Electrostatic Dominoes: Long Distance Propagation of Mutational Effects in Photosynthetic Reaction Centers of *Rhodobacter capsulatus*†

Pierre Sebban,‡ Péter Maróti,§ Marianne Schiffer,|| and Deborah K. Hanson*,||

Photosynthèse Bactérienne, Centre de Génétique Moléculaire, Bât. 24, Centre National de la Recherche Scientifique, 91198 Gif/Yvette, France, Institute of Biophysics, József Attila University Szeged, Egyetem utca 2, Szeged, Hungary H-6722, and Center for Mechanistic Biology and Biotechnology, Argonne National Laboratory, 9700 South Cass Avenue, Argonne, Illinois 60439

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ABSTRACT: Two point mutants from the purple bacterium *Rhodobacter capsulatus*, both modified in the M protein of the photosynthetic reaction center, have been studied by flash-induced absorbance spectroscopy. These strains carry either the M231Arg → Leu or M43Asn → Asp mutations, which are located 9 and 15 Å, respectively, from the terminal electron acceptor Q_B. In the wild-type *Rb. sphaeroides* structure, M231Arg is involved in a conserved salt bridge with H125Glu and H232Glu and M43Asn is located among several polar residues that form or surround the Q_B binding site. These substitutions were originally uncovered in phenotypic revertants isolated from the photosynthetically incompetent L212Glu-L213Asp → Ala-Ala site-specific double mutant. As second-site suppressor mutations, they have been shown to restore the proton transfer function that is interrupted in the L212Ala-L213Ala double mutant. The electrostatic effects that are induced in reaction centers by the M231Arg → Leu and M43Asn → Asp substitutions are roughly the same in either the double-mutant or wild-type backgrounds. In a reaction center that is otherwise wild type in sequence, they decrease the free energy gap between the Q_A^- and Q_B^- states by 24 ± 5 and 45 ± 5 meV, respectively. The pH dependences of K_2, the $Q_A^-Q_B \leftrightarrow Q_AQ_B^-$ equilibrium constant, are altered in reaction centers that carry either of these substitutions, revealing differences in the pK_as of titratable groups compared to the wild type. These results confirm that interactions among distant residues influence the electrostatic potential in the immediate vicinity of Q_B to ensure the efficient conduction of protons through the protein matrix and their delivery to the reduced quinone. It is possible that these influences are propagated over such large distances by mutation-induced realignments of salt bridges within a network of acidic and basic residues that is located in this region of the reaction center, which could serve as a relay mechanism to partially relocate the new negative charge much closer to the quinone.

In photosynthetic reaction centers (RC), light induces primary electron transfer reactions coupled to proton uptake by the protein. A major step toward the understanding of these processes has been achieved with the successful crystallization and structure determination of these proteins from two species: *Rhodopseudomonas (Rps.) viridis* (Deisenhofer et al., 1985, 1995) and *Rhodobacter (Rb.) sphaeroides* (Allen et al., 1988; Chang et al., 1991; Ermler et al., 1994; Arnoux et al., in press). Three subunits—L, M, and H—form the protein core of this complex. The primary electron donor P, a dimer of bacteriochlorophyll, is situated on the periplasmic side of the complex. The first stable electron acceptors are quinone molecules Q_A and Q_B which are situated near the cytoplasmic side of the complex. In *Rb. sphaeroides* and *Rb. capsulatus*, both Q_A and Q_B are ubiquinone$_{10}$ (UQ_{10}). Q_B is bound in a polar region of the L protein, whereas Q_A is found in a hydrophobic part of the M protein. Q_B functions as a two-electron acceptor which leaves the RC in its doubly reduced and doubly protonated state, Q_BH_2, following the delivery of protons into the buried Q_B binding site. Q_BH_2 is replaced by an oxidized quinone molecule from the quinone pool present in the membrane.

The formation of Q_B^- on the first flash induces shifts in the pK_as of several amino acid residues in the Q_B environment. This leads to partial proton uptake by these residues. The pH dependence of the stabilization of the energy level of Q_B^- by these protonations was determined by measurements of proton uptake stoichiometries (Maróti & Wraight, 1988; McPherson et al., 1988) and the kinetics of $P^+Q_B^-$ charge recombination (Kleinfeld et al., 1984; Sebban & Wraight, 1989; Baciou et al., 1990, 1993; Takahashi & Wraight, 1992). L213Asp and possibly L210Asp were suggested to participate in the stabilization of Q_B^- at low pH in RCs of *Rb. sphaeroides* (Takahashi & Wraight, 1990; Okamura & Feher, 1992) and *Rb. capsulatus* (Maróti et al., 1994). At high pH, deprotonation of L212Glu was proposed to be directly involved in the observed increase in the free energy of Q_B^- in *Rb. sphaeroides* (Paddock et al., 1989).

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* Author to whom correspondence should be addressed.
‡ Centre National de la Recherche Scientifique.
§ József Attila University Szeged.
|| Argonne National Laboratory.

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However, the specific assignment of this pH dependence to L212Glu is still a matter of debate since a similar pH dependence was also observed in modified RCs from *Rb. capsulatus* where L212Glu was replaced by Ala (Hanson et al., 1992b; Maróti et al., 1994). L212Glu and L213Asp have been suggested to be components of the pathway for proton delivery to Q_B^- in wild-type *Rb. sphaeroides* [for a review, see Okamura and Feher (1992)].

