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ORIGINAL PAPER

The redox midpoint potential of the primary quinone of reaction centers in chromatophores of *Rhodobacter sphaeroides* is pH independent

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Abstract The redox midpoint potential (E_m) of the primary quinone of bacterial reaction centers, Q_A, in native membranes (chromatophores) measured by redox potentiometry is reported to be pH dependent (−60 mV/pH) up to a highly distinctive pK_a (9.8 in *Rba. sphaeroides*) for the reduced state. In contrast, the E_m of Q_A in isolated RCs of *Rba. sphaeroides*, although more variable, has been found to be essentially pH-independent by both redox potentiometry and by delayed fluorescence, which determines the free energy (ΔG_{P^*A}) of the $P^+Q_A^-$ state relative to P*. Delayed fluorescence was used here to determine the free energy of $P^+Q_A^-$ in chromatophores. The emission intensity in chromatophores is two orders of magnitude greater than from isolated RCs largely due to the entropic effect of antenna pigments "drawing out" the excitation from the RC. The pH dependence of ΔG_{P^*A} was almost identical to that of isolated RCs, in stark contrast with potentiometric redox titrations of Q_A. We considered that Q_A might be reduced by disproportionation with QH_2 through the Q_B site, so the titration actually reflects the quinone pool, giving the −60 mV/pH unit dependence expected for the Q/QH_2 couple. However, the parameters necessary to achieve a strong pH-dependence are not in good agreement with expected properties of Q_A and Q_B. We also consider the possibility that the time scale of potentiometric titrations allows the reduced state (Q_A^-) to relax to a different conformation that is accompanied by stoichiometric H^+ binding. Finally, we discuss the choice of parameters necessary for determining the free energy level of $P^+Q_A^-$ from delayed fluorescence emission from chromatophores of *Rba. sphaeroides*.

Keywords Redox chemistry · Delayed fluorescence · Bacterial photosynthesis · Chromatophore · Reaction center protein · Quinones

Abbreviations

E_m	Redox midpoint potential
Q	Quinone
RC	(Bacterial) Reaction center

Introduction

Bacterial photosynthetic reaction centers (RC) have been very widely used to develop and test our understanding of fundamental biological processes, including long distance electron transfer (Moser et al. 2003), and proton transfer (Wraight 2006). The cofactors of the RC (4 bacteriochlorophylls (BChl), 2 bacteriopheophytins, and 2 quinones) are arranged in a symmetrical fashion, in two very similar chains, only one of which is active in electron transfer. This functional asymmetry provides an ideal model system for studying the origins of specific properties of cofactors in proteins. In *Rhodobacter* (*Rba.*) *sphaeroides*, the two quinones are both ubiquinone, but the primary quinone (Q_A) functions only in one-electron chemistry, while the

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secondary quinone (Q_B) is both singly and doubly reduced (Parson 1978; Wraight 1979a, 1982). If the reduction of either quinone is functionally coupled to proton uptake, its redox midpoint potential (E_m) will be pH dependent, potentially modifying the free energy of any reaction it is involved in.

Special interest has been focused on Q_A, which is the first stable electron acceptor, as the free energy level of Q_A/Q_A^- determines the maximum yield of photochemical conversion of the absorbed light energy: $PQ_A \rightarrow P^+Q_A^-$. Equilibrium redox potentiometry on native chromatophore membranes of *Rba. sphaeroides* shows $E_m(Q_A/Q_A^-) = -20$ mV at pH 7 ($E_{m,7}$), but it is strongly pH dependent, with a slope of −60 mV/pH unit up to a pK_a = 9.8 for the reduced species, i.e., the reduction is coupled to the uptake of one proton per electron (Prince and Dutton 1976, 1978). However, the semiquinone is anionic, as indicated by optical spectroscopy (Bowyer et al. 1981; Verméglio 1982; Lavergne et al. 1999), and the reduced form can be designated $Q_A^-(H^+)$ to indicate that protons are bound to the protein, rather than to the quinone, but coupled to Q_A reduction. At high pH the midpoint potential becomes pH independent with $E_{m,pH} = -180$ mV (Prince and Dutton 1976, 1978). Qualitatively similar behavior is encountered in other species. All exhibit a similar E_m at high pH, $E_{m,pH > pK} = -170 \pm 30$ mV (Prince and Dutton 1978), even when the quinone used for Q_A is different, e.g., menaquinone in *Thermochromatium vinosum* and *Blastochloris viridis* (Parson 1978). However, the location of the pK_a varies widely, ranging from 6.5 in *Thiocapsa pfennigii* (Prince 1978) to 9.8 in *Rba. sphaeroides* (Prince and Dutton 1976, 1978).

It was suggested that, in normal light activated turnover, forward electron transfer from Q_A^- was too fast to allow the coupled proton uptake and that the "operating midpoint potential" of Q_A/Q_A^- at all pH values was that determined above the pK_a (Prince and Dutton 1978). A lower E_m for Q_A would add substantially to the initial yield of the energy conversion in the reaction center. However, it is now known that a single electron equilibrates rapidly between the two quinones, $Q_A^-Q_B \leftrightarrow Q_AQ_B^-$, and remains in the acceptor quinone complex until a second electron arrives from the photochemical events (Wraight 1977, 1982; Verméglio and Clayton 1977).

