Effect of pH and surface potential on the rate of electric potential generation due to proton uptake by secondary quinone acceptor of reaction centers in *Rhodobacter sphaeroides* chromatophores

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An electrometric method was used to investigate the effect of pH and ionic strength on the second flash-induced formation of the transmembrane electric potential difference ($\Delta\psi$) arising from proton uptake by the quinone complex of the photosynthetic reaction center (RC) in chromatophores of non-sulphur purple bacterium *Rhodobacter sphaeroides* (wild-type). The characteristic time of this $\Delta\psi$ phase is approx. 70 μs at pH 7.5 in the presence of 20 mM KCl. Increases in both pH and salt concentration were found to slow the time of generation of the electric potential due to quinol formation ($Q_B H_2$) after the second flash by more than 10-times. At higher pH, the effects of salts were more pronounced. The pH dependence of the Q_B^{2-} protonation rate is explained by the change in the surface proton concentration near the RC protein. The density of surface charges calculated on the basis of Gouy-Chapman theory is approx. 0.002 e/$\mathring{A}^2$ (pH 7) or approx. 10 negative charges per RC protein from the cytoplasmic side of the membrane, in good agreement with previous estimates for chromatophores. The sensitivity of the rate constant of the second flash-induced RC protonation to the salt concentration probably resolves the contradiction among results reported by different laboratories (Wraight, C.A. (1979) Biochim. Biophys. Acta 548, 309–327; Kleinfeld, D., Okamura, M.Y. and Feher, G. (1985) Biochim. Biophys. Acta 809, 291–310) for the pH dependence of the rate constant of the reaction $Q_A^- Q_B^- \rightarrow Q_A Q_B H_2$.

Introduction

In recent years much progress has been made in the investigation of the structure and function of the photosynthetic reaction centers of purple bacteria.

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Abbreviations: RC, photosynthetic reaction center; P, bacteriochlorophyll dimer in the RC; Q_A, Q_B, the primary and secondary quinone-type acceptors in the RC; TMPD, N,N,N',N'-tetramethyl-p-phenylenediamine; E_h, redox potential of the medium; pH_s, pH_v, the pH at the protein surface and in the bulk volume, respectively; $\Delta\psi$, transmembrane electric potential difference; $\Delta\psi_s$, surface potential; σ, surface density of charges.

From the X-ray structure analysis of crystalline RCs of *Rhodopseudomonas viridis* [1–3] and *Rhodobacter sphaeroides* [4–7] cofactors of the RC proteins have been localized and the roles of individual amino-acid residues in cofactor binding were made clear. Following RC excitation with a light quantum, a photo-activated electron is passed from the dimer of bacterio-chlorophyll, P870 (P), to the primary quinone acceptor (Q_A) and then to the secondary quinone acceptor (Q_B) (reviewed in Refs. 8–10). The character of the electrogenic reactions in the complex of quinone acceptors of RC is dependent on the pH of the medium, as well as on the presence of electron donors [11,12].

Under neutral conditions (pH 7.5) a small amount of protons is trapped by RC preparations after the first flash due to the generation of the state $P^+ Q_A Q_B^-$ [12,13]. No essential transmembrane potential difference ($\Delta\psi$), aside from the phase associated with electron transfer between P870 and Q_A (PQ$_A$ phase), is

generated [14,15] when the electron is transferred from Q_A to Q_B, since the two quinones are practically equidistant from the membrane surface, as determined by X-ray crystal structure data of the RCs [3,7]. Only following the second flash, when the formation of a doubly-reduced quinone species Q_BH_2 occurs, trapping of two protons takes place and an additional electrogenic phase of $\Delta\psi$ with an amplitude corresponding to 15–20% of the PQ_A phase is formed [15–21].

Under alkaline conditions (9 < pH < 10) proton trapping also occurs on the first flash due to creation of the state $P^+Q_AQ_B^-(H^+)$ [12,13,22]. This proton uptake correlates well with the first flash-induced electrogenic phase that is sensitive to atrazine [15]. Hence, under alkaline conditions each electron transfer to Q_B is compensated (in the micro- to millisecond time domain) by trapping of a proton(s) from the medium [11,12].

In the present work, chromatophore membranes of *Rb. sphaeroides* were used to investigate the influence of pH and surface potential on the kinetics of the second flash-induced $\Delta\psi$ generation due to the formation of Q_BH_2.

Materials and Methods

Conditions for the growth of *Rb. sphaeroides* cells (wild-type, strain R1), as well as isolation of chromatophores from French-press-disrupted cells are described elsewhere [23].

Photo-electric activity was measured electrometrically with the use of phospholipid-impregnated nitrocellulose film [23–25]. The film was made by placing a drop of 1% nitrocellulose solution in isoamiloacetate on water surface and then drying for more than 1 h. The film was then impregnated from both sides with 20 μl decane solution of azolectin (120 mg/ml) and ubiquinone-10 (20 mg/ml). The latter was added to restore the function of secondary quinone acceptor which is lost during fusion of chromatophores with azolectin impregnated nitrocellulose membrane [20,21]. A chromatophore suspension was placed in one compartment of a teflon cell separated from the other compartment with a nitrocellulose membrane impregnated by phospholipid and incubated in the presence of 20 mM $CaCl_2$ for 4 h. The incubation medium was then changed to a buffer free of $CaCl_2$.

Saturating light pulses were delivered from a Quantel Nd laser (France) ($\lambda = 530$ nm, $\tau = 15$ ns). The data recording and processing system consisted of a DL-1080 transient recorder (Data Lab) coupled to a Nova-3D computer (Data General). For exponential deconvolution of kinetic curves, the routine DIS-CRETE was used [26].

