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Flash-induced changes in buffering capacity of reaction centers from photosynthetic bacteria reveal complex interaction between quinone pockets

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Abstract

A novel method was applied to determine light-induced protonation in reaction centers from photosynthetic purple bacteria. Changes in buffering capacities upon flash excitation were detected in (0.03% Triton X-100) detergent solution of reaction centers from *Rhodobacter* (*Rb.*) *sphaeroides* and *Rb. capsulatus* wild type and mutant strains with empty or occupied secondary quinone (Q_B) binding sites in the presence of an external electron donor. The light-induced differences in buffering capacities between pH 4 and 11 were analyzed in terms of protonatable residues. Due to its differential nature, this method is more sensitive to the position and shift of pK_a of the individual groups than the direct method based on proton uptake measurements. Out of the four different ionizable residues which were used to fit the curves, the two groups with apparent (dark) pK_a values between 8.4–8.8 and 9.5–10.0 (depending on the species and conditions) disappeared when the native ubiquinone$_{10}$ was replaced by menadione at the primary quinone (Q_A) binding site of *Rb. sphaeroides* or when the key protonatable residues (L212Glu and L213Asp) were replaced by non-protonatable alanines in the Q_B binding site of the AA+M43D mutant from *Rb. capsulatus*. The experimentally observed acidic and neutral residues remained unchanged. These results obtained from modifications in both quinone sites reveal the origin of the alkaline pH groups: they reflect the interaction of Q_A^- and the cluster of ionizable residues around L212Glu in the Q_B binding pocket. The involvement of two residues with close pK_a values reflects the complex titration of the cluster. The interaction between the quinone pockets is best described qualitatively as a network of ionizable residues extending from the Q_A site to the Q_B site.

Keywords: Photosynthesis; Bacterial reaction center; Electrostatic interaction; Proton binding; Site-specific mutagenesis

1. Introduction

The membrane-spanning reaction center (RC) protein in photosynthetic organelles sets the stage for vital bioenergetic processes by converting light into other forms of free energy, namely redox potential, ion (proton)-electrochemical gradient, and phosphate potential. In the purple photosynthetic bacteria *Rho-*

Abbreviations: LDAO, lauryl dimethylamine *N*-oxide; MD, menadione; P, bacteriochlorophyll dimer; Q_A and Q_B, ubiquinones; TX-100, Triton X-100

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dobacter sphaeroides and *Rhodobacter capsulatus*, this set of processes is initiated by light absorption and electronic excitation of a bacteriochlorophyll dimer (P) followed by transmembrane electron transfer to the primary ubiquinone electron acceptor, Q_A: $PQ_A \rightarrow P^+Q_A^-$. If quinone ($Q_B$) is available at the secondary quinone binding site, then the electron is transferred to it and the charge pair is stabilized in the form of $P^+Q_B^-$. In the absence of an electron donor to the oxidized dimer, the $P^+Q_A^-$ and $P^+Q_B^-$ charge pairs recombine within 0.1 s and 1 s, respectively. In the presence of an external electron donor, the charge recombination is impeded by fast re-reduction of the oxidized dimer (for a review, see [1]).

The stabilization against charge recombination is facilitated by uptake of H^+ by the RC as the bound protons contribute to the solvation of the electron in the protein matrix (reviewed in [2–4]). An interacting network of protonatable residues and water molecules is located between the quinone binding sites in the region of the RC complex that is near the cytoplasmic face of the membrane [5–10]. Neither of the semi-reduced quinones are protonated directly, but their interaction with this network of protonatable amino acid residues increases the pK_a values of some of those residues resulting in sub-stoichiometric proton binding [11]. The question of whether the interaction is distributed among a large number of groups with correspondingly smaller pK_a changes or is restricted to a few residues with larger pK_a shifts is an important and current debate [12–14]. It has previously been proposed that L213Asp and L212Glu are directly involved in proton uptake observed in the acidic and alkaline pH range, respectively [2–4,15], and constitute an important (but not irreplacable [16]) part of the delivery pathways of the first [17,18] and second [19–21] protons to Q_B, respectively.

