

Temperature dependence of the electrogenic reaction in the Q_B site of the *Rhodobacter sphaeroides* photosynthetic reaction center: the $Q_A^- Q_B \rightarrow Q_A Q_B^-$ transition

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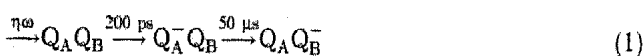
Abstract The temperature dependencies for the kinetics and relative amplitudes of electrogenic reaction(s) coupled with the first reduction of the secondary quinone acceptor Q_B were measured with dark-adapted chromatophores of *Rhodobacter sphaeroides*. The kinetics, while acceptably fitted by a single exponent at room temperature, clearly split into two components below 15°C (rise times, 25 μ s and 300 μ s at pH 7.0 and 10°C) with the slow phase ousting the fast one at pH > 9.0. The activation energies of the fast and slow phases were estimated at pH 7.0 as < 10 kJ/mol and 60–70 kJ/mol, respectively. To explain the kinetic heterogeneity of the $Q_B \rightarrow Q_B^-$ transition, we suggest two possible conformations for the neutral oxidized ubiquinone at the Q_B site: one with a hydrogen bond between the side chain carboxyl of Glu-L212 and the methoxy oxygen at C₃ of the Q_B ring (Q_B -H-Glu centers) and the other one, without this bond (Q_B :Glu⁻ centers). The fast phase is attributed to $Q_A^- Q_B$ -H-Glu \rightarrow $Q_A Q_B$ -H-Glu transition, whereas the slow one to the $Q_A^- Q_B$:Glu⁻ \rightarrow $Q_A^- Q_B$ -H-Glu \rightarrow $Q_A Q_B^-$ -H-Glu transition.

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Key words: Purple photosynthetic bacterium; Photosynthetic reaction center; Ubiquinone; Electron transfer; Proton transfer; Electrogenesis; (*Rhodobacter sphaeroides*)

1. Introduction

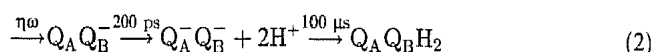
The photosynthetic reaction center (RC) of purple bacterium *Rhodobacter sphaeroides* catalyses conversion of light energy into chemical free energy by stabilizing the products of photochemical separation of electric charges. After the first flash on a dark-adapted RC, an electron is transferred across the membrane from *P* (a bacteriochlorophyll dimer which is located on the interface between two membrane-embedded L and M subunits of the RC [1–3]), via porphyrin-type electron carriers, first to the primary quinone acceptor (ubiquinone Q_A bound to the M subunit) and then to the secondary quinone acceptor (ubiquinone Q_B located on the L subunit) with formation of a tightly bound semiquinone Q_B^- :



Delivery of the second electron to Q_B^- triggers trapping of

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two protons from the surrounding medium and formation of an ubiquinol which can exchange rapidly with the membrane ubiquinone pool:



Both the electron transfer from *P* to quinone acceptors and transfer of protons from the outer surface to Q_B contribute to the generation of electrical potential difference across the photosynthetic membrane ($\Delta\psi$). The kinetics and extent of the electrogenesis which was coupled with Reaction 2 have been monitored by electrometric techniques [4–6] and spectrophotometrically [7]. Further it has been demonstrated that even the transfer of the first electron to Q_B (Reaction 1) is coupled with an electrogenic event [8–10]. The rise time of this electrogenic phase was similar to the characteristic time of $Q_A^- Q_B \rightarrow Q_A Q_B^-$ electron transfer; the relative amplitude of the phase, while small at the neutral pH, increased significantly at acidic and alkaline pH values [8–10]. The observed electrogenic reaction has been attributed to a proton transfer from the bulk to an amino acid residue(s) located in the vicinity of Q_B which changed their pK value(s) after Q_B^- formation (pK_{QB} \rightarrow pK_{QB}⁻-shift). From the studies of the L212EQ and L213DN mutants the carboxy groups of Glu-L212 and Asp-L213 may be identified as the main proton acceptors. The former accepts protons at pH > 9, whereas the latter seems to perform this function at pH < 6 [10,11].

