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Electrogenic proton transfer in *Rhodobacter sphaeroides* reaction centers: effect of coenzyme Q_{10} substitution by decylubiquinone in the Q_B binding site

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Abstract An electrometric technique was used to investigate the effect of coenzyme Q_{10} (UQ), substitution by decylubiquinone (dQ) at the Q_B binding site of reaction centers (UQ-RC and dQ-RC, respectively) on the electrogenic proton transfer kinetics upon Q_B reduction in *Rhodobacter sphaeroides* chromatophores. Unlike dQ-RC, the kinetics of the second flash-induced proton uptake in UQ-RC clearly deviated from the mono-exponential one. The activation energy (about 30 kJ/mol) and the pH profile of the kinetics in dQ-RC were similar to those in UQ-RC, with the power law approximation used in the latter case. The interpretation of the data presumed the quinone translocation between the two binding positions within the Q_B site. It is proposed that the native isoprenyl side chain (in contrast to decyl chain) favors the equilibrium binding of neutral quinone at the redox-active 'proximal' position, but causes a higher barrier for the hydroquinone movement from 'proximal' to 'distal' position.

Key words: Chromatophore; Electrogenic reaction; Photosynthetic reaction center; Ubiquinone binding site; Proton transfer; *Rhodobacter sphaeroides*

1. Introduction

The photosynthetic reaction center (RC) of purple bacteria is a membrane-bound pigment–protein complex that catalyzes conversion of light energy into chemical energy. Following light excitation of RC, electrons are transferred from the bacteriochlorophyll dimer (P) through a number of cofactors to a ubiquinone molecule Q_A ($\tau \approx 0.2$ ns) and then to another ubiquinone Q_B. The latter operates as a two-electron gate. The first reduction step of Q_B ($\tau \approx 100$ μs in *Rhodobacter sphaeroides* RC, pH 7) does not involve its protonation at physiological pH:

$$Q_A Q_B \xrightarrow[h\nu(1)]{} Q_A^{\bullet -} Q_B \leftrightarrow Q_A Q_B^{\bullet -} \quad (1)$$

The electric field of $Q_B^{\bullet -}$ induces substoichiometric proton uptake by the protein (see [1,2]). The second reduction step involves the net binding of two protons, and QH_2 release into the membrane:

$$Q_A Q_B^{\bullet -} \xrightarrow[h\nu(2)]{} Q_A^{\bullet -} Q_B^{\bullet -} + 2H^+ \rightarrow Q_A(Q_B H_2) \rightarrow Q_A + QH_2 \quad (2)$$

The mechanism of reaction (Eq. 2) studied in the isolated RCs includes the transient protonation of $Q_B^{\bullet -}$ by the first proton (H_I^+), the rate-limiting electron transfer to $Q_B H^{\bullet}$, and attraction of the second proton (H_{II}^+)by $Q_B H^-$ [3]. The Glu-L212, Asp-L213, and Ser-L223 residues, adjacent to Q_B, are crucial for its efficient protonation; they are connected to the cytoplasmic surface of RC through a number of hydrogen-bonded pathways [1,2]. The proton translocation along the pathways contributes to the generation of the transmembrane electric potential difference ($\Delta\psi$) which was measured by the electrometric technique [4,5]. A small phase, B1, in the $\Delta\psi$ generation kinetics, induced by the first flash (Eq. 1), was attributed to the proton transfer between amino acid residues due to their pK shift [6–8]. A larger $\Delta\psi$ phase, B2, induced by the second flash (Eq. 2), was attributed to the transfer of two protons to Q_B [4,5,9,10]. Although in chromatophores the rate of this phase as well as of the second electron transfer to Q_B ($\tau \approx 100$ μs, pH 7 [11]) was about one order of magnitude higher than that in isolated RCs, the same mechanism was suggested to be valid in both types of preparations [10].

X-ray crystallography of *R. sphaeroides* and *Rhodopseudomonas viridis* RCs [12,13] revealed two positions for quinone binding at the Q_B site: the 'proximal' ($Q_{B,prox}$, close to the non-heme Fe^{2+} atom) and 'distal' ($Q_{B,dist}$, 5 Å apart). It was suggested that $Q^{\bullet -}$ binds only to the 'proximal' position, but neutral Q, and QH_2 shuttle between both positions [2]. Only 'proximal' position supports electron transfer between Q_A and Q_B. Shuttling between the positions requires displacement and rotation of ubiquinone, displacement of bound water molecules, and re-arrangement of hydrogen bonds around Q_B. These processes can account for the large activation barriers for Eqs. 1 and 2 (see [10]).