The time response of measuring electrodes at differ-

ent salt concentrations was faster than 1 μs, indicating that resolution of our measuring system does not interfere with the measurements of kinetics of electric potential with $\tau \geq 10$ μs.

Results

Photo-electric response due to Q_B protonation after the second flash

Illumination of dark-adapted *Rb. sphaeroides* chromatophores with a series of flashes in the presence of an exogenous electron donor for P870 induces formation of semiquinone Q_B^- on odd-numbered flashes and Q_BH_2 on even-numbered flashes [23,27–29]. In agreement with this binary oscillation behavior of Q_B the additional electrogenic phase is observed after each even-numbered flash with a rise time of 0.1–0.2 ms at pH 7 [15,21,23].

Presented in Fig. 1 are the representative photo-electric responses of *Rb. sphaeroides* chromatophores induced by the first (a) and the second (b) flash, as well as the difference between the two (c). The first fast phase of $\Delta\psi$ ($\tau < 0.1$ μs) observed after each flash with amplitude of approx. 12 mV corresponds to the charge separation between P870 and Q_A [23–25]. After the second flash, an additional electrogenic phase with a rise time of approx. 0.1 ms with an amplitude approx. 15% of that for the fast phase is observed. It has earlier been demonstrated that the $\Delta\psi$ component with τ approx. 0.1 ms appears only after even-numbered flashes and accelerates at low pH [15,20,21,23]. It is sensitive to *o*-phenanthroline [21], an inhibitor of electron transfer from Q_A to Q_B, and is insensitive to the inhibitors of the bc_1 complex, antimycin A and myxothiazol [23]. Therefore, it is reasonable to ascribe this constituent to the protonation of the secondary quinone acceptor [15,20,21,23,30] during Q_BH_2 formation.
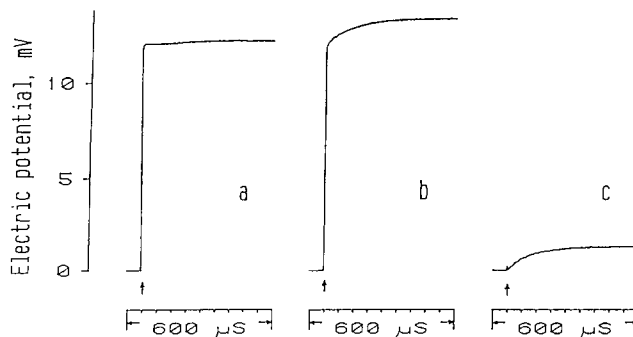

Fig. 1. Generation of electric potential by *Rb. sphaeroides* (wild-type) chromatophores induced by the first (a) and second (b) flash, and the difference of the two responses (c). The time between two flashes is 2 s. Incubation medium: 20 mM Hepes (pH 7), 1 mM potassium ferrocyanide, 30 μM TMPD, 5 μM methylene blue, 4 μM myxothiazol and 5 μM antimycin A.

Photo-electric response after first flash, arising from protein protonation at alkaline pH

Under alkaline conditions one can also see a small additional phase of electric potential induced by the first flash with a rise time approx. 0.1 ms (Fig. 2a). This phase is sensitive to atrazine [15], but unlike the electrogenic phase seen after the second flash the rate constant of this phase is only slightly pH dependent up to pH 9.5 (Fig. 2c). At the end of measurements each sample was 'killed' by addition of herbicide to determine electrogenic phase due to $Q_A^- Q_B \rightarrow Q_A Q_B^-$ reaction and, hence, each point in Fig. 2c corresponds to a new preparation of chromatophores.

The maximal amplitude of this phase is reached at pH approx. 9.6 and is equal to approx. 7% of the amplitude of the phase due to charge separation between P870 and Q_A. This phase of $\Delta\psi$ was connected with protonation of an amino-acid residue located near Q_B in response to electron transfer to Q_B [15] (see Discussion).

Effect of salts on the amplitude and kinetics of quinone phase of $\Delta\psi$

The addition of salts to the incubation medium slowed down the second flash-induced quinone phase of the photoelectric response of the chromatophores.

Fig. 3 shows kinetic curves of $\Delta\psi$ formation (each being similar to that shown in Fig. 1c) induced by the second flash for various concentrations of $La(NO_3)_3$. This quinone phase of $\Delta\psi$ slows down with increasing

Fig. 3. The dependence of the second flash-induced photo-electric response of *Rb. sphaeroides* chromatophores on $La(NO_3)_3$ concentration. Conditions: 20 mM Hepes (pH 7), 50 μM TMPD, 4 μM methylene blue, 4 μM myxothiazol, 5 μM antimycin A. The numbers near curves indicate the concentration of $La(NO_3)_3$ in mM.
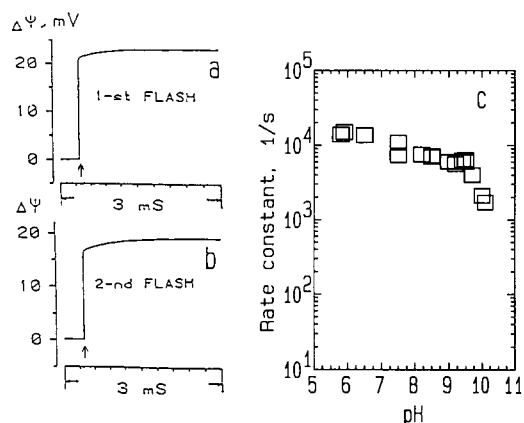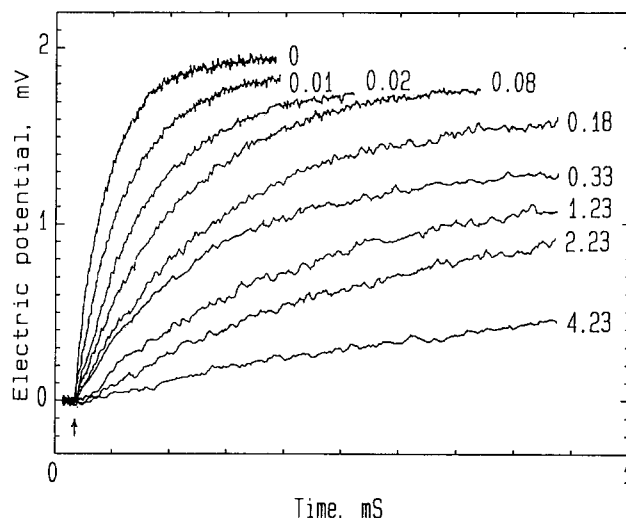