In isolated RCs from *Rba. sphaeroides*, Q_A reduction also yields the anionic semiquinone (Clayton and Straley 1972; Slooten 1972; Verméglio 1977; Wraight 1977), but it is probably not pH dependent, or only weakly so. Titrations of the $g = 1.82$ EPR signal of Q_A^- showed it to be pH-independent, with $E_m = -45$ mV; the E_m was also unresponsive to occupancy of the Q_B site by o-phenanthroline, whereas the pH-dependent species in chromatophores is sensitive to o-phenanthroline (Jackson et al. 1973; Dutton et al. 1973). However, some titrations of the flash-induced optical signal of P^+ formation in isolated RCs, either incorporated into phospholipid vesicles (Wraight 1981) or in Triton X-100 suspension (Maróti and Wraight 1988b), showed two types of redox titration behavior, one pH-dependent (i.e., with a slope approaching −60 mV/pH), which was responsive to Q_B site inhibitors such as o-phenanthroline and terbutryn, the other pH-independent and unresponsive to inhibitors. Direct measurements of proton uptake by isolated RCs in the Q_A^- state do not support strong pH-dependence, showing only sub-stoichiometric H^+ binding (Maróti and Wraight 1988a, b; McPherson et al. 1988; Shinkarev et al. 1992). The 0.2–0.3 H^+ bound on Q_A reduction would be expected to yield an E_m shift of −15 to −20 mV per pH unit.

The pH independence of Q_A reduction in RCs is supported by measurements on the delayed fluorescence emission in isolated RCs, which monitors the free energy gap between P* and $P^+Q_A^-$ state (Arata and Parson 1981; Arata and Nishimura 1983; McPherson et al. 1990; Turzó et al. 1998, 2000). However, it has also been reported that the intensity of delayed light emission from chromatophores is only weakly pH-dependent (Arata and Nishimura 1983; Wraight 2004). Since the E_m of P^+/P is pH independent (Dutton and Jackson 1972; Dutton and Prince 1978), this implies that the mid-point potential of Q_A/Q_A^- is also essentially pH-independent, in marked contrast to the potentiometric titrations of Q_A. This leaves the stoichiometry of the proton uptake coupled to Q_A reduction and its operating E_m still unresolved. The origin of these discrepancies between isolated RCs and chromatophores and between potentiometric titrations, delayed fluorescence and proton uptake measurements is unknown.

In this work, the E_m of Q_A in chromatophore was determined by measurement of the delayed fluorescence (DF) at different pH values. The observed, very weak pH-dependence will be discussed based on equilibrium binding and redox models.

Materials and methods

Three strains of the photosynthetic bacterium *Rba. sphaeroides* were used for this work. Reaction centers were isolated from the carotenoidless strain R-26 and carotenoid-containing strain Ga, both grown under photoheterotrophic, anaerobic conditions in Sistrom's medium. The carotenoid-containing, non-photosynthetic strain, CYCAI, a deletion strain derived from *Rba. sphaeroides*, 2.4.1, that lacks both cytochrome c_2 and iso-cytochrome c_2, was grown aerobically in the dark in Hutner medium supplemented with the antibiotics kanamycin and spectinomycin corresponding to the resistance cassettes associated with the deletions (Rott

et al. 1993). CYCAI was a kind gift of Prof. T. Donohue (Madison, Wisconsin USA).

To prepare chromatophores from CYCAI, the cells were washed, suspended in 100 mM KCl and 10 mM Tris buffer (pH 8.0) and broken in a French pressure cell. Unbroken cells were eliminated by centrifugation (17,000*g*, 20 min). The supernatant was spun at 200,000*g* for 90 min. The chromatophore pellet was resuspended in a minimum volume of 100 mM KCl, 1 mM Tris (pH 8.0). Bacteriochlorophyll content was determined after extraction in acetone/methanol (7:2 v/v), using an extinction coefficient of 75 mM^{-1} cm^{-1} at 770 nm. Samples for delayed fluorescence measurements were diluted to approximately 30 μM total BChl. The reaction center content in the chromatophore samples was determined by the flash-induced change at 602 nm, indicative of P^+ formation, using an extinction coefficient of 19 mM^{-1} cm^{-1} (Dutton et al. 1975). A saturating flash was delivered from a xenon flash lamp.

Isolation of RC from R-26 and Ga chromatophores followed standard procedures (Maróti and Wraight 1988a). The protein was solubilized by ionic detergent LDAO (N,N-dimethyldodecylamine-N-oxide, Fluka) and purified by subsequent ammonium sulfate precipitation steps followed by DEAE Sephacel (Sigma) anion exchange column chromatography. The fractions of purity index (OD_{280}/OD_{802}) ≤ 1.30 were collected. To exchange the ionic detergent (LDAO) for non-ionic one (Triton X-100), the RC preparation was dialyzed against 1 mM Tris buffer (pH 8.0) and 0.03% Triton X-100 overnight at 4°C under heavy stirring. RC concentration was determined from the absorbance at 802 nm, using an extinction coefficient of 288 mM^{-1} cm^{-1} (Straley et al. 1973).

