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Rapid Report

In bacterial reaction centers protons can diffuse to the secondary quinone by alternative pathways

Deborah K. Hanson [a], Laura Baciou [c], David M. Tiede [b], Sharron L. Nance [a], Marianne Schiffer [a] and Pierre Sebban [c]

[a] *Biological and Medical Research Division Argonne National Laboratory, Argonne, IL (USA),* [b] *Chemistry Division Argonne National Laboratory Argonne, IL (USA) and* [c] *UPR 407, CNRS, Gif sur Yvette (France)*

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The mechanisms of proton conduction to the reduced secondary quinone in bacterial reaction centers were studied in wild-type and genetically modified reaction centers from *Rhodobacter capsulatus*. In the L212–213AA double mutant (L212Glu → Ala, L213Asp → Ala), reaction center function is severely altered. However, a photocompetent revertant of this strain which carries a third 'compensating' mutation, M231Arg → Leu, at about 15 Å from the secondary quinone, displays the normal proton binding function of the reaction center. Furthermore, the apparent pK values of group(s) involved in the stabilization of the semiquinone anion are restored by that mutation. We conclude that L212Glu and L213Asp are not obligatory residues for proton donation to Q_B in *Rb. capsulatus*. We suggest that protons can be delivered to the Q_B site from the cytoplasm via a network of proton channels activated by compensatory mutations, possibly involving water molecules bound in the interior of the reaction center.

In purple photosynthetic bacteria, light excitation energy is converted into electrochemical free energy in the reaction center (RC) protein. The first product of the RC that is stable for tens of milliseconds is a photooxidized bacteriochlorophyll dimer, P^+, and a reduced quinone in the primary site (Q_A^-). However, in less than a millisecond, before the $P^+Q_A^-$ state can decay, Q_A transfers an electron to a secondary quinone, Q_B. The Q_A and Q_B sites are located near the cytoplasmic side of the membrane but are shielded from the external aqueous medium in part by the L and M polypeptides, and also the H polypeptide which caps the sites and extends into the aqueous medium. The redox catalysis at the Q_B site is distinct from that observed at the Q_A site in that Q_B can accept electrons from Q_A in sequence, forming first transiently Q_B^-, and then Q_B^{2-}, which requires the obligatory binding of two protons to form the final product Q_BH_2. The quinol molecule can then leave the site, enter the quinone pool, and be replaced by a quinone to allow the process to occur again. One of the principal questions regarding redox catalysis at the Q_B site is how protons are translocated from the aqueous medium through the protein to the Q_B site to form Q_BH_2.

Correspondence to: P. Sebban, UPR 407, Bat. 24, CNRS, Gif sur Yvette 91198, France.

Site-specific mutagenesis of RCs has been used in an attempt to elucidate this mechanism. Results suggest that L213Asp and L212Glu may be important in donating protons to reduced Q_B in wild-type *Rhodobacter sphaeroides* RCs [1–3]. It was shown that when L212Glu and L213Asp were changed, either singly or together to the nonprotonable amino acids Gln and Asn, respectively, proton binding was severely impeded [1–3]. From their results, Takahashi and Wraight [3] for the L212Glu-L213Asp → Gln-Asn double mutant of *Rb. sphaeroides*, and Paddock et al. [1] for the L212Glu → Gln mutant of *Rb. sphaeroides* have suggested an obligatory role for L213Asp and L212Glu in electron and proton transfer to Q_B. In the present work, we have characterized a photosynthetically incompetent (PS^-) double mutant, L212Glu-L213Asp → Ala-Ala, of *Rhodobacter capsulatus* and a photocompetent (PS^+) revertant of this strain that carries the L212Ala-L213Ala mutations as well as a compensatory mutation, M231Arg → Leu, far from Q_B. Therefore, our data on the revertant strain suggest that L212Glu and L213Asp are not obligatory intermediates in the pathways for proton donation to Q_B.

