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Proton Uptake of *Rhodobacter capsulatus* Reaction Center Mutants Modified in the Primary Quinone Environment^{††}

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ABSTRACT

Flash-induced absorbance spectroscopy was used to analyze the proton uptake and electron transfer properties of photosynthetic reaction centers (RC) of *Rhodobacter capsulatus* that have been genetically modified near the primary quinone electron acceptor (Q_A). M246Ala and M247Ala, which are symmetry-related to the positions of two acidic groups, L212Glu and L213Asp, in the secondary quinone electron acceptor (Q_B) protein environment, have been mutated to Glu and Asp, respectively. The pH dependence of the stoichiometry of proton uptake upon formation of the $P^+Q_A^-$ ($H^+/P^+Q_A^-$) and PQ_A^- (H^+/Q_A^-) (P is the primary electron donor, a noncovalently linked bacteriochlorophyll dimer) states have been measured in the M246Ala $\rightarrow$ Glu and the M247Ala $\rightarrow$ Asp mutant RC, in the M246Ala–M247Ala $\rightarrow$ Glu–Asp double mutant and in the wild type (WT). Our results show that the introduction of an acidic group (Glu or Asp) in the Q_A protein region induces notable additional proton uptake over a large pH region (~ 6 – 9), which reflects a delocalized response of the protein to the formation of Q_A^- . This may indicate the existence of a widely spread proton reservoir in the cytoplasmic region of the protein. Interestingly, the pH titration curves of the proton release caused by the formation of P^+ (H^+/P^+ : difference between $H^+/P^+Q_A^-$ and H^+/PQ_A^- curves) are nearly superimposable in the WT and the M246Ala $\rightarrow$ Glu mutant RC, but substantial additional proton release is detected between pH 7 and 9 in the M247Ala $\rightarrow$ Asp mutant RC. This effect can be accounted for by an increased proton re-

lease by the P^+ environment in the M247Ala $\rightarrow$ Asp mutant. The M247Ala $\rightarrow$ Asp mutation reveals the existence of an energetic and conformational coupling between donor and acceptor sides of the RC.

INTRODUCTION

Reaction center (RC) proteins of photosynthetic bacteria convert light energy into chemical free energy. This is achieved through a series of electron transfer reactions which lead, in about 200 ps, to a transmembrane charge separation between a dimer of bacteriochlorophyll molecules (primary electron donor [P]) situated on the periplasmic side of the membrane and the primary quinone electron acceptor (Q_A), at a distance of ~ 30 Å from P (1–4), bound in a relatively hydrophobic part of the cytoplasmic side of the protein. Q_A functions as a one-electron acceptor and is never protonated in its semiquinone state (Q_A^-) under physiological conditions. A secondary quinone electron acceptor, Q_B , is also found in the same region of the protein. Although chemically identical to Q_A (both ubiquinone₁₀) in *Rhodobacter sphaeroides* and *Rhodobacter capsulatus*, Q_B functions as a two-electron gate, which accepts two electrons after successive photochemical events and two protons from the cytoplasmic bulk solvent to form the Q_BH_2 species.

Whereas, in the past, a great deal of emphasis has been placed on probing the Q_B environment because it is thought to be more relevant to the functional proton transfer events, more recent data have shown that the formation of Q_A^- is a key step in initiating subsequent events in the protein complex (5–10). First, it has been shown recently that upon formation of Q_A^- , at neutral pH, some residues present in the Q_B environment and interacting with Q_A^- shift their pK_a s, leading to partial proton uptake that favors the $Q_A^- \rightarrow Q_B$ electron transfer process (11). In addition, L212Glu (or alternatively, its cluster of acidic groups), which is at a distance of 5 Å from Q_B , was shown to be directly involved in the same process at high pH (6,12,13). It is also of interest that the kinetics of proton uptake as a result of the formation of Q_A^- has been suggested to reflect a gated process triggered by protein rearrangement events (5). This is reflected at the level of the $Q_A^- \rightarrow Q_B$ electron transfer rate (7,14,15). Lastly, correlations between the arrangement of the Q_B pock-

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Abbreviations: CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; $H^+/P^+Q_A^-$, proton uptake stoichiometry induced by the $P^+Q_A^-$ state; H^+/Q_A^- , proton uptake stoichiometry induced by the Q_A^- state; MES, 2-(*N*-morpholino)ethanesulfonic acid; P , primary electron donor, a noncovalently linked bacteriochlorophyll dimer; Q_A and Q_B , primary and secondary quinone, respectively; RC, reaction center; UQ_6 , ubiquinone-6; WT, wild type.