Delayed fluorescence measurements were performed as described by Turzó et al. (2000). The free energy drop from P* to $P^+Q_A^-$, ΔG_{P^*A}, was calculated by comparison of the delayed and prompt fluorescence yields, according to Arata and Parson (1981):

$$\frac{\int F_d(t)dt}{\int F_p(t)dt} = \frac{k_f \phi_p}{k_d \phi_f} \exp\left(\Delta G_{P^*A}/k_B T\right) \quad (1)$$

$\int F_d(t)dt$ and $\int F_p(t)dt$ are the integrated intensities of delayed and prompt fluorescence, measured in the same sample but at very different excitation intensities (both in the linear region) to give similar emission intensities. $\int F_d(t)dt$ is determined by a one-exponential fit to the decay of the delayed fluorescence signal; $\int F_p(t)dt$ is determined by electronic integration of the prompt fluorescence, using a time constant (0.1 s) similar to that of the delayed fluorescence decay time. $k_B T$ is the Boltzmann factor (25 meV at room temperature), k_f is the radiative rate constant, ϕ_p is the quantum yield of photochemical trapping, k_d is the rate of decay of the delayed fluorescence signal, and ϕ_f is the prompt fluorescence yield.

The use of an exponential fit to the decay of the emission for computing the integration ($\int F_d(t)dt = A_0 \cdot \tau$, where A_0 is the amplitude of emission at $t = 0$, and τ is the lifetime of the exponential decay) introduces some systematic errors in the determination of ΔG_{P^*A} from delayed fluorescence. The $P^+Q_A^- \rightarrow PQ_A$ decay kinetics are not strictly exponential (McMahon et al. 1998), although the deviation at room temperature is very small. Nevertheless, the choice of window over which the delayed fluorescence is fitted, as may be determined by the opening time of the photomultiplier shutter, does affect the numerical outcome. The use of very low light intensity is also essential, to ensure that the delayed and prompt fluorescence emissions are in the linear region. These probably account for most of the discrepancy in ΔG_{P^*A} values reported in the literature, which range from −860 to −910 meV at pH 8.0 (Arata and Parson 1981; Turzó et al. 2000; Rinyu et al. 2004). However, providing a consistent protocol is employed, results are highly reproducible and allow good comparisons between different samples.

Measurements were routinely made in 100 mM KCl, plus 2–10 mM pH buffers Mes, Mops, Tris, Ches and Caps (all from Sigma) to stabilize the pH of the sample solution at different pH values. Valinomycin (5 μg/mL) was always added to chromatophore samples to ensure that any light induced membrane potential was rapidly dissipated before the shutter opened. However, no difference was detected without it, presumably because the flash intensity was too low to provide significant activation. For fluorescence measurements on chromatophores, the total BChl concentration was varied from 7–40 μM, with no influence on the calculated value of ΔG_{P^*A}, indicating that any impact of self absorption is identical for both prompt and delayed fluorescence, as expected if the emission spectra are the same. Reaction center function of each sample was characterized by the near-infrared absorbance spectrum and by measurement of the flash-induced P^+ signal amplitude and $P^+Q_A^-$ recombination kinetics before and after the lengthy delayed fluorescence measurement. Absorbance spectroscopy was performed on a kinetic spectrophotometer of local design.

Results

Figure 1 shows the kinetics of delayed fluorescence (DF) after flash excitation of isolated RCs of *Rba. sphaeroides*, strain R-26, and of chromatophores from the cytochrome deficient mutant (CYCAI). Identical DF characteristics are observed for isolated RCs from the carotenoid-containing

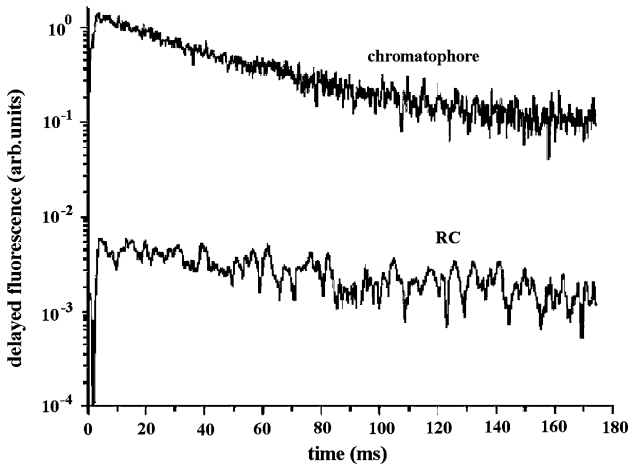


Fig. 1 Decay of delayed fluorescence of bacteriochlorophylls from chromatophores (CYCAI) and isolated RCs (strain R-26) after flash excitation. Average of 128 traces. *Top* trace: (RC) ~ 0.32 μM, 0.02% Triton X-100, 0.5 mM Ches, 100 mM NaCl, and 100 μM terbutryne. *Bottom* trace: Chromatophores (40 μM BChl, 0.32 μM RCs), 10 mM Tris, 10 mM Ches, 100 mM KCl, 5 μM valinomycin and 100 μM terbutryne. Both measurements at pH 9.05, $T = 298$ K