In this work, we studied the kinetics of Q_B reduction monitored as the electrogenic charge translocation in *R. sphaeroides* chromatophores in which native coenzyme Q_{10} (UQ) was substituted by its synthetic analogue, decylubiquinone (dQ). The latter has been widely used as a substrate for various quinone binding enzymes (for review, see [14]). In bacterial RC, the influence of the ubiquinone side chain structure

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Abbreviations: RC, reaction center; UQ, coenzyme Q_{10} (2,3-dimethoxy-5-methyl-6-decaisoprenyl-1,4-benzoquinone); dQ, decylubiquinone (2,3-dimethoxy-5-methyl-6-*n*-decyl-1,4-benzoquinone); $\Delta\psi$, transmembrane electric potential; E_a, activation energy; τ, relaxation time constant

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on equilibrium binding, and redox properties of Q_B were extensively studied [15–17]; however, its effect on the functional activity of RC has not been studied yet.

2. Materials and methods

The photoelectric activity of *R. sphaeroides* chromatophores was measured as in [10]. Chromatophores were immobilized in the presence of 25 mM $CaCl_2$ onto one side of a thin Teflon film (instead of previously used nitrocellulose film), impregnated with soybean phosphatidylcholine (type II-S, 150 mg/ml) dissolved in *n*-decane. Then the unattached chromatophores, and $CaCl_2$ were removed by volume change providing >10 times dilution. Without exogenously added quinone, the native UQ was extracted from the chromatophores and removed from the Q_B site of RCs, while the Q_A site retained UQ [18]. To restore Q_B, either UQ or dQ was added to the lipid phase of the samples (UQ-RC and dQ-RC, respectively). Although the lipid:water partition coefficient for dQ (log*P* 4.8–7.3) is much lower than for UQ ($\log P \gg 10$) [16,17,19], the dQ loss from the lipid phase should not exceed 10–15% in our system. 1,1′-Dimethylferrocene (dMF) provided re-reduction of P^+ ($\tau < 20$ ms) between flashes. Methylene blue (3,7-bis-[dimethylamino]-phenazothionium chloride, MB) provided slow ($\tau \geq 1$ min) re-oxidation of $Q_A^{\bullet-}$ and $Q_B^{\bullet-}$. Terbutryn was used as the Q_B site inhibitor. All chemicals were from Sigma-Aldrich.

The Nd:YAG laser flash-induced (>99% saturation, FWHM 15 ns) voltage changes were recorded by a PC-driven set-up (time resolution, 0.1 μs) [10]. The kinetics were analyzed with a non-linear least-square algorithm using the multi-exponential (Eq. 3) or the power law (Eq. 4) fit functions:

$$\psi(t) = \sum_i \psi_i \cdot (1 - \exp[-k_i t]); \quad \psi_{tot} = \sum_i \psi_i \tag{3}$$

$$\psi(t) = \psi_{tot} \cdot (1 - [1 + (k_0/n)t]^{-n}) \tag{4}$$

where ψ_i and k_i are amplitudes and rate constants, ψ_{tot} is the total amplitude, k_0 is the 'effective' rate constant (the average of the γ-type rate distribution), n is the distribution form factor (see [20], section 18.8-5). The factor n characterizes the kinetics deviation from an exponential: at small ($n < 5$) values, the kinetics become broadly distributed.

To analyze the temperature dependencies of the rate constant of the electrogenic phase B2 (k_{B2}), the p*K* shift effect of buffers was taken into account. Linear coefficients, δpK (pH units/°C), were used for pH correction: $pH^*(T) = pH_0 + \delta pK \cdot (T - T_0)$, where T_0 and T are the standard (25°C), and ambient temperatures, pH_0 and pH* are measured at T_0, and corrected pH values, respectively. For each given T, the observed k_{B2} value was corrected using the pH dependencies of k_{B2} which were measured at several 'reference' temperatures, T_{ref} (3, 10, 25, and 37°C):

$$k_{B2}(T, pH^*) = k_{B2}(T, pH_0) \times k_{B2}(T_{ref}, pH^*)/k_{B2}(T_{ref}, pH_0) \tag{5}$$