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et (empty or occupied, presence of inhibitor, nearby mutations, *etc.*) and the physicochemical and spectroscopic properties of Q_A have also been reported (16–21). Whereas this interdependence between the phenomena occurring at Q_A and Q_B has been demonstrated in these works, the possibility of similar linkage between P and Q_A has also been recently suggested in the native RC (22).

In a similar way, as the formation of either semiquinone state leads to proton uptake by the protein, it was reported that, conversely, the formation of P^+ induces proton release (23–25). However, the way in which these protons are released is not clearly established. A pure electrostatic effect could account for a downward shift of the pK_a s of neighboring groups, therefore releasing protons. However, the hypothesis of a light-activated conformational change leading to the modification of the water content of the protein in the periplasmic region or to a different exposure of ionizable groups to the solvent (or both) has also been proposed (25).

In the present study, we have introduced acidic residues at positions M246 and M247 near Q_A and have examined their effects on the function of the Q_A site. The alanine residues at these two sites in the wild type (WT) RC are symmetry-related to L212Glu and L213Asp, respectively, in the Q_B binding pocket (26,27). We have examined RC derived from the photosynthetically incompetent M246Ala–M247Ala → Glu–Asp double mutant and from two photo-competent phenotypic revertants of this strain; the revertants carry either the M246Ala → Glu or M247Ala → Asp single mutations.

We present for the first time, data on the pH dependencies of the stoichiometries of proton uptake by the PQ_A^- and $P^+Q_A^-$ states in RC mutants modified in the Q_A protein pocket. Our data show that the introduction of ionizable residues in the Q_A region substantially modifies the interactions of Q_A^- with the protein groups over a wide pH range, and that the response of the protein is probably not localized. Furthermore, our results suggest that the protein environment of P^+ and Q_A^- mutually interact over a distance of nearly 30 Å.

EXPERIMENTAL PROCEDURES

Bacterial strains and cell culture. Construction of the M246Ala–M247Ala → Glu–Asp double mutant and isolation and genotypic characterization of the phenotypic revertants have been described previously (28,29) (Fig. 1). Cells were grown semiaerobically in the dark on RPYE medium (27) containing 30 $\mu\text{g mL}^{-1}$ kanamycin to select the plasmid. RC were isolated as previously described (30).

Charge recombination kinetics. The kinetics of $P^+Q_B^- \rightarrow PQ_B$ and $P^+Q_A^- \rightarrow PQ_A$ charge recombinations were measured at 430 nm with a home-made spectrophotometer (31). The absorbance changes were triggered by saturating flashes from a Q-switch YAG laser at 532 nm.

Electron transfer kinetics. The kinetics of the first electron transfer from Q_A^- to Q_B were measured at 750 nm in the electrochromic bandshift of the bacteriopheophytins. These experiments were done in the presence of 75 μM ubiquinone-6 (UQ_6) and $\sim 1 \mu\text{M}$ RC.

The kinetics of transfer of the second electron from Q_A^- to Q_B^- were measured at 450 nm in the presence of reduced cytochrome *c* (20 μM), 75 μM UQ_6 and $\sim 2 \mu\text{M}$ RC.

pH titrations. For the different pH titrations, buffers (10 mM) used were 2-(*N*-morpholino)ethanesulfonic acid (MES; Sigma Chemical Co., St Louis, MO) between pH 5.5 and 6.5; 1,3-bis[tris(hydroxymethyl)methylamino]propane (Sigma) between pH 6.3 and 9.5; and 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS; Calbiochem) above pH 9.5.

Proton uptake. Proton uptake was measured by using a glass pH

Figure 1. The relative positions of P, Q_A , Q_B and residues M248Glu, M249Asp, L212Glu and L213Asp are shown in the RC structure from *R. sphaeroides* (4) (PDB code: 1AIG) (equivalent residue numbers in *R. capsulatus* are M246 and M247, respectively). The distance between the midpoint of the dimer and the nonheme iron atom is indicated. The M248Ala → Glu and M249Ala → Asp mutated residues are located in a helical region; their side chains were modeled by using the program Turbo-Frodo. The modeled positions of the mutated residues are given in the absence of a major conformational change.

electrode and pH-sensitive dyes (at 585 nm, at the isosbestic point for P^+/P absorbance changes). The conditions were as follows: 2 μM RC, 50 mM NaCl, 0.03% Triton X-100, 200 μM ferrocene, 100 μM terbutryn and 20 μM dye (bromocresol purple, phenol red, cresol red or chlorophenol red, depending on the pH). The H^+/Q_A^- proton uptake stoichiometries were measured in the presence of 100 μM ferrocene to reduce P^+ . In all measurements, the buffer concentrations were kept below 10 μM by extensive dialysis (36 h at 4°C). In order to avoid any electrochromic dye effect, the buffered (~ 10 mM MES, MOPS, TRIS or CAPS) traces were systematically subtracted from the unbuffered traces.