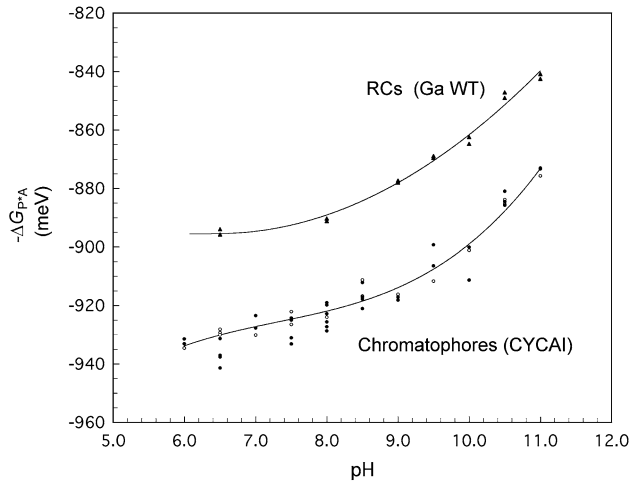


Fig. 2 pH-dependence of free energy gap between P* and $P^+Q_A^-$ determined from delayed fluorescence from two different preparations of chromatophores (*filled circle*, *open circle*) from cytochrome deficient mutant (CYCAI), and isolated RCs (*filled triangle*) of wild type (Ga strain) *Rba. sphaeroides*. For both chromatophores and RCs, the free energy, ΔG_{P^*A}, was calculated using parameters for RCs, as discussed in the text

strain, Ga (Rinyu et al. 2004). The delayed light was extremely weak, with a maximum in the near infrared spectral region (920 nm), and decayed with the same kinetics as the $P^+Q_A^-$ state of the RC (charge recombination) from which it originated. Using the same RC concentration, the intensity of the DF from chromatophores was two orders of magnitude larger than for isolated RCs, indicating a role for the antenna pigments in intensifying the DF.

Delayed fluorescence comes from thermal re-population of the excited primary donor of the RC (P*) from the charge separated states of the RC, and the free energy gap (ΔG_{P^*A}) between P* and a given charge separated state can be determined from the integrated intensity of the delayed fluorescence over the lifetime of the charge separated state, compared to the integrated intensity of the prompt fluorescence (Arata and Parson 1981; Woodbury and Parson 1984). The component observed in the 10–100 ms time range originates from $P^+Q_A^-$, and k_d in Eq. (1) is equivalent to k_P^{obs}, the observed rate constant for decay of P^+ by recombination. The free energy gap (ΔG_{P^*A}) between P* and $P^+Q_A^-$ was determined from the measured DF in isolated RCs and chromatophores at different pH values (Fig. 2). Although offset by 35–40 mV, the pH dependencies are very similar for the two data sets, indicating that the free energy of $P^+Q_A^-$ is only very weakly pH dependent in chromatophores, as previously found for isolated RCs (McPherson et al. 1990; Turzó et al. 2000).

Discussion

The magnitude of the free energy difference, ΔG_{P^*A}, between the initial ($P^+Q_A^-$) and final (P*) states of the delayed fluorescence precursor reaction, is dependent on a number of parameters, as given by Eq. (1). Some of these are uncertain, especially in chromatophores, but none are likely to exhibit significant pH-dependence. Thus, the pH dependence of ΔG_{P^*A} is reliably due to the pH dependence of the free energy level of $P^+Q_A^-$, and we discuss this first. We will then return to the origin of the enhancement of DF emission in chromatophores, and the magnitude of ΔG_{P^*A}.

The midpoint redox potential of P^+/P is well characterized in both isolated RCs and in chromatophores, and is only very weakly pH dependent (Maróti and Wraight 1988b; Dutton and Jackson, 1972; Dutton and Prince 1978). Consequently, the observed weak pH dependence of ΔG_{P^*A} indicates that the redox midpoint potential of Q_A/Q_A^- in chromatophores is also very weakly pH dependent, as it is in isolated RCs. This is in contrast to all the reported potentiometric data for chromatophores (see, e.g., Prince and Dutton 1978). Attention is focused on $E_m(Q_A/Q_A^-)$ rather than $E_m(P^+/P)$ because the primary donor is readily accessible to water soluble redox mediators and exhibits well-behaved ($n = 1$) and reversible titrations under all conditions.

We consider first that the observed pH dependence of $E_m(Q_A/Q_A^-)$ in potentiometric titrations might be an artifact of redox equilibration with the Q pool through the Q_B site,

as suggested earlier (Wraight 2004). A similar effect was proposed for the apparent existence of cyt b_{150} in the bc_1 complex (Crofts et al. 1995), in which the high potential b-heme (b_H or b_{50}) is reduced by ubiquinol binding at the Q_i site, forming the state $b_H^-Q_i^-$. Similarly, a redox link between the pool quinones and the primary quinone through the secondary quinone binding site in chromatophores can produce a strong pH-dependence of the measured E_m of the primary quinone. Alternatively, the pH-dependence in chromatophores might arise from slow equilibrations that are absent in isolated RCs.

Redox link between Q_A and the pool: dismutation in the Q_B site

An artifactual origin of the potentiometric E_m can arise if redox mediators access Q_A only via the Q_B site, and if direct redox mediation to the one-electron states of Q_B is negligible. This is readily imagined since, when bound, Q_B would block access to Q_A while the hydrophobic tail of Q_B blocks access to its own headgroup. Q_A is then additionally reducible through disproportionation with bound Q_BH_2:

$$Q_A(-) + QH_2 \leftrightarrow Q_AQ_BH_2 \leftrightarrow Q_A^-Q_B^- + 2H^+. \quad (2)$$

The quinone in the pool undergoes two electron/two proton reduction:

$$Q + 2e^- + 2H^+ \leftrightarrow QH_2 \quad (3)$$

characterized by a $n = 2$ Nernst titration curve and pH-dependence of the redox midpoint potential, $E_m(Q/QH_2)$, with a slope of −60 mV/pH unit between the highest and lowest pK_a values of the oxidized and reduced forms, respectively, which encompasses the whole range of experimentally accessible pH (5–11) (Takamiya and Dutton 1979).