In RC mutants and phenotypic revertants of *Rb. capsulatus*, we have recently shown that the electrostatic potential "seen" by Q_B^- results from interactions involving distant residues (9−15 Å from Q_B^- in the wild-type *Rb. sphaeroides* RC structure) in addition to those with nearby residues L212Glu (about 5 Å from Q_B) and L213Asp (about 6 Å from Q_B). These long range electrostatic interactions were uncovered when photocompetent derivatives of the nonphotosynthetic (PS^-) L212Ala-L213Ala double mutant were characterized. Two of those derivatives were found to carry distant second-site mutations, either M231Arg → Leu or M43Asn → Asp [class 3 or 4 suppressor, respectively (Hanson et al., 1992a,b, 1993)], that suppressed the PS^- phenotype. For example, loss of M231Arg, which is situated about 15 Å from Q_B and forms salt bridges with H125Glu and H232Glu in the wild-type *Rb. sphaeroides* structure, was shown to increase the free energy gap between Q_A^- and Q_B^- by about 30 meV at pH 7 in RCs of *Rb. capsulatus*, whereas loss of the nearby residue L213Asp increases it by about 60 meV (Maróti et al., 1994). With the aim of understanding these electrostatic effects more fully and detemining the mechanisms by which these distant substitutions suppress the PS^- phenotype of the L212Ala-L213Ala strain, we have analyzed here two strains which carry, in an otherwise wild-type background, either the M231Arg → Leu or M43Asn → Asp mutation. The analysis of these mutants gives us insight into the effects of distant negatively charged residues on electron transfer kinetics in the wild type.

MATERIALS AND METHODS

Plasmids carrying the M231Arg → Leu or M43Asn → Asp mutations were constructed from plasmids that were recovered from the class 3 or 4 (respectively) phenotypic revertant strains (Hanson et al., 1993). The revertant plasmids carried the L212Ala-L213Ala substitutions in addition to the M231Leu or M43Asp mutations. In each case, the M gene suppressor mutation was separated from the L gene mutations by digestion of the revertant plasmids with *Kpn*I and *Sac*I (Bylina & Youvan, 1986; Bylina et al., 1989; Hanson et al., 1992a). The *Kpn*I-*Sac*I fragment carrying the M gene mutation was then switched for the corresponding fragment in a *Bgl*II-tagged version of the wild-type plasmid, yielding plasmids that carried the single M231Leu or M43Asp mutation in an otherwise wild-type background. These plasmids were transferred to *Rb. capsulatus* deletion strain U43 (Youvan et al., 1985; LHI^-, $LHII^-$, RC^-) from *Escherichia coli* donor strain S17-1 (Simon et al., 1983) by conjugal transfer (Davis et al., 1988). Growth phenotypes were determined as described (Robles et al., 1990). Mutant plasmids were recloned from the U43 strains for dideoxy sequencing of double-stranded DNA (Sequenase kit, U.S. Biochemical Corp.) to confirm the presence of the desired mutation. All strains were routinely maintained by chemoheterotrophic culture (semiaerobic, dark, 34 °C).

Large scale cultures for RC preparation were grown under chemoheterotrophic conditions on RPYE medium (Hanson et al., 1992b) containing kanamycin to ensure presence of the plasmid. RCs of the two mutant strains were prepared as previously described for the wild type (Baciou et al., 1993). Occupancy of the Q_B site was restored by the addition of 50−100 μM UQ_6 (Sigma).

Spectrophotometric experiments were carried out at 21 °C (Baciou et al., 1993). $P^+Q_A^-$ and $P^+Q_B^-$ decay kinetics were measured at 865 nm. The first-electron transfer rate was determined at 397 nm as suggested by Takahashi and Wraight (1992). The second-electron transfer rate was determined at 450 nm in the presence of 40 μM cytochrome *c* and 0.5−2 mM sodium ascorbate as previously described (Maróti et al., 1994). Depending on the pH range, buffers used in the charge recombination and second-electron transfer experiments were MES [2-(*N*-morpholino)ethanesulfonic acid], bis-Tris-propane [1,3-bis[tris(hydroxymethyl)methylamino]propane], or CAPS [3-(cyclohexylamino)-1-propanesulfonic acid].

Molecular modeling of the RC was done using the computer graphics program O (Jones et al., 1991) with the wild-type *Rb. sphaeroides* RC structure (Chang et al., 1991). Residue numbering refers to the amino acid sequence of the *Rb. capsulatus* RC subunits.

RESULTS

pH Dependence of the Rate Constant of $P^+Q_B^-$ Charge Recombination, k_{BP}, and the $Q_A^-Q_B \leftrightarrow Q_AQ_B^-$ Equilibrium Constant, K_2. The pH dependences of k_{BP} in RCs from the M231Arg → Leu and M43Asn → Asp mutants are shown in Figure 1, in comparison with the wild type. The pH dependences of k_{BP} in RCs that carry these mutations in the double-mutant background [class 3 phenotypic revertant (L212Ala-L213Ala-M231Leu) and class 4 phenotypic revertant (L212Ala-L213Ala-M43Asp)], which were determined previously (Maróti et al., 1994), are also included for comparison. In the M43Asn → Asp mutant, k_{BP} increases from ca. 0.14 s^{-1} at pH 3.3 to ca. 1.5 s^{-1} at pH 7. This sharp pH dependence is reminiscent of that observed in the wild type and is a different pattern from that measured in the RCs from the M231Arg → Leu mutant where two waves of k_{BP} variation are detected. In the M231Arg → Leu mutant, k_{BP} varies from ca. 0.6 s^{-1} at pH 3.3 to ca. 1.1 s^{-1} at pH 5.5 and starts to further increase at pH 6 to reach a value of ca. 3.3 s^{-1} at pH 8. Quinone was weakly bound above pH 9 in both mutants.