The following buffers were separately used (δpK are shown in brackets): potassium acetate (0.000), MES (−0.011), potassium phosphate (−0.003), MOPS (−0.006), HEPES (−0.014), Tris–HCl (−0.031), CHES (−0.009), CAPS (−0.009) [21,22]. Unless k_{B2} is corrected, the activation energies (E_a) will be overestimated (up to 30%) due to the character of the k_{B2} pH dependencies (see Fig. 3). When E_a were determined in the presence of different buffers at the same pH, the values after k_{B2} correction (Eq. 5) were similar within the error range (±3 kJ/mol).

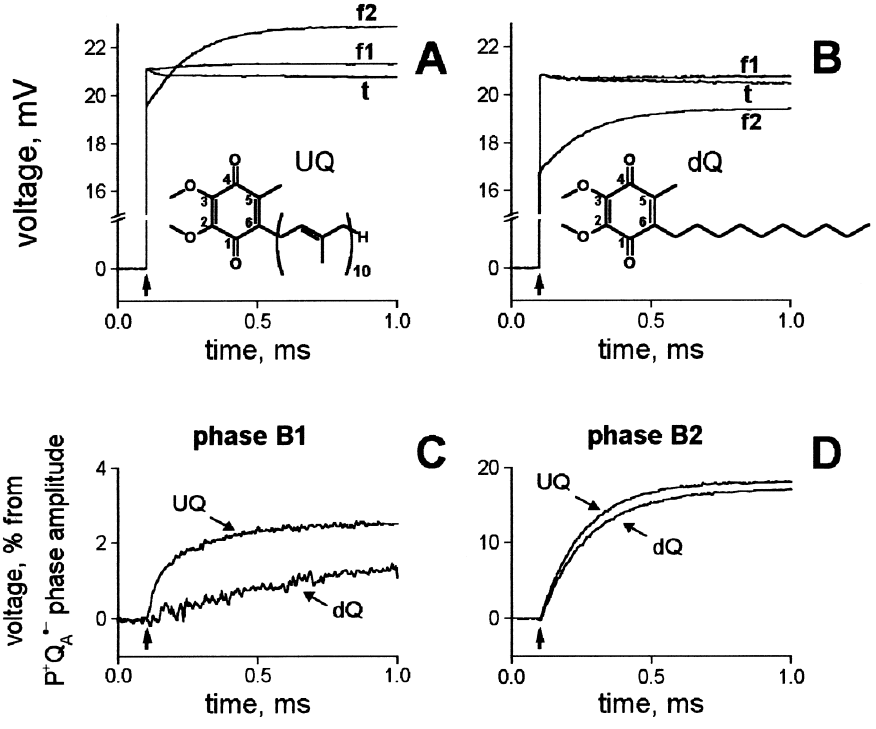

Fig. 1. Flash-induced voltage changes of *R. sphaeroides* chromatophores. (A) The voltage transients in UQ-RC after the first (**f1**) and the second (**f2**) sequential flashes in the absence of inhibitor, and after the first flash in the presence of 5 μM terbutryn (**t**). (B) The same as in (A), but in dQ-RC. (C) Kinetic phase B1. (D) Kinetic phase B2. Phases B1 and B2 were obtained by point by point subtraction of normalized **t** transient from **f1** and **f2** transients, respectively, and expressed as a percentage from the fast phases due to $P^+Q_A^{\bullet-}$ formation, A1 and A2, which amplitudes are taken as 100% (see Section 3). Conditions: 20 mM HEPES, pH 7.4, 50 mM KCl, 100 μM dMF, 2 μM MB, 5 μM myxothiazol, 5 μM antimycin A, E_h +360 mV (potassium ferrocyanide/ferricyanide, 2 mM), 25°C. Chromatophores were incubated with oligomycin A (200 μg/ml) before use. Either UQ or dQ, each at 20 mg/ml, were added to the lipid phase of the samples (see Section 2). The dark period between the first and the second flashes, 1 s. Bold arrows indicate laser flashes.