The primary quinone in the RC is a one electron couple:

$$Q_A + e^- \leftrightarrow Q_A^- \quad (4)$$

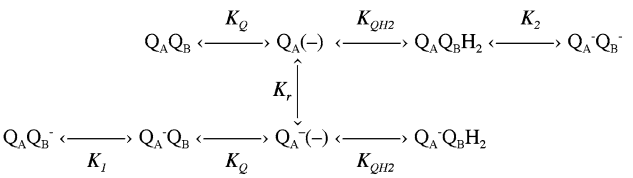
described by a $n = 1$ redox titration with pH independent $E_m(Q_A/Q_A^-)$ (Dutton et al. 1973).

The time scale of a redox titration allows binding and unbinding of quinone and quinol to and from the Q_B binding site (the respective dissociation constants are K_Q and K_{QH2}) and equilibration of the first and second electron in the quinone complex (the electron equilibrium constants are denoted by K_1 and K_2) (Scheme 1). The reduction of Q_A can be readily analyzed by introducing the redox equilibrium constant K_r that can be derived from the Nernst equation. The basic picture of equilibration via the Q_B site can also be extended to include competitive binding of inhibitors, such as o-phenanthroline and triazines (not shown). In order to illustrate the essential features, we assume that the dissociation constants for Q and QH_2 are independent of the redox state of Q_A and therefore the redox midpoint potential of Q_A/Q_A^- is independent of the occupancy of the Q_B site. In chromatophores, but not RCs, some dependence has been shown for inhibitors binding to the Q_B site, but the effect in *Rba. sphaeroides* is small and is, in any case, possibly of the same artifactual origin as the pH dependence.

In a potentiometric titration, binding of QH_2 leads to reduction of Q_A according to Eq. (2), to an extent that depends on the prevailing redox potential, E_h, and the size of the Q pool, which together determine the concentration of QH_2, and the E_m of the Q_B^-/Q_BH_2 redox couple. The latter is also determined by the E_m of the Q pool and the relative affinities (dissociation constants) for Q^- and QH_2 binding at the Q_B site. The pH-dependence of the reduction of Q_A through Q_BH_2 depends on the pK_a values for the bound Q_B states, which are not well known but, at least in the range pH 6–9, the behavior can be expected to follow that associated with $Q_B/Q_B^-(H^+)_m$, $Q_B^-(H^+)_m/Q_BH^-(H^+)_n$ and $Q_BH^-(H^+)_n/Q_BH_2$ (m, $n \leq 1$), where (H^+) indicates protons taken up by the protein in response to the electrostatic influence of the charged quinone states (Wraight 1979b; Maróti and Wraight 1988a, b; McPherson et al. 1988; Beroza et al. 1995; Alexov and Gunner 1999). These yield E_m versus pH slopes of $\leq |-60|$ mV/pH, and we approximate them all as −60 mV/pH.

For illustrative calculations, the following values were taken for the concentrations and dissociation constants: the RC concentration was taken as 0.3 μM, with 30 quinone molecules in the pool per RC, and Q_B-site dissociation constants of 1 μM for quinone and 10 μM for quinol (see McComb et al. 1990). The values for $E_m(Q_A/Q_A^-)$, K_1 and K_2 are taken with some license but in general accord with the literature (Wraight 1979b; Kleinfeld et al. 1984a, 1985; McPherson et al. 1994), as is E_m(Q pool) (Takamiya and Dutton 1979). At each value of the redox potential (E_h), the concentrations of RCs with oxidized and reduced primary quinone were calculated and redox titration curves were constructed at different pH (Fig. 3). Over much of the pH range, Q_A is reduced through the Q_B site and the titration exhibits $n = 2$ behavior, with the pH dependence of the Q/QH_2 pool, i.e., −60 mV/pH unit. At both low and high pH, the direct titration of Q_A begins to become apparent.

In principle, the model described here can account for an apparent pH-dependence of the reduction of Q_A, as well as a



Scheme 1

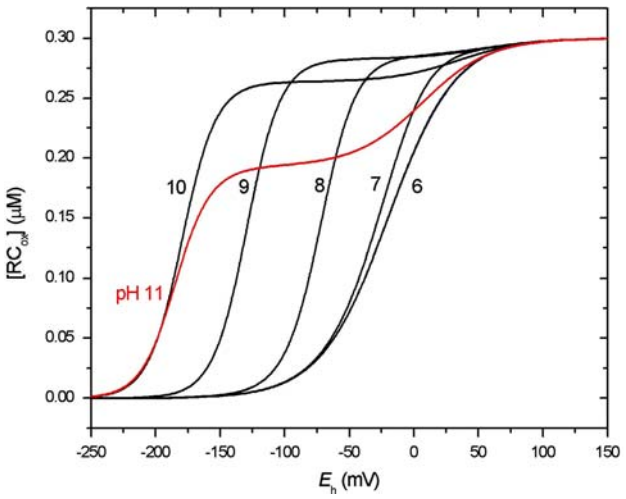


Fig. 3 pH-dependence of the observed midpoint potential of the primary quinone of RC in chromatophore membrane calculated from the equilibrium model (Scheme 1). Conditions: 0.3 μM RC, 10 μM quinone pool, $E_m(Q_A/Q_A^-) = -20$ mV, $E_m(Q/QH_2,\ pool) = 50 + 60\ (7.0 - pH)$ mV, $K_Q = 1$ μM, $K_{QH2} = 10$ μM, $K_1 = [20\ (pH < 9);\ 18\ (pH = 9);\ 8\ (pH = 10);\ 2\ (pH = 11)]$ and $K_2 = 100 \times 10^{(7.0\ -\ pH)}$