The kinetics of Reaction 1 are quite acceptably fitted by a single exponent when measured at the room temperature [8,9]. However, when the $Q_A^- Q_B \rightarrow Q_A Q_B^-$ transition have been monitored by IR-spectroscopy at the C–O mode of Q_B at 1478 cm⁻¹ and at 4°C, two kinetic components were revealed [see [12] and references therein]. To further clarify this point, we attempted here to resolve the kinetic components of the $Q_A^- Q_B \rightarrow Q_A Q_B^-$ reaction by measuring the accompanying $\Delta\psi$ changes at various temperatures.

2. Materials and methods

Cells of *Rb. sphaeroides* (wild-type strain 2R from the collection of the Moscow University) were grown anaerobically at 30°C in the light and chromatophores were isolated by a French-press treatment as described in [13,14]. The fast kinetics of the laser flash-induced $\Delta\psi$ generation were investigated using the direct electrometry [15,16]. In this study chromatophores of *Rb. sphaeroides* were adsorbed onto a surface of a nitrocellulose film impregnated with the solution of soybean asolectin and ubiquinone-10 in *n*-decane. The film was clamped between two chambers of an electrometric cell and the photoelectric signals were recorded by Ag/AgCl electrodes as described in [8,14]. Chromatophores were pre-adapted in complete darkness for 10–15

min before applying a series of light pulses from a Q-switched neodymium YAG laser YG-481 (Quantel, France, $\lambda = 532$ nm, pulse half-width, 15 ns; 24 mJ/cm² per flash). Kinetic traces with the time resolution of 100 ns per address were processed using GIM software package developed by A.L. Drachev. The traces were analyzed by DISCRETE algorithm (kindly provided by Dr. S. Provencher) and by Microcal Origin (USA).

3. Results

A short laser flash on dark-adapted chromatophores of *Rb. sphaeroides* caused $\Delta\psi$ generation which was monitored electrometrically (Fig. 1A). The fastest phase of $\Delta\psi$ formation (phase A, reflects the electron transfer between *P* and Q_A [4], characteristic time < 1 ns) was followed by a decay that was due to the passive discharge through the chromatophore membrane. Hereafter we use the amplitude of phase A for the estimation of relative amplitudes of other electrogenic phases.

To discriminate the charge displacements coupled to $Q_A^-Q_B \rightarrow Q_AQ_B^-$ transition from (i) the electrogenic reduction of *P*⁺ by cytochrome *c*₂ [17] and (ii) the charge displacement coupled with Q_A^- formation [18], we applied Q_B antagonists. Although terbutryn was used routinely throughout the study, the results were the same, when atrazine or stigmatellin, two other Q_B antagonists with quite different structures and binding modes in the Q_B site [19], were tested. This excludes the possibility of artefacts not coupled with the inhibition of the $Q_A^-Q_B \rightarrow Q_AQ_B^-$ electron transfer. The difference between the kinetic curves obtained in the absence of inhibitor (Fig. 1A, upper trace) and in the presence of it (Fig. 1A, lower