The problem of light-induced protonation has been approached by different experimental methods including direct H^+ detection by pH electrodes [12,13] and pH sensitive dyes [11,12,14], electric conductance measurements [14,22], infrared spectroscopy [23,24] and direct electrometry [15]. Here a different technique, light-minus-dark pH-metric titration was used. Recently, we were able to carry out pH-metric titration of water-insoluble RCs solubilized in different detergents [25]. The light-minus-dark difference pH-metric titration presented here allows the determination of the number and pK_a values of protonatable residues influenced by electrostatic interaction with the semiquinones. The pK_a values and their shifts in native RCs of *Rb. sphaeroides* and *Rb. capsulatus* were compared with those measured in RCs in which the native primary quinone was replaced by menadione and in mutant RCs in which the protonatable residues L212Glu and L213Asp in the vicinity of Q_B^- were replaced by non-protonatable alanines.

2. Materials and methods

The wild type and mutant strains of *Rb. sphaeroides* and *Rb. capsulatus* were cultivated and harvested and the RCs isolated and purified as described earlier [12,26–28]. The buffer (10 mM Tris) and the ionic detergent (0.1% LDAO) were removed and replaced by 0.03% non-ionic detergent Triton X-100 by extensive dialysis (48 h). Removal of quinones from the Q_A and Q_B sites and reconstitution of quinone activity by native ubiquinone$_{10}$ or by menadione (MD) were done according to Kálmán and Maróti [29]. To eliminate the secondary ubiquinone activity, 100 μM terbutryne was added which blocked the interquinone electron transfer. The very low binding affinity of MD for the Q_B site compared to that for the Q_A site resulted in essentially no secondary quinone activity in MD-reconstituted samples. The isolation of the AA+M43D mutant (L212Glu-L213Asp-M43Asn → Ala-Ala-Asp) of *Rb. capsulatus* was described in [27].

The buffering capacity was measured as the pH change upon addition of a small quantity (3 μl) of strong acid or base from a series of stock concentrations of 5, 10 and 20 mM to a 3 ml solution of the RC in its dark (PQ) or light-adapted (PQ^-) states. When the light experiment is done, the acid from the Hamilton syringe is injected into the stirred solution immediately after the flash. On the time scale used in this study, all these events are practically at time 0. The PQ^- redox state of the RC was achieved by a single and saturating xenon flash excitation (EG&G FX-200) in the presence of 200 μM ferrocene, as external electron donor. The rate of re-reduction of P^+ by the ferrocene, $(250\ ms)^{-1}$, at pH 8 was much

larger than that of charge recombination from $P^+Q_A^-$ state, $(70\ ms)^{-1}$. The concentration of the RC was determined by flash-induced absorbance change due to P^+Q^- at 430 nm [12,14]. The pH electrode (Orion 91-03) was shielded from the direct illumination of the xenon flash.

The temperature was set to 25°C in all measurements.

3. Theory

Fig. 1 demonstrates the correlation between the light-induced direct H^+ binding and changes of buffering capacity in the case of a single protonatable residue, R. The pH dependence of mole fraction of the group with bound H^+ (RH) is described by the Henderson-Hasselbalch expression

$$[RH] = \frac{1}{1 + 10^{pH-pK}} \quad (1)$$

with $pK = pK_D$ and $pK = pK_L$ in the dark (PQ) and light (PQ^-) states of the RC, respectively. The difference of the two Henderson-Hasselbalch type curves offers the directly observable uptake of protons upon illumination. The buffering capacity due to 1 mol of the protonatable group R can be derived from the