The data points presented in Figs. 4 and 5 for the H^+/Q_A^- and $H^+/P^+Q_A^-$ proton uptake represent the average of several (up to 12) measurements performed with different RC preparations.

RESULTS

pH dependence of $-\Delta G_{AB}^\circ$, the free energy barrier between Q_A^- and Q_B^-

The pH dependencies of the $-\Delta G_{AB}^\circ$ values in WT RC (reproduced from [32]) and in RC of the M246Ala → Glu, M247Ala → Asp and M246Ala–M247Ala → Glu–Asp mutants are presented in Fig. 2. The values of $-\Delta G_{AB}^\circ$ at each pH were calculated from the rate constants of the $P^+Q_A^-$ (k_{AP}) and $P^+Q_B^-$ (k_{BP}) charge recombination reactions for each type of RC. No more than 30% variability was observed in the k_{AP} rate constant values between the various RC. To calculate the $-\Delta G_{AB}^\circ$ values, we used the formula $-\Delta G_{AB}^\circ = -kT \ln(K_2)$, where k represents the Boltzmann constant, T the absolute temperature and K_2 the one-electron equilibrium constant between the $P^+Q_A^-$ and $P^+Q_B^-$ free energy states. K_2 is derived from the formula initially suggested (33)

Figure 2. pH dependence of the free energy gap, $-\Delta G_{AB}^\circ$, between the Q_A^- and Q_B^- states in the RC ($\sim 1 \mu M$) of M246Ala $\rightarrow$ Glu ($\circ$), M247Ala $\rightarrow$ Asp ($\square$) and WT ($\bullet$) (30). Data points from the M246Ala–M247Ala $\rightarrow$ Glu–Asp ($\blacksquare$) double mutant strictly refer to the right axis. The very slow $P^+Q_B^- \rightarrow PQ_B$ decay observed in this mutant suggests a direct route (not through Q_A) for the charge recombination process. The $-\Delta G_{AB}^\circ$ values are deduced from the rates of the $P^+Q_A^-$ and $P^+Q_B^-$ charge recombination processes. Conditions: 430 nm, 0.05% LDAO (pH 7–11) or 0.03% Triton X-100 (pH 5–8), 75 mM UQ₆, 21°C. Buffers (10 mM), see text.

$$1 + K_2 = k_{AP}/k_{BP} \quad (1)$$

The pH dependence of $-\Delta G_{AB}^\circ$ for the M246Ala $\rightarrow$ Glu mutant RC ($\circ$ in Fig. 2). Below pH ~ 6 , $-\Delta G_{AB}^\circ$ values display a net decrease from about -65 meV at pH ~ 6.0 to about -75 meV at pH 4.6. Interestingly, above pH 6.0, $-\Delta G_{AB}^\circ$ is no longer pH-dependent and remains constant with a value of about -65 meV, up to pH 10.8. At pH 7.8, k_{BP} equals ~ 0.70 s $^{-1}$.

The pH dependence of $-\Delta G_{AB}^\circ$ for the M247Ala $\rightarrow$ Asp mutant RC ($\square$ in Fig. 2). A plateau is observed between pH 7 and 9 with a value of about -80 meV. This value is slightly more negative than that of the M246Ala $\rightarrow$ Glu mutant RC. Below pH 7, $-\Delta G_{AB}^\circ$ smoothly titrates to reach a value of -90 meV at pH 5.9. Above pH 9, an increase of $-\Delta G_{AB}^\circ$ is observed, reaching a value of -70 meV at pH 9.5. Because of the instability of the RC from the M247Ala $\rightarrow$ Asp mutant and the low occupancy of the Q_B site at very high pH, it was difficult to collect data above this pH.

