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Properties of the primary and secondary quinone electron acceptors in RC/LH_I complexes from the purple sulfur bacterium *Ectothiorhodospira mobilis*

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Reaction centers (RCs) with one antenna complex attached (RC/LH_I complexes) were isolated from the alkaliphilic and moderately halophilic, phototrophic purple sulfur bacterium *Ectothiorhodospira* (*E.*) *mobilis* and analyzed with respect to quinone composition and charge recombination kinetics, using time-resolved (low- and room-temperature) spectroscopy and thin-layer chromatography. The RCs contain menaquinone as primary and ubiquinone as secondary quinone electron acceptor, Q_A and Q_B, respectively. Q_B is lost during isolation of the RC/LH_I complexes. P⁺Q_A⁻ charge recombination kinetics, which were shown to be pH independent, were only slightly temperature dependent, indicating that this recombination proceeds via the direct (electron tunnelling) route. At room temperature, its average lifetime was 34.5 ms. These decay kinetics were shown to be monophasic at room temperature and biphasic at low temperature. Addition of an excess of UQ₆ to RC/LH_I complexes resulted in retardation of the P⁺ recovery, due to charge recombination of the state P⁺Q_AQ_B⁻. Functional reconstitution of Q_B was also evident from flash-induced binary oscillations at 450 nm, which could be observed in RC/LH_I complexes in the presence of an excess of UQ₆. This reconstitution of Q_B activity was, however, incomplete and decreased with increasing pH. The P⁺Q_AQ_B⁻ decay kinetics were pH independent; the average lifetime was about 6 s at room temperature. The apparent equilibrium constant K_2 between the states Q_A⁻Q_B and Q_AQ_B⁻, and consequently the free energy difference between these states, were relatively large and pH independent. K_2 was on average 167, whereas the mean value of the free energy difference between the two states was 130 meV. Finally, a scheme is presented for the kinetics and free energy changes of the electron transfer steps in isolated *E. mobilis* RCs.

Introduction

The family of the Ectothiorhodospiraceae, comprising anoxygenic phototrophic purple sulfur bacteria,

contains a single genus, *Ectothiorhodospira* [1], which is characterized by its dependence on saline and alkaline conditions [2] (exception for *E. marismortui* [3]). In contrast to the Chromatiaceae, to which they formerly belonged, the Ectothiorhodospiraceae accumulate sulfur globules outside the cells when sulfide is used as electron donor (reviewed in Ref. 4).

In purple bacteria a cyclic electron transfer chain, involving membrane-spanning photochemical RCs and a cytochrome *bc*₁ complex as well as *c*-type cytochrome(s), is supposed to be primarily responsible for the conversion of light energy into an electrochemical proton gradient, which can subsequently be used for, for example, synthesis of ATP or reduction of NAD⁺ (see, for example, Ref. 5). Most studies on cyclic electron transport have, however, been performed in representatives of the α - or β -subclass of the Proteobacteria. Much less is known (except for *Chromatium* (*Chr.*))

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Abbreviations: BChl, bacteriochlorophyll; BPh, bacteriopheophytin; diaminodurene, 2,3,5,6-tetramethyl-1,4-phenylenediamine; $\Delta G_{(AB)}^0$, free energy difference (between P⁺Q_A⁻Q_B and P⁺Q_AQ_B⁻); Hepes, *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethane-sulfonic acid]; LH_{I(II)}, light-harvesting complex I (II); MQ_{*n*}, menaquinone (vitamin K₂) with tail length of *n* isoprenoid units; octyl glucoside, *n*-octyl β -D-glucopyranoside; *o*-phenanthroline, 1,10-phenanthroline; P^(*), (excited singlet state of) primary donor (special pair BChl); P⁺Q_{A(B)}⁻, simplified notation of the state P⁺Q_A⁻Q_B (P⁺Q_AQ_B⁻); Q_{A(B)}, primary (Q_A) or secondary (Q_B) quinone electron acceptor; RC, reaction center; R_F, relative migration distance; UQ_{*n*}, ubiquinone with tail length of *n* isoprenoid units.

vinosum) about members of the γ -subclass, the purple sulfur bacteria. In this communication we present a characterization of the light-induced electron transfer kinetics, occurring on the millisecond to second time-scale, in RCs of the BChl a -containing *Ectothiorhodospira mobilis*.