The rate constant of $P^+Q_A^-$ charge recombination in the mutants, measured as a function of pH, was within 5% of that of the wild-type RCs. Therefore, the pH dependence of k_{AP} measured in the wild type (Baciou et al., 1993) was used to determine the pH dependence of K_2, the $Q_A^-Q_B \leftrightarrow Q_A\,Q_B^-$ equilibrium constant, by using the relationship derived by Mancino et al. (1984): $1 + K_2 = k_{AP}/k_{BP}$. The pH dependences of K_2 for the M43Asn → Asp and M231Arg → Leu mutants are shown in Figure 2, in comparison with the wild type. The lines are fitted according to an iterative procedure using the following equation:

$$K_2(\mathrm{pH}) = K_{2H^+} \times \frac{1 + 10^{(\mathrm{pH}-\mathrm{p}K_{a1Q_B^-})}}{1 + 10^{(\mathrm{pH}-\mathrm{p}K_{a1Q_B})}} \times \frac{1 + 10^{(\mathrm{pH}-\mathrm{p}K_{a2Q_B^-})}}{1 + 10^{(\mathrm{pH}-\mathrm{p}K_{a2Q_B})}} \quad (1)$$

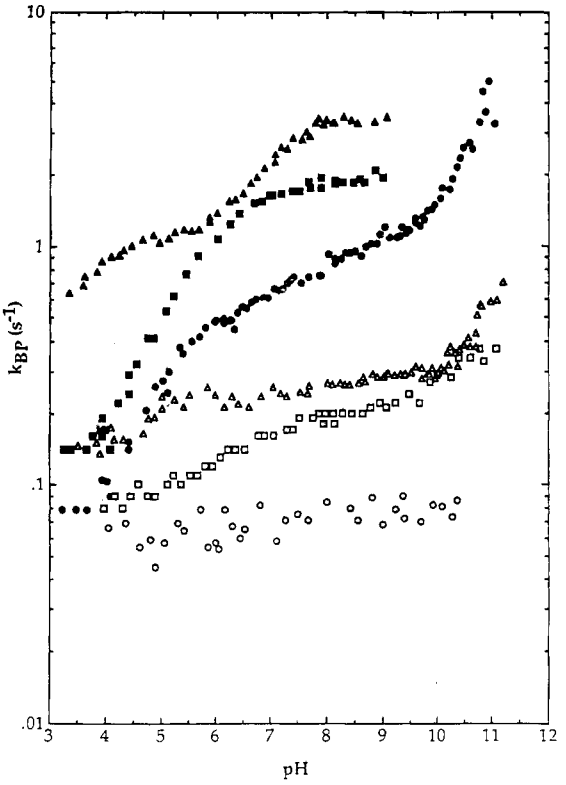

FIGURE 1: pH dependence of k_{BP}, the rate constant of $P^+Q_B^-$ charge recombination, in RCs (0.5 μM) from the M231Arg → Leu (▲) and M43Asn → Asp (■) single mutants, compared to the wild type (●) as previously measured (Maróti et al., 1994). Curves for the L212Ala-L213Ala double mutant (○) and the class 3 (L212Ala-L213Ala-M231Leu; △) and 4 (L212Ala-L213Ala-M43Asp; □) photocompetent supressor strains that were isolated from it are included for comparison (Maróti et al., 1994). The absorbance changes are detected at 865 nm. Conditions: 10 mM buffer (described in Materials and Methods), 0.03% Triton X-100, and 100 μM UQ_6.

Table 1: Apparent pK_a Values Measured at Low pH in RCs from the Wild-Type and Modified Strains of *Rb. capsulatus*

	pK_{a1Q_B}	$pK_{a1Q_B^-}$	pK_{a2Q_B}	$pK_{a2Q_B^-}$
wild type	4.3 ± 0.10	5.15 ± 0.10		
M43Asn → Asp	4.35 ± 0.10	5.40 ± 0.10	6.80 ± 0.10	6.95 ± 0.10
M231Arg → Leu	2.35 ± 0.30	3.30 ± 0.30	6.35 ± 0.10	6.95 ± 0.10
class 3 suppressor	4.60 ± 0.10	4.85 ± 0.10		
class 4 suppressor	5.85 ± 0.10	6.10 ± 0.10		