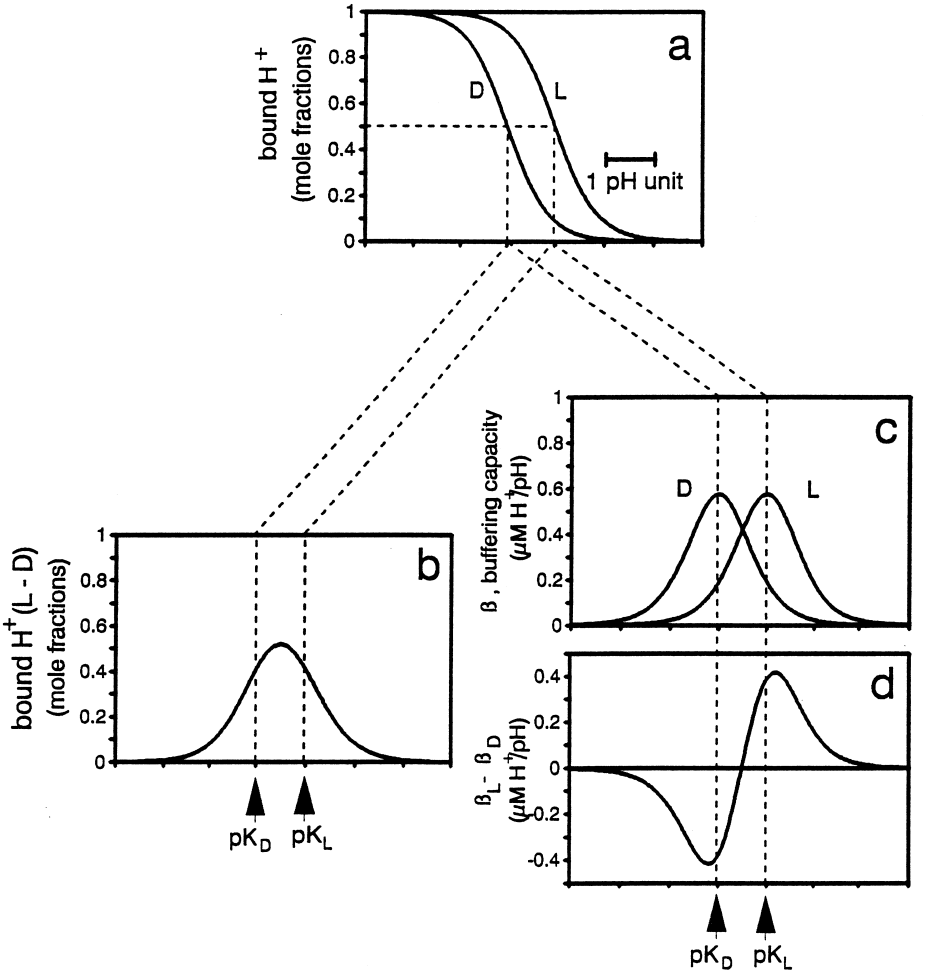

Fig. 1. pH dependence of proton binding and changes in buffering capacity due to dark (D)-light (L) transition for a single protonatable residue. (a) Titration curves (Eq. 1) with 1 pH unit pK_a shift ($pK_L - pK_D = 1$). (b) Light-induced proton uptake as the difference of the two titration curves (L−D). (c) Buffering capacities (β) of the dark (D) and light (L) adapted states (Eq. 2). (d) Light-minus-dark difference of the buffering capacities ($\beta_L - \beta_D$).

Henderson-Hasselbalch equation using the definition of $\beta = -d[H^+]/d(pH)$:

$$\beta = \frac{2.3}{f} \cdot \frac{1}{2 + 10^{pK-pH} + 10^{pH-pK}} \quad (2)$$

Here f ($= a_H/[H^+]$) is the activity coefficient of H^+ in the solution and a_H ($= 10^{-pH}$) is the activity of H^+. The buffering capacity is a bell-shaped curve centered at $pH = pK$ in accordance with the expectations of negligible buffering capacity by substantially protonated or deprotonated states of the residue. The light-induced change is described by the difference of two curves with pK_a values of pK_L and pK_D. It has two extreme values: a (negative) minimum around $pH = pK_D$ and a (positive) maximum around $pH = pK_L$. Due to this sophisticated pH dependence and narrow double peaks, the decomposition of the measured light-induced buffering capacity will be more sensitive to the position of the components and their light-induced shifts than the data of proton uptake measurements.