The RC of purple bacteria is a membrane-spanning pigment-protein complex containing four BChls, two bacteriopheophytins (BPh), a non-heme iron atom and two quinones, the primary and secondary electron acceptors (Q_A and Q_B , respectively). These components are symmetrically arranged by two protein subunits (L and M), which together with a third subunit (H), comprise the RC protein structure (recently reviewed in Refs. 6 and 7). After excitation of the special pair BChl, an electron is transferred from the excited singlet state of the primary donor (P^*) via BPh_L to Q_A (the subscript L refers to the L branch of the RC). Whether monomeric BChl is involved in electron transfer from P^* to Q_A , is not clear yet (see, for example, Refs. 8 and 9). Charge separation is subsequently stabilized by transfer of the electron from Q_A to Q_B , resulting in formation of $P^+Q_A^-Q_B^-$ ($P^+Q_B^-$). Electron transfer between Q_A and Q_B is reversible, leading to an equilibrium between the states $Q_A^-Q_B$ and $Q_AQ_B^-$. Despite the symmetric organization of the co-factors in the RC, electron transfer proceeds only via one branch (see Refs. 6,10,11 for recent reviews).

The negative charge on Q_A and Q_B induces pK shifts of nearby amino acid residues, resulting in protonation of the RC and stabilization of the charge on the Q_B^- semiquinone [12–14]. After the second turnover of the RC, Q_B^- is reduced further and Q_BH_2 is formed after a second proton has been taken up (recently reviewed in Ref. 15). Q_BH_2 diffuses out of the Q_B -site of the RC and is replaced by quinone from the Q-pool, due to a difference in affinity for Q_B and Q_BH_2 of this site [16].

In the absence of electron donors, light-induced charge separation is followed by charge recombination. Determination of charge recombination of the states $P^+Q_A^-$ and $P^+Q_B^-$ provides detailed information about the functionality of the RCs. $P^+Q_B^-$ recombination usually proceeds by more than 95% via state $P^+Q_A^-$ [17,18]. In *Rhodobacter (Rb.) sphaeroides*, $P^+Q_A^-$ recombines via a direct, electron-tunnelling process [19,20] and is only slightly temperature-dependent [19]. In *Rhodospseudomonas (Rps.) viridis* $P^+Q_A^-$ recombination occurs via two parallel routes: direct recombination between P^+ and Q_A^- (like in *Rb. sphaeroides*), and a thermally activated recombination via the intermediate state P^+BPh^- [21]. In this organism hence a strong temperature dependence of the $P^+Q_A^-$ charge recombination kinetics is observed [22]. The thermally activated process dominates at high temperature (above 250 K), while the direct electron-tunnelling process

becomes dominant at temperatures below 250 K. In *Rb. sphaeroides*, $P^+Q_A^-$ recombination is only slightly temperature-dependent.

In this study we report on the nature of Q_A and Q_B , and on the temperature and pH-dependence of the charge recombination kinetics of RCs of the alkaliphilic and moderately halophilic, purple sulfur bacterium *E. mobilis*. The results are discussed in the light of data reported for more extensively characterized species like *Rb. sphaeroides* and *Rps. viridis*.

Materials and Methods

Isolation of intracellular membranes and RC/antenna complexes

E. mobilis strain BN9903 was grown as described previously [23]. *Chloroflexus (C.) aurantiacus* strain OK 70-fl was grown anaerobically at 52°C, essentially as reported in Ref. 24. *Rhodospirillum (R.) rubrum* strain G9 was grown at 30°C in degassed Hutner medium (essentially as described in Ref. 25). Intracellular membranes (chromatophores)¹ were isolated from all strains as reported previously [23]. Chromatophores were resuspended in 50 mM Na-Hepes (pH 8.0), 5 mM $MgCl_2$ and 1 mM phenylmethylsulfonylfluoride (PMSF). For *E. mobilis* this buffer was supplemented with 500 mM NaCl. Isolation of both RC/LH $_I$ and RC/LH $_I$ /LH $_{II}$ complexes from chromatophores with the non-ionic detergent *n*-octyl β -D-glucopyranoside (octyl glucoside), was performed as described previously [26]. Both chromatophores and RC/antenna complexes were stored in liquid nitrogen until use.

Spectroscopy

Kinetics of donor recovery ($P^+ \rightarrow P$) were measured at 813.8 nm (unless stated otherwise) as described previously [27]. The decay curves were analyzed using software developed by Dr. J. Lavorel (C.E. de Cada-rache, France). Elementary simplex algorithms were used to determine τ_i , by least-square analysis, in the model function $\sum \alpha \exp(-\tau/\tau_i)$ [28]. The laser artefact was subtracted from the decay curves. Semiquinone oscillations were measured at 450 nm on a home-built spectrophotometer as reported [29].

The buffers used for the spectroscopic measurements were: Mes between pH 5.5 and 6.5, Hepes between pH 7.0 and 8.5, glycylglycine at pH 9.0, CAPS between pH 9.5 and 11.0.

¹ The intracellular membranes isolated from the Ectothiorhodospiraceae do not form closed vesicles, but rather have the form of thylakoid stacks. They are termed chromatophores for convenience only.