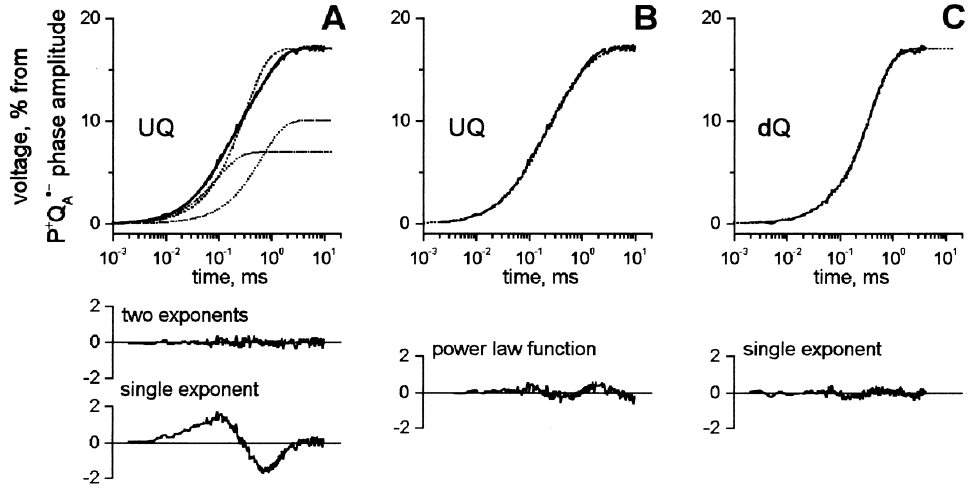

Fig. 2. Kinetic analysis of the phase B2. (A) The best one-exponential fit ($\tau = 310$ μs, dashed line), and the best two-exponential fit (solid line, and the exponentials, $\tau_1 = 95$ μs, 45%, and $\tau_2 = 625$ μs, 55%, dotted lines) in UQ-RC. (B) The best power law fit ($\tau = 210$ μs, $n = 1.1$, dashed line) for the same transient as in (A). (C) The best one-exponential fit ($\tau = 345$ μs, dashed line) for dQ-RC. Conditions: as in Fig. 1, except temperature, 10°C. The residuals of the fits are shown at the bottom.

3. Results

Absorption of light by RC results in transmembrane charge separation between P and Q_A, and $P^+Q_A^{\bullet-}$ formation, that causes fast, unresolved rise ($\tau \ll 0.1$ μs) in $\Delta\psi$ kinetics [4] (the rise phases designated as A1 and A2 following the first and the second flash, respectively, Fig. 1A,B). The phase A1 amplitude (ψ_{A1}) was notably larger than the A2 amplitude (ψ_{A2}). Under conditions of effective P^+ re-reduction, the ratio $\alpha = \psi_{A2}/\psi_{A1}$ represents a fraction of RC in which the second electron is transferred to $Q_B^{\bullet-}$. The quinone concentration dependencies of this ratio as well as that of the amplitude of voltage phase B2 (not shown) revealed that before the second flash, Q_B sites had attained an apparent saturation above 10 mg of each of the quinones per ml of the lipid solution. Since at higher quinone concentrations the α value in dQ-RC was smaller than that in UQ-RC (0.80 ± 0.03, and 0.92 ± 0.02, respectively, pH 7.4), then the $dQ_B^{\bullet-}$ state was destabilized relatively to the $UQ_B^{\bullet-}$ state. The respective free energy changes of the first electron transfer ($\Delta G° \approx -59 \cdot \log[\alpha/(1-\alpha)]$) for dQ-RC and UQ-RC were −35 meV and −63 meV. Similar $dQ_B^{\bullet-}$ destabilization was observed within the pH range from 4 to 10 (data not shown).

In addition to fast phases A1 and A2, slower, terbutryn-sensitive phases, B1 and B2, were also observed in the μs to ms time domain following the first and the second flashes, respectively. The phase B1 in dQ-RC was significantly slower than in UQ-RC ($\tau > 2$ ms and ≈ 100 μs, respectively, Fig. 1C), the former not depending on dQ concentration. The phase B2 in dQ-RC was slightly slower than that for UQ-RC ($\tau = 185$ and 140 μs, respectively, Fig. 1D).