trace) is shown in the inset to Fig. 1A. It is denoted hereafter as phase BI. As one can see, the difference kinetic trace which was obtained at 25°C is acceptably fitted by a single exponent in agreement with previous observations [8,9]. The pH-dependencies of the onset time and of the relative amplitude of phase BI measured at 25°C (see the dashed lines in Fig. 2A,B) are in accordance with those published previously [8–10]. Upon decreasing the temperature, the overall rate of phase BI slowed down and two kinetic components were clearly discriminated in its onset (Fig. 1B). Fig. 2A,B shows the pH-dependencies of relative amplitudes and kinetics of two kinetic components of phase BI as measured at 10°C. The relative amplitudes of two components, being almost equal at neutral pH, exhibited opposite pH dependencies: the fast phase was ousted by the slow one at pH > 8.5 but prevailed at pH < 6.0, so only one component could be resolved at pH > 9.5 and at pH < 5.0 (Fig. 2A). The rate of the fast component was almost pH-independent; the rate of the slow one while pH-independent at pH < 9.5, started to decrease approximately 10-fold per pH unit when pH was increased above 9.5 (Fig. 2B). Upon decreasing the temperature to 3°C, the total amplitude of phase BI (and, respectively, that of both kinetic components) decreased by a factor of 1.5 (not shown). We attribute this to the weaker quinone binding at low temperatures [see [20] and references therein]. At 3°C the pattern of the pH-dependence of the relative amplitudes was the same as that observed at 10°C (the latter is shown in Fig. 2A), whereas the splitting of two kinetic components increased at lower temperature (Fig. 2B).

