'molecular', triplet state of the primary donor, ^3P. In blocked reaction centers there are two main decay channels for P^+I^-: recombination of $^1[P^+I^-]$ to yield the ground state, and recombination of $^3[P^+I^-]$ to yield ^3P. The triplet recombination rate constant, k_t, is significantly faster than that for the singlet recombination, k_s [52,55], but the triplet pathway is normally rate limited by the spin rephasing process, represented by the coupling constant, ω. Thus, in reaction centers from *Rb. sphaeroides*, blocked by chemical reduction of Q_A, at room temperature and in zero magnetic field, ^3P is formed with a yield of 10–20% [45,54]. When a weak magnetic field is applied ($B < 0.1$ T), the spin rephasing is slowed down further by removal of the triplet degeneracy, i.e., the T_{+1} and T_{-1} levels split away from the nearly isoenergetic T_0 and ^1S (singlet) levels [54,55]. This results in a substantial decrease in the triplet (^3P) yield and a slight (20%) slowing of the rate of decay of P^-I^- as a whole [45,46,52].

In *Rb. sphaeroides* reaction centers blocked by extraction of Q_A, the triplet yield is higher (30–50%) than in Q_A-reduced reaction centers

[51–53]. This reflects, in part, an influence on the singlet-triplet rephasing by the local magnetic field of Q_A^-. However, Norris and coworkers [53,55] have shown that the singlet decay constant is also modified: $k_s = 7.7 \cdot 10^7\ s^{-1}$ or $3.7 \cdot 10^7\ s^{-1}$ in Q_A-reduced or Q-extracted reaction centers, respectively [53]. Similarly, Chidsey et al. [51] concluded that, in Q-depleted reaction centers, k_s is less than $5.9 \cdot 10^7\ s^{-1}$ and they have used $5 \cdot 10^7\ s^{-1}$ in their work.

In Q-extracted *Rb. sphaeroides* reaction centers, the rate of decay of ^3P is also decreased by a weak, external magnetic field [43], indicating that, at room temperature, the molecular triplet decays predominantly by reexcitation to $^3[P^+I^-]$, followed by spin rephasing to $^1[P^+I^-]$ and decay to the ground state via the singlet channel. Thus, in blocked reaction centers, although P^+I^- disappears via both singlet and triplet pathways, return to the ground state occurs primarily through the singlet channel. In the state $P^+Q_A^-$, formed in open reaction centers, the spin coupling between the electron on Q_A^- and the iron atom (Fe(II), $S = 2$) of the acceptor complex provides for very rapid spin dephasing [22,51–53]. Thus, the spin distribution of $[P^+Q_A^-]$ should quickly attain equilibrium (25% $^1[P^+Q_A^-]$, 75% $^3[P^+Q_A^-]$) and P^+I^-, generated by back electron transfer, should exhibit the same preponderance of the triplet form. If the decay of P^+I^- to the ground state is limited to the singlet pathway, then the effective rate constant for ground state repopulation during charge recombination from $P^+Q_A^-$ will be diminished by the partition between singlet and triplet channels, modified by the extent to which these two states interconvert during the P^+I^- lifetime. Since the singlet–triplet mixing process is much slower than the forward electron transfer from I^- to Q_A in open reaction centers, the effective rate, k_d, will be approximately one-fourth of k_s. Thus, a value of $(1–2) \cdot 10^7\ s^{-1}$ may be the best choice for substitution in our calculations.

If P^+I^- is indeed the intermediate state in the thermally activated recombination of $P^+Q_A^-$ in *Rps. viridis* then, following the same arguments as above, one might expect some sensitivity of the recombination rate to an applied magnetic field. However, in the presence of a field of 25 mT, provided by a permanent magnet, we could detect no significant (less than 10%) change in the decay kinetics of the $P^+Q_A^-$ state (not shown). Following back transfer to P^+I^-, the subsequent spin rephasing, necessary for decay of the triplet population through the singlet channel, will be retarded by the application of a magnetic field. However, although the significance of singlet–triplet mixing in $[P^+I^-]$ is evident when forward electron transfer is blocked (Q_A removed or reduced), when Q_A is oxidized, reformation of $P^+Q_A^-$ is so far the dominant pathway that spin rephasing will effectively occur by cycling through the rapidly equilibrating state, $^{1,3}[P^+Q_A^-]$, possibly obviating any effect of singlet–triplet mixing in $^{1,3}[P^+I^-]$.