shift in E_m in the presence of Q_B inhibitors (not shown). However, the pH dependent region is limited, and to extend this behavior to the range seen in the experimental studies (pH 5–10) requires significant departures from the properties of Q_A or Q_B known or expected from studies on isolated RCs. Although, the value of $E_m(Q_A/Q_A^-)$ in chromatophores is open to speculative choices, being the subject of this study, it is partly constrained by the free energy gap, ΔG_{P^*A}, and by the measured $E_m = 450$ mV for P^+/P. As discussed below, ΔG_{P^*A} is on the order of -860 meV. With an excited state energy of 1.38 eV, this places $P^+Q_A^-$ at about 0.52 eV above the ground state. Thus, at the least, we can expect $E_m(Q_A/Q_A^-) < 0$, consistent with the value of -45 mV for isolated RCs (Dutton et al. 1973).

In isolated RCs, the 2nd electron transfer equilibrium constant, K_2, is about 100 at pH 7, implying that $E_{m,7}$ (Q_B^-/Q_BH_2) is 120 mV more positive than $E_m(Q_A/Q_A^-)$ (Kleinfeld et al. 1985; Takahashi and Wraight 1992). In order to see significant reduction of Q_A by this route, the midpoint potentials of Q_A/Q_A^- and Q_B^-/Q_BH_2 must be significantly closer than this. This could be achieved by stronger binding of Q compared to QH_2 at the Q_B site, as might be expected from a functional point of view, but these parameters are not independent, and changing K_Q/K_{QH2} substantially gives rise to unrealistic values for K_1 and K_2 at one or other end of the pH range. The opposite assumption of stronger binding of QH_2 to the Q_i site of the cytochrome bc_1 complex was made by Crofts in order to account for the appearance of a high potential *b*-cytochrome (cyt b_{150}) (Crofts et al. 1995).

Also at odds with experimental results, which report potentiometric titrations of Q_A in chromatophores to follow a one-electron curve (Prince and Dutton 1978), the simulated titration of Q_A through the Q_B site has clear $n = 2$ character. Much work remains to be done to fully explore this model of Q_A reduction through the Q_B site, but at the present time we must view it with some skepticism.

Slow relaxation of the protein in the Q_A^- state

An alternative explanation of the discrepancy between the pH independent free energy of $P^+Q_A^-$ and the pH dependent potentiometric E_m of Q_A is that the chemically reduced state, Q_A^-, in chromatophores undergoes slow conformational changes in the time necessary for redox equilibration (usually many minutes per data point), and relaxes to a new condition in which stoichiometric proton uptake occurs. In both isolated RCs and in chromatophores, the light induced semiquinones are very long lived in the PQ^- state, i.e., when P^+ is rereduced, but no studies have been reported to say whether the electron transfer equilibria, for example between Q_A and Q_B, or proton uptake change over such time scales. However, it is noteworthy that when isolated RCs are maintained in the $P^+Q_A^-$ state, by long exposure to light in the absence of secondary donors to P^+, substantial changes in proton uptake and release occur over a period of minutes (Kálmán and Maróti 1997). There is, in fact, a substantial literature on slow, and mostly hysteretic, changes in RC behavior during prolonged maintenance of the $P^+Q_A^-$ state (Gouscha et al. 1997, 2004; Andréasson et al. 2003; Agostiano et al. 2005), and, even at cryogenic temperatures, prolonged illumination is reported to induce detectable structural changes (Katona et al. 2005).

Equilibrium conformational states of RCs are also evident in biexponential or non-exponential decay kinetics of $P^+Q_A^-$ and $P^+Q_B^-$. These reflect preexisting populations in the ground state, prior to single turnover flash activation and have been extensively reported (Parot et al. 1987; Sebban and Wraight 1989; Gao et al. 1991; Schoepp et al. 1992; Fufezan et al. 2005). Although possibly related mechanistically, these are observationally distinct from the dynamic conformational distributions that are apparent in the very fast (picosecond) kinetics of charge separation (Kirmaier and Holten 1990), the rapid (nanosecond) recombination from the P^+I^- state (I is the A-branch bacteriopheophytin) (Woodbury and Parson 1984), or in states that can be trapped at low temperature by freezing during illumination (Kleinfeld et al. 1984b; McMahon et al. 1998; Xu and Gunner 2001).

The charge recombination kinetics of *Blastochloris (Blc.) viridis* are well known to be biphasic, and are interpreted as reflecting an equilibrium between two states of the RC (Parot et al. 1987; Sebban and Wraight 1989;

Gao et al. 1991; Schoepp et al. 1992). In this species, the back reaction occurs via the P^+I^- state (Shopes and Wraight 1987) and the biphasic kinetics arise from populations with distinct P^+I^- energy levels (Gao et al. 1991). The equilibrium between conformational states in the ground state (PQ_A) is complete in 10 s (Gao et al. 1991), but the time scale is unknown for the charge separated states, except that it is very slow compared to the $P^+Q_A^-$ and $P^+Q_B^-$ back reactions. More recently it was found that the potentiometric E_m of Q_A is correlated with the relative populations of these two conformers, leading to the suggestion that it reflects a weighted average of two distinct midpoint potentials (Fufezan et al. 2005). However, relating the pH dependence of the conformational equilibrium to the pH dependence of the Q_A potentiometric midpoint potential is not straightforward, with many parameters involved.