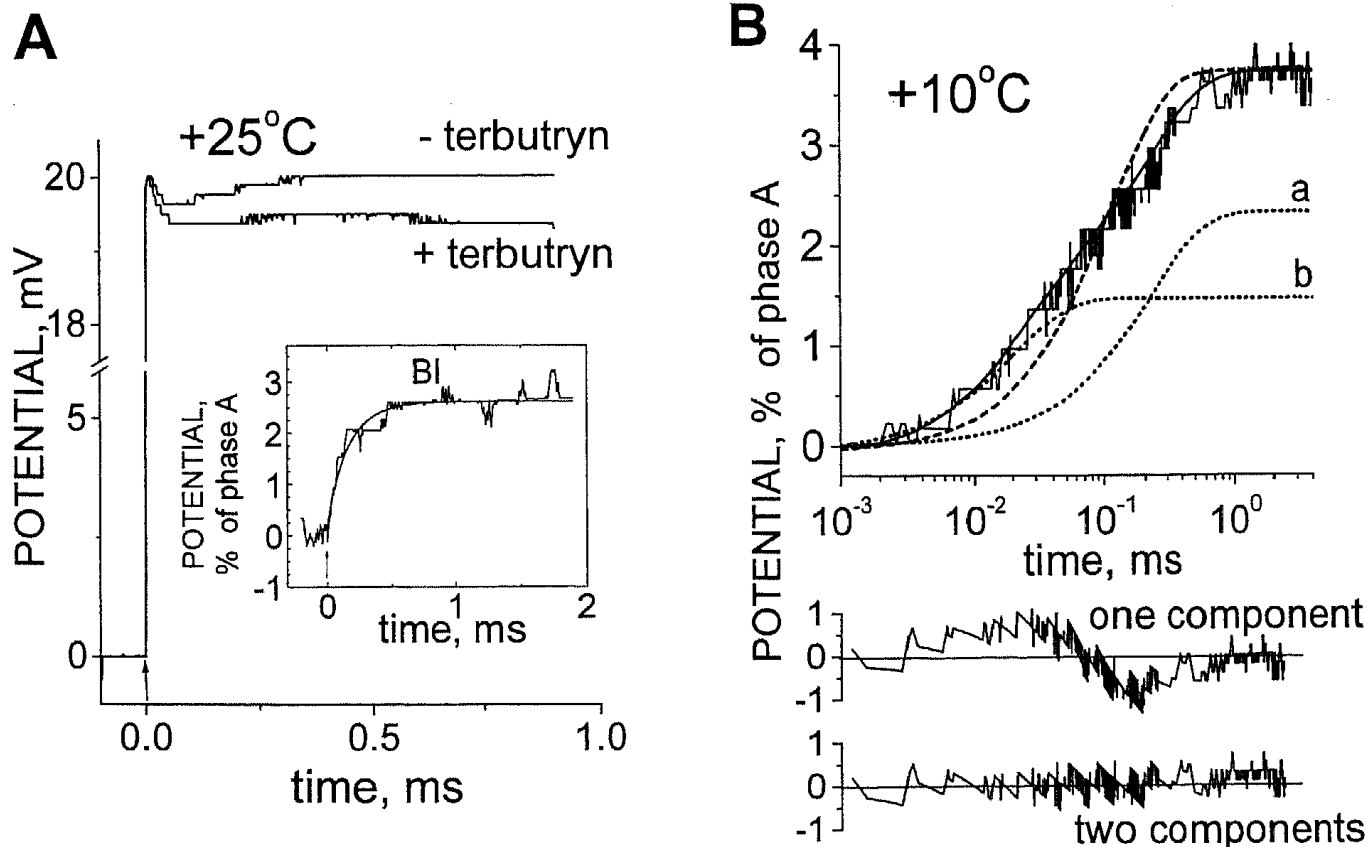


Fig. 1. Photoelectric responses coupled with the $Q_A^-Q_B \rightarrow Q_AQ_B^-$ transition as measured in chromatophores of *Rb. sphaeroides* at different pH values and at different temperatures. A: Photoelectric responses induced by the first flash of light in the absence and in the presence of terbutryn; the difference trace fitted with a single exponent is shown in the inset. B: A difference trace obtained at 10°C and at pH 7.0; the trace was fitted either by one exponent (shown as dashes) or by two exponents (a and b trace, shown as dots). The noise residuals between the experimental and theoretical curves are shown at the bottom of the traces. For the incubation medium see Fig. 2.

Using the data obtained at three temperature values (Fig. 2B) we estimated roughly the activation energies of the fast and slow components as <10 kJ/mol and 60–70 kJ/mol, respectively, at pH about 7.0. Because the slow component speeded up sharper than the fast one when the temperature was increased, two components became unseparable above 15°C.