The system of plasmids described by Bylina et al. [4,5] was used for the construction of the double site-specific mutant L212Glu-L213Asp → Ala-Ala. For the strains discussed here, *Rb. capsulatus* deletion strain U43 served as the host [6]. U43 complemented *in trans*

by plasmid pU2922 [5] is defined as the 'wild-type'. The construction and initial characterization of the PS^- double mutant strain (U43[pR212–213AA]) and the isolation and characterization of the PS^+ revertant strain (U43[pLL7]) will be reported elsewhere (Hanson, D.K., Tiede, D.M., Nance, S.L. and Schiffer, M., unpublished observations). Mapping of the second-site plasmid-borne suppressor mutation was performed as described previously [7]. All strains used for RC preparations were propagated under chemoheterotrophic (semi-aerobic, dark) conditions on either MPYE [8], RCV [9] or 'RPYE' medium (2/3 RCV, 1/3 MPYE). Double-stranded DNA sequencing was performed according to directions supplied with a kit (Sequenase, United States Biochemical Corporation).

RCs were prepared essentially as previously described [10,11] except that the concentration of lauryldimethylamine oxide (LDAO) used to solubilize the chromatophore membrane was 1%. The absorbance changes related to the $P^+Q_B^-$ decay kinetics were followed at 865 nm on a homemade spectrophotometer. In order to calculate the $Q_A^-Q_B \leftrightarrow Q_AQ_B^-$ equilibrium constant values, the pH dependences of the rate constants of $P^+Q_A^-$ charge recombination decays (k_{AP}) were also measured at 865 nm.

Cytochrome oxidation in a series of flashes was detected at 550 nm in the presence of 40 μM cytochrome c_2 and 200 μM sodium ascorbate. Semiquinone oscillations were detected at 450 nm in the presence of 500 μM diaminodaurene (DAD) and 1 mM sodium ascorbate.

Depending on the pH range, the pH buffers were Mes (2-[*N*-morpholino]ethanesulfonic acid), Bistris-propane (1,3-bis[tris(hydroxymethyl)methylamino]propane) or CAPS (3-cyclohexylamino-1-propanesulfonic acid).

Although the amino-acid substitutions were modeled by using the *Rb. sphaeroides* structure, numbering of the residues corresponds to the *Rb. capsulatus* sequence [12].

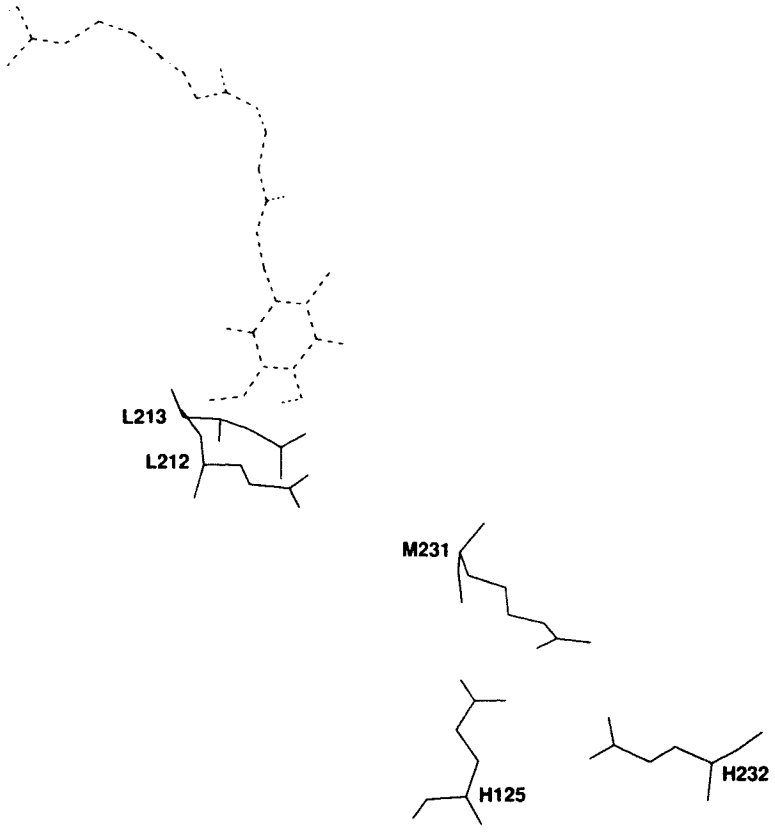

Fig. 1. Molecular model, using the *Rb. sphaeroides* structure [13], showing the interactions between the amino acids described in the text. The wild-type version is pictured (L212Glu-L213Asp-M231Arg). The quinone molecule is represented by dashed lines; numbers refer to the *Rb. capsulatus* sequence [12]. In the photosynthetically incompetent double mutant strain, L212Glu and L213Asp are replaced by alanines. In the revertant strain, L212Ala and L213Ala are still present and a distant amino-acid substitution, M231Arg → Leu, disrupts the salt bridges formed between M231Arg, H125Glu and H232Glu. This substitution acts as an intergenic suppressor of the double mutation in the Q_B site, restoring the photosynthetic phenotype.