Fig. 2. Photoelectric response of *Rb. sphaeroides* chromatophores induced by the first (a) and second (b) flashes at pH 9 and pH dependence of the rate constant of $\Delta\psi$ generation induced by the first flash (c). Incubation medium in cases a and b contained: 20 mM bis-tris-propane (pH 9), 1 mM potassium ferrocyanide, 50 μM N,N,N',N'-tetramethyl-p-phenylendiamine, 5 μM antimycin A and 3 μM myxothiazol. The second flash was given 2 s after the first. In case c the incubation medium contained 20 mM of one of the following pH buffers: Mes, Mops, Tricine, Bistris-propane and CAPS. Each buffer was used at a pH value close to its pK. The characteristic time was obtained by deconvolution of kinetic curve obtained after subtraction of the first flash-induced photoelectric response in the absence and presence of 200 μM of atrazine, which inhibits the electron transfer between Q_A and Q_B.

salt concentration. The kinetics of $\Delta\psi$ at high salt concentrations is characterized by a lag-phase.

It is important to note that absolute amplitude of second flash-induced quinone phase of $\Delta\psi$ as well as amplitude of the PQ_A phase, arising from charge separating between P870 and Q_A, are reduced with increasing concentration of all salts studied (not shown). The amplitude of quinone phase of $\Delta\psi$ normalized to PQ_A phase decreases less than 2-times for concentrations of KCl up to 2 M, $MgSO_4$ and $CaCl_2$ up to 200 mM and more than 2-times for 3 mM $La(NO_3)_3$.

Fig. 4 shows the concentration dependence of the characteristic rise time of the second flash-induced quinone phase of $\Delta\psi$ induced by addition of various salts. The data were obtained by deconvolution of kinetic curves similar to those presented in Fig. 3. The trivalent lanthanum salt $La(NO_3)_3$ appeared to produce the greatest slowing effect for the same concentration. The salts of divalent metals $CaCl_2$ and $MgSO_4$ appeared less effective. The least effective for the same concentration was the salt of monovalent metal, KCl. The explanation for the behavior patterns of the concentration dependence of the rise time of the second flash-induced quinone phase of $\Delta\psi$ is given in the Discussion in terms of Gouy-Chapman theory. The experimental points are fitted by theoretical curves, obtained on the basis of this theory. The experimental data for all salts studied agree qualitatively and in some cases even quantitatively with theoretical curves.

pH dependence of the kinetics of the second flash-induced quinone phase of $\Delta\psi$ at different concentrations of salts

The rate constant for the disproportionation of semiquinones, $Q_A^- Q_B^- (H^+) \rightarrow Q_A Q_B H_2$, is known to

decrease as pH increases [11,31,32]. In agreement with this observation, the rate constant of the second flash-induced quinone phase of $\Delta\psi$ generation also decreases with decreasing proton concentration [15,20,21]. The cause of this pH dependence is not yet clear. One possibility is the change in surface charge density which modulates the surface proton concentration. Indeed, consistent with this suggestion, the pH dependence of the rate constant of second flash-induced quinone phase of $\Delta\psi$ generation is dependent on the salt concentration. Fig. 5 shows the pH dependence of the rate constant of the $\Delta\psi$ generation measured in the presence of 20 mM and 2 M KCl. As the concentration of salt is increased, the observed pH dependence of this rate constant is stronger. The above dependences also show similar pattern as pH is lowered. The difference between rate constants for 20 mM and 2 M KCl is absent at pH approx. 5.5 and reaches one order of magnitude for alkaline conditions.

Dependence of equilibrium constant of electron transfer between Q_A and Q_B on surface potential

Increasing the salt concentration causes decreasing of amplitude of the fast electrogenic phase due to $P870^+Q_A^-$ formation induced by the second flash $(\Delta\psi_{PA}(2))$ relative to that seen after the first flash $(\Delta\psi_{PA}(1))$. The 2-s interval between flashes is enough to reduce $P870^+$ formed after the 1st flash by TMPD before the second flash $(E_h$ approx. 320 mV). Hence, the lower $\Delta\psi_{PA}(2)$ value is due to an increase of the

Fig. 5. The pH dependence of rate constant of quinone phase induced by the second flash for low (20 mM) and high (2 M) KCl concentration in *Rb. sphaeroides* chromatophores. For a given salt concentration, all points were obtained for the same sample of chromatophore preparation. Incubation medium: 2 mM Mes, Hepes, Tris, CHES and CAPS each, 50 μM TMPD, 5 μM methylene blue, 4 μM myxothiazol, 5 μM antimycin A. The experimental points are fitted by solid lines calculated from Eqn. 11.

RC fraction with Q_A^- before the second flash, arising from decreasing the equilibrium constant, L_{AB}, of the reaction $Q_A^-Q_B \leftrightarrow Q_AQ_B^-$.