Analytical procedures

All chemicals were obtained from commercial sources. RC concentrations were determined as described previously [26]. Separation of quinones was performed on thin-layer silica-gel plates, containing fluorescent indicator (Kieselgel 60 F254, Merck, Germany), with petroleum ether/diethyl ether (9:1, v/v) as eluent [30]. The quinone spots were detected with UV light (red fluorescent spots), or by spraying with Methylene blue or Nile blue [30].

Results

Identification of the primary and secondary quinones

To investigate the nature of the primary and secondary quinones in the RCs of *E. mobilis*, we extracted and identified quinones from various samples, using silica gel thin-layer chromatography. Relative migration rates (R_F values) of the quinones are shown in Table I. The different spots were identified by using membranes from other organisms and synthetic quinone as a reference.

In chromatophores of *E. mobilis* both ubiquinone and menaquinone could be detected (presumably MQ₇ and UQ₇ c.f. Ref. 31). Ubiquinone was, however, lost during isolation of the RC/antenna complexes from the photosynthetic membranes of *E. mobilis*. Only a weakly visible spot could be observed in extracts from RC/LH_I/LH_{II} complexes, whereas no ubiquinone at all could be detected in RC/LH_I complexes. Only menaquinone was observed in these latter complexes (Table I). Besides the spots corresponding to ubiquinone and menaquinone, additional spots were visible in all lanes containing extracts from RC preparations. These were presumably due to carotenoids and ergos-

TABLE I

R_F values of quinones present in various reaction center preparations

The error in the R_F values was approx. 5%. The spots corresponding to ubiquinone and menaquinone were detected by fluorescence under UV light or by spraying with Methylene blue (fast detection of ubiquinone, much slower detection of menaquinone (about 30 min after spraying; c.f. Ref. 30)) or Nile blue. Preparation of neutral lipid extracts was performed as described in Materials and Methods. * slightly visible; –, not detectable.

Organism	Sample	UQ	MQ
<i>E. mobilis</i> ^a	chromatophores	0.26	0.75
<i>E. mobilis</i>	RC/LH _I /LH _{II} complexes	0.26 *	0.75
<i>E. mobilis</i>	RC/LH _I complexes	–	0.75
<i>C. aurantiacus</i> ^b	chromatophores	–	0.79
<i>R. rubrum</i> G9 ^c	chromatophores	0.31	–
	UQ ₆ (synthetic)	0.22	–

^a *E. mobilis* cells contain UQ₇ and MQ₇ [31].

^b *C. aurantiacus* cells contain MQ_{8–10} [32].

^c *R. rubrum* G9 cells contain UQ₁₀ [31].

terol (c.f. Ref. 30). These results indicate that *E. mobilis* RCs contain menaquinone as Q_A and ubiquinone as Q_B, the latter being lost during isolation of RC/antenna complexes (see below).

$P^+Q_A^-$ charge recombination kinetics at room temperature

At pH 6 in the presence of *o*-phenanthroline (to inhibit reduction of residual Q_B) and without secondary donors present that could reduce P⁺, the charge-separated state of *E. mobilis* RC/LH_I complexes was studied after single saturating flashes. The state P⁺Q_A[–] decayed biphasically with a lifetime τ_1 ($1/k_{\text{fast}}$) of 35 ms for the fast phase and a lifetime τ_2 ($1/k_{\text{slow}}$) of 174 ms for the slow phase (Fig. 1A). The

Fig. 1. pH dependence of P⁺Q_A[–] charge recombination kinetics in *E. mobilis* RC/LH_I complexes at room temperature. (A) Decay curve measured at pH 6, $\tau_1 = 35.0$ ms (92%), $\tau_2 = 174.1$ ms (8%); (B) The lifetime of the fast phase of P⁺ recovery, τ_{Q_A} , determined from a biexponential non-linear least-squares fit to the data; the error in these values was $\leq 5\%$. The contribution of the fast phase was $\geq 91\%$ over the entire pH range, the slow phase is omitted from the figure (see text). Conditions: 0.6 μ M RC in 20 mM buffer, 5 mM KCl, 4.5 mM octyl glucoside, 4.5 mM sodium deoxycholate and 2 mM *o*-phenanthroline; no exogenous donor was present. The measurements were performed at room temperature as described in Materials and Methods.

amplitude of the fast phase of P^+ recovery was strongly dominant ($\geq 91\%$) in the entire range between pH 6 and 11. Both the amplitude and the lifetime of the fast and the slow phase of P^+ recovery were pH-independent over the pH range studied. The average lifetime of τ_1 over this pH range was 34.5 ms (Fig. 1B); the slow phase was neglected (see below).