Genetic mapping and DNA sequencing has confirmed that the revertant strain described here (U43[pLL7]) actually carries both of the original site-specific mutations (L212Ala-L213Ala) as well as a second-site suppressor mutation at M231 (Arg → Leu) that compensates for the loss of L212Glu and L213Asp (Hanson, D.K., Tiede, D.M., Nance, S.L. and Schiffer, M., unpublished observations). In the wild-type *Rb. sphaeroides* structure, M231Arg is involved in conserved ion pair interactions with H125Glu and H232Glu (Fig. 1 [13]). Disruption of these ion pair interactions by the M231Arg → Leu mutation changes the charge distribution in this region of the RC by removing a positively charged residue, thus freeing up a potential negative charge. The oxygen atoms of the carbonyl groups of H125Glu and H232Glu are 4–6 Å apart and their closest distances from the carbonyl oxygen (O2) of Q_B are about 14 Å and 18 Å, respectively.

Fig. 2 shows cytochrome oxidation induced by a series of near saturating flashes, activating RCs of the wild-type, the double mutant and the revertant. The ability of the RCs to photooxidize exogenous cytochrome c_2 after each of several flashes is related to the capacity of the Q_A and Q_B acceptors. Thus, the oxidation pattern displayed by the wild-type RCs corresponds to an unrestricted electron transfer from Q_A to Q_B and the quinone pool. However, cytochrome oxidation by the RCs from the PS^- double mutant strain are restricted: the amplitudes of the oxidation after the first and second flashes are virtually unaltered, while those of the third and subsequent flashes are dramatically smaller. Examination of the revertant strain yielded the results also shown in Fig. 2. This revertant yielded results strikingly similar to those from the wild-type. Measurement of the steady-state rate of cytochrome oxidation in RCs of the revertant had previously determined that multiple fast turnovers occurred, indicating rapid equilibration of Q_BH_2 with the quinone pool (Tiede, D.M., Flory, J. and Hanson, D.K.,unpublished observations).

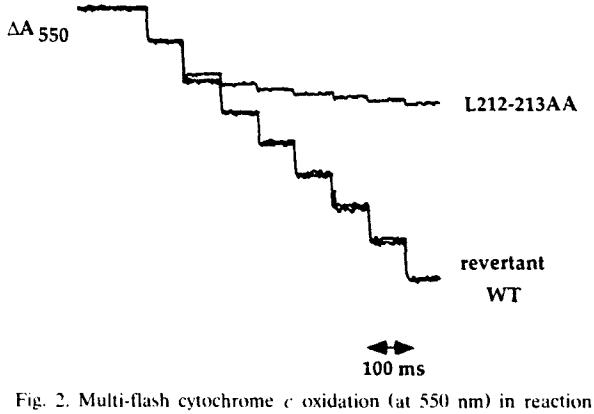

Fig. 2. Multi-flash cytochrome *c* oxidation (at 550 nm) in reaction centers isolated from the wild-type, the L212–213AA double mutant and the revertant (L212A-L213A-M231L) strains of *Rb. capsulatus*. 10 mM Tris (pH 7.8), 0.05% LDAO, 200 μM sodium ascorbate, 40 μM cytochrome *c*.