Slow attainment of a proton-linked conformational equilibrium is an attractive contender to account the pH-dependence discrepancy we report here for the free energy level of $P^+Q_A^-$ compared to the E_m of Q_A. However, the $P^+Q_A^-$ back reaction in *Rba. sphaeroides* is usually reported as monophasic, except under special circumstances, e.g., with artificial, low potential quinones as Q_A (Sebban 1988) or at low temperature (Franzen and Boxer 1993; McMahon et al. 1998). Thus, an influence of equilibrium conformations on the charge recombination reaction has not been seen in *Rba. sphaeroides*. This may be because $P^+Q_A^-$ charge recombination in *Rba. sphaeroides* occurs by direct electron tunneling from Q_A^- to P^+, rather than via the P^+I^- state, or simply because the kinetics are approximately 10-fold slower. Nevertheless, one should, perhaps, expect similar underlying conformational phenomena regardless of their observable influences on the recombination kinetics.

Even under low light conditions, the quinone states are expected to turnover on the seconds time scale or faster, and the functional relevance of conformational equilibria will depend on the kinetics of interconversion between states. In *Rba. sphaeroides*, at physiological, near-neutral pH, the difference between the transient, pH-independent free energy level of Q_A^- and the equilibrated, pH-dependent E_m is not large, although it becomes significant at more extreme pH values. In other species, however, because of differences in the redox pK_a values, the distinction could be substantial and may be more readily studied.

Intensification of DF by the membrane environment–effects of lipids and the light harvesting antenna

At the same RC content, the intensity of delayed fluorescence from chromatophores was two orders of magnitude larger than for RCs solubilized in detergent (Fig. 1). Only a negligible part of this huge difference can arise from the somewhat different spectral properties of the fluorescence of the bacteriochlorophylls in RC (P) and in the antenna: their peaks of emission are at 915 nm and 890 nm, respectively (Zankel et al. 1968; De Klerk et al. 1969; Zankel and Clayton 1969), but the DF was measured here through a red cutoff filter (RG-850 nm) in both cases and the change in the spectral response of the red-sensitive photomultiplier (Hamamatsu R3310–03) is not significant in this spectral range (<10%). Rather, major reasons are likely to be a change in Q_A energetics due to the membrane environment and the much higher yield of BChl fluorescence in the antenna than in the RC.

The potentiometric midpoint potential reported for Q_A in chromatophores becomes pH-independent above the pK_a of the reduced form ($E_{m,>pK} = -180$ mV, in *Rba. sphaeroides*). This low value was proposed to represent the operating potential of Q_A (Prince and Dutton 1976), based on the presumption that forward electron transfer from Q_A^- occurred much faster (<100 μs) than H^+ uptake (Chance et al. 1970). This would substantially decrease the energy gap to P* and a shift of −120 mV would account for the observed two orders of magnitude larger intensity of the DF in chromatophore than in isolated RC. This was the motivation for an earlier study by Arata and Nishimura (1983), who found that the free energy level of $P^+Q_A^-$ in chromatophores was almost identical to that in chromatophores (0.51 eV above the ground state). However, they considered this to be most likely due to strong electrostatic interaction between P^+ and Q_A^- in chromatophores, which canceled out the lower E_m of Q_A.

In contrast to the kinetic basis of the "operating potential" concept, it is now known that the first electron is shared between Q_A and Q_B in $(Q_AQ_B)^-$ with a lifetime of many minutes when P^+ is rereduced (Wraight 1977, 1979b; Verméglio 1977, 1982). Furthermore, the state $P^+Q_A^-$, from which the DF originates in the absence of functional Q_B, decays in 100 ms, which is very much longer than the time for any functional H^+ uptake (0.1–1 ms) (Petty et al. 1979; Maróti and Wraight 1988a; Takahashi et al 1992; Maróti and Wraight 1997). We find here that the free energy level of Q_A^- is independent of pH and the large shift determined from redox potentiometry must be attributed to an alternative origin, such as the pool quinones or a very slow relaxation of the protein in reduced state. However, the E_m of Q_A might be different in chromatophores in a pH-independent fashion, arising from the specific influence of certain lipids. We have previously reported that cardiolipin enhances the intensity of delayed fluorescence from isolated RCs by a factor of ten, and other phospholipids have similar but smaller effects (Rinyu et al. 2004; Nagy et al. 2004). Cardiolipin is present at high levels in chromatophores from *Rba. sphaeroides* (Onishi and Niederman

1982), and is also one of a few lipids seen in specific contact with the RC in crystal structures (McAuley et al. 1999; Wakeham et al. 2001; Camara-Artigas et al. 2002; Fyfe et al. 2004). Other influences of the native membrane environment might be expected from interactions with the light harvesting pigment proteins that surround the RC (Dezi et al. 2007). These observations clearly indicate that the membrane environment of the RC can contribute to the intensification of the observed delayed fluorescence in chromatophores.