At room temperature, $P^+Q_A^-$ charge recombination in *Rps. viridis* RCs shows biphasic kinetics [21]. The lifetime as well as the amplitude of both phases of $P^+Q_A^-$ charge recombination are strongly pH-dependent in *Rps. viridis* [33–34] (also in the presence of *o*-phenanthroline), indicating that the slow phase of $P^+Q_A^-$ recombination with an amplitude $\leq 9\%$ in the *E. mobilis* RCs (Fig. 1A) very likely arose from a small, instrumental base-line drift. We conclude that the $P^+Q_A^-$ charge recombination kinetics in *E. mobilis* RCs are apparently monophasic at room temperature as described for *Rb. sphaeroides*, *Chr. minutissimum* and *Rubrivivax (Rv.) gelatinosus* (formerly *Rhodocyclus gelatinosus*) [20,35,36].

$P^+Q_A^-$ charge recombination kinetics at low temperature

Fig. 2 displays the temperature dependence of $P^+Q_A^-$ charge recombination kinetics in RC/LH₁ complexes of *E. mobilis*. The rate constant gradually increased 2-fold after lowering the temperature from 295 to 125 K; below this temperature, it remained constant. These data demonstrate that $P^+Q_A^-$ charge recombination in *E. mobilis* RCs occurs almost exclu-

Fig. 2. Temperature dependence of the lifetime of $P^+Q_A^-$ charge recombination, τ_{QA^-} , in *E. mobilis* RC/LH₁ complexes. RC/LH₁ complexes (pH 8) were mixed with glycerol and *o*-phenanthroline to give final concentrations of 67% (v/v) glycerol, 2 mM *o*-phenanthroline and 0.6 μ M RCs, respectively; no exogenous donor was present. The data were analyzed with a monoexponential fit; the error in the lifetimes was $\leq 15\%$. The measurements were performed as described in Materials and Methods.

Fig. 3. Kinetics of $P^+Q_A^-$ charge recombination in *E. mobilis* RC/LH₁ complexes at low temperature, measured near the isosbestic point at 798 nm. The lifetimes ($\geq 75\%$) were 25 ms at 797.8 nm (A), and 10 ms at 798.3 nm (C). Temperature: 108 K, other conditions as in Fig. 2.

sively via the direct electron-tunnelling route, as is also the case for *Rb. sphaeroides* [19,20], and not via two parallel routes, as observed in *Rps. viridis* [21], because in the latter case, the rate constant decreases with decreasing temperature (down to about 240 K, whereafter the rate between 240 K and 80 K is temperature-independent).

Besides the biphasic nature of $P^+Q_A^-$ charge recombination kinetics observed in *Rps. viridis* at room temperature, it has been reported [27,37] that lowering the temperature induces biphasicity of the $P^+Q_A^-$ charge recombination kinetics in *Rb. sphaeroides* and *R. rubrum*, organisms that show monophasic kinetics at room temperature. This temperature-induced heterogeneity in the decay kinetics is thought to be due to the existence of 'fast' and 'slow' conformational RC states, possessing different rates of charge recombination and slightly distinct light-induced difference spectra [27,37]. At room temperature a fast equilibrium (compared to the recombination) occurs between these two conformational states, and the two states thus tend to follow a common monophasic recombination reaction [28].

The $P^+Q_A^-$ charge recombination kinetics in *E. mobilis* RC/LH₁ complexes, which are essentially monophasic at room temperature (see above), became also biphasic after lowering the temperature (Fig. 3). The absorbance changes observed around the isosbestic point (at approximately 798 nm) in the light-induced difference spectrum of the *E. mobilis* RCs, revealed complex kinetics. At the isosbestic point, two decays were observed in the *E. mobilis* RCs with opposite signs (Fig. 3B). At a slightly shorter wavelength, at 797.8 nm, a slow component with a lifetime of 25 ms ($\approx 75\%$) could be observed (Fig. 3A), while at a slightly longer wavelength, at 798.3 nm, a fast component was observed with a lifetime of 10 ms ($\approx 75\%$), see Fig. 3C.

At 813.8 nm, the wavelength at which most of the measurements were performed, biexponential fitting of the $P^+Q_A^-$ decay curves measured at low temperature (≈ 100 K), revealed two phases with approximately the same lifetimes as around the isosbestic wavelength, $\tau_1 = 11.9$ ms ($\approx 20\%$) and $\tau_2 = 24.2$ ms ($\approx 80\%$). This supports the existence of two conformational states of the RCs of *E. mobilis* too, as has been suggested previously for *Rb. sphaeroides* and *R. rubrum* [27,37]. The overall lifetime measured at 813.8 nm at low temperature was comparable to that observed at 880 nm (not shown; see also Ref. 27).

$P^+Q_B^-$ charge recombination kinetics

Above, we reported that Q_B is lost during isolation of RC/LH_I complexes from the photosynthetic membranes of *E. mobilis*. This suggestion is supported by the kinetics of P^+ recovery in *E. mobilis* RC/LH_I complexes in the absence of *o*-phenanthroline and exogenous donor. The kinetics of P^+ recovery in RC/LH_I complexes, either in the presence or absence of *o*-phenanthroline, were comparable; the lifetime was about 35 ms at pH 7 in the absence of inhibitor (compare with Fig. 2).