4. Results

The experimental determination of the buffering capacity of a micellar solution of RCs is an inherently slow process as it requires establishment of a new equilibrium after its perturbation by addition of a small amount of acid. In the present experiments, care was taken to avoid overlap of the mixing time and response time of the electrode with lifetime of the redox state of the RC. Fig. 2 demonstrates typical kinetic traces of acid mixing calibration at three different pH values where the buffering capacities of the solution are different. This is reflected by different pH changes upon addition of an identical amount of H^+ (5 μM). Although the kinetics of the transient changes and the offsets of the baselines display large differences, the mixing and the response of the electrode to added acid occurs within 15 s at all pH values investigated. Because all of the redox states studied here (except of PQ_A^- at $pH > 10$ where corrections for Q_A^- decay were made) had larger lifetimes (see [29] for *Rb. sphaeroides*), the experimental design assured sufficient time for determination of

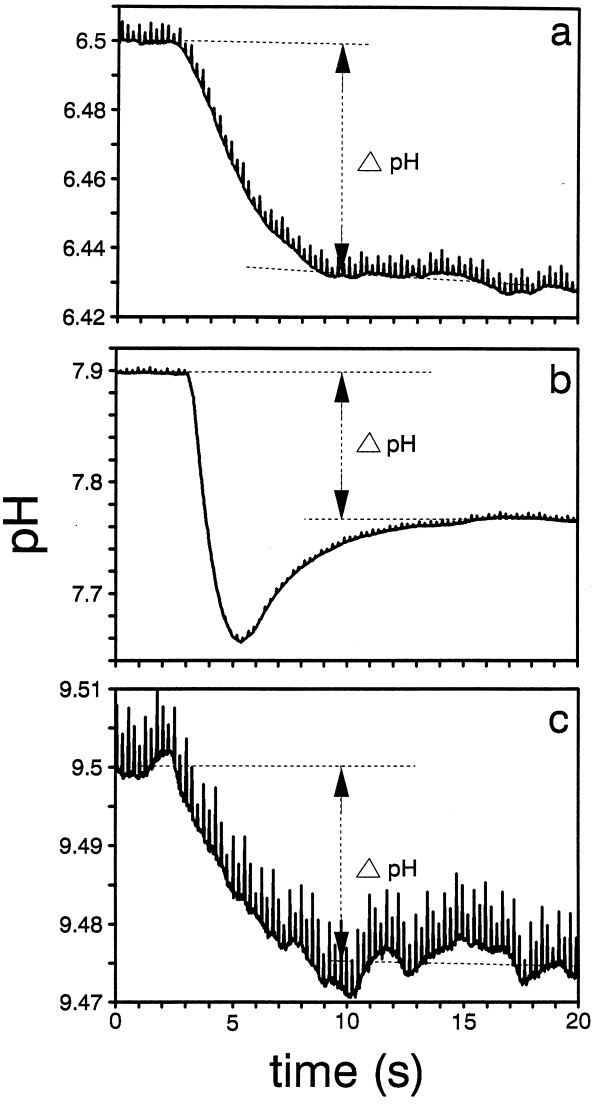

Fig. 2. Kinetic traces of observed pH changes in acid mixing calibration of RC at redox state PQ_A at three different pH values: 6.5 (a), 7.9 (b) and 9.5 (c). 5 μM H^+ was injected and stirred in aqueous solution of 1 μM RC from *Rb. sphaeroides*, 0.03% TX-100, 100 mM NaCl, 200 μM ferrocene and 100 μM terbutryne.

the buffering capacity of the micellar solution of the RC.

The magnitude of light-induced changes in buffering capacity of bacterial RCs is very small at any pH value. It is at the limit of detection as it amounts only a few percent of the buffering capacity of the solution in either the dark or the light. Fig. 3 demonstrates the experimental determination of light-induced changes as the difference of the buffering ca-