In accordance with our previous work [10], the B2 kinetics in UQ-RC fitted a single exponent above 25°C, whereas at lower temperatures, it stretched and it was a good two-exponential fit (Fig. 2A) reflecting two sequential protonation steps of Q_B. Besides this approach, we also applied the analysis using the power law function (Eq. 4) which has been used before for fitting of the non-exponential charge recombination kinetics in isolated RCs [23]. This approach implies that the transfer of both protons is controlled by a single, kinetically

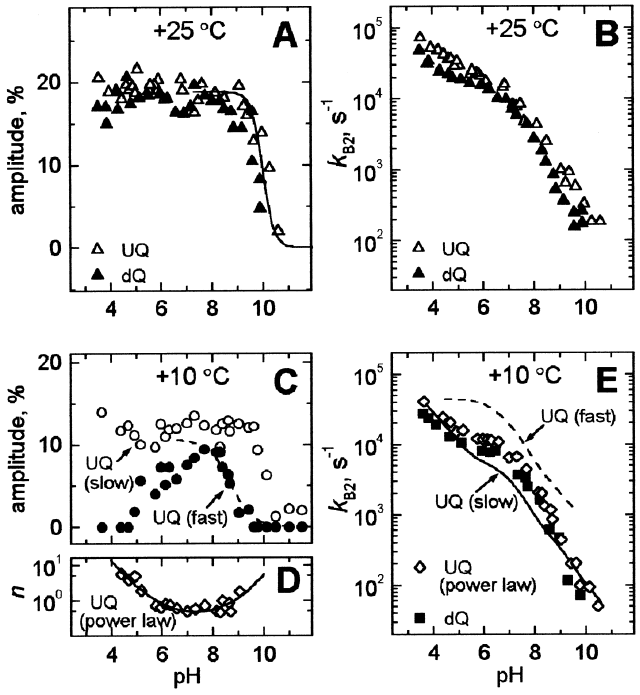

Fig. 3. The pH dependencies of the phase B2 kinetics. (A, B) The results of one-exponential fits for UQ-RC (△) and dQ-RC (▲): amplitudes (A), rate constants, k_{B2} (B), temperature, 25°C. (C–E) The results of two-exponential fits for UQ-RC (● or dashed line for fast, and ○ or solid line for slow components), the power law fits for UQ-RC (◇), and one-exponential fits for dQ-RC (■): amplitudes (C), factor n (D), k_{B2} (E), temperature, 10°C. Lines represent the quadratic, pK^{app} 10.2 (A), and the linear, pK^{app} 8.5 (C), titration curves and data splines for the factor n (D) and k_{B2} for the fast (dashed line) and slow (solid line) components for UQ-RC (E). Conditions: as for Fig. 1, except that (i) the incubation medium contained 20 mM of one of the buffers (see Section 2) depending on pH region; (ii) the MB concentration varied from 0.5 μM at alkaline pH up to 40 μM at acidic pH. On (A, C), Y-axes are as on Fig. 2.

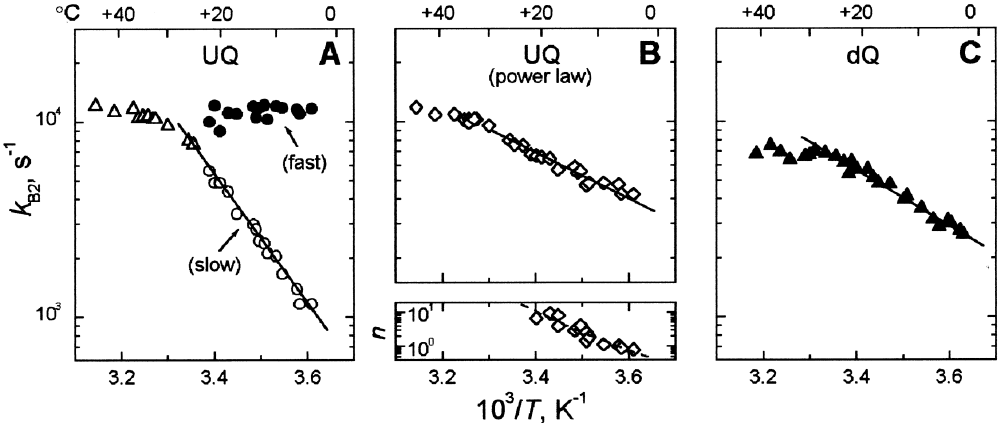

Fig. 4. Temperature dependence of k_{B2}. (A) The results of one- (△), or two-exponential (●, fast, and ○, slow components) fits for UQ-RC. (B) The results of the power law fit for UQ-RC (◇); the values of factor n and the data spline are shown at the bottom. (C) The results of one-exponential fit for dQ-RC (▲). The slopes of the fit lines yielded E_a of 55 kJ/mol (A, slow component), 28 kJ/mol (B), 32 kJ/mol (C). Conditions: as for Fig. 1, except that the concentration of MB varied from 1 to 16 μM.