where K_{2H^+} is the value of K_2 at very low pH. pK_{a1Q_B} and $pK_{a1Q_B^-}$ represent the pK_as of one or two protonatable groups interacting with Q_B and Q_B^-, respectively. For the M43Asn → Asp mutant, this analysis leads to $pK_{a1Q_B} = 4.35 \pm 0.10$, $pK_{a1Q_B^-} = 5.40 \pm 0.10$, $pK_{a2Q_B} = 6.80 \pm 0.10$, and $pK_{a2Q_B^-} = 6.95 \pm 0.10$. The ΔpK_a measured for the first group ($pK_{a1Q_B^-} - pK_{a1Q_B} = 1.05$) suggests an interaction energy of ca. 65 meV between this group and Q_B^-. This is similar to what is observed in the wild type, where only one group is necessary to fit the K_2 curve with $pK_{a1Q_B} = 4.30 \pm 0.10$ and $pK_{a1Q_B^-} = 5.15 \pm 0.10$. The situation is different in the M231Arg → Leu mutant. In the pH range 3.3−5.5, K_2 decreases only slightly from ca. 9 to ca. 5. K_2 starts decreasing again above pH 6. In the pH 6−8 range, this parameter varies from 5 to ca. 1.5. Below pH 3.3 in this mutant, K_2 increases, but measurements in this range are not possible due to denaturation of the RC complex. In the fitting procedure using eq 1, we have arbitrarily fixed K_{2H^+} to a value of 50, in agreement with that of the M43Asn → Asp strain and slightly smaller than that of the class 3 and 4 phenotypic revertant strains. This procedure results in the determination of $pK_{a1Q_B} = 2.35 \pm 0.30$, $pK_{a1Q_B^-} = 3.30 \pm 0.30$, $pK_{a2Q_B} = 6.35 \pm 0.10$, and $pK_{a2Q_B^-} = 6.95 \pm 0.10$. Fitting the curves obtained previously (Maróti et al., 1994) for the class 3 and 4 phenotypic revertants leads to pK_{a1Q_B}

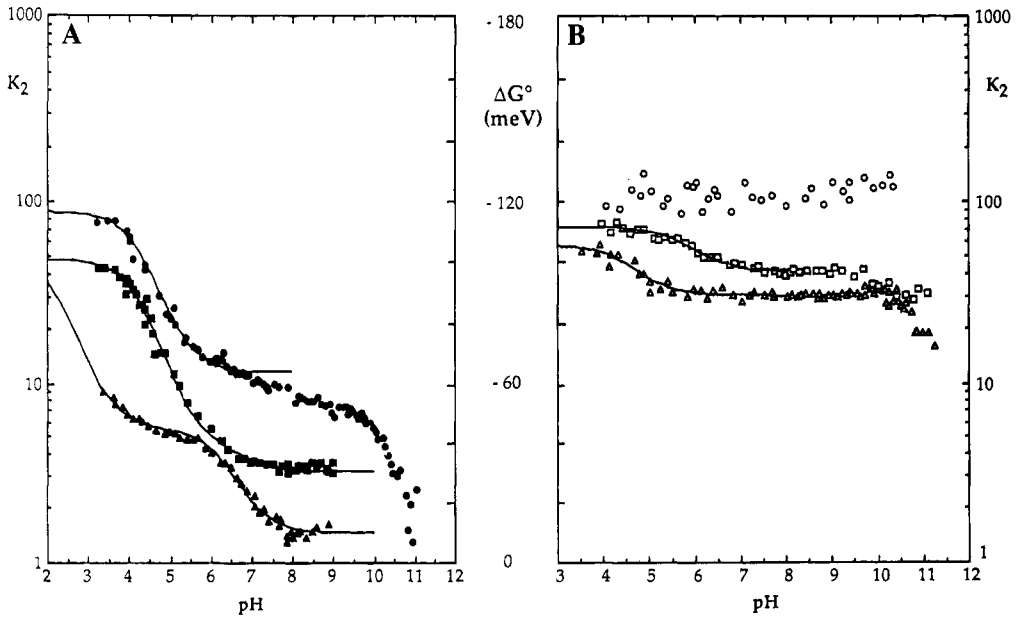

FIGURE 2: pH dependence of the equilibrium constant K_2 and the free energy gap, $\Delta G°$, between the $P^+Q_A^-$ and $P^+Q_B^-$ states. (A) RCs from the M231Arg → Leu (▲) and M43Asn → Asp (■) single mutants, compared to the wild type (●) as previously measured (Maróti et al., 1994). In panel B we have presented the data already measured in Maróti et al. (1994) for these mutations in the double-mutant background: the class 3 (L212Ala-L213Ala-M231Leu; △) and 4 (L212Ala-L213Ala-M43Asp; □) suppressor strains, compared to the L212Ala-L213Ala double-mutant strain (○). These $\Delta G°$ values were derived from independent measurements of k_{AP} and k_{BP}, by $\Delta G° = -kT \ln(k_{AP}/k_{BP} - 1)$ (Maróti et al., 1994). Conditions: as in Figure 1.

Table 2: Changes in the Free Energy Gap (meV) at pH 8 between Q_A^- and Q_B^- Due to the M43Asn → Asp and M231Arg → Leu Mutations in RCs of *Rb. capsulatus*

wild type	−54 ± 2
M43Asn → Asp	−30 ± 2
M231Arg → Leu	−9 ± 2
double mutant (L212Ala-L213Ala)	−122 ± 2
class 4 suppressor (L212Ala-L213Ala + M43Asn → Asp)	−93 ± 2
class 3 suppressor (L212Ala-L213Ala + M231Arg → Leu)	−85 ± 2

Table 3: Kinetics of First- and Second-Electron Transfer Measured in the RCs from the Wild-Type and Mutant Strains of *Rb. capsulatus*

	$1/k_{AB}(1)$, pH 8 (μs)	$1/k_{AB}(2)$, pH 8 (ms)	$1/k_{AB}(2)$, pH 7 (ms)
wild type	60 ± 10	0.58 ± 0.04	0.28 ± 0.02
M43Asn → Asp	250 ± 50	2.05 ± 0.02	0.38 ± 0.04
M231Arg → Leu	320 ± 50	5.90 ± 0.04	0.95 ± 0.01

= 4.60 ± 0.10 and $pK_{a1Q_B^-}$ = 4.85 ± 0.10 in the class 3 strain and pK_{a1Q_B} = 5.85 ± 0.10 and $pK_{a1Q_B^-}$ = 6.10 ± 0.10 in the class 4 strain.