This effect of salts is similar to the effect of alkaline pH which can be seen, for example, from comparison of kinetic traces induced by the first and second flashes at pH 7 and pH 9 in Figs. 1 and 2, respectively. At pH 9 $\Delta\psi_{PA}(2)$ is approx. 0.8 $\Delta\psi_{PA}(1)$. The pH dependence of equilibrium constant L_{AB} has been determined for *Rb. sphaeroides* RCs preparations [22,38,58] as well as for chromatophores [22,33].

The ratio of the amplitudes of $\Delta\psi$ arising from charge separation between P and Q_A after second and first flash allow estimation of the equilibrium constant L_{AB} of reaction $Q_A^-Q_B \rightarrow Q_AQ_B^-$ by the equation:

$$L_{AB} = \Delta\psi_{PA}(1)/(\Delta\psi_{PA}(1) - \Delta\psi_{PA}(2)) \tag{1}$$

In chromatophores of *Rb. sphaeroides* at neutral pH and high quinone concentration (> 20 mg/ml) the value of the fast phase induced by the second flash $(\Delta\psi_{PA}(2))$ is approx. 0.97–0.98 of that induced by the first one $(\Delta\psi_{PA}(1))$ (see, for example, Fig. 1). Hence, from Eqn. 1:

$$L_{AB} = 33\text{–}50 \tag{2}$$

This value is slightly less than the value of approx. 60 (pH 7) obtained from kinetics of the $P870^+$ dark relaxation in *Rb. sphaeroides* chromatophores [22,33], probably due to a fraction of RCs in which Q_B is absent in its binding site.

For example, in the presence of 2.23 mM La^{3+} the amplitude of PQ_A phase after second flash is approx. 88% of that after first flash. So, the equilibrium con-
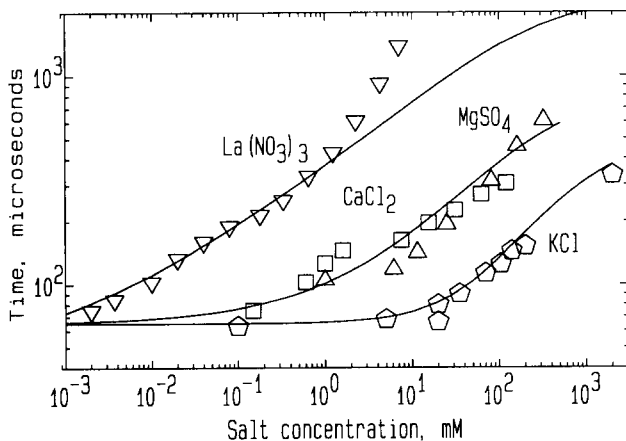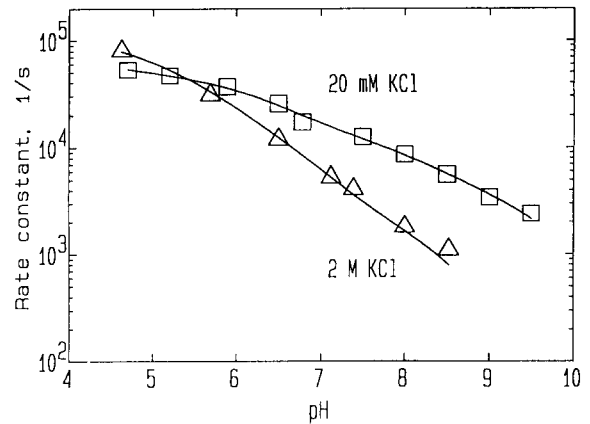

Fig. 4. The dependence of characteristic time of second flash-induced quinone phase of the photo-electric response of *Rb. sphaeroides* chromatophores induced by the second flash on concentration of KCl (pentagons), $MgSO_4$ ($\triangle$), $CaCl_2$ ($\square$) and $La(NO_3)_3$ ($\triangledown$). Conditions: 20 mM Hepes, 1 mM potassium ferrocyanide, 50 μM TMPD, 5 μM methylene blue, 4 μM myxothiazol, 5 μM antimycin A (pH 7.5) for all salts, excluding $La(NO_3)_3$ where pH = 7. The experimental points are fitted by theoretical curves (Eqn. 11), based on the Gouy-Chapman theory, with surface charge density equal to 0.0015 elementary charge per Å^2. The rate constant k'_1 (Eqn. 6) is equal to $6 \cdot 10^{10}$ $M^{-1}s^{-1}$ (KCl), $3.6 \cdot 10^{10}$ $M^{-1}s^{-1}$ ($CaCl_2$ and $MgSO_4$) and $5 \cdot 10^9$ $M^{-1}s^{-1}$ ($La(NO_3)_3$).

stant, estimated from Eqn. 1, is approx. 8.4 for pH 7, i.e., about 5-times less than in the absence of the salt. The effect of salts on equilibrium constant L_{AB} is similar to the effect of the lipophylic cation tetram-ethyl-phosphonium which decreases the equilibrium constant of electron transfer between Q_A and Q_B under alkaline conditions [22].

Discussion

The electric potential generation during Q_B reduction

In spite of the general understanding that ubiquinol is the final product formed in the acceptor side of the reaction center of purple bacteria after the second flash, there are some uncertainties concerning the real reactants of this reaction (reviewed in Refs. 34, 35). Maroti and Wraight showed by analysis of absorption changes of pH-indicator dyes, as well as by conduc-timetry, that in the presence of electron donor, the amount of protons which is taken up by *Rb. sphaeroides* RCs after the first and the second flashes is approxi-mately the same in the pH range 6–9.5 [12]. This means that the process of $\Delta\psi$ generation induced by the second flash under these conditions may be consis-tent with uptake of only one proton. At the same time the flash-induced proton uptake measured by glass pH electrode indicates that the amount of protons taken up after the first flash at pH 7.5 in the presence of high salt concentration is one order less than that taken up after the second flash [36], more consistent with uptake of two protons after second flash. The data presented in Fig. 1 show large differences in amplitude of quinone electrogenic phases induced by the first and second flashes at neutral pH. So measurements of electric potential in chromatophores at neutral pH are also consistent with uptake of two protons after the second flash [11]. Hence, we will assume that after the second flash two protons are transferred to the secondary quinone acceptor (at neutral and low pH).