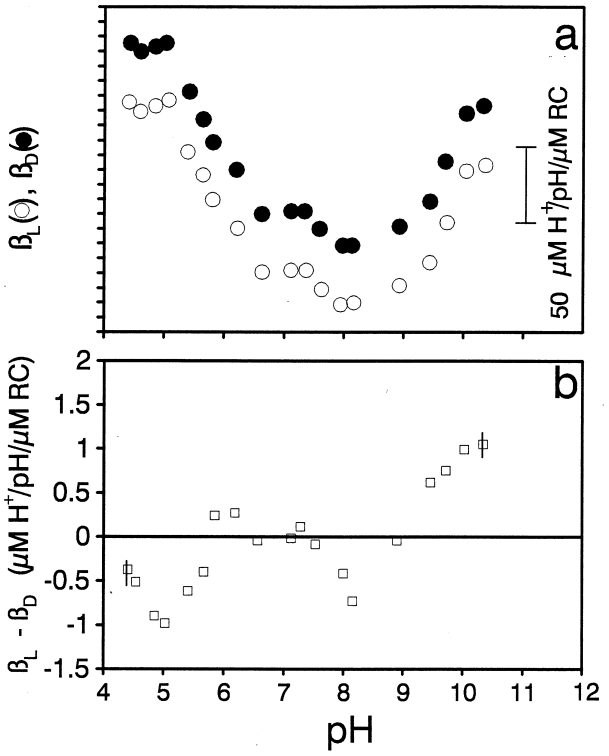

Fig. 3. pH dependence of the observed buffering capacity (β) of RC from *Rb. sphaeroides* in light (○) and dark (●) adapted states (a) and their difference ($\beta_L-\beta_D$, b). The experimental data at particular pH values were determined as shown in Fig. 2. The pH was adjusted by strong acid (HCl) and base (NaOH). Conditions are the same as in Fig. 2, except 3 μM RC.

pacities of the micellar solution measured in the light- (PQ^-) and in the dark-adapted (PQ) redox states of the RCs across a wide pH range.

The PQ^- redox form was achieved by fast re-reduction of P^+ by an external electron donor after flash excitation. From the point of view of buffering capacity measurement, the PQ^- state had at least two advantages compared to the P^+Q^- state: it had a significantly longer lifetime (5–60 s, depending on the strain and the pH) during which the determination of the buffering capacity by acid/base mixing