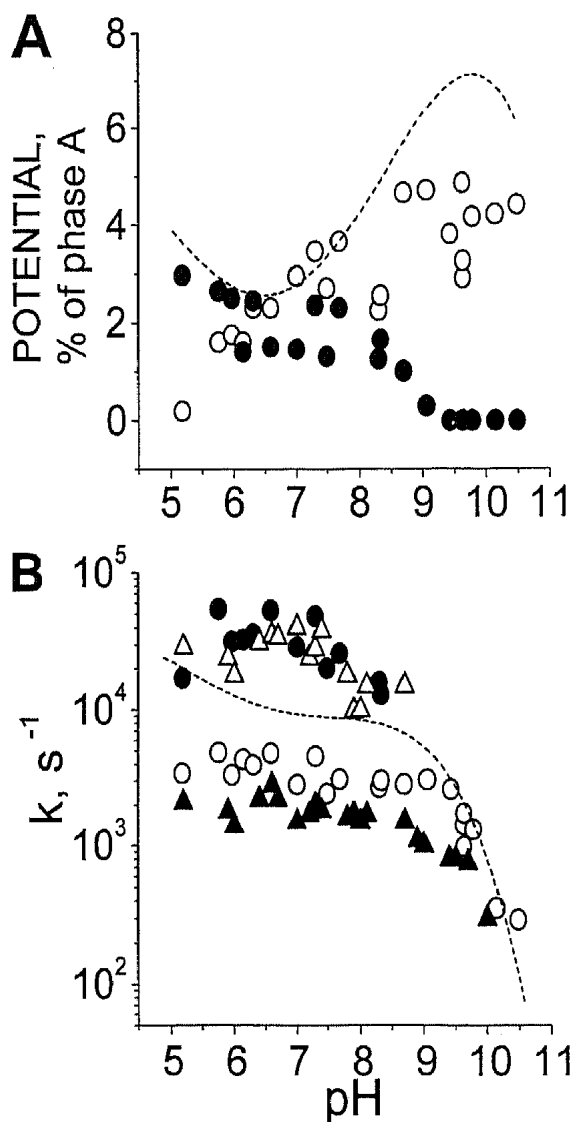


Fig. 2. Dependencies of the relative amplitudes and of the rate constants of phase BI components on pH. A, the pH dependence of the relative amplitudes of the fast (●) and slow (○) components of phase BI measured at 10°C. The pH-dependence of the relative amplitude for the single component which was revealed at 25°C is shown as a dashed line and is in accordance with the previously published ones [8,9]. B: pH-Dependencies of the rate constants of the fast (●, ▲) and slow (○, △) components of phase BI measured at 3°C (△, ▲) and 10°C (○, ●). The pH-dependence of the rate constant of the single component which was revealed at 25°C is shown by a dashed line and is in accordance with the previously published ones [8,9]. Incubation medium contained: 20 mM buffer solution (acetic acid, MES, MOPS, HEPES, Tris-HCl, CHES, and CAPS were used), 50 mM KCl, 2 mM potassium ascorbate, 50 μM TMPD, 0.1–20 μM methylene blue, 4 μM myxothiazol, 2 μM antimycin A. The concentration of methylene blue was determined experimentally for each temperature and pH region to achieve (1) full dark adaptation in 10–15 min; (2) a negligible oxidation of Q_B^- in 0.5 s between 1st and 2nd flashes [see [12] for discussion of factors influencing the life time of Q_B^-].

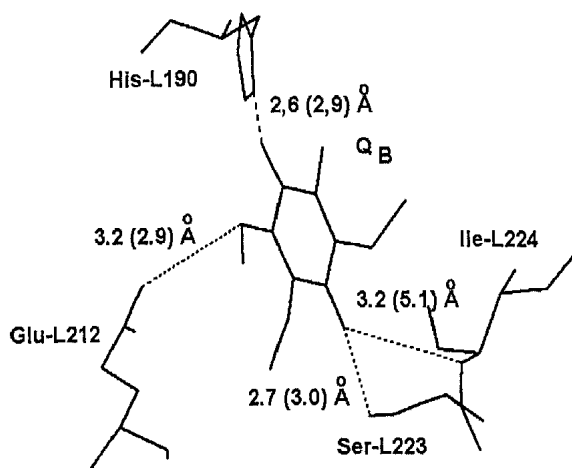


Fig. 3. Structure of the Q_B site in the RC of *Rb. sphaeroides* [from [1]]. The numbers indicate the distances as obtained from the crystal structures published in [1] and [2]; the values for the latter structure are shown in brackets. Note that the mode of binding of the distal carbonyl of the Q_B ring in the RC of *Rb. sphaeroides* is still controversial [see [29] for the latest survey].

4. Discussion

The pH-behavior of two components of the phase BI which we revealed at low temperatures (Fig. 2A,B) resembles the pH-behavior of two components which have been monitored by IR-spectroscopy at the C–O mode of Q_B at 1478 cm^{-1} and which have been attributed to the $Q_A^-Q_B \rightarrow Q_AQ_B^-$ transition [see [12] and references therein]. At 4°C the kinetics displayed two phases: a fast component of $\sim 200\ \mu\text{s}$ dominated at neutral pH values, whereas a slower component with τ of $\sim 1\text{ ms}$ ousted the fast one as the pH value was increased from 6 to 10 [12]. Two similar components were also found in the kinetics of Glu-L212 protonation which have been monitored at 1725 cm^{-1} [12]. It has been suggested that the fast step involved electron transfer to Q_B in RC in which Glu-L212 was pre-protonated while the slow step involved a combination of electron and proton transfer to RC in which Glu-L212 was pre-ionized [12]. The pH behavior of the latter residue remains still rather unclear. The unusually high pK_{Q_B} value of about 10 which was determined for Glu-L212 by the functional measurements [10,11,20–22] is in contradiction with electrostatic calculations that have predicted an acidic pK_{Q_B} value for a carboxy amino acid residue in the L212 position [23,24]. Moreover, the IR-measurements cited above have shown that only a fraction of Glu-L212 (monitored directly with IR-spectroscopy at 1725 cm^{-1} [12,25] and with FTIR spectroscopy at 1728 cm^{-1} [26]) was protonated in response to the formation of Q_B^- . This fraction increased gradually from 0.3 to 0.6 as the pH value was elevated from 6 to 10. Based on these observations the *partially* protonated state of Glu-L212 in the pH range between 6 and 10 has been postulated and it has been suggested, that this state is sustained by the electrostatic interactions with neighboring polar amino acid residues [12,26].