distributed step. The two-exponential (Fig. 2A) and the power law function (Fig. 2B) approximations exhibited similar fits. Thus, the fitting comparison alone does not allow distinguishing between these models. In contrast to UQ-RC, the B2 kinetics in dQ-RC within the temperature range from 3 to 47°C were well fitted by one exponent (see Fig. 2C).

The phase B2 amplitude maintained at a constant level at pH below 9; at higher pH, it sharply decreased (Fig. 3A). In UQ-RC, the fast and slow components of phase B2 at 10°C gave similar contributions at neutral pH (Fig. 3C). The power law analysis of the data revealed a single kinetic phase. The factor n values indicated broadly distributed kinetics at neutral pH, whereas at pH < 5 and pH > 9, the kinetics approached to mono-exponential (Fig. 3D). The rate constant, k_{B2}, was weakly pH-dependent below pH 7, followed by a steeper dependence at higher pH (Fig. 3B,E, cf. the pH profiles, obtained from the two-exponential fittings of k_{B2} in UQ-RC at 10°C [10]).

The Arrhenius plots for k_{B2} are shown on Fig. 4. Below 25°C, the slope of the slow component for UQ-RC yielded E_a of 55 kJ/mol (Fig. 4A). The power law analysis of the same data yielded the E_a value of ~30 kJ/mol (Fig. 4B; the factor n is shown on the insert at the bottom). A similar E_a value was obtained in dQ-RC (Fig. 4C). The E_a values did not depend on pH within a range from 4.0 to 8.5 (data not shown).

4. Discussion

In this work, we investigated the effect of UQ substitution by dQ in the Q_B binding site of RC on the kinetics of electrogenic proton transfer in *R. sphaeroides* chromatophores. Isoprenyl and decyl radicals cause similar electronic induction effects on the ubiquinone ring. Redox properties of UQ and dQ are similar both for aprotic solvents [24] and ethanol–water mixtures (P. Rich, personal communication); however, we have found that the kinetics of electrogenic reduction of Q_B in dQ-RC differ from those in UQ-RC. Therefore, we addressed the issue by what means the ubiquinone side chain influences Q_B function.

As it follows from Section 3, the midpoint potential of $dQ_B/dQ_B^{\bullet-}$ pair is ~30 mV lower than that of $UQ_B/UQ_B^{\bullet-}$ (pH 7.4). This is consistent with the observation that the equilibrium constant of the first electron transfer in isolated RC declined as the isoprenyl side chain of ubiquinone was shortened [16]. A plausible explanation for the destabilization of $dQ_B^{\bullet-}$ is that the transition of neutral dQ from the 'distal' to the redox-active 'proximal' position is less favorable than that of UQ:

$$Q_A^{\bullet-}Q_{B,dist} \leftrightarrow Q_A^{\bullet-}Q_{B,prox} \rightarrow Q_AQ_{B,prox}^{\bullet-} \quad (6)$$

The same argument may explain the retardation of the electrogenic phase B1 kinetics in dQ-RC (Fig. 1C), since conformational gating was suggested to be rate-limiting for the first electron transfer to Q_B [8,25,26]. Moreover, the X-ray data for *R. viridis* RCs indicate that a short-chained ubiquinone in the neutral state binds preferably to the 'distal' position [12]. However, we cannot exclude that the 'proximal' positions in $dQ_B^{\bullet-}$ and $UQ_B^{\bullet-}$ are different thereby accounting for the difference in both the redox potentials and the electron transfer rates.