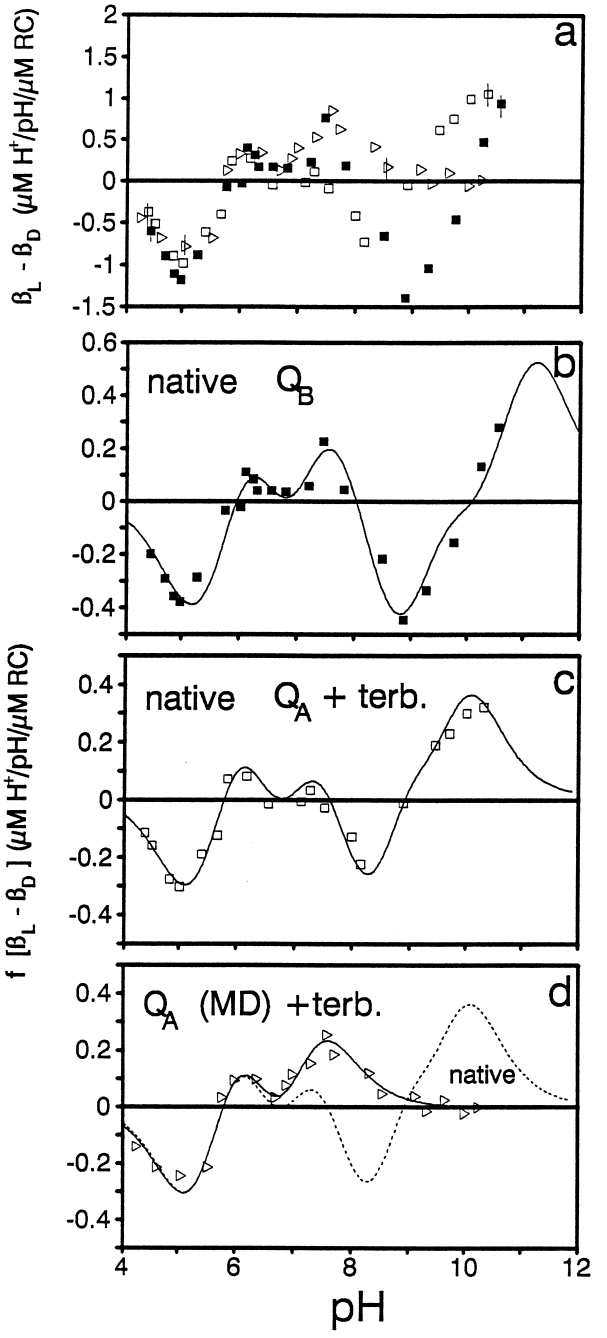

Fig. 4. pH dependence of flash-induced changes in buffering capacity of RCs from *Rb. sphaeroides* strain R-26 solubilized by detergent TX-100. The buffering capacities were determined by pH electrode in dark (PQ) and light (PQ^-) adapted states of the RC and the difference was plotted. Typical standard deviations of the data are indicated by error bars. The observed data (a) were corrected for the proton activity coefficient ($f_H = 0.33$) (b–d). Native quinones at the Q_A and Q_B binding sites (b, filled squares), native quinone at the Q_A site but the Q_B binding site is filled with terbutryne (c, open squares) and menadione (MD) at the Q_A site (d, open triangles). Best fit curves using Eq. 2 with pK_a values from Table 1 (continuous line). The dashed line in panel d represents the fit with pK_as for Q_A active native RC. Conditions: 3 μM RC, 0.03% TX-100, 100 mM NaCl, 200 μM ferrocene and in panels c and d 100 μM terbutryne.