It is of interest to calculate the pH dependence of the free energy gap between the $P^+Q_A^-$ and $P^+Q_B^-$ states ($\Delta G°$) in the mutants in comparison to the wild type. Using the relation $\Delta G° = -kT \log K_2$ leads to the $\Delta G°$ values for the curves presented in Figure 2. We have shown the $\Delta G°$ curves of these two mutants together with the wild type and compared them with the previously presented (Maróti et al., 1994) $\Delta G°$ curves of the class 3 (L212Ala-L213Ala-M231Leu) and 4 (L212Ala-L213Ala-M43Asp) phenotypic revertants compared to the L212Ala-L213Ala double-mutant strain. At pH 8, i.e., where the acidic group(s) responsible for stabilization of Q_B^- (relative to the Q_A^- state) at low pH are deprotonated, it appears that the M43Asn → Asp and M231Arg → Leu mutations destabilize Q_B^- by ca. 24 ± 5 and 45 ± 5 meV, respectively, compared to the wild type. The coulombic interaction energies between Q_B^- and M43Asp or Q_B^- and the acidic residues liberated by the replacement of M231Arg by Leu, respectively, match those derived from the comparison between the phenotypic revertants and the double-mutant strain (Figure 2B). In this case, values of 29 ± 5 and 37 ± 5 meV, respectively, were calculated (Maróti et al., 1994). However, as previously mentioned (Maróti et al., 1994), the $\Delta G°$ values calculated for the double mutant may be somewhat underestimated because of the very slow $P^+Q_B^-$ charge recombination in this mutant ($k_{BP} \approx 0.07\ s^{-1}$).

First-Electron Transfer Rate. The first-electron transfer rate, $k_{AB}(1)$, was measured at 397 nm and pH 8 in the wild type and and the two single mutants. As displayed in Table 3, $k_{AB}(1)$ is slowed in the mutants by a factor of about 5: $k_{AB}(1) = (60 \pm 10\ \mu s)^{-1}$ in the wild type; $k_{AB}(1) = (250 \pm 50\ \mu s)^{-1}$ and $(320 \pm 50\ \mu s)^{-1}$ in the M43Asn → Asp and M231Arg → Leu mutants, respectively.

Second-Electron Transfer Rate. The pH dependences of the rate constant of the second-electron transfer rate, $k_{AB}(2)$, for the M231Arg → Leu and M43Asn → Asp mutants and the wild type are presented in Figure 3. Again, the curves for the L212Ala-L213Ala double mutant along with the class 3 and 4 phenotypic revertants are included for comparison of the effects of the M231Leu and M43Asp substitutions in both a wild-type and double-mutant background. As we have

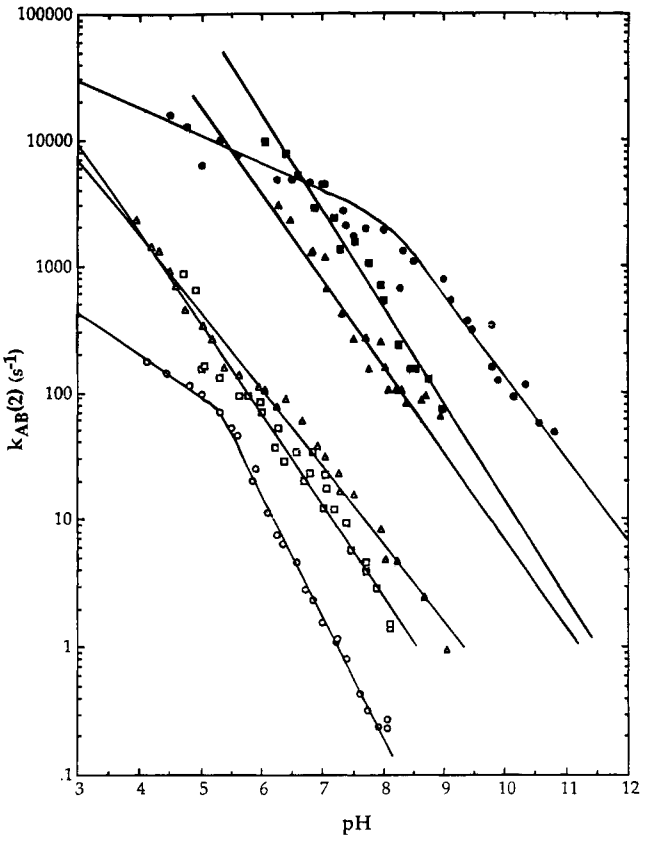

FIGURE 3: pH dependence of $k_{AB}(2)$, the rate constant of the second-electron transfer in the RCs (≈1 μM) from the M231Arg → Leu (▲) and M43Asn → Asp (■) single mutants, compared to the wild type (●) as previously measured (Maróti et al., 1994). Curves for the L212Ala-L213Ala double mutant (○) and the class 3 (L212Ala-L213Ala-M231Leu; △) and 4 (L212Ala-L213Ala-M43Asp; □) photocompetent supressor strains that were isolated from it are included for comparison (Maróti et al., 1994). Conditions: 40 μM cytochrome *c*, 1 mM ascorbic acid, 100 μM UQ_6, and 0.03% Triton X-100. Buffers: MES, MOPSO, and Tris depending on the pH.