The M246Ala–M247Ala $\rightarrow$ Glu–Asp double mutant RC. In this case we have displayed, in Fig. 2, the pH dependence of the $k_{AP}/k_{BP} - 1$ term and not that of $-\Delta G_{AB}^\circ$. This should be done because the small values of k_{BP} measured in this mutant suggest that an important part of the $P^+Q_B^-$ charge recombination process may occur through a direct pathway between P and Q_B instead of the indirect route *via* repopulation of the $P^+Q_A^-$ state (34). Indeed, the negative (or partly negative) charges carried by the mutations present in the Q_A environment in the double mutant destabilized the Q_A^- state. In other words, the midpoint redox potential of the Q_A^-/Q_A couple is very likely to be more negative in the mutant than in the WT. As a consequence, $-\Delta G_{AB}^\circ$ notably increases in the mutant, leading to a substantial decrease of k_{BP} . Indeed, the observed k_{BP} value is close to the value previously determined for the rate of the direct pathway (34). At pH 8, $k_{BP} \sim 0.14$ s $^{-1}$ ($k_{AP}/k_{BP} - 1 = \sim 55$). In the pH range 8–10, the $(k_{AP}/k_{BP} - 1)$ term is roughly constant. A sharp and continuous decrease is detected below pH 8. At pH 7, this factor is about 130. At this pH, the $-\Delta G_{AB}^\circ$ value in this mutant

Table 1. Energetic and kinetic parameters of electron transfer in the WT and the RC mutants at pH 7.8

Strain	k_{BP} (s $^{-1}$) ($\pm 10\%$)	$k_{AB}(1)$ (s $^{-1}$) ($\pm 10\%$)	$k_{AB}(2)$ (s $^{-1}$) ($\pm 10\%$)
WT	0.90	15 400	1900
M246Ala $\rightarrow$ Glu	0.70	12 500	2150
M247Ala $\rightarrow$ Asp	0.31	7 400	2000
M246Ala/M247Ala $\rightarrow$ Glu/Asp	0.11	6 250	1400

must, therefore, be greater than 120 meV, *i.e.* more than 60 meV greater than that measured in WT RC.

Rate of transfer of the first electron in mutant RC

The rate of the first electron transfer reaction from Q_A^- to Q_B was measured for the RC mutants, at pH 7.8 and 750 nm, which monitors the electrochromic bandshift of both bacteriopheophytins. We previously measured this rate in RC of WT *R. capsulatus* and found it to be $(65 \mu s)^{-1}$ (30). In mutant RC carrying the M246Ala $\rightarrow$ Glu, M247Ala $\rightarrow$ Asp or M246Ala–M247Ala $\rightarrow$ Glu–Asp mutations, we measured, respectively, $(80 \pm 10 \mu s)^{-1}$, $(135 \pm 20 \mu s)^{-1}$ and $(160 \pm 20 \mu s)^{-1}$ (Table 1). The observed modification of the $-\Delta G_{AB}^\circ$ values in the mutant RC is, therefore, not accompanied by significant changes of the first electron transfer rate. The first electron transfer process from Q_A^- to Q_B has been suggested to be kinetically limited by protonation or conformational rearrangements (or both) of the protein (35). Therefore, one does not expect $k_{AB}(1)$ to sharply vary (as in a Marcus-type plot) as a function of $-\Delta G_{AB}^\circ$. This explains the weak $k_{AB}(1)$ variations observed here between the WT and the various mutants.

pH dependence of the second electron transfer rate, $k_{AB}(2)$, in the mutant RC

The pH dependence of the rate for the second electron transfer reaction in the mutant RC is shown in Fig. 3, together with that previously reported for the WT (32). No major

Figure 3. pH dependence of the rates of the second electron transfer from Q_A^- to Q_B^- (450 nm) in the RC ($\sim 2 \mu M$) for M246Ala $\rightarrow$ Glu ($\circ$), M247Ala $\rightarrow$ Asp ($\square$) and M246Ala–M247Ala $\rightarrow$ Glu–Asp ($\blacksquare$) mutants. The data points from the WT RC ($\bullet$) were taken from (32). Conditions: 0.05% LDAO or 0.03% Triton X-100 buffers as indicated in the text. 20 μM cytochrome *c*, 200 μM sodium ascorbate, 75 μM UQ₆. Buffers (10 mM), see text.