In addition to direct physical interactions between the RC and its environment in the chromatophore membrane, the excitation energy of P (P*) can escape from the RC, via energy transfer to the antenna pigment bed. Once in the antenna, any of the BChls have the chance to emit the excitation energy as a photon of fluorescence with relatively large yield ($\phi_f = 0.002$–0.02 (Zankel and Clayton 1969; van Grondelle and Duysens 1980; Arata and Nishimura 1983; van Grondelle 1985). Because P* in the RC has to compete with the very effective photochemistry ($\phi_p \approx 1.0$ (Wraight and Clayton 1973)), the yield of fluorescence is very low ($\phi_f < 5 \times 10^{-4}$ (Zankel et al. 1968; Woodbury et al. 1985)). However, the RC is only a shallow trap for the excitation energy and the excitation can readily escape to the antenna. The resulting entropy increase serves to draw it out from the RC. The enhancement in DF emission (approx 120-fold) is remarkably similar to the size of the light harvesting antenna (125 ± 5 BChl per RC), but the close agreement is largely fortuitous, since differences in the free energy of $P^+Q_A^-$ clearly exist, as discussed above. Differences in the excited state energy of the RC and antenna BChl* may also contribute, as we now discuss.

The free energy gap ΔG_{P^*A} in chromatophores

Estimating the absolute energy level of $P^+Q_A^-$ in chromatophores, relative to an excited state, involves appropriate choice of several parameters in Eq. (1). For reaction centers, k_f has been taken as $8 \times 10^7\ s^{-1}$, from the Strickler-Berg relationship (Zankel et al. 1968; Ross 1975; Arata and Parson 1981); ϕ_p has been determined to be effectively 1.0 (Wraight and Clayton 1973); k_d is equivalent to the measured rate of decay of $P^+Q_A^-$, the DF precursor state in the 10–100 ms time range, with a typical value of about $10\ s^{-1}$ in wild type RCs; and ϕ_f is the prompt fluorescence yield of P^* ($4.0 \pm 1.5 \times 10^{-4}$ (Zankel et al. 1968) and 3.2×10^{-4} (Woodbury et al. 1985)). The uncertainties in these parameters are small and amount to a maximum effect on the calculated ΔG_{P^*A} that is less than 15 meV. Some systematic uncertainties are potentially more significant, including the use of a single exponential to describe the decay of the DF emission (Turzó et al. 2000), and adherence to a very low light regime for DF measurements, and may generate differences between results from different laboratories. Values from different studies range from −860 to −910 meV (Arata and Parson 1981; Turzó et al. 2000; Rinyu et al. 2004), but within a single experimental set up, the results are highly reproducible (see discussion in Rinyu et al. 2004).

For chromatophores the corresponding parameters are less well established, but for most the range of values is not large. The radiative lifetimes for the BChl pigments have been estimated to be very similar to that of RCs: $k_f = 5$–$8 \times 10^7\ s^{-1}$ (Zankel et al. 1968; Ross 1975; Arata and Nishimura 1983; van Grondelle 1985); ϕ_p has been determined to be greater than 0.9 in chromatophores (Loach and Sekura 1968; van Grondelle 1985); k_d is measured directly and is similar to that in RCs. The uncertainties in these values are of trivial consequence to the calculated value of ΔG_{P^*A}. The main uncertainty lies in the value of ϕ_f, the prompt fluorescence yield of $BChl^*$ in the antenna pigments. In *Rba. sphaeroides*, it was originally reported to range from 0.02 at low light intensity to 0.05 at high light intensity (Zankel et al. 1968; Wang and Clayton 1971). However, subsequent measurements have given lower values of 0.002–0.01, possibly due to the use of lower light intensities (Borisov and Godik 1972; Arata and Nishimura 1983; van Grondelle 1985). Since it is the low light regime that is relevant to calculating ΔG_{P^*A}, we choose a value of $\phi_f = 0.005$. A 5-fold uncertainty in ϕ_f translates to ±20 meV in the calculated ΔG_{P^*A}. Importantly, there is no basis for considering any of these physical parameters to be pH dependent.

Figure 2 shows the values of ΔG_{P^*A} for RCs and chromatophores, *both* calculated using the better known parameters for RCs. The effect of using a value of ϕ_f more suitable for chromatophores (0.005), rather than RCs (4×10^{-4}), is to raise the free energy level of $P^+Q_A^-$ by 65 meV, i.e., to about 860 meV below the excited state, at pH 8. This is about 30 meV above our value for RCs and very similar to that determined by Arata for chromatophores [−870 meV (Arata and Nishimura 1983)] and for RCs (−860 meV (Arata and Parson 1981)). The question arises, however, what is the excited state to which the chromatophore value refers? The fluorescence detection is through a broad-band filter and the emission spectrum arises from both B870 (emission peak at 890 nm) and B850–800 (emission peak at 865 nm) (Zankel and Clayton 1968; Wang and Clayton 1971). At ambient temperatures, the excitation is close to thermal equilibrium (Zankel 1978; van Grondelle 1985), so the relative contributions favor B870 emission, as determined by a Boltzmann distribution. The 0-0 transition for B870 is similar to that of RCs (Zankel et al. 1968), but that for B850–800 is about

35 meV higher. Although a more detailed analysis might be warranted, it can be concluded that the free energy level of $P^+Q_A^-$ is about 0.52 meV above the ground state, similar to that reported by Arata, but with an uncertainty of ±40 meV, as described above. Taking $E_m = 450$ mV for P/P^+ (Kuntz et al. 1964; Dutton and Prince 1978) places $E_m(Q_A/Q_A^-)$ at -70 ± 40 mV. This does not take into account any interaction energy between P^+ and Q_A^-, but Ginet and Lavergne (2000) have concluded that this is less than 28 mV in the closely related species *Rba. capsulatus*. The value of $E_m(Q_A/Q_A^-) = -45$ mV in isolated RCs is at the high end of this range, but can be considered consistent with it.

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