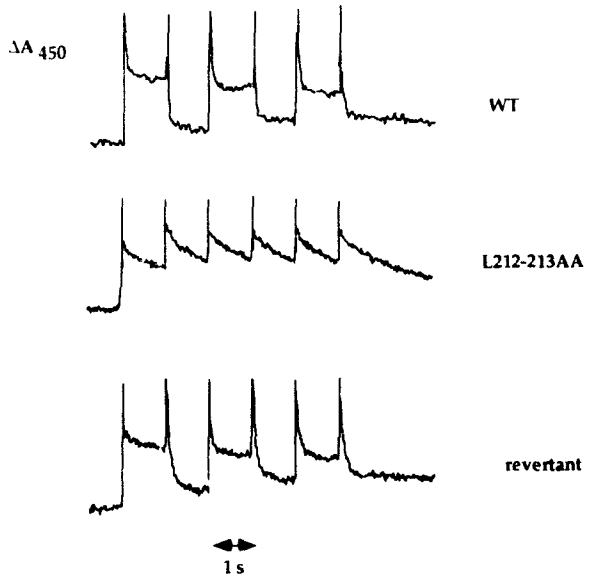

Fig. 3. Semiquinone oscillations in reaction centers isolated from the wild-type, the L212–213AA double mutant and the revertant strains (L212A-L213A-M231L) of *Rb. capsulatus*. 10 mM Tris (pH 7.8), 0.05% LDAO, 1 mM ascorbate, 500 μM DAD.

Another more direct way of examining the reaction is to follow the formation of the semiquinone in the Q_A and Q_B sites. Fig. 3 shows, at pH 7.8, that in the presence of exogenous donors (DAD) to P^+, the wild-type RCs display the typical semiquinone oscillations observed in *Rb. sphaeroides* [14,15]. In RCs from the PS^- double mutant, these oscillations are not observed. Instead, a slow semiquinone decay is observed on each flash, suggesting that Q_B cannot accept a second electron. These results are consistent with those derived from the multiple flash cytochrome oxidation measurements. The observed signals are likely to reflect the formation of Q_A^- and Q_B^-, which do not require proton uptake. However, formation of Q_B^{2-} is energetically unfavorable without provision of protons, hence all further reactions are prevented. This result agrees with the work of Takahashi and Wraight [3]. However, in RCs from the PS^+ revertant, the semiquinone oscillations are recovered (Fig. 3). The oscillations show that proton uptake does occur in the revertant with a rate that does not significantly limit the reduction of Q_B^-, and appears comparable to that of the wild-type. This observation corroborates the steady-state cytochrome oxidation observations described above, and clearly shows that RCs isolated from the revertant function well in the absence of L212Glu and L213Asp.

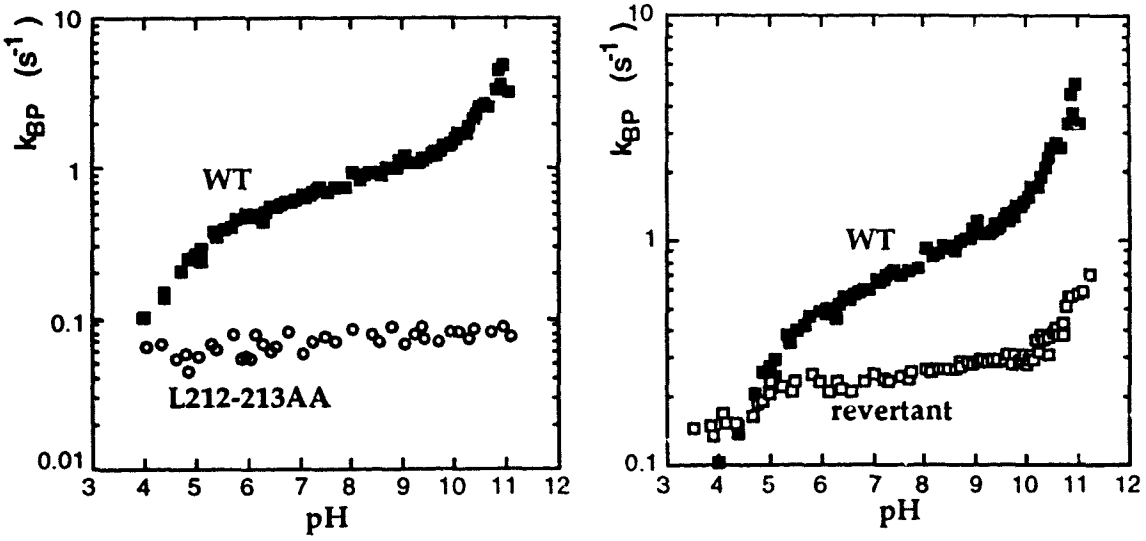

Fig. 4. pH dependence of k_{BP}, the $P^+Q_B^-$ charge recombination, in reaction centers from the wild-type, the L212–213AA double mutant and the revertant (L212A-L213A-M231L) strains of *Rb. capsulatus*. Buffers are as described in the methods, 0.05% LDAO.