To explain our data we find it helpful to consider a working model which, besides the electrostatic interaction of Glu-L212 with chargeable amino acids, takes also into account its chemical interaction with the Q_B ring (see Fig. 3 for the arrangement of the Q_B site). Namely, we suggest that two energetically close conformations of the Q_B pocket are in an

equilibrium with each other: in one state the methoxy oxygen at C₃ of the Q_B ring (O_{3m}) forms a hydrogen bond with the side chain carboxyl of Glu-L212 as shown in Fig. 3 (Q_B–H–Glu centers) while in the other this bond is absent (Q_B:Glu[−] centers). If in the state Q_B:Glu[−] the ubiquinone forms an alternative hydrogen bond with some other amino acid residue (a hydrogen bonding of the distal carbonyl of Q_B with Ser-L223 and/or the backbone nitrogen of Ile-L224 may be considered as plausible, see Fig. 3), the equilibrium between Q_B–H–Glu and Q_B:Glu[−] states may only moderately depend on pH and a partially protonated state of Glu-L212 may be sustained at 5 < pH < 9.5. As the carboxyl of Glu-L212 acts as a proton donor in this system, the hydrogen bond stabilizes the protonated state of Glu-L212 and shifts its pK_{QB} value into the alkaline region (in the Q_B–H–Glu fraction of RC). The magnitude of this shift is determined by the hydrogen bond energy and may be as high as 3–4 pH units. Hence, a formation of a hydrogen bond between Glu-L212 and the O_{3m} of Q_B may be (at least partly) responsible for the uniquely high pK_{QB} value of the former. Interestingly, an aspartic acid when introduced in the L212 position, with a shorter side chain not reaching Q_B, had a pK value of ~5 (estimated in [27]). This value is in line with electrostatic calculations noted above [23,24] and may be used as an estimate for pK_{QB} of Glu-L212 in the Q_B:Glu[−] state (when no hydrogen bond with O_{3m} is formed). As the appearance of negative charge of Q_B[−] shifts the pK value of Glu-L212 to more than 12.5 [12,28], the reduction of Q_B is thermodynamically coupled with Glu-L212 protonation (Q_A[−]Q_B:Glu[−] → Q_A[−]Q_B–H–Glu → Q_AQ_B[−]–H–Glu transition) causing the experimentally observable proton binding [12,28].

The following observations support further the suggested model.

(1) In two available crystal structures of the *Rb. sphaeroides* RC, which were crystallized with oxidized Q_B, the distances between Glu-L212 and the O_{3m} of Q_B are 2.9 Å [2] and 3.2 Å [1], respectively. The latter value is still in the range of hydrogen bonding if one takes into account that the mean error for a distance between two atoms may be estimated as 0.6 Å for this structure. That the exact position of Q_B is poorly determinable in all crystal structures of the RC [see [29] for a review] may reflect the existence of several conformational states of the Q_B pocket with close energies.

(2) Breton et al. [30] have concluded from the Q_B/Q_B[−] FTIR difference spectra that O_{3m} of Q_B is anchored by protein. Glu-L212 seems to be the only anchor that is compatible with the crystal structure.