Because Q_B is located deep inside the protein, the involvement of amino-acid residues of RC protein in transfer of proton(s) to quinone acceptor is highly probable. The data of X-ray structural analysis [3,7] and site-directed mutagenesis [38–43] strongly support the involvement of amino-acid residues Glu L212 [38,39], Asp L213 [38,41] and Ser L223 [40] in this process (reviewed in Refs. 34, 35, 42). But the possibil-ity of involvement of other amino-acid residues [6], as well as water molecules [3] can not be ruled out.

Now it is clear that the first flash-induced electro-genic phase with τ approx. 0.1 ms, observed at alkaline conditions [15] (see Fig. 2), is due to the protonation of Glu L212 in response to electron transfer to Q_B. This conclusion follows from the observation that in mutant reaction centers, in which Glu L212 is altered to Gln, generation of the first flash-induced electric potential

with τ approx. 0.1 ms [37], as well as proton uptake [38] are absent. Hence, it is reasonable to assume that first flash-induced electrogenic phase observed at alkaline conditions is due to proton transfer to Glu L212 (E) as result of the compensation of the Q_B^- charge:

$$Q_A^- E^- Q_B + H^+ \rightarrow Q_A EHQ_B^- \tag{3}$$

This phase of $\Delta\psi$ is not seen at pH < 8.5 because Glu L212 is already protonated. The rate of this reaction is only slightly pH dependent below pH 9.5 (Fig. 2c), indicating involvement other amino-acid residues (or water molecules) in this reaction.

The second flash-induced electrogenic phase is probably due to transfer of two protons from cytoplas-mic side of membrane to quinone during its reduction (neutral conditions):

$$Q_A^- Q_B^- + 2H^+ \rightarrow Q_A Q_B H_2 \tag{4}$$

Because the final product of RC after two turnovers are quinol, the amount of protons taken up by RCs in Eqns. 3 and 4 does not exceed 2 per RC.

Analysis of the various RC mutants has shown the presence of two pathways of proton delivery to Q_B during the reaction as given in Eqn. 4 (reviewed in Refs. 34, 35, 42). The first proton is transferred through pathway involving Ser L223 [40] and Asp L213 [38,41] while the second proton transfer occurs through a pathway involving Glu L212 [35,38,39] and possibly Asp L213 [38]. The delivery of the first proton to Q_B^- is enough for fast semiquinone disproportionation [38]. In the absence of the second proton, the doubly-re-duced quinone is stabilized in the binding site and is not released from RC protein [38].

The generation of electric potential after second flash arising from the protonation of the secondary quinone is a multistage process. We will approximate the observed time (τ) of the electrogenic protonation of Q_B after the second flash by the sum of times (τ_i) of various steps:

$$\tau = (\tau_1^1 + \tau_1^2) + (\tau_2^1 + \tau_2^2) + \tau_3 \tag{5}$$

Here τ_1^1 and τ_1^2 represent the time of the reaction of protons with the surface group(s) of protein for the first and the second route of proton transfer. τ_2^1 and τ_2^2 are the times of the limiting step of proton movement through the protein for first and second route and τ_3 is the time of the electron transfer between Q_A^- and Q_B^- itself, which is not limited by proton uptake.

Depending on conditions, each of these steps can be limiting. At alkaline conditions the protonation of groups at the protein surface will be the limiting factor ($\tau_1 \gg \tau_2, \tau_3$). At acidic conditions the limiting step will probably be the electron transfer between Q_A^- and Q_B^- itself (semiquinone disproportionation) ($\tau_3 \gg \tau_1, \tau_2$).

In some mutant RCs the limiting step could be the proton transfer through protein $(\tau_2 \gg \tau_1, \tau_3)$ [38].

For simplicity of presentation we will assume that $\tau_1^1 = \tau_1^2 \tau_1$ and $\tau_2^1 = \tau_2^2 = \tau_2$. We also assume that the rate constant of the surface protonation is proportional to the surface proton concentration, $[H^+]$:

$$\tau_1 = 1/k_1 = 1/k'[H^+]_s \tag{6}$$

where the bimolecular rate constant k' is the diffusion-limiting constant $((3-6) \cdot 10^{10} \text{ M}^{-1}\text{s}^{-1})$ [44].

τ_2 can depend on the pH of the medium, because of the effect the protonation state of amino-acid residues of proton channel. However, we will not consider this dependence here. We will suggest that the rate constant of electron transfer between Q_A^- and Q_B^- (disproportionation of semiquinones) does not depend on pH when proton uptake is not limiting, although some pH-dependence due to the difference in local charge density between Q_A and Q_B binding sites cannot be ruled out.

Using Eqn. 6, Eqn. 5 for the characteristic time of quinone protonation after second flash could be rearranged to the form:

$$\tau = 2/k_1'[H^+] + \tau_{23} \tag{7}$$

where $\tau_{23} = 2\tau_2 + \tau_3$.

Surface charge density of RC protein as function of pH and salt concentration

For description of the effect of salts and pH we will use the theory of double layer of Gouy-Chapman, which has been proven as a useful model for membrane research [45,46].