The recovery of RC function in the revertant prompted us to examine the pH dependence of the rate constant of $P^+Q_B^-$ charge recombination decay kinetics (k_{BP}). The k_{BP} value is directly related to the amount of semiquinone stabilization. The pH dependence of k_{BP} therefore identifies pK values of group(s)

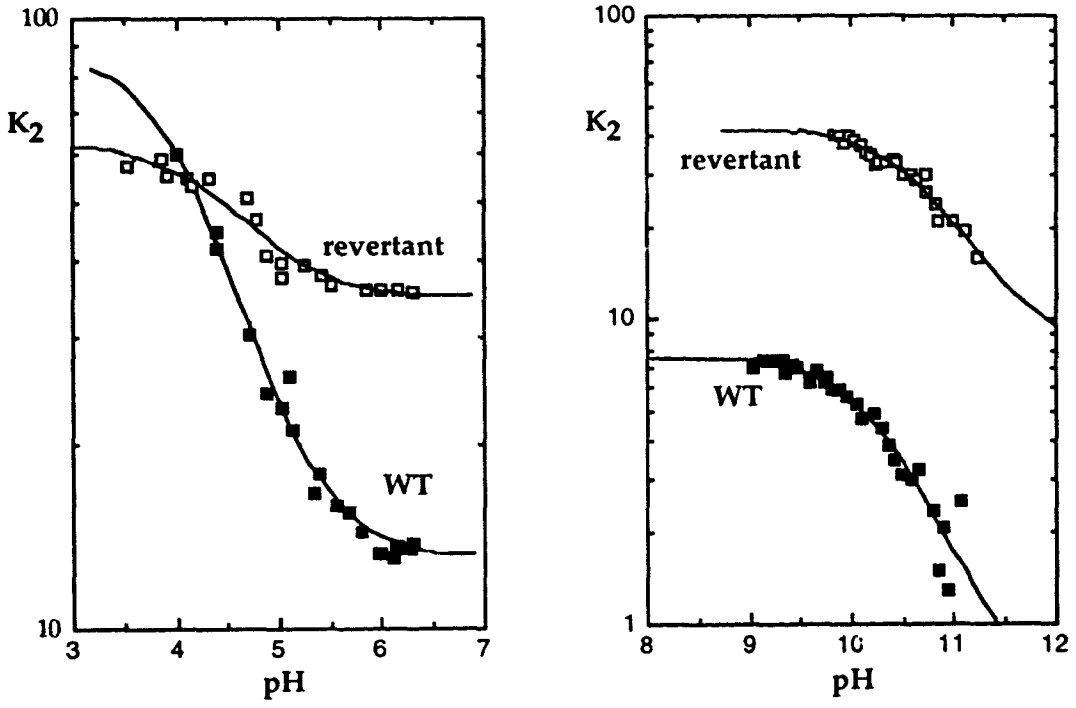

Fig. 5. pH dependence of K_2, the $Q_A^- \leftrightarrow Q_B^-$ electron transfer equilibrium constant, in the wild-type and revertant (L212A-L213A-M231L) strains of *Rb. capsulatus*. Lines are drawn according to equation 1. At high pH, the derived pK values are $pK_{Q_A^-Q_B} = 10.3 \pm 0.1$ and $pK_{Q_AQ_B^-} = 11.6 \pm 0.3$ for the wild-type; for the revertant, $pK_{Q_A^-Q_B} = 10.7 \pm 0.3$ and $pK_{Q_AQ_B^-} = 11.5 \pm 0.3$. At low pH, in the wild-type, $pK_{Q_A^-Q_B} = 4.2 \pm 0.2$ and $pK_{Q_AQ_B^-} = 5.1 \pm 0.1$; in the revertant, $pK_{Q_A^-Q_B} = 4.3 \pm 0.2$ and $pK_{Q_AQ_B^-} = 4.8 \pm 0.1$. Note the large difference in the magnitudes of the K_2 variation in the wild-type versus the revertant at low pH. The variation of K_2 in the revertant is significantly smaller.

involved in the semiquinone stabilization. The pH titrations of k_{BP} for the wild-type, the double mutant, and the revertant are shown in Fig. 4.

In the pH range 4–7, a marked variation of k_{BP} is observed (Fig. 4). In *Rb. sphaeroides*, this substantial destabilization of Q_B has previously been attributed to deprotonation of L213Asp [2,3]. Above pH 10, destabilization of Q_B is also observed here in *Rb. capsulatus*. In *Rb. sphaeroides*, that process was suggested to be due to deprotonation of L212Glu [1].