Implications of the identity of M with P^+I^-

The possibility of a recombination pathway for $P^+Q_A^-$ via P^+I^- was first suggested by Gunner and Dutton [26,27] for reaction centers from *Rb. sphaeroides,* with low-potential quinones acting as Q_A. A detailed study of that system, by Woodbury et al. [22], has used delayed fluorescence to provide free energy and enthalpy differences between P* and the charge storage states P^+I^- and $P^+Q_A^-$ in quinone-substituted reaction centers. They concluded that for sufficiently low potential quinones, $P^+Q_A^-$ recombines via an intermediate state similar to P^+I^-, but of lower free energy than the initial charge separation state (P^F, also P^+I^-) formed in the forward, photochemical pathway. In the case of 1-amino-5-chloroanthraquinone-substituted reaction centers, which exhibited a recombination rate ($450\ s^{-1}$) similar to that observed here for *Rps. viridis*, the discrepancy between the free-energy levels of M and P^+I^- was about 0.15 eV [22]. This takes into account an 0.05 eV relaxation in P^+I^- that was inferred, in earlier studies, from multiple components of the decay of nanosecond delayed fluorescence [47,50], but the remaining discrepancy implies the need for substantial further relaxation on longer time-scales, in *Rb. sphaeroides*. Our cruder estimates of the relative energy levels of M and P^+I^- in *Rps. viridis* do not indicate such a large discrepancy. The magnitudes of the calculated energy difference between M and P^+I^- largely reflects the higher energy level of P^+I^-, relative to the ground state, in *Rb. sphaeroides* compared to *Rps. viridis*. This seems most likely attributable to the chemical nature of

the chromophores – BChl *b* and BPheo *b* in *Rps. viridis*, BChl *a* and BPheo *a* in *Rb. sphaeroides*. However, it is not clear why the two species should differ in the extent of relaxation of P^+I^- on the micro-to-millisecond time-scales.

In their study of *Rb. sphaeroides* reaction centers, Woodbury et al. [22] determined that the enthalpy difference between P* and M ($\Delta H^* = 0.32$ eV) should have placed M at a level sufficient to allow population of the molecular triplet, ^3P ($\Delta H^* = 0.40$ eV [44]). However, ^3P could not be detected even for the most rapidly recombining preparations, with 2,3-dimethyl-anthraquinone as Q_A. One possibility, of course, is that this reflects an unfavorable free-energy difference between ^3P and M. This would imply a substantially more relaxed, P^F-like intermediate (M) when accessed from $P^+Q_A^-$ than when formed directly from P*. It is noteworthy that Chidsey et al. [43] reported that P^+I^-, repopulated from ^3P on the microsecond time-scale, was 0.28 eV below P* in enthalpy. They erroneously quoted Woodbury and Parson's work (Ref. 47) as showing an enthalpy drop (ΔH^*) of 0.15 eV between P* and P^+I^- in the forward pathway, apparently in reasonable agreement with their value obtained for reexcitation from ^3P. However, Woodbury and Parson actually report an enthalpy drop of only 0.015 eV (but a free energy drop of 0.15 eV). It is conceivable that the substantially larger ΔH^* observed on the microsecond time-scale of the triplet lifetime, compared to the nanosecond time-scale of delayed fluorescence, reflects additional relaxations of the reaction center and may point to even greater changes on the longer time-scales of the $P^+Q_A^-$ recombination, possibly coupled to the electron transfer from I^- to Q_A.