The proton concentrations at the surface (subscript s) of protein and in the bulk volume (subscript v) are related through the Boltzmann equation:

$$[H^+]_s = [H^+]_v e^{-\Delta\psi_s F/RT} \text{ or } pH_s = pH_v + \Delta\psi_s F/(2.3RT) \tag{8}$$

where $\Delta\psi_s$ is the surface electric potential (it is assumed that $\psi = 0$ at infinity), F is the Faraday constant, R is the gas constant, T is the absolute temperature and pH_s and pH_v are the surface and volume pH values, respectively.

Using Eqns. 7 and 8, one could estimate the value of the surface potential:

$$\Delta\psi_s F/RT = 2.3[pK_1 - pH_v + \log(\tau - \tau_{23})] \tag{9}$$

where $pK_1 = -\log(2/k_1')$.

According to the Gouy-Chapman theory, for a symmetrical z-z electrolyte (KCl or MgSO₄ type), the surface charge density, σ, (expressed as the number of elementary charges per Å^2), surface potential, $\Delta\psi_s$ (in

mV), and salt concentration, c (in mol/l), are related as (see, for example, Refs. 45, 46):

$$\sigma = 0.0073\sqrt{c} \sinh(zy/2) \tag{10}$$

where $y = F\Delta\psi_s/RT$, $T = 295$ K, $\sinh(u)\overset{\text{def}}{=}(e^u - e^{-u})/2$.

Substituting the surface potential expressed according to Eqn. 9 in Eqn. 10 yields a relationship which connects the time of electrogenic phase (τ), pH of the medium (pH_v), salt concentration (c) and surface charge density (σ):

$$\sigma = 0.0073\sqrt{c} \sinh(2.3z(pK_1 - pH_v + \log(\tau - \tau_{23}))/2) \tag{11}$$

From this relationship it is possible to describe the dependence of the surface charge density (σ) on salt concentration and pH of the medium in terms of the measured time of electrogenic phase.

For small values of surface potential, when $\sinh(u)$ is approx. u, Eqn. 11 can be reduced at fixed pH and $\tau_{23} = 0$ to the following equation:

$$\log k = \log k^0 - \text{const} \cdot \sigma/(z\sqrt{c}) \tag{12}$$

used earlier in Refs. 47 and 48 for estimation of the charge density of chloroplast membrane from kinetics of reduction of acceptors by PS II. Our approach differs from that used earlier [47,48] by consideration of nonlinear relationship between $\Delta\psi$ and σ, by including pH as independent variable, as well as by introducing the additional term τ_{23}, responsible for electron transfer between Q_A and Q_B. It is noteworthy to indicate that dependence of the logarithm of the rate constant of quinone electrogenic phase on $1/\sqrt{c}$, used earlier [47] to determine the value of surface
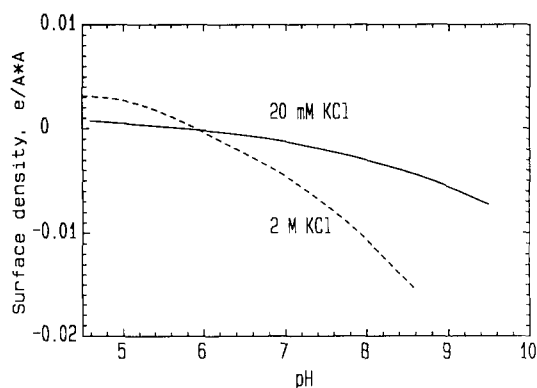

Fig. 6. Predicted pH dependence of *Rb. sphaeroides* chromatophore surface charge density calculated from Eqn. 11 and experimental dependences shown in Fig. 5 for 20 mM and 2 M KCl. The diffusion-limited rate constant of protonation was taken to be $6 \cdot 10^{10}$ $\text{M}^{-1}\text{s}^{-1}$ [44]. The value of τ_{23}, corresponding to the rate of electron transfer between Q_A and Q_B at low pH, was taken as 20 μs.

charge density, is non-linear for our data, indicating inadequacy of linear approximation (12) in case of bacterial RCs.

Presented in Fig. 6 is the pH dependence of the reaction center protein surface charge density, calculated from Eqn. 11 using the data of Fig. 5 for 20 mM and 2 M KCl. The characteristic time $\tau_{23} = 2\tau_2 + \tau_3$ at low pH was taken to be 20 μs. The value of the rate constant k_1 of reaction at the protein surface was considered as the diffusion-limited constant, which is equal to approx. $6 \cdot 10^{10}$ M^{-1} s^{-1} [44]. Fig. 6 shows that there is a monotonic decrease in the charge density from $+0.003$ at pH 5 to -0.014 elementary charge per Å^2 at pH 9.5 with zero surface charge density around pH 6. The calculated value of σ, approx. 0.0017 e/Å^2 (approx. 2.7 μC/cm^2), at pH 7 is in reasonable agreement with values 1.6–4.6 μC/cm^2, obtained for chromatophores of Rb. sphaeroides [49,50], especially if one considers many simplifications made.

The reaction center of Rb. sphaeroides has more than 150 charged amino-acid residues [51]. Their locations are precisely determined by X-ray structural analysis. The cytoplasmic side of membrane contains more than 100 charged amino-acid residues, so the upper limit of the surface charge density at cytoplasmic side of membrane is approx. 0.02 e/Å^2, if one takes the surface of RC protein as approx. 5500 Å^2. This simple estimation indicates that only charges of RC protein could be responsible for observed effects of pH and salts, although it is difficult to exclude the involvement of negative charges of acid phospolipids, especially at low salt concentration. The value of surface charge density approx. 0.0017 elementary charge per Å^2 (pH 7) for 20 mM KCl is equivalent to approx. 10 negative charges per RC on the cytoplasmic side of membrane (for surface 5500 Å^2).