Fig. 4 also shows the very substantial stabilization of the semiquinone in the double mutant. At pH 7, the ratio between the k_{BP} values measured in the wild-type and the double mutant RCs is about 10, suggesting that Q_B is stabilized by an additional 60 meV in the double mutant compared to the wild-type. The same value was obtained by Takahashi and Wraight in the L213Asp → Asn mutant of *Rb. sphaeroides* [2], suggesting that there are similar dielectric distances (ϵ_r) between the side chain of L213Asp and Q_B in *Rb. sphaeroides* and *Rb. capsulatus*.

The other main difference between the double mutant and the wild-type arises from the absence of pH dependence of k_{BP} in RCs of the double mutant. The results are the same as measured for the L212Glu-L213Asp → Gln-Asn double mutant of *Rb. sphaeroides* [3] and would apparently confirm that L213Asp and L212Glu are responsible for the marked k_{BP} variations observed in the pH range 4–7 and above pH 10, respectively, in the wild-type.

In the revertant, above pH 5, Q_B^- is stabilized compared to the wild-type, but much less than in the double mutant. This is most likely due to the absence of the positive charge of M231Arg in the revertant. At pH 7, k_{BP} is about 0.25 s^{-1} in the revertant, compared to 0.65 s^{-1} in the wild-type. Much to our surprise, the behavior of k_{BP} in the revertant again mimics the wild-type in displaying a 2.5-times increase in the pH range 10–11.2. Although smoother than in the wild-type, a low pH variation of k_{BP} is also observed in the revertant. Neither pH variation, especially that detected at high pH, can be explained in this case by the specific deprotonation of L213Asp and L212Glu since in the revertant both residues were replaced by alanine.

In order to determine the pK values associated with the variations of Q_B^- stabilization, we have calculated the pH dependence of the $Q_A^-Q_B \leftrightarrow Q_AQ_B^-$ equilibrium constant ($K_2 + 1 = k_{AP}/k_{BP}$; [16]), leading to the pH titration curves for K_2 shown in Fig. 5. It was thus necessary to measure the pH dependence of k_{AP}. For the wild-type and the revertant, the charge recombination decay (k_{AP}) curves are superimposable (data not shown). A smooth regular pH dependence is observed in the pH range 4–11.5 $(k_{AP})^{-1}$ varying from 160 ms to about 80 ms. This probably accounts for a direct route of charge recombination from the $P^+Q_A^-$ state in *Rb. capsulatus*. If only one protonation event occurs in the Q_B vicinity in the pH range where the curves are analyzed, one can fit the K_2 curves as follows:

$$K_2(\mathrm{pH}) = K_2^{\mathrm{H}+} \cdot \frac{1 + 10^{(\mathrm{pH} - \mathrm{p}K_{Q_AQ_B})}}{1 + 10^{(\mathrm{pH} - \mathrm{p}K_{Q_AQ_B})}} \qquad (1)$$

where K_2^{H+} represents the K_2 value at low pH, $pK_{Q_AQ_B}$ and $pK_{Q_AQ_B}$ are the pK values of the protonable groups close to Q_B when the electron is on Q_B and on Q_A, respectively. For simplification, as has been previously done [3], we neglect any direct interactions between this group and Q_A.

Fitting the K_2 curves of Fig. 5B by Eqn. 1 leads to similar pK values for the wild-type and the revertant, at high pH. For the wild-type, $pK_{Q_AQ_B} = 10.3 \pm 0.1$ and $pK_{Q_AQ_B} = 11.6 \pm 0.3$; for the revertant, $pK_{Q_AQ_B} = 10.7 \pm 0.3$ and $pK_{Q_AQ_B} = 11.5 \pm 0.3$. Although the p$K$ values have some uncertainty because neither titration is complete, the courses of the titrations are close enough in the wild-type and the revertant to suggest that the groups involved in that deprotonation behave in a similar manner. Clearly this residue can not be L212Glu, since L212 is an alanine in the revertant.