Our conclusions, for native reaction centers of *Rps. viridis* are similar to those of Woodbury et al. for *Rbs. sphaeroides* [22], although the precision and availability of the *Rps. viridis* data do not strongly indicate that the free-energy level of the intermediate state, M, populated on the milliseconds time-scale, is substantially below that of P^+I^- generated in the forward photochemical pathway. There are, of course, serious gaps in our current knowledge of the parameters governing the proposed charge recombination pathway in *Rps. viridis*, and the uncertainties in our determination are considerable. Nevertheless, the identification of the intermediate state, M, as P^+I^-, or some closely related entity, is reasonably well supported by this study. However, in order to reconcile this identity with the equilibrium redox properties of Q_A/Q_A^- we have found it necessary to suggest that the operating E_m of I/I^- is pH dependent and that the protonation state of Q_A^-, in IQ_A^-, may be carried over in I^-Q_A during the electron-transfer equilibrium between the two redox centers. The E_m of I/I^- is, therefore, effectively modulated by the redox and protonation state of Q_A. This is not unreasonable given their close association, as revealed by the X-ray crystallographic structure of the reaction center from *Rps. viridis*. Furthermore, the pair exhibit magnetic coupling [34,57] and the reduction of Q_A induces substantial electrochromic shifts in the BPheo absorption spectrum [8]. The involvement of protolytic reactions of the electron acceptors provides a likely source of the additional relaxation processes, on the micro-to-millisecond time-scales, suggested by the free-energy comparison between M and P^F in *Rps. viridis* (this work) and, with more precision, in *Rb. sphaeroides* [22,47]. It should be noted that if M is a charge separation state other than P^+I^-, the properties that we have deduced for I, such as the pH dependence of the operating midpoint potential of I/I^-, should still be applicable to the intermediate electron acceptor species.

Gopher et al. [20], who have previously reported a positive temperature dependence for *Rb. sphaeroides* reaction centers with anthraquinone substituted as Q_A, also determined that the recombination process was sensitive to an electric field applied across the reaction centers while incorporated into a lipid bilayer. This behavior is fully consistent with recombination via an intermediate state. Assuming this state to be P^F, they used a decay constant, k_d, of $8 \cdot 10^7\ s^{-1}$ (taken from the P^F lifetime for reduced reaction centers) to analyze the temperature dependence of the recombination kinetics, but did not allow for a low-temperature limit to the reaction rate. The value of ΔS reported was small (0.03 meV per degree) compared to the value found here for *Rps. viridis*, but also of opposite sign. Application of our Eqn. 3, with a finite low-temperature recombination rate ($k_T = 8$

s^{-1}, see Fig. 3), and use of a smaller value for k_d ($2 \cdot 10^7$ s^{-1}), increases ΔS substantially (0.36 meV per degree, see Table II). Whether this distinction between the two species is significant is not certain, especially as the data for *Rb. sphaeroides* are for a limited pH range and the reliability of the partition of total ΔG between ΔH and ΔS, in a single experiment, is suspect. For *Rps. viridis*, however, we found that the relative contributions of enthalpy and entropy change considerably over the pH range so that, at some pH values, the entropy contribution must be substantial.