To check whether the activity of Q_B could be restored by adding exogenous quinone, P^+ recovery was measured in RC/LH_I complexes of *E. mobilis* in the presence of an excess of UQ_6 . These P^+ recovery kinetics could clearly be resolved into two exponential decays. The fast decay had a lifetime, τ_1 , of 34.6 ms at pH 7 (Fig. 4A; amplitude approx. 55%) and of 33.9 ms at pH 11 (Fig. 4B; amplitude approx. 70%), and is due to recombination of the state $P^+Q_A^-$ (see above). The slower decay had a lifetime, τ_2 , of 5.9 s at pH 7 (Fig. 4A; amplitude approx. 45%) and of 4.8 s at pH 11 (Fig. 4B; amplitude approx. 30%). The contribution of this slow phase was very sensitive to the inhibitor *o*-phenanthroline, indicating that the retardation of P^+ recovery was due to charge recombination from the state $P^+Q_B^-$.

The decay of $P^+Q_B^-$ can be described by two parallel routes, either via the equilibrium population with

Scheme 1.

$P^+Q_A^-$, or via direct recombination between P^+ and Q_B^- [17], as shown in Scheme I (see also Fig. 8, which is further discussed below). It has been demonstrated [17–18] that in both *Rb. sphaeroides* and *Rps. viridis* direct recombination between P^+ and Q_B^- (k_{dir} , Scheme I) is negligible and that decay of $P^+Q_B^-$ is strongly dominated by charge recombination via $P^+Q_A^-$ over a wide range of pH values (k_{BP}).

If the electron transfer rates between the quinones are fast compared to charge recombination (i.e., $k_{AB}, k_{BA} \gg k_{AP}$, see Scheme I), an equilibrium will be established between the $P^+Q_A^-Q_B$ and $P^+Q_AQ_B^-$ states, in accordance with the free energy difference ($-\Delta G_{AB}^0$) between these two states [17]. Giangiacomo and Dutton [38] have shown that the free energy difference between the states $P^+Q_A^-Q_B$ and $P^+Q_AQ_B^-$ strongly influences the apparent affinity of the Q_B site for quinone: $-\Delta G_{AB}^0$ has to be sufficiently large to detect quinone at the Q_B site. By using UQ_6 , $-\Delta G_{AB}^0$ appeared to be sufficiently large in the *E. mobilis* RCs (see below). The contribution of the slow phase of P^+ recovery in the absence of inhibitor (Fig. 4) is indicative for the relative binding affinity of the exogenous UQ_6 for the Q_B site (c.f. Refs. 35,39). Addition of UQ_{10} instead of UQ_6 , did not increase the relative contribution of the slow phase in RC/LH_I complexes. Charge recombination kinetics measured in chromatophores of *E. mobilis* revealed about the same proportion of slow and fast phases as observed in the isolated RC/LH_I complexes (Fig. 4), suggesting that the relatively low ratio of functional Q_B over P observed in the

Fig. 4. Kinetics of P^+ recovery in *E. mobilis* RC/LH_I complexes at room temperature in the presence of excess UQ_6 . (A) decay curve measured at pH 7, $\tau_1 = 34.6$ ms ($\approx 55\%$), $\tau_2 = 5.9$ s ($\approx 45\%$); (B) pH 11, $\tau_1 = 33.9$ ms ($\approx 70\%$), $\tau_2 = 4.8$ s ($\approx 30\%$). The curves are plotted on two time scales. Conditions: 75 μ M UQ_6 , no inhibitor present, other conditions as in Fig. 1; no exogenous donor was present.

Fig. 5. pH dependence of P⁺Q_B⁻ charge recombination kinetics in *E. mobilis* RC/LH₁ complexes at room temperature. (A) Relative amount of P⁺Q_B⁻ charge recombination. This amount was determined by dividing the amplitude of the slow phase of P⁺ recovery in the absence of inhibitor, by the total amplitude (i.e., charge recombination in the presence of 2 mM *o*-phenanthroline); (B) Lifetime of P⁺Q_B⁻ recombination, τ_{Q_B}; the error in these values was ≤15%. Conditions as in Fig. 4.

isolated RC/antenna complexes was not due to the use of synthetic UQ₆ instead of natural UQ₇. The contribution of the slow phase of P⁺ recovery in RC/LH₁ complexes in the presence of an excess of UQ₆ decreased with increasing pH (Figs. 4 and 5A), indicating a pH dependence of the binding of UQ₆ at the Q_B site. This was also observed in *Chr. minutissimum* and some mutants of *Rb. sphaeroides* [39,40].