The K_2 variation curves at low pH are displayed in Fig. 5A. Fitting the data by Eqn. 1 yields $pK_{Q_AQ_B} = 4.2 \pm 0.2$ and $pK_{Q_AQ_B} = 5.1 \pm 0.1$, in the wild-type, in agreement with the determination done in *Rb. sphaeroides* [3]. In the revertant, the $pK_{Q_AQ_B} = 4.3 \pm 0.2$ and $pK_{Q_AQ_B} = 4.8 \pm 0.1$. However, the average p$K$ of the titrations is about the same in the two strains: ≈ 4.6. This value suggests that again, the groups responsible for this deprotonation are likely to have similar properties and could be the same in both strains. Therefore, this argues against the mandatory participation of L213Asp in the semiquinone stabilization at low pH in *Rb. capsulatus*. The smaller calculated value of ΔpK ($pK_{Q_AQ_B}$ minus $pK_{Q_AQ_B}$) in the revertant reflects a lower interaction energy between Q_B^- and the protonatable group(s) and is responsible for the much smaller variation in K_2 that was observed (Fig. 5A). This may result from a higher screening effect in that strain, a slightly higher local dielectric constant, and/or a greater distance between Q_B and the protonatable group(s), possibly H125Glu and/or H232Glu, in the revertant.

The highly deleterious effects on Q_B function observed in this work that result from the alteration of L212Glu and L213Asp to alanines agree with the earlier findings that led to the suggestion that these residues are required for the final stages of intraprotein proton translocation from the external aqueous medium to the reduced quinone at the Q_B site in the wild-type [1–3].

The results obtained on the revertant are therefore very surprising. In this strain, the M231Arg → Leu mutation is sufficient to restore photosynthetic growth to a level similar to that of the wild-type (not shown). Our results show that the RC function that requires delivery of protons to the Q_B site, which is lost in the PS^- L212–213AA double mutant, is totally recovered in the RCs from the revertant. Moreover, the dependency on pH of k_{BP} behaves in a manner that resembles the wild-type and suggests that protons are effectively driven to Q_B in the revertant.

Whether the effect of the absence of M231Arg in the PS^+ revertant described here is direct or indirect, it influences the charge balance of the RC. The replacement of M231Arg by the aliphatic leucine is sufficient to partially restore the apparent pK values of the two 'resultant' groups, possibly H125Glu and H232Glu, responsible for the high and low pH dependence of k_{BP}. How the M231Arg → Leu alteration, some 15 Å from the Q_B site, restores function is not certain at the present time. However, M. Gunner (personal communication) has demonstrated that there is an electrostatic interaction between M231 Arg and Q_B (−1.5 kcal/mol). The wild-type *Rb. sphaeroides* structure shows that M231Arg forms salt bridges with a pair of acidic residues in the H chain, H125Glu and H232Glu. Based upon the probable structural similarity between the *Rb. capsulatus* and *Rb. sphaeroides* reaction centers, the M231Arg → Leu mutation could restore a 'di-acid' that could be functionally equivalent to the original L212Glu-L213Asp pair, albeit ~ 15 Å away from the Q_B site. The *Rb. sphaeroides* structure shows that the distances between the oxygen atoms of the carboxyl groups of L212Glu-L213Asp and H125Glu-H232Glu are similar (5–6 Å and 4–6 Å, respectively). The role played by water molecules located between Q_B and H125Glu-H232Glu could also be relevant. In *Rhodopseudomonas viridis* RC crystals, channels of water molecules exist between the cytoplasmic side of the protein and the Q_B site (Diesenhofer, J. and Michel, H., personal communications).

Taken all together, our results strongly suggest that L212Glu and L213Asp can be replaced by other residues in the revertant for the events leading to proton donation to Q_B. While L212Glu and L213Asp are important in wild-type *Rb. sphaeroides* and *Rb. capsulatus*, they are only part of a larger network of protonatable amino acids in the Q_B vicinity. It is interesting to note that in *Rps. viridis* RCs, where L213Asp is naturally asparagine, a very substantial acceleration of k_{BP} is observed from pH 4 to pH 8 in a very similar way as in *Rb. sphaeroides* and *Rb. capsulatus* [17,18]. This observation also suggests that it seems unlikely that a particular behavior of the protein can be assigned to a single amino acid (see also Ref. 7).

We think that in bacterial reaction centers, proton diffusion is likely to take place through several possible pathways from the cytoplasmic side of the protein to the secondary quinone. Different proton transfer circuits may be activated by suppressor mutations in the revertant strains to restore function. Those pathways could involve bound water molecules.

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