observed previously (Maróti et al., 1994), the second-electron transfer process is notably accelerated in the class 3 and 4 phenotypic revertants compared to the double mutant. However, as shown in Table 3, at pH 7 $k_{AB}(2)$ is slightly decreased in the M43Asn → Asp RCs compared to the wild type and $k_{AB}(2)$ is decreased by an even greater amount in the M231Arg → Leu RCs. This observation is unexpected in light of the roles of these two substitutions in restoring photocompetence to the L212Ala-L213Ala double-mutant strain. Above pH 7, the slope of the $k_{AB}(2)$ curve is nearly the same (≈−0.7) in the three strains, with $k_{AB}(2)$ values substantially smaller in the M43Asn → Asp and M231Arg → Leu mutants. These data for the M43Asn → Asp mutant differ from those of Rongey et al. (1993) who have measured an increase in the second-electron (and proton) transfer processes in an M44Asn → Asp mutant of *Rb. sphaeroides* (residue M43 in *Rb. capsulatus*). Both groups have done the measurements at the same wavelength using the same experimental protocol. At the present time, we cannot explain this apparent discrepancy.

DISCUSSION

The quinone acceptor complexes of the RCs of *Rb. capsulatus* and *Rb. sphaeroides* share very similar functional properties and primary amino acid sequences. The functional

similarities have been demonstrated for the wild type as well as for various genetically modified strains of both species. In either organism, the lack of L213Asp results in a PS^- phenotype due to the impairment of the first-proton and second-electron transfer capabilities (Takahashi & Wraight, 1990; Hanson et al., 1992b; Rongey et al., 1993). A very substantial stabilization of the free energy level of the Q_B^- state is also observed in such mutants. Mutation of M231Arg (M233 in *Rb. sphaeroides*) restores the PS^+ phenotype to strains that lack L213Asp (Hanson et al., 1992b; Beroza et al., 1992). Similarly, the M43Asn → Asp mutation, when selected in *Rb. capsulatus* (Hanson et al., 1992a) or constructed in *Rb. sphaeroides* (Rongey et al., 1993), can compensate for the loss of L213Asp; M43Asp may substitute for L213Asp in the RCs of *Rps. viridis*, *Rhodospirillum rubrum*, and *Chloroflexus aurantiacus* where residue L213 is asparagine. It must also be noted that in both species L212Glu seems to have a very high pK_a, ≈9.8 in *Rb. sphaeroides* (Paddock et al., 1989; Takahashi & Wraight, 1992) and 10.1 in *Rb. capsulatus* (Hanson et al., 1992b; Maróti et al., 1994). Since they may be rationalized to the *Rb. sphaeroides* RC structure, studies of *Rb. capsulatus* RCs are therefore of general interest in that they may also apply to the RCs of other species which show functional and structural homology in the absence of complete sequence homology.

We have previously reported the impairment of the photosynthetic capability that results from the replacement of L212Glu and L213Asp by alanines in RCs of *Rb. capsulatus* (Hanson et al., 1992b; Maróti et al., 1994). We showed that these mutations yield a PS^- strain in which the rate of transfer of the second electron from Q_A to Q_B is decreased by 4 orders of magnitude, presumably due to the interruption of the pathway for proton delivery to Q_B^- (Maróti et al., 1994). At the same time, the energy level of the Q_B^- state was stabilized by more than 60 meV compared to the wild type, probably due to the specific absence of L213Asp.

Two PS^+ phenotypic revertants derived from this double mutant carried second-site mutations that compensated for the loss of acidic residues at L212 and L213 to reestablish proton delivery to the Q_B site. In addition to the initial site-specific alanine substitutions at the L212 and L213 sites, these phenotypic revertants carried the M231Arg → Leu or M43Asn → Asp substitutions [class 3 and 4 suppressor strains, respectively (Hanson et al., 1993)] present in the single mutants studied here. The effect of both of the suppressor mutations is to increase the negative electrostatic environment in this region of the RC. In the class 4 strain, this is accomplished by substitution of an aspartate for M43Asn about 9 Å from Q_B. In the class 3 strain, a basic residue is lost, disrupting the H125Glu-M231Arg-H232Glu salt-bridged cluster that is 15−20 Å from Q_B. In the phenotypic revertants we have shown that the effect of each of these second-site mutations was to recover part of the 60 meV stabilization energy induced by the L213Asp → Ala mutation and to increase simultaneously the rate of the second-electron transfer by 10−15-fold compared to the L212Ala-L213Ala double mutant.