The pH dependence of the P⁺Q_B⁻ charge recombination kinetics (τ_{BP} = 1/k_{BP}, Scheme I, and Fig. 8) is shown in Fig. 5B. In the range studied, pH 6–11, no pH dependence of τ_{BP} could be observed. The average lifetime of P⁺Q_B⁻ charge recombination over this range was approx. 6 s, comparable to the lifetime of 8 s in *Chr. minutissimum* [35], but significantly longer than the lifetimes observed for *Rps. viridis* and *Rb. sphaeroides* (≈ 0.1 and ≈ 1 s, respectively [17,34]).

$Q_A^-Q_B \leftrightarrow Q_A Q_B^-$ equilibrium

When semiquinone behaviour was examined in *E. mobilis* RC/LH₁ complexes at pH 7, using multiple saturating flashes in the presence of an exogenous donor (diaminodurene) and an excess of UQ₆, the typical flash-induced binary oscillations [41] of the absorbance at 450 nm were observed. These oscillations reflected the formation of the semiquinone Q_B⁻ species on odd-numbered flashes and quinol, Q_BH₂, on even-numbered flashes (Fig. 6). The amplitude of the semiquinone signal after the second flash was smaller than expected from the extinction coefficients given in Ref. 42. This was probably due to loss of Q_B (see above). The signal size was, however, in reasonable agreement with the amplitude of P⁺Q_B⁻ recombination demonstrated by the contribution of the slow phase of P⁺ recovery in Fig. 4A.

The lifetime of P⁺Q_B⁻ decay in *E. mobilis* RCs (Figs. 4 and 5) was interpreted in terms of an equilibrium of the states P⁺Q_A⁻Q_B and P⁺Q_AQ_B⁻. The apparent equilibrium constant, K₂, between both states is given by [43]:

$$K_2 = \frac{k_{AP}}{k_{BP}} - 1 = \frac{\tau_{BP}}{\tau_{AP}} - 1 \quad (1)$$

As shown in Fig. 7, no effect of the pH on the apparent equilibrium constant K₂ was observed, due to the pH-independence of both P⁺Q_A⁻ and P⁺Q_B⁻ charge recombination kinetics (Figs. 1B and 5B, respectively). The mean value of K₂ over the pH range 6–11 was 167. This high value for K₂ in RCs of *E. mobilis* is comparable to that observed in both *Chr. minutissimum* and *Rps. viridis* (at neutral pH), but an order of

Fig. 6. Semiquinone oscillations at pH 7 in *E. mobilis* RC/LH₁ complexes. The absorbance at 450 nm was measured respectively 100 μs, 0.5, 1 and 1.5 s after the flashes (●); the flashes are indicated by arrows. Conditions: 3 μM RC in 20 mM buffer, 5 mM KCl, 4.5 mM octyl glucoside, 4.5 mM sodium deoxycholate, 75 μM UQ₆ and 5 mM diaminodurene (exogenous donor). The measurements were performed at room temperature as described in Materials and Methods.

Fig. 7. pH dependence of K_2 , the apparent $Q_A^- Q_B \leftrightarrow Q_A Q_B^-$ equilibrium constant, as derived from relation (1) and the data from Figs. 1B and 5B. The error in the K_2 values is $\leq 20\%$.

magnitude higher than found for RCs of *Rb. sphaeroides* [17].

Using K_2 , the free energy difference between the states $P^+ Q_A^- Q_B$ and $P^+ Q_A Q_B^-$ can be calculated [17]:

$$K_2 = \frac{k_{AB}}{k_{BA}} = \exp(-\Delta G_{AB}^0 / kT) \quad (2)$$

where k is Boltzmann's constant and T the absolute temperature. From this equation we obtain for this free energy difference:

$$-\Delta G_{AB}^0 = kT \ln(K_2) \quad (3)$$

Using the mean value of 167 for the apparent equilibrium constant K_2 , this leads to a free energy difference, $-\Delta G_{AB}^0$, of 130 meV. This value of $-\Delta G_{AB}^0$ suggests, compared to *Rb. sphaeroides* ($-\Delta G_{AB}^0 = 60$ meV [39]), an increased stabilization on the state $P^+ Q_A Q_B^-$ relative to the $P^+ Q_A^- Q_B$ in *E. mobilis*.

Discussion

Nature of primary and secondary quinone and quinone binding

The data presented in this paper clearly demonstrate that the RCs of the alkaliphilic and moderately halophilic, purple sulfur bacterium *E. mobilis* contain menaquinone as the primary and ubiquinone as the secondary quinone electron acceptor. The fact that Q_B is an ubiquinone is consistent with the high midpoint potential of cytochrome c_1 found in this bacterium [44], which is indicative for ubiquinone, rather than menaquinone, dominating the quinone pool in the membranes of *E. mobilis*. This is in agreement with the higher concentration of ubiquinone relative to menaquinone in the membranes of *E. mobilis* [31].