At first it is surprising that the surface charge density increases with increasing salt concentration at constant pH value (Fig. 6). Note that such an increase in the surface charge density has been reported in the literature [52–54], although in most investigations the surface charge density has been assumed constant and not varied with salt addition (see, e.g., Ref. 46). The increase in the surface charge density with increasing of the salt concentration may be explained in terms of the alteration of proteolytic equilibrium of the amino-acid residues of the RC protein. Indeed, increasing the salt concentration causes decreasing of the surface potential, which in turn changes the apparent dissociation constants for the proton-exchangeable amino-acid residues. This in turn changes the surface charge density. The final charge-density value becomes higher than the initial value and the surface potential is lowered.

Hence, for the explanation of the pH and salt effects, instead of Eqn. 11, it is necessary to use the system of equations below (we consider, for simplicity, only the KCl effect):

$$\sigma = 0.0073\sqrt{c} \, \sinh(y/2) \tag{13}$$

$$\sigma = \sum_i \sigma_i / (1 + 10^{(pH_v + 2.3y/2 - pK_i)}) \tag{14}$$

Here, Eqn. 13 is the Gouy-Chapman equation for 1:1 salt; Eqn. 14 describes changes in surface charge density with pH and surface potential; $y = F\Delta\psi/RT$; σ_i is the maximal surface charge density due to i-th type amino-acid residues, with $pK = pK_i$ at zero surface potential; σ_i is positive for amino-acid residues which become positively charged after protonation and negative for amino-acid residues which become neutral. We consider for simplicity that each type of amino-acid residue has the same pK.

Numerical solutions of this set of equations may be obtained, for example, by varying surface potential (y in Eqns. 13 and 14) as independent parameter to get equal values of σ. The pH dependence of surface charge density for different salt concentrations calculated on the basis of Eqns. 13 and 14 agrees qualitatively with that presented in Fig. 5, in spite of many simplifications (not shown).

So, by acknowledging the dependence of surface potential density on pH and salt concentration, we were able to explain the observed pH dependence of the rate of the second flash-induced quinone phase of the photo-electric response at different salt concentrations.

Binding of anions and cations

Previous estimations of surface charge density of RC are based on the assumption that binding of cations and anions to the RC protein are absent. At the same time the preliminary data of refined X-ray structural analysis for Rhodopseudomonas viridis RCs indicate the presence of seven anions bound to the protein [3], which may be due to high concentration of $(NH_4)_2SO_4$ used during the crystallization procedure. At this moment the question of ion binding to the RC protein in vivo is still open.

Different proteins usually bind anions at low pH and cations at alkaline conditions [53,55]. For example, lysozyme binds up to 50 Cl$^-$ per protein at low pH, drastically changing the titration curve of the protein [55]. The analysis of salt and pH effects on purple membranes of Halobacterium halobium indicates the existence of five cation-binding sites in this protein (reviewed in Ref. 53).

The effect of divalent cations on quinone electrogenic phase also partially can be explained by specific binding of cations to the protein. However, more experiments are needed to check this suggestion in more detail.

Addition of La^{3+} was found to slow the Q_B^{2-} protonation rate after the second flash more than predicted by the simple electrostatic theory (Fig. 4). This may be caused by the interaction of La^{3+} with carboxylic acid residues involved in the proton transfer, as well as by breakdown of the hydrogen bonding inside of proton trap in the RC protein.

The estimation of proton transfer distances

The electrometric technique is characterized by a high signal-to-noise ratio (approx. 200) which allows estimation of the electric distance (r/ϵ) of charge transfer in the RC with high accuracy. The distance between P870 and Q_A is known with high precision and can be used for calibration of all electrogenic processes in the native membrane. The main uncertainty of this approach is the value of the effective dielectric constant, ϵ.

In Table I, the electrogenic reactions in RCs measured by the electrometric method in *Rb. sphaeroides* chromatophores at high redox potential (E_h approx. 320 mV) are indicated.

The electric potential difference measured after the first flash at alkaline pH in the microsecond time domain is probably due to proton transfer from water on cytoplasmic side of membrane (subscript W) to Glu L212 (subscript E) in response to semiquinone Q_B^- generation, i.e.:

$$\Delta\psi(1) = \Delta\psi_{WE} \tag{15}$$

The electric potential difference with τ approx. 0.1 ms, measured after the second flash, is due to transfer of two protons from water to quinone, which is necessary for QH_2 generation.

The electric potential corresponding to the transfer of two protons from water to quinone after second flash $(\Delta\psi(2))$ can be subdivided among the electric potential generation due to proton transfer from Glu L212 to Q_B $(\Delta\psi_{EB})$ and proton transfer from water to Glu L212 $(\Delta\psi_{WE})$:

$$\Delta\psi(2) = 2(\Delta\psi_{EB} + \Delta\psi_{WE}) \tag{16}$$

Combining Eqns. 15 and 16 results in the following:

$$\Delta\psi_{EB} = \Delta\psi(2)/2 - \Delta\psi(1) \tag{17}$$

Eqn. 17 states that electric potential difference due to the proton transfer between Glu L212 and Q_B is equal to half the amplitude of electric potential generated after second flash minus maximal amplitude of electric potential generated after the first flash.