Effects of the M231Arg → Leu and M43Asn → Asp Single Mutations on the Energy Level of the Q_B^- State and the $Q_A^-Q_B \leftrightarrow Q_AQ_B^-$ Equilibrium. In light of their effects in the double-mutant background, it was of interest to determine the specific influence of each compensatory mutation on these two parameters in an RC that is otherwise wild type in sequence. The electrostatic effects of the mutations in the wild-type background roughly match those induced by the same mutations in the double-mutant background. At pH 8, where deprotonations of groups that titrate at low pH are completed, the M43Asn → Asp mutation induces a destabilization of 29 ± 5 meV in the class 4 phenotypic revertant compared to the L212Ala-L213Ala double mutant, which is close to the value of 24 ± 5 meV detected in the M43Asn → Asp single mutant compared to the wild type.

The similarity of k_{BP} curves of the M43Asn → Asp mutant and the wild type in the pH 3−6 range, which differ essentially by an overall increase of k_{BP} in the mutant, suggests that the pK_a of M43Asp is below the pH range covered in these experiments. The effective pK_a of a group in a protein is determined by the degree of its interaction with neighboring groups [for a discussion, see Yang et al. (1993)]. The pK_a of an acidic residue that is buried in a hydrophobic part of the protein is abnormally high. The pK_a increases if the residue interacts with another negatively charged residue, and the degree of the increase depends on the interaction energy between the two groups. When two acidic groups having initially different pK_as interact, the lowest pK_a does not change but the highest pK_a shifts to an even higher pH value. The pK_a of an acidic residue is decreased, compared to its value in solution, if it forms a salt bridge or a hydrogen bond with a neighboring residue. The small distance between L217Arg and M43Asn (4 Å) in the *Rb. sphaeroides* RC supports the probability of a very low pK_a for M43Asp. Therefore, the decrease in the free energy difference between Q_A^- and Q_B^- that is measured in the M43Asn → Asp mutant very likely reflects the influence of ionized M43Asp on Q_B^-.

In the class 3 phenotypic revertant, the M231Arg → Leu mutation destabilizes Q_B^- (relative to Q_A^-) by 37 ± 5 meV compared to the double mutant, and it destabilizes Q_B^- by 45 ± 5 meV in the M231Arg → Leu single mutant versus the wild type. This additivity of the electrostatic effects may seem logical *a posteriori*, but it shows that no major structural change has occurred in the modified strains compared to the wild type and that the direct comparison of the various electrostatic effects is valid. It is worth noting that the magnitudes of the relative electrostatic influences on the energy level of Q_B^- due to the M43Asn → Asp and M231Arg → Leu mutations do not reflect their respective distances from Q_B. M43Asn and M231Arg are situated at ca. 9 and 15 Å from Q_B, respectively. The apparently stronger effect of the removal of the M231Arg may be understood in the terms of the very polar environment of M43Asp in the M43Asn → Asp mutant (in the *Rb. sphaeroides* structure M43Asn is 5 Å from L213Asp and 4 Å from L217Arg), resulting in a greater screening of the effects of M43Asp. In addition, residue M43 is situated close to the water interface, which probably increases the local effective dielectric constant.

In both the M43Asn → Asp and M231Arg → Leu single mutants, the occupancy of the Q_B binding site is strongly diminished above pH 8.5 compared to the wild type. The pH dependences of k_{BP} in the two RC mutants show a destabilization of Q_B^-, suggesting a more negative environment in this part of the protein due to the mutations. This environment yields values for the midpoint redox potentials

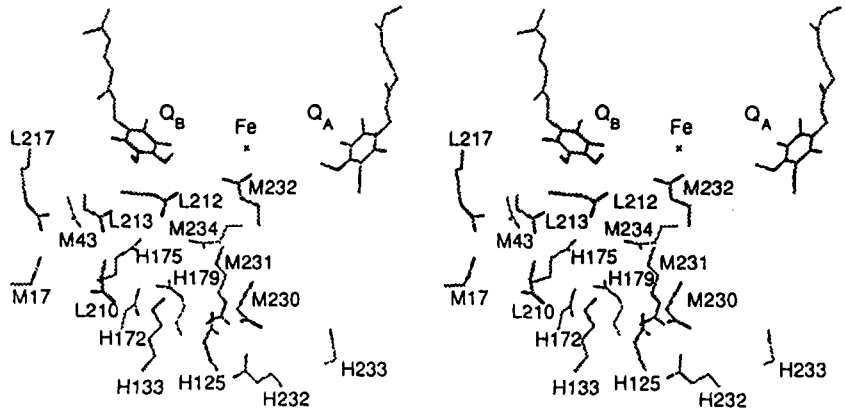

FIGURE 4: Stereoview of the network of charged residues surrounding Q_B (side chains only). M43Asn is also included. Residue numbers refer to the *Rb. capsulatus* amino acid sequence.

of the Q_B/Q_B^- couple in the mutants that are more negative than that of the wild type. The change of this physicochemical parameter, which reflects the differential binding affinity for the Q_B and Q_B^- forms, is not explained by a higher binding of Q_B in the M43Asp and M231Leu mutant strains. Therefore, our data are consistent with a substantial decrease in the binding of semiquinone in the M43Asp and M231Leu mutants above pH 8.5, i.e., when the electrostatic potential at the level of the Q_B protein pocket is more negative than that of the wild type.