It is clear that Q_B is lost during isolation of RC/LH₁ complexes from the photosynthetic membranes of *E.*

mobilis. Strikingly, loss of Q_B during isolation is reported for many photosynthetic bacteria that possess a mixed system of quinone electron acceptors in the RC [22,35,36,45–47]. Bacteria containing ubiquinone for both Q_A and Q_B appear to have a more stable Q_B site [48]. Strong variation in the Q_B -binding affinity, independent of ΔG^0 values, was, however, observed [39] in herbicide-resistant mutants of *Rb. sphaeroides* (one of the organisms that contains only ubiquinone in its RC). In addition to this, it has been observed for herbicide-resistant mutants of *Rps. viridis* (one of the organisms that contains a mixed system of quinones in its RC), that structural changes decreased the affinity of the Q_B site for quinone [49], but they had no effect on the electron transfer kinetics from Q_A^- to Q_B , nor on the recombination rates [18,50]. Further studies are required to clarify this issue of Q_B -binding.

$P^+ Q_A^-$ charge recombination kinetics

The average $P^+ Q_A^-$ charge recombination time was shown to be 34.5 ms in RC/LH₁ complexes of *E. mobilis*. This is comparable to decay times reported for other photosynthetic bacteria that possess menaquinone as Q_A and ubiquinone as Q_B , like *Chr. minutissimum*, *Rv. gelatinosus* and *Chr. vinosum* [35,36,46], but faster than observed for *Rb. sphaeroides* [17]. The lower redox potential of menaquinone compared to ubiquinone is, however, not the only reason for these differences in the $P^+ Q_A^-$ charge recombination rate, since the lifetime observed in RCs of *Rps. viridis* (also having MQ as Q_A and UQ as Q_B) is much faster; $1/k_{\text{fast}}$ is about 0.6 ms and $1/k_{\text{slow}}$ equals 2.7 ms [33]. In addition to the nature of Q_A , the effective midpoint potential, which is subtly modulated by the protein environment in the RC, and the standard free energy gap between $P^+ Q_A^-$ and P^* , are important in determining the charge recombination rate from $P^+ Q_A^-$ [20].

In RCs of *Rb. sphaeroides* in which Q_A had been replaced by anthraquinone, resulting in a free energy gap between $P^+ Q_A^-$ and P^* of less than 0.8 eV [51,52], charge recombination occurred almost exclusively via an indirect, thermally activated process via the state $P^+ BPh^-$, while in RCs with a free energy gap of more than 0.8 eV, the decay proceeded via a temperature-insensitive, activationless process of electron tunnelling [20]. The temperature dependence of charge recombination in RCs of *Rb. sphaeroides* containing anthraquinone as Q_A became qualitatively similar to that observed for *Rps. viridis* RCs [21]. The smaller free energy gap in *Rps. viridis* RCs compared to *Rb. sphaeroides* is assigned to the lower excitation levels of BChl_b and BPh_b compared to BChl_a and BPh_a and to the lower redox potential of Q_A [53].

Although we did not determine exact redox midpoint potentials, the energy gap between P^* and $P^+ Q_A^-$ is very likely larger than 0.8 eV in *E. mobilis* RCs as

found for *Rb. sphaeroides* (note that *E. mobilis* also contains BChl_a and BPha). Furthermore, this conclusion is confirmed by the observed temperature dependence of the $P^+Q_A^-$ recombination kinetics in *E. mobilis* (Fig. 2), which is indicative for a direct, electron tunnelling process.

In contrast to the biphasicity of the $P^+Q_A^-$ charge recombination kinetics in *Rps. viridis* RCs, these kinetics are essentially monophasic in *E. mobilis*. At low temperature, however, we did observe heterogeneous $P^+Q_A^-$ recombination kinetics in *E. mobilis*, comparable to the temperature-induced heterogeneity described for *Rb. sphaeroides* and *R. rubrum* [27,37]. Although both types of heterogeneity (i.e., the temperature-induced and the temperature-independent heterogeneity) are supposed to be caused by a similar phenomenon, i.e., 'fast' and 'slow' conformational states of the RC, the precise relation between them is still unclear [37,53].

$P^+Q_B^-$ charge recombination kinetics

Fig. 8, summarizes the results presented above on the kinetics and free energy changes of the electron transfer steps occurring in isolated *E. mobilis* RCs. The kinetics of P^+ reduction by *c*-type cytochromes will be described in a following paper. From the quinone-oscillation experiments (Fig. 6), the rate of