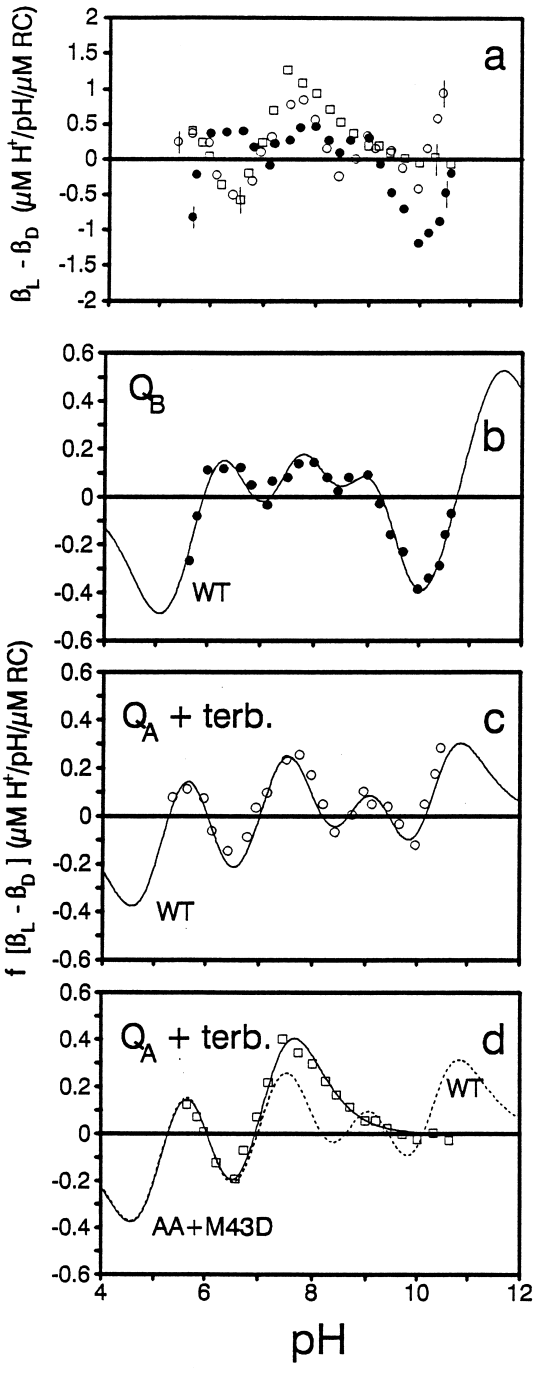

Fig. 5. pH dependence of flash-induced changes in buffering capacity of wild-type and mutant RCs from *Rb. capsulatus* solubilized by detergent TX-100. The buffering capacities were determined by pH electrode in dark (PQ) and light (PQ^-) adapted states of the RC and the difference was plotted. The observed data with typical errors indicated by error bars (a) were corrected for the proton activity coefficient ($f_H = 0.33$) (b–d). Wild-type RC with secondary quinone activity (b, filled circles), native quinone at the Q_A site but the Q_B binding site is filled with terbutryne of the wild-type RC (c, open circles) and AA+M43D mutant with native quinone at the Q_A site only (d, open squares). Best fit curves using Eq. 2 with pK values from Table 1 (continuous line). The dashed line in panel d represents the fit with pK values for Q_A active wild-type RC. Conditions: 2.3 μM RC, 0.03% TX-100, 100 mM NaCl, 200 μM ferrocene and in panels c and d 100 μM terbutryne.
←

could be carried out [25,29], and the stoichiometry of the proton uptake was higher due to the elimination of opposite effects caused by P^+ [2–4,11–13]. The pH dependence of light-induced changes of the buffering capacity showed both positive and negative regions in RCs from *Rb. sphaeroides* (Fig. 4) and *Rb. capsulatus* (Fig. 5). The data taken directly from the experiments (panels a) had to be corrected for the activity coefficient of the H^+ ($f_H = 0.33$, in 0.03% Triton X-100 [18]) to correlate with the sum of individual components described by Eq. 2. The pH dependence of the light-induced changes of buffering capacity in both bacterial strains with (panels b) and without (panels c) active secondary quinone could be decomposed into the sum of four apparent residues with pK_a values listed in Table 1. According to their 'dark' apparent pK_a values in the pH range, these groups can be classified as acidic (group 1), neutral (group 2) and alkaline (groups 3 and 4). Of special interest are the replacements of native ubiquinone$_{10}$ by menadione at the Q_A binding site of the RC of *Rb. sphaeroides* [29] and the protonatable L212Glu and L213Asp residues by non-ionizable alanines at the Q_B binding site in the RC of *Rb. capsulatus* (panels d). (In the L212Ala-L213Ala double mutant, photocompetence was achieved by an additional suppressor mutation, Asn-Asp, at residue M43 [27,30]). In both cases, compared to the wild-type RCs, the residues whose apparent pK_a values are in the acidic and neutral ranges were unaffected but the two groups with apparent pK_a values in the alkaline region disappeared.

5. Discussion

We report here, for the first time, light-induced changes in buffering capacity of detergent-solubilized RCs from several strains. Both wild-type RCs and

RCs carrying modifications in the Q_A or Q_B binding sites were studied. The measured difference titrations were fitted with the difference of the mathematical expression of Eq. 2 using a least-squares procedure. Good fits were obtained using four independent protonatable groups, whose apparent pK_a values and shifts were treated as adjustable parameters in wild-type strains of *Rb. sphaeroides* and *Rb. capsulatus*. These data are in good agreement with earlier results obtained from measurements of direct proton binding [4,12,13,29], and changes in conductance [22] and electrogenicity [15] in *Rb. sphaeroides* and *Rb. capsulatus* [4,31,32]. The light-induced pH-metry offered more well-resolved peaks in the *Rb. capsulatus* data of Fig. 5 than the previous proton uptake measurements [4]. The application of this method resulted also in higher upper pK_a value for *Rb. capsulatus* than for *Rb. sphaeroides* (Table 1). The two apparent alkaline groups and the acidic and neutral groups reflect different interactions with the semiquinones which will be discussed in more detail below.

In the RCs of *Rb. sphaeroides*, the native ubiquinone$_{10}$ in the primary quinone site was replaced by menadione. The presence of menadione as Q_A caused a dramatic change in the high pH behavior of the flash-induced buffering capacity, in good accordance with earlier proton binding results which showed no proton uptake by the PQ_A^- state in MD-reconstituted RCs [29]. It can be concluded that menadione in the Q_A site does not establish the interaction with the network of ionizable residues around Q_B observed in the wild-type RC [4,32,33]. Since the distances between Q_A and the ionizable residues near Q_B are so large (>15 Å), effects other than direct electrostatics must also be involved in the interaction. However, the structural and quantitative explanation of modification of the interquinone network in the MD-reconstituted RC is beyond our present capacity.