Using data of Table I and Eqn. 17, the value of relative amplitude of electrogenic phase due to the proton transfer between Glu L212 and Q_B is:

$$\frac{\Delta\psi_{EB}}{\Delta\psi_{AP}} = \frac{\Delta\psi(2)/2}{\Delta\psi_{AP}} - \frac{\Delta\psi(1)}{\Delta\psi_{AP}}$$

$$= (0.15 - 0.18) - (0.1 - 0.12) = 0.05 \pm 0.03 \tag{18}$$

TABLE I

Main electrogenic phases in RCs of chromatophores of purple bacteria Rb. sphaeroides measured in this work

Reaction	Time	Amplitude	Characteristics
$PQ_A \rightarrow P^+Q_A^-$	not resolved (< 0.1 μs)	100%	Nonsensitive to herbicides, diminished at $E_h > 450$ mV and at $E_h < -100$ mV
$Q_B^- E^- + H^+ \rightarrow Q_B^-$ (EH)	0.1 ms [b] (pH 9)	$< 7\%$ [a]	Seen only after first flash in alkaline conditions, sensitive to herbicides, is due to Glu L212 protonation
$Q_A^- Q_B^- + 2H^+ \rightarrow Q_A Q_B H_2$	0.1 ms [d] (pH 7.5)	15–20% [c]	Sensitive to herbicides, seen only after second flash, slowdown at increasing pH and ionic strength

[a] The measured amplitude of this reaction is less than maximum amplitude in approx. 1.2–1.4 times, due to the fact that it is obtained as difference of the two titration curves separated by approx. 1.5–2 units of pH.

[b] Rate of this reaction is pH dependent at pH ≥ 9.

[c] The amount of $Q_B H_2$ generated after the second flash, as a rule, is less than one per reaction center. This is influenced by such factors as time between flashes, intensity of flash, concentration of redox mediator, finite value of equilibrium constant between Q_A and Q_B and others. The recalculation which considers influences all of these factors showed that the maximal amplitude of quinone phase induced by the second flash may be as large as 30% of that for the PQ_A phase [10].

[d] Rate of this reaction depends on pH [20,21].

i.e., $\Delta\psi_{EB} = (0.05 \pm 0.03)\Delta\psi_{AP}$. This means that electric potential resulting from proton transfer between Glu L212 and Q_B consists of approx. 0.05 of electric potential arising from charge separation between P and Q_A.

It is important to stress that in Eqn. 18 we used maximum amplitude of the second flash-induced phase which is about 2-times higher than the observed value (see footnote to Table I).

The flat capacitor formula, $\Delta\psi = r/(4\pi\epsilon\epsilon_o S)$, for electric potential difference and Eqn. 18 will yield:

$$\epsilon_{AP} = 0.05\epsilon_{EB}r_{AP}/r_{EB} \qquad (19)$$

The values of the distances between Q_A and P870, as well as between Glu L212 and Q_B determined from X-ray structural analysis of RCs crystals are approx. 27 Å and approx. 6 Å, respectively [4]. The value of dielectric constant for hydrophobic region of RC is approx. 4 [56]. So, from Eqn. 19 the effective dielectric constant near Q_B can be estimated as:

$$\epsilon_{EB} \simeq 20 \qquad (20)$$

Hence, the value of effective dielectric constant is higher than for the membrane region of RC. This agrees well with the estimated ϵ of approx. 20, based on the consideration of the shift of the pK of Glu L212 after the arrival of electron on Q_B (from 9.8 to 11.2) and known distance (approx. 6 Å) between Glu L212 and Q_B [34,35,57].

Comparison of different measurements of semiquinone disproportionation

In chromatophores of *Rb. sphaeroides* wash out from Ca^{2+} the rise time of $\Delta\psi$ formation at pH 7, as measured electrometrically (approx. 60 μs), is close to that measured from electrochromic shift of carotenoids [21] or obtained by direct measurement of the disproportionation reaction at 450 nm in chromatophores [28]. The characteristic times of quinone phase measured earlier by the same method in *Rb. sphaeroides* [30] and *Rhodospirillum rubrum* [20] chromatophore preparations were 2–4-times slower than that measured in this work. This difference could be explained by the presence of a small amount of residual Ca^{2+} used for fusion of chromatophores with lipid-impregnated nitrocellulose membrane in previous works. The effect of residual cations is more pronounced at alkaline conditions. It is possible that the same reason could explain differences of the times of second flash-induced quinone electrogenic phase measured in *Rhodopseudomonas viridis* proteoliposomes (400 μs [59]) by this method and in whole cells, measured by the light-gradient method (40–80 μs [60]).

The pH dependence of semiquinone disproportionation

At this time, the differences in pH dependences of the rate constant of the reaction $Q_A^- Q_B^- + 2H^+ \rightarrow Q_A Q_B H_2$ measured in various laboratories at alkaline conditions [11,31,32] are still unresolved. For example, the rate constant determined in Ref. 11 was approx. 7-times less at pH 10 than that determined in Ref. 32 for the same strain of *Rb. sphaeroides R-26*. The results obtained in this work indicate that observed contradiction can be resolved partially by considering the differences in salt concentrations, as well as the differences in charge surface density due to the type and concentrations of detergent used. Indeed, in Ref. 11 the assay solution contained 100 mM NaCl, while in Ref. 32 it contained only 3.3 mM KCl. The pH dependences mentioned above have a tendency to converge at low pH, in good agreement with the model proposed in this work, as well as experimental results of salt effect on the pH dependence of the electrogenic Q_B protonation (see Fig. 5).

Conclusion

The second flash-induced formation of the transmembrane electric potential difference arising from proton uptake by the quinone complex of the photosynthetic reaction center of *Rb. sphaeroides* is sensitive to pH and salt concentration. The Gouy-Chapman approach to the kinetics of second flash-induced phase of $\Delta\psi$ can be used for semi-quantitative explanation of (1) different sensitivity of $\Delta\psi$ to the mono-, di- and trivalent salts; (2) pH dependence of electrogenic phase at constant salt concentration and (3) both pH and salt effects on $\Delta\psi$ simultaneously. It is appears that main effects of pH, as well as salts, on the second flash-induced electrogenic phase is due to the decreasing of surface proton concentration which is determined by (1) pH of bulk volume; (2) density of charges in protein (depending on pH) and by (3) the screening effect of salts in manner similar to that considered in the Gouy-Chapman theory.

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