Figure 4. pH dependence of the stoichiometries of proton uptake by the PQ_A^- state in RC of M246Ala $\rightarrow$ Glu ($\circ$), M247Ala $\rightarrow$ Asp ($\square$) and M246Ala-M247Ala $\rightarrow$ Glu-Asp ($\blacksquare$) mutants. The previously measured WT data points ($\bullet$) (36) are also shown for comparison. The lines represent the fittings of the stoichiometry of proton uptake using Eq. 2, with the assumption of four groups with apparent pK_a . Conditions: $\sim 2 \mu M$ RC, 0.03% Triton X-100, 100 μM ferrocene, 50–75 μM UQ₆, 50 mM NaCl, 100 μM terbutryn and 40 μM dye (bromocresol purple, phenol red, cresol red or chlorophenol red, depending on the pH). Buffers ($< 10 \mu M$), see text.

changes are observed—the values of $k_{AB}(2)$ obtained for RC of the M246Ala $\rightarrow$ Glu and M247Ala $\rightarrow$ Asp mutants are almost superimposable with those of the WT between pH 6 and 8. The rate is the same for all at pH 7. However, the slopes of the pH dependence for both the M246Ala $\rightarrow$ Glu and M247Ala $\rightarrow$ Asp mutant RC are slightly smaller than the slopes for the WT RC. As a result, slightly higher values of $k_{AB}(2)$ are observed for the single mutants at high pH, *i.e.* above the break point for the WT RC. In the case of the M246Ala-M247Ala $\rightarrow$ Glu-Asp double mutant, the steeper slope indicates that $k_{AB}(2)$ values in this mutant are nearly two times smaller than in the WT or single mutant RC (Table 2).

pH dependence of H^+/Q_A^- proton uptake

The pH dependencies of the H^+/Q_A^- (PQ_A^- state) proton uptake stoichiometries, as measured for the WT RC (previously published in [36]) and for the mutant RC are displayed in Fig. 4. These data have been obtained in the presence of terbutryn (inhibitor of the $Q_A^- \rightarrow Q_B$ electron transfer process) and ferrocene (electron donor to P^+). In each case, the lines drawn through the data points represent a fit, assuming that the net proton uptake is the sum of proton binding by independent ionizable residues, represented by four apparent pK_a s. Assuming that an ionizable group, i , interacting with Q_A^- , undergoes a pK_a shift from in the Q_A state to upon Q_A^- formation, the net proton binding may be expressed as

$$H^+/Q_A^- = \sum_i \left(\frac{1}{1 + 10^{[pH - pK_{a,Q_A^-}]}} - \frac{1}{1 + 10^{[pH - pK_{a,Q_A}]} } \right) \quad (2)$$

This is the simplest model that fits the data. However, because we are aware of the limitation of this model, in the following discussion we only considered, for comparison, the highest pH pK_a group, called $group_{high}$. In each case (the WT and the two single mutants), we called pK_{highQ_A} and $pK_{highQ_A^-}$ the respective values of this pK_a in the Q_A and Q_A^- forms, and $\Delta pK_{high} = pK_{highQ_A^-} - pK_{highQ_A}$.

The M246Ala $\rightarrow$ Glu and M247Ala $\rightarrow$ Asp mutants. As

Figure 5. pH dependence of the stoichiometries of proton uptake by the $P^+Q_A^-$ state in RC of M246Ala $\rightarrow$ Glu ($\circ$), M247Ala $\rightarrow$ Asp ($\square$) and WT ($\bullet$). The lines represent the fittings of the stoichiometry of proton uptake using Eq. 2, with the assumption of four groups with apparent pK_a . Conditions: same as those in Fig. 4, but no ferrocene. Buffers ($< 10 \mu M$), see text.

shown in Fig. 4, in a wide pH region, the H^+/Q_A^- proton uptakes for the M246Ala $\rightarrow$ Glu and the M247Ala $\rightarrow$ Asp RC mutants are notably larger than in the WT. In the pH range 6–8, about 0.35–0.40 and 0.65–0.45 H^+ are respectively taken up in both mutants, (~ 0.20 – $0.25 H^+$ in the WT). Because of the low occupancy of the Q_A pocket (in the Q_A^- state) above pH 9.7, in RC of these mutants, the intrinsic H^+/Q_A^- uptake measurements become more difficult. Therefore, to reliably achieve these high pH values, we repeated them five times more than the measurements done at neutral pH. At high pH, $group_{high}$ —whose apparent pK_a s in the WT are ~ 10.20 (pK_{highQ_A}) and ~ 10.60 ($pK_{highQ_A^-}$)—is also present in the two single mutants with similar values for ΔpK_{high} (~ 0.3 – 0.4) as in the WT RC.