Fig. 8. Scheme of the kinetics and free energy changes of electron transfer steps in reaction centers, isolated from *E. mobilis*. Light excites the special pair BChl₂ (denoted P) to form the excited singlet state P* (open arrow). Decay from this state can be nonradiative or by fluorescence, the overall rate constant of decay is estimated to be 10^9 s^{-1} for *Rb. sphaeroides* [54]. Charge separation occurs, yielding P^+ and Q_A^- (denoted $P^+Q_A^-Q_B$). Other energy levels (termed intermediate states) including BPh and possibly monomeric BChl (see Refs. 10–11 for discussion) are involved in electron transfer from the singlet to the $P^+Q_A^-Q_B$ state. This latter state reduces Q_B , yielding the state $P^+Q_A^-Q_B$. Charge recombination of $P^+Q_A^-Q_B$ proceeds either via the thermally excited state $P^+Q_A^-Q_B$ at k_{BP} or directly at k_{dir} . Transfer between the states $P^+Q_A^-Q_B$ and $P^+Q_A^-Q_B$ proceeds at k_{AB} or k_{BA} . Either in the absence of Q_B , or after inhibition of electron transfer to Q_B , decay from $P^+Q_A^-Q_B$ to the ground state occurs at k_{AP} . The numbers refer to reaction rate constants (per second) at room temperature. Energies are approximately on scale. Data taken from *Rb. sphaeroides* [17] are in parentheses.

electron transfer from the state $P^+Q_A^-$ to $P^+Q_B^-$ could be estimated, by determining the time constant of the increase of the absorbance at 450 nm after the first flash (the extinction coefficient of Q_B^- is larger than of Q_A^- [42]). At pH 7, the rate constant of electron transfer from the state $P^+Q_A^-$ to $P^+Q_B^-$ (k_{AB}) in *E. mobilis* RCs was approximately $1.5 \cdot 10^5$ (not shown). Using the average value of 167 for K_2 (Fig. 7) and Eqn. 2, a time constant of 10^3 was calculated for k_{BA} . See the legend to Fig. 8 for further details.

The absence of a clear biphasicity of the $P^+Q_B^-$ recombination kinetics in the *E. mobilis* RC/LH_I complexes, supports the conclusion that the $P^+Q_A^-$ recombination kinetics are monophasic at room temperature.

The apparent pH-independence of the $P^+Q_B^-$ recombination kinetics in *E. mobilis* RC/LH_I complexes over the range of pH 6–11 is in contrast to the pH dependence observed in RCs of both *Rb. sphaeroides* and *Rps. viridis* [17,34,53]. The onset of the pH dependence at about pH 9 in *Rb. sphaeroides* RCs has been attributed to ionization of Glu^{L212} with an apparent pK value of 9.3, and subsequent electrostatic interaction with Q_B/Q_B^- [55]. Mutation of Glu^{L212} → Asp in *Rb. sphaeroides* RCs resulted in elimination of the pH dependence of the $P^+Q_B^-$ recombination kinetics over the range of pH 6–11. The high-pH transition changed from a pK_a of 9.3 to a pK_a < 5, presumably due to changes in the order of ionization of strongly interacting residues like Glu^{L212} and Asp^{L210} (reviewed in Ref. 15).

Since we did not determine τ_{BP} in the range below pH 6, the reason for the apparent pH-independence of the $P^+Q_B^-$ recombination kinetics in *E. mobilis* remains to be established. The apparent pH independence of τ_{BP} over the range of pH 6 up to pH 11 may be related to the alkaline growth conditions of *Ectothiorhodospira*. Preliminary results indicate that *E. mobilis* cells are not able to keep their internal pH constant over the pH range 5–10. Their intracellular pH increased by increasing the external pH (Leguijt, T., Bart, A., Hellingwerf, K.J., unpublished results).

An alternative explanation for the apparent pH-independence of τ_{BP} in *E. mobilis* RCs might be direct recombination between P^+ and Q_B^- (k_{dir}). The $P^+Q_B^-$ recombination in *E. mobilis* is sufficiently slow to exclude a priori direct recombination. Direct recombination has been suggested for *Chr. minutissimum* at low pH [35], and for mutants of *Rb. sphaeroides* in which the ionizable amino acids Glu^{L212} and/or Asp^{L213} have been changed to respectively Gln and Asn [40]. Thus, the tendency towards pH-independence of τ_{BP} in *E. mobilis* may be misleading. The possibility of direct recombination also has implications for the mean value of 130 meV calculated for the free energy difference between the states $P^+Q_A^-$ and $P^+Q_B^-$ in *E. mobilis*.

The large value of τ_{BP} may not reflect a large value for the equilibrium constant K_2 , as implied by relation (1), and the value calculated for ΔG_{AB}^0 may reflect a minimum.

With the data presented in this paper, the nature as well as the kinetic properties of the primary and secondary quinone electron acceptors in the RCs of the alkaliphilic and moderately halophilic purple sulfur bacterium *E. mobilis* have been characterized. The observed properties resemble those described for organisms of the β - and γ -subclass of the proteobacteria (e.g., *Rv. gelatinosus* and *Chromatium*, respectively) rather than organisms of the α -subclass like *Rb. sphaeroides* or *Rps. viridis*.

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