(3) The half-width of the flash-induced IR signal at 1725 cm^{−1}, which has been attributed in [12] to the protonated Glu-L212 in the presence of Q_B[−] (see above), was less than 10 cm^{−1} indicating "fixed position and restricted rotational freedom for the Glu-L212" [12]. This would be exactly the case if the COOH group of Glu-L212 is fixed by the hydrogen bond with Q_B[−].

(4) Existence of the Q_B–H–Glu hydrogen bond provides a plausible explanation for the lower stabilization of Q_B[−] in the L212EQ mutant which could not be explained electrostatically [20]. Although glutamine in the L212 position may still sustain a hydrogen bond, its strength must be lower than that of the one provided by a glutamic acid.

(5) The decrease in the Q_B binding at alkaline pH values was explained in [31] by the participation of a group the pK

value of which, while in the neutral range in the absence of quinone, shifts to ~9 after binding of quinone. Glu-L212 has been considered as the most plausible candidate. Consideration of the possibility of a hydrogen bonding of Q_B by Glu-L212 helps to rationalize this set of observations.

The slow phase in the kinetics of the Q_A[−]Q_B → Q_AQ_B[−] transition monitored by IR-spectroscopy and FTIR spectroscopy was attributed in [12,25,26] to the protonation of COO[−] group of Glu-L212. (In the framework of our model this corresponds to the Q_A[−]Q_B:Glu[−] → Q_A[−]Q_B–H–Glu → Q_AQ_B[−]–H–Glu transition.) The slow component of the BI phase in Figs. 1B and 2 may be directly attributed to this slow component measured by IR-spectroscopy as the proton transfer from the bulk to Glu-L212 has been shown to be electrogenic [10]. The onset time of the slow component of phase BI was similar to the one obtained in the IR-measurements (~1 ms in both cases at 3–4°C); also in the similar way it ousted the fast component at alkaline pH values (Fig. 2A,B). In [12] the rate of the slow protonation of Glu-L212 has been reported to be pH-independent at all pH intervals studied (6 < pH < 10) and hence not limited by the proton transfer. In our hands, the rate of the slow component while also almost constant at pH < 9.5, started to slow down at pH > 9.5 (Fig. 2B). In the framework of our model it is possible to speculate that the rate-limiting step at pH < 9.5 is the bringing of O_{3m} of Q_B and Glu-L212 to a hydrogen bond distance in the course of Q_A[−]Q_B:Glu[−] → Q_A[−]Q_B–H–Glu transition and that the slowing at pH > 9.5 may be attributed to the decrease in the concentration of the electron-accepting Q_A[−]Q_B–H–Glu state at pH values above its pK_{QB}.

The fast component in the IR and FTIR measurements has been attributed in [12] to the pre-protonated COOH group of Glu-L212 changing its stretching mode after Q_B[−] formation. The kinetics and the pH dependence of fast component of phase BI measured at 3°C (see Fig. 2A,B) were similar with those of the fast phase from the IR-measurements [12]. Hence, in our model we attribute this component to the Q_A[−]Q_B–H–Glu → Q_AQ_B[−]–H–Glu transition. Although the reaction itself is not electrogenic, some electrogenic events may be coupled with it, particularly: (i) the electrogenic proton binding by some proximally located residues in response to the formation of Q_B[−] (a 'basal' proton binding has been observed even with the mutants lacking both Glu-L212 and Asp-L213 [11]), and (ii) the internal redistribution of proton(s) in response to Q_B[−] formation. It is quite plausible that they may move from Glu-H173 in the direction of Asp-L210. The flash-induced partial protonation of Asp-L210 carboxyl in response to Q_B[−] formation was observed with IR- and FTIR-spectroscopy at neutral pH values [24,25]. Consideration of the closely located Glu-H173 as a proton donor may explain why the substitution of Glu-H173 for Gln decreased the stability of Q_B[−] [32]. (iii) Electrogenic displacements of polar amino acid side chains may also contribute to the relaxation in response to Q_B[−] formation [33].

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