The M246Ala-M247Ala $\rightarrow$ Glu-Asp double mutant. Because of the low quinone occupancy at high pH and the instability of this mutant RC below pH 7.5, we could measure the H^+/Q_A^- proton uptake only in the pH range 7.5–8.8. We found proton uptake values as high as 0.65–0.72 H^+ . It is of interest that the difference in the stoichiometry of proton binding between this mutant RC and the WT measured at the same pH (*e.g.* at pH 8, $0.70 - 0.18 = 0.52 H^+/Q_A^-$) roughly represents the sum of the additional H^+/Q_A^- uptake observed in the M246Ala $\rightarrow$ Glu (~ 0.17) and in the M247Ala $\rightarrow$ Asp mutant (~ 0.30) compared to the WT.

pH dependence of the $H^+/P^+Q_A^-$ proton uptake

The pH dependencies of the $H^+/P^+Q_A^-$ ($P^+Q_A^-$ state) proton uptake stoichiometries, measured in the presence of terbutryn but in the absence of ferrocene, for RC of the WT, the M246Ala $\rightarrow$ Glu mutant and the M247Ala $\rightarrow$ Asp mutant are displayed in Fig. 5. The data were fitted in a similar way as for the H^+/Q_A^- data points (see earlier).

The wild type. The $H^+/P^+Q_A^-$ stoichiometry shown here for the native *R. capsulatus* RC has not been reported before. The profile of the curve is very similar to that previously shown for the RC from *R. sphaeroides* (23,24). The maximum amplitude of proton uptake measured here near pH 10, $\sim 0.27 H^+$, is lower than that of *R. sphaeroides* RC ($\sim 0.4 H^+$) at pH 10. The titration of $H^+/P^+Q_A^-$ leads to a similar behavior at high pH as was shown previously for H^+/Q_A^-

Figure 6. H^+/P^+ apparent proton release (shown in positive) obtained by subtracting the H^+/PQ_A^- curves of Fig. 5 from the $H^+/P^+Q_A^-$ curves of Fig. 4. The error bars reflect the respective error bars of each set of measurements. (○), M246Ala → Glu mutant; (□), M247Ala → Asp mutant; and (●), WT.

with, however, a notable decrease of about 0.5 pH units of both pK_{highQA^-} and pK_{highQA^-} but with a comparable value for ΔpK_{high} (~ 0.43). The $H^+/P^+Q_A^-$ titration below pH 9 shows a continuous decrease. At pH 6, almost no net protons are taken up by the $P^+Q_A^-$ state.

The M246Ala → Glu and M247Ala → Asp mutants. Similarly to the WT, RC of the M246Ala → Glu and the M247Ala → Asp mutants display similar patterns of H^+/Q_A^- and $H^+/P^+Q_A^-$ proton uptake at high pH. Indeed, compared to the H^+/Q_A^- curves, no changes of the ΔpK_{high} (~ 0.40) occur in the presence of P^+ as well as for the pK_{highQA^-} and pK_{highQA^-} values themselves. Below pH 9.2, the overall amount of proton uptake is notably lower than in the Q_A^- state. Compared to the WT, both mutant RC take up more protons. Consistent with the observation for the H^+/Q_A^- curves, the M247Ala → Asp mutant takes even more than the M246Ala → Glu mutant in the pH range 6.5–9.0.

Proton release by P^+ in native and mutant RC

The difference between the $H^+/P^+Q_A^-$ and H^+/Q_A^- proton uptake curves measured for the RC from each of the three strains affords the contribution resulting from proton release following the formation of the P^+ state. The difference curves are presented in Fig. 6 for the WT, the M246Ala → Glu mutant and the M247Ala → Asp mutant. As previously reported for *R. sphaeroides* RC (23,24), the proton release caused by P^+ formation is nearly zero above pH 9, and it continuously increases at lower pH to reach a value of about 0.3 H^+ released per P^+ at pH 6.5. Our present data obtained for *R. capsulatus* RC agree with these figures, although the absolute value of H^+/P^+ at pH 6.5 is lowered to ~ 0.22 .

The attribution of these curves to the pH dependence of the H^+/P^+ proton release implicitly assumes that protonation events occurring upon P^+ formation do not depend on the redox state of Q_A . Likewise, it should also be assumed that proton uptake induced by Q_A^- formation is the same in the P and P^+ states. Yet, our data show that this is not the case. The patterns observed for the H^+/P^+ proton release are nearly the same for RC of the WT and the M246Ala → Glu mutant. At variance, substantial additional proton release is detected in the M247Ala → Asp mutant compared to the RC

of the WT and the M246Ala → Glu mutant. This effect is dominant around pH 8.5.