To explain the deviation of the phase B2 kinetics from mono-exponential in UQ-RC, we have suggested in the preceding work [10] that H_I^+ binding to Q_B (Eq. 7) is controlled by the activationless electron transfer, and highly activated H_{II}^+ binding (Eq. 8) is controlled either by hydroquinone movement or by protein conformation change required for the proton transfer.

$$Q_A^{\bullet-}Q_B^{\bullet-} + H_I^+ \leftrightarrow Q_AQ_BH^- \quad (7)$$

$$Q_BH^- + H_{II}^+ \leftrightarrow Q_BH_2 \rightarrow QH_2 \quad (8)$$

However, the kinetics for dQ-RC remained mono-exponential in the whole temperature range studied (Fig. 4). Therefore, we suggested that in dQ-RC, either (i) binding of H_{II}^+ (Eq. 8) is accelerated and the electron transfer becomes rate-limiting for binding of both protons or (ii) the equilibrium of Eq. 7 is shifted backward and one of the subsequent reactions (Eq. 8) becomes the rate-limiting step. In the first case, E_a characteristic for the electron transfer in dQ-RC is rather high (~30 kJ/mol), although it sharply decreases above 30°C (Fig. 4). In the second case, the pK^{app} of the reversible H_I^+

binding coupled to the electron binding (Eq. 7) in dQ-RC should be shifted from 8.5 (as for UQ-RC [10], see Fig. 3C, dashed line) to the acidic pH by at least three pH units. According to Tandori et al. [27], in purified *R. sphaeroides* RC the second electron transfer to Q_B has E_a of $\sim$55 kJ/mol (pH 8.1). Since the reaction was shown to be rate-limited by electron tunneling [3], this E_a value seems to be unexpectedly high. However, this should be observed if the pK^{app} (Eq. 7) was below pH 8.1 and H^+_{II} binding, rather than electron transfer, was rate-limiting.

As it was mentioned in Section 3, we cannot exclude an alternative explanation for the deviation of the phase B2 kinetics from mono-exponential. The continuum (rather than discrete) distribution of RC sub-states may result in non-exponential electron transfer kinetics that were analyzed using the power law function [23]. The k_{B2} pH profiles (Fig. 3E) and E_a values (Fig. 4B,C) obtained by the power law analysis in UQ-RC, and by mono-exponential approximation for dQ-RC, were rather similar. The non-exponential kinetics could be a consequence of either static distribution, or time-dependent changes of the free energy due to the relaxation processes along the electron transfer path [28]. The structural uniformity for both Q_A and Q_B semiquinones (see [2]) excludes the possibility of large structural changes during Eq. 7; however, minor relaxations can strongly affect the electron transfer rate, and thus may account for high E_a values. Note that unlike chromatophores, in isolated RC the second electron transfer kinetics were mono-exponential even at low temperature [29]. Note also that in a recent work by Palazzo et al. [30], the non-exponential charge recombination kinetics in RCs incorporated in UQ-containing proteoliposomes were interpreted in terms of the quinone distribution among the vesicles. However, this approach can hardly be applied to the second electron transfer kinetics since in the latter case, the Q_B sites seem to be fully saturated by non-exchangeable semiquinone anion before the second flash-induced reaction.

The data on the equilibrium binding free energies showed that only the first two, and a part of the third isoprenyl units of ubiquinone side chain (this precisely corresponds to the decyl chain length of dQ) interact with the Q_B binding pocket of RC [15–17]. This interaction is clearly seen on the X-ray structures with the 'proximal' but not with the 'distal' position of ubiquinone (e.g. see PDB entries, 1AIG and 1AIJ); in the latter case, the side chain is almost fully extruded out of the pocket. Decyl radical provides many more degrees of freedom as compared to the RC-interacting part of isoprenyl radical; the entropy loss for the decyl radical transfer from the hydrophobic phase to the 'proximal' position is about 20 kJ/mol (300 K) larger than that for isoprenyl radical [16,17]. This energy is enough for the break of a few hydrogen bonds, accompanying the transition between the two positions. Therefore, dQ (or dQH_2) is less stable at the 'proximal' position than UQ (or UQH_2); moreover, the same reason may provide the difference between binding positions of $dQ^{\bullet-}_B$ and $UQ^{\bullet-}_B$. As long as rotation of the rigid isoprenyl chain has high activation energy [31], then the flexibility of decyl chain facilitates hydroquinone rotation during its dissociation. We proposed that the native isoprenyl chain favors binding of the substrate, UQ, at redox-active 'proximal' position but hampers the movement of UQH_2 from the 'proximal' to the 'distal' position required for its release from the site.

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