The pH dependence of the activation parameters in *Rps. viridis* provides a somewhat elaborate test of the reliability of our analysis. We measured the temperature dependence of the rate of recombination at several pH values between pH 6 and pH 11.5 under the same conditions as in Fig. 1. The values found for ΔH ranged from 0.27 to 0.17 eV (Fig. 7). Using $k_d = 2 \cdot 10^7$ s^{-1}, we determined ΔS from the intercept of the temperature dependence at each pH. The values calculated for ΔS ranged from -0.02 to -0.32 meV per degree ($-T\Delta S \approx 0.005$–0.095 eV) and those for ΔG varied from 0.28 to 0.27 eV (Fig. 7). We then used Eqn. 3 to calculate the rate of recovery from the $P^+Q_A^-$ state at each pH value, at 295 K (open squares of Fig. 5). The pH dependence of the rate, calculated with the parameters determined form the temperature dependence, fits well with the pH dependence measured at constant temperature (closed squares in Fig. 5). This seems to support the reliability of the data and the consistency of our analysis, but it does not provide proof of the identity of M. Indeed, the value chosen for k_d is unrestricted – any other value would merely be compensated by a shift in the absolute magnitude of ΔS, while the range of intercepts in the temperature dependences remains the same, thus yielding an identical pH dependence. It must be emphasized, however, that, in order to fit the data, a pH dependent ΔS (or pH dependent k_d) is needed to offset the substantial pH dependence of ΔH. For example, if we simply assume that $\Delta S \approx 0$, not only would the value needed for k_d be an order of magnitude slower than that found for the decay of P^F, but the calculated pH dependence of the rate would not match the measured pH dependence.

Entropic contributions to the energetics of primary processes have frequently been ignored, sometimes with the presumption that they are, in fact, small. This assumption is not supported in the few cases where data are available. In *Rb. sphaeroides*, the free-energy level of P^F in reduced reaction centers is about 0.15–0.20 eV below P*, whereas the enthalpy levels are very close [44,47] or even inverted [45]. The entropic contribution to the free-energy drop from P* to $P^+Q_A^-$ is of similar magnitude, although it is now overwhelmed by a much larger enthalpy change [22,32]. Dielectric relaxation is one of the few obvious phenomena which one may associate with the entropic effects. On longer time-scales, protonation events, and accompanying changes in charge and solvent mobility, have been suggested, by Parson and coworkers [32,58], to account for changes in the entropy of metastable charge separation states. In

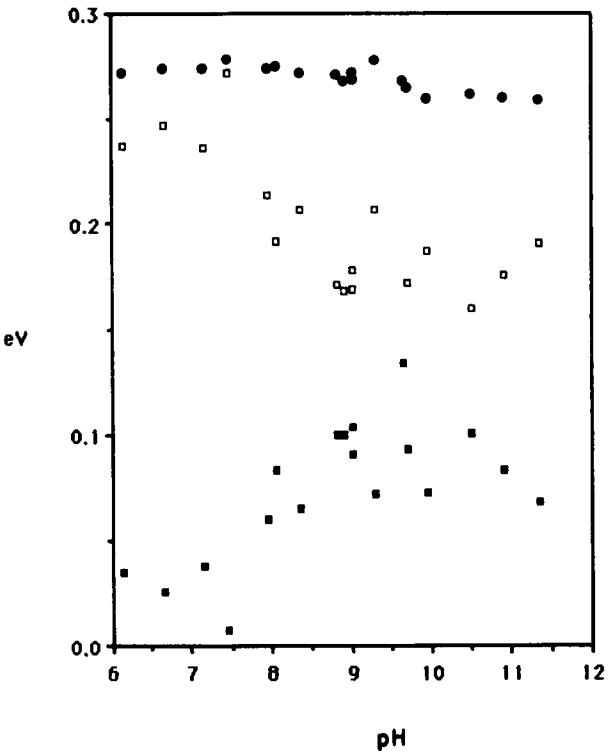


Fig. 7. Dependence on pH of the enthalpy, ΔH (□), entropy, $-T\Delta S$ (■), and free energy, ΔG (●), at 296 K, for the activation of the thermal route of charge recombination of the $P^+Q_A^-$ state. Conditions as for Fig. 1. The activation parameters were determined from individual temperature dependences at each pH value, over the range 275–310 K, and were calculated, using Eqn. 3, as described in the text, with $k_d = 2 \cdot 10^7$ s^{-1} and $k_T = 100$ s^{-1}.

the present work, the viscosity dependence of the recombination reaction, and pH dependence of the entropy/enthalpy contributions to the activation process, show that such processes can influence states closely related to the primary intermediates, and suggest this may be an interesting topic for further examination.

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