DISCUSSION

The electrostatic and functional connections between the protein environments of the two quinone species have previously been highlighted (11,16,18,21,33,37–40). The idea that the formation of the Q_A^- species might be an important step for the function of the RC complex was initially suggested on the basis of experiments performed with RC isolated from *R. capsulatus* (10) and with *R. sphaeroides* RC incorporated in lipid monolayers (8). These studies attributed the functional connection between Q_A^- and the secondary quinone site to residue L212Glu or to an acidic cluster in which L212Glu participates (or to both). In addition, proton uptake measurements attributed the electrostatic response of the protein to the formation of Q_A^- to groups localized in the Q_B environment or to groups involved in the network connecting Q_A^- and Q_B^- (or to both) (5,11,37). Because all these studies pointed to the predominant role of L212Glu—possibly in conjunction with its participation in a cluster of strongly interacting acidic residues including L213Asp and L210Asp—we were prompted to study how, conversely, the introduction of ionizable residues at the analogous positions in the Q_A pocket would be experienced by the protein.

Energetic effects of the introduction of Glu or Asp (or both) in position M246 or M247 (or both). The measure of the pH dependence for proton uptake resulting from the formation of the Q_A^- or Q_B^- state allows us to probe the specific energetic effects of acidic residues on nearby or distant protein environments. As compared to the WT, in RC of the M246Ala → Glu and M247Ala → Asp mutants, the main trend is a notable increase of the H^+/Q_A^- and $H^+/P^+Q_A^-$ proton uptake values over quite extended pH regions (5.8–10.5 and 6.5–10.2, respectively)(see Fig. 2).

Most of the literature for proton uptake comes from measurements made with WT RC following the light-induced formation of the Q_A^- or Q_B^- states. Some data have been reported for RC from mutants of *R. capsulatus* (6,41) and *R. sphaeroides* (13,42) that lack L212Glu. In all these studies, the absence of L212Glu resulted in the drop of proton uptake above pH 8 in the Q_A^- as well as the Q_B^- states; neither pattern of proton uptake was affected at neutral and low pH. However, infrared spectroscopy experiments, which are more specific to follow the ionization state of a particular carboxylic group, have rather suggested that L212Glu is partly ionized at neutral pH, and that the ionization of L212Glu takes place over a wide pH range (43). In strong support of this hypothesis, electrostatic calculations have shown that the titration of carboxylic groups, especially within clusters of interacting acidic residues, is necessarily extended over very wide pH ranges (38–40,44–48). In other words, these calculations have shown that the titrations of these groups do not follow classical Handerson–Hasselbach curves, but rather that a particular group may statistically carry part of a proton over several pH units.

It is likely that M246Glu and M247Asp have similar behavior in the Q_A pocket. This is probably why the increase of H^+/Q_A^- and $H^+/P^+Q_A^-$ values in the M246Ala → Glu and

M247Ala → Asp single mutants extends over the whole pH range measured here.

The small but constantly higher $-\Delta G_{AB}^\circ$ value (~ 20 meV) measured in the M247Ala → Asp mutant (Fig. 2) as compared to the WT suggests that M247Asp is partly ionized over the whole pH range measured here. A full ionization of M247Asp and a distance of about 6 Å between Q_A^- and the carboxyl group of M247Asp would be consistent with a dielectric constant value higher than 80. This obviously too high value strongly suggests that the amount of ionization of M247Asp is very low.