In the other experiment, key protonatable residues L212Glu and L213Asp in the Q_B site were mutated to non-protonatable alanines. These mutant RCs also have lost the light-induced buffering capacity changes at high pH region indicating that proton uptake induced by reduction of either Q_A or Q_B should occur to amino acid residues in the Q_B pocket. The observed changes in flash-induced buffering capacities on the whole pH range were in good agreement with direct proton binding data carried out recently on a set of protonation mutants [4,16,31–33]. In these earlier studies, the analysis concentrated on the highest pH group only. However, a closer look at these data suggest the involvement of more than one alkaline group in the protonation of mutants where only residue is changed, suggesting that the pK_a of another residue has shifted in response to the mutation. This observation is supported and quantified by the data reported in this study (see Table 1). Because the decomposition of the pH dependence of the flash-induced buffering capacity assumed independent residues, the contribution of two groups with closely spaced alkaline pK_a values can be interpreted in terms of interacting residues, which is consistent with data from mutant strains [32]. Indeed, recent kinetic IR [23] and FTIR [24] measurements, theoretical calculations [8,9], structural data [5–7] and mutational studies ([21]; M. Valerio-Lepiniec, M. Schiffer, D.K. Hanson, P. Sebban, unpublished observations) support

Table 1
Apparent pK_a values of non-interacting protonatable groups (1–4) in the oxidized (pK_{aQ}) and reduced (pK_{aQ^-}) states of the quinone acceptor complex (Q_A/Q_B) in different strains and mutants of purple bacteria obtained from light-induced changes of buffering capacity

Strain		Q_A/Q_B	Group 1		Group 2		Group 3		Group 4	
			pK_Q	pK_{Q^-}	pK_Q	pK_{Q^-}	pK_Q	pK_{Q^-}	pK_Q	pK_{Q^-}
Rb. sphaeroides R-26		UQ_{10}/UQ_{10}	5.4	6.2	6.9	7.5	8.8	9.8	10.0	11.2
		UQ_{10}/terb.	5.4	6.0	6.9	7.3	8.4	9.1	9.5	10.0
		MD/terb.	5.4	6.0	6.9	7.3	–	–	–	–
Rb. capsulatus	WT	UQ/UQ_{10}	5.2	6.4	6.9	8.0	8.5	9.0	10.0	11.5
	WT	UQ_{10}/terb.	4.9	5.6	6.6	7.5	8.5	8.9	10.0	10.6
	AA+M43D	UQ_{10}/terb.	4.9	5.6	6.6	7.5	–	–	–	–

the concept of a network of amino acids around L212Glu, thus explaining the distributed (complex) interaction with Q_A^-.

The absence of large changes in the pH range between 5 and 8 (see Table 1) indicates that residues titrating in this range do not interact specifically with the quinones, i.e. the response is distributed among many possible distal residues, each of which displays a correspondingly small change in pK_a ('diffused proton uptake'). A similar conclusion can be drawn from salt titration experiments based on accessibility of protonatable residues to ionic screening in different pH regions [34]. As a consequence of the diffused (delocalized) interaction in the pH range 5–8, recent FTIR studies were unable to identify a specific carboxyl group whose protonation state changed at pH 7 in response to the formation of Q_A^- in the *Rhodopseudomonas viridis* RC (E. Nabedryk, J. Breton, unpublished results).

In summary, the cross-talk between the two quinone pockets via electrostatic interaction between Q_A^- and the network of protonatable amino acids around Q_B was tested in this study from both sides of the participants. The results described above show that the interaction between the two quinone sites was localized to residues with apparent pK_a values in the alkaline pH range and that this interaction could be canceled by appropriate modifications in either of the two quinone sites.

In conclusion, light-minus-dark difference pH-metry, in the form presented in this work, provides direct information on the number and pK_a values of protonatable groups involved in electrostatic interaction with both of the semiquinones. Its advantage is that it monitors the change in the derivative of the proton uptake rather than the change in proton binding. Combined with other techniques, it can shed some light on the complex pattern of interaction of the primary semiquinone and the cluster of residues around L212Glu.

Acknowledgements

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