On the other hand, in the M246Ala → Glu mutant, Glu is likely to be protonated below pH 6 and more and more deprotonated at higher pH. As shown in Fig. 2, $-\Delta G_{AB}^\circ$ in the M246Ala → Glu RC is pH-independent from pH ~ 6.0 to ~ 10.8 . In this mutant RC, $-\Delta G_{AB}^\circ$ is about the same as that of the WT at pH 6. However, the smooth increase of $-\Delta G_{AB}^\circ$ observed in the WT up to pH 9.7 and its strong increase above this pH value are absent in this mutant. This observation can be explained if M246Glu has a similar pH titration as the acidic cluster (L210Asp–L212Glu–L213Asp) in the Q_B^- pocket. Indeed, the electrostatic effect of M246Glu must be to increase the free energy level of the Q_A^- state to a similar extent and pH dependence as the above cluster does on Q_B^- . Therefore, the combination of both effects compensate and lead to the lack of a pH dependence for $-\Delta G_{AB}^\circ$ at high pH. In support of this effect (ionization of M246Glu and increase of the Q_A^- free energy state), we have noticed that the occupancy of the Q_A site continuously decreases with pH in this mutant, with a dependence that roughly matches that of the ratio between the $-\Delta G_{AB}^\circ$ curves of RC of the WT and of the M246Ala → Glu mutant (data not shown). The increasing charge on M246Glu as pH increases is likely to progressively destabilize the Q_A^- state and may concomitantly displace Q_A^- from its site. It is of interest to associate this effect to recent calculations, which have suggested that the proximal position of Q_B (in the Q_B^- state) in the WT RC more easily accommodates L212Glu that is fully protonated (49). Indeed, M246Glu is situated at the symmetry-related position of L212Glu. The relative position of Q_A with regard to M246Glu is similar to Q_B in its proximal position relative to L212Glu. It is therefore interesting that Q_A^- is less stable (progressive unbinding upon increasing pH) as M246Glu deprotonates at high pH similar to Q_B^- when L212Glu gets ionized at similar pH values. This underlines the structural–functional analogy between events occurring at both quinone sites.

Proton uptake data (Figs. 4 and 5) show the same amplitudes for the M246Ala → Glu mutant and the WT RC at pH 10. This may seem to contradict the statement that M246Glu partly titrates at high pH. Indeed, in that case, higher H^+/Q_A^- and $H^+/P^+Q_A^-$ values would have been expected at high pH in the mutant compared to the WT. It is in fact possible that the charge that exists on M246Glu at high pH (and detected in the charge recombination data) is buffered by an internal pool of protons, which, therefore, is not associated, at the same pH, with concomitant proton uptake from outside the protein and detected by the dyes. This available internal reservoir of protons may be delocalized over the Q_A – Q_B region. The high pH region (above pH 8.0) may be a signature of this proton pool, which may be common for

the Q_A and Q_B regions of the protein. Such a reservoir, whose role could be to buffer the total response of the protein, may involve mobile and structured networks or clusters of water molecules, as we have recently suggested (50). This highlights the likely functional connections between the two quinone sites of the RC protein, as suggested previously (11,16,18,21,38,40).

Increase of the P^+ proton release in the M247Ala → Asp. The observed proton uptake is influenced by charges that are present in the RC molecule, which include P^+ , Q_A^- and the ionized residues of the complex. As shown in Fig. 6, the pH-dependence curves for the apparent proton release (H^+/P^+) because of the formation of P^+ are nearly superimposable for the WT and the M246Ala → Glu mutant RC but differ significantly from that of the M247Ala → Asp mutant RC. This result shows that the apparent proton release by P^+ , derived by a simple subtraction of the H^+/PQ_A^- proton uptake curve from the $H^+/P^+Q_A^-$ curve, is affected by the M247Ala → Asp Q_A -site mutation. This observation that the difference between the two curves is not a constant—that it is dependent on mutation—suggests the existence of an interaction in the native RC between P^+ and Q_A^- and the protein environment surrounding them. The M247Ala → Asp mutation reveals this interaction in the pH range that we monitor.

Should the interaction producing the observed effect, *i.e.* increased proton release by the P environment, in the M247Ala → Asp mutant be electrostatic, it would be higher than each two pH units (in ΔpK units) in order to reveal groups that are not observed in the WT or in the M246Ala → Glu mutant. This is quite unlikely. We rather favor the hypothesis that the additional proton release observed in M247Ala → Asp mutant arises from a conformational modification of the protein, triggered by the P^+ formation and leading to a change in the exposure to the bulk phase of protonatable groups or internal water molecules (or both) in the P^+ surrounding. This has been reported for the WT (25) and could be amplified in the M247Ala → Asp mutant. This is consistent with recent work carried out with chromatophores of *R. capsulatus*, where notable interactions between the donor and acceptor sides of the RC complex have been demonstrated to exist and lead to conformational destabilization of the $P^+Q_A^-$ state (22).

CONCLUSIONS

The more we know about the functional behavior of the RC protein, the more we are forced to interpret our experimental and theoretical data with the concept that static and dynamic interactions are delocalized over extended regions of the protein. The example given here of the “dialogue” between P^+ and Q_A^- through the protein milieu reflects the fact that the protein is a complex unit. One main challenge in the future will be to understand the observed or calculated phenomena in the whole protein within the frame of their spatial and temporal interdependence.

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