Charged residues that surround Q_B affect the pH dependence of the equilibrium constant K_2. In the *Rb. sphaeroides* structure (Chang et al., 1991), nearby residues (Figure 4) include L212Glu (5.2 Å), L213Asp (6.2 Å), L217Arg (8.3 Å), H175Glu (8.5 Å), and L210Asp (12.5 Å). The assignment of one of these particular residues to the pH titration curve of K_2 in the pH range 4−6 in wild-type *Rb. sphaeroides* is still a matter of debate and is based on observations of pK_a shifts in site-specific mutants. Takahashi and Wraight (1992) have attributed the pH 4−6 variations to deprotonation of L213Asp. This assignment was based on data obtained with RCs of L213Asp → Asn mutants which display a pH independence for k_{BP} below pH 7. However, the substantial stabilization of Q_B^- in these RCs, which is due to the absence of L213Asp, results in a very slow rate of $P^+Q_B^-$ charge recombination (<0.06 s^{-1}). It was therefore suggested that in these mutants, $P^+Q_B^-$ charge recombination proceeds directly to the ground state by an activationless tunneling process. The rate constant of such an electron transfer mechanism is not expected to be very sensitive to the free energy level of Q_B^-. Therefore, the absence of variation in k_{BP} at low pH in the L213Asp → Asn mutant is not neccessarily due to the specific lack of deprotonation of L213Asp. In addition, in *Rps. viridis*, where a substantial pH dependence for k_{BP} is also detected in the pH range 5−7 (Baciou et al., 1990), a nonprotonatable Asn is located in position L213. On the other hand, Okamura and Feher (1992) have reported that this low-pH dependence of K_2 is also totally suppressed in the L210Asp → Asn RC mutant of *Rb. sphaeroides*. This observation could assign the titration of K_2 at low pH to the interaction between ionized L210Asp and Q_B^-. However, the absence of L210Asp may allow L217Arg, whose NH group is only 5 Å distant from L213Asp, to interact more strongly with this residue and therefore could shift the pK_a of L213Asp to a much lower value in the L210Asp → Asn mutant. Finally, in the class 3 and 4 phenotypic revertants, the absence of L213Asp does not result in the absence of the pH dependence of k_{BP} below pH 7. Instead, both types of RCs display a noticeable pH dependence for K_2 below this pH. The ΔpK_a calculated at low pH in these strains could be attributed to the remaining L210Asp residue and may suggest the participation of this amino acid in the pH titration of K_2 in the wild type. Okamura and Feher (1992) have suggested that L213Asp has a slightly lower pK_{aQ_B} than L210Asp, but about the same $pK_{aQ_B^-}$. Since the data available in this field do not clearly favor any hypothesis, we infer that both L210Asp and L213Asp contribute to the pH titration of K_2 detected between pH 3 and 6.

The respective influence of the M231Arg → Leu and M43Asn → Asp mutations on the dependence of K_2 at low pH is quite different. In the M43Asn → Asp mutant, the pK_a of the main group titrating at low pH (pK_{a1Q_B}) is essentially the same for the oxidized form of the quinone as that observed in the wild type (≈4.3). However, with the formation of the semiquinone, the pK_a of this group is shifted to a higher pH ($pK_{a1Q_B^-} \approx 5.4$) than in the wild type ($pK_{aQ_B^-} \approx 5.15$). The higher ΔpK_a observed in the mutant (≈1.05 in the M43Asn → Asp mutant vs 0.85 in the wild type) reflects a slightly increased interaction in this mutant of Q_B^- with L210Asp and/or L213Asp, due to its (their) interaction(s) with ionized M43Asp. Inspection of the structure of the *Rb. sphaeroides* RC (Figure 5; Chang et al., 1991) suggests that there would be a distance of about 5 Å between the carboxylic acid groups of M43Asp and L213Asp and about 10 Å between M43Asp and L210Asp. It is therefore likely that the pK_a of L213Asp is shifted more than that of L210Asp. However, considering that the distance between M43Asp and L213Asp would be short, it is surprising that the detected pK_a shift is so small. This small shift may again arise from strong screening of electrostatic effects in the vicinity of M43, which is rich in charged amino acid residues (Figure 5).

In the M231Arg → Leu mutant, a different pattern is observed for the pH dependence of K_2, which in this case displays two protonation waves ($pK_{a1Q_B^-} \approx 3.30$, $pK_{a2Q_B^-} \approx 6.95$). In the absence of M231Arg, which is salt-bridged with H125Glu and H232Glu in the wild-type *Rb. sphaeroides* structure (Figure 6), the negative electrostatic influence of the latter two groups is readily observable at the level of Q_B^- in both the wild-type and double-mutant backgrounds; thus, it is likely to increase the pK_as of the acid residues

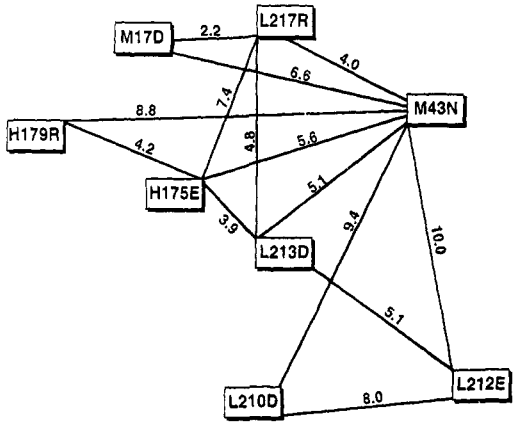

FIGURE 5: Schematic representation of the network of charged residues whose pK_as could be affected by the M43Asn → Asp mutation. Numbers represent the shortest distances, in Å, between side chain functional groups (O and N). Distances greater than 10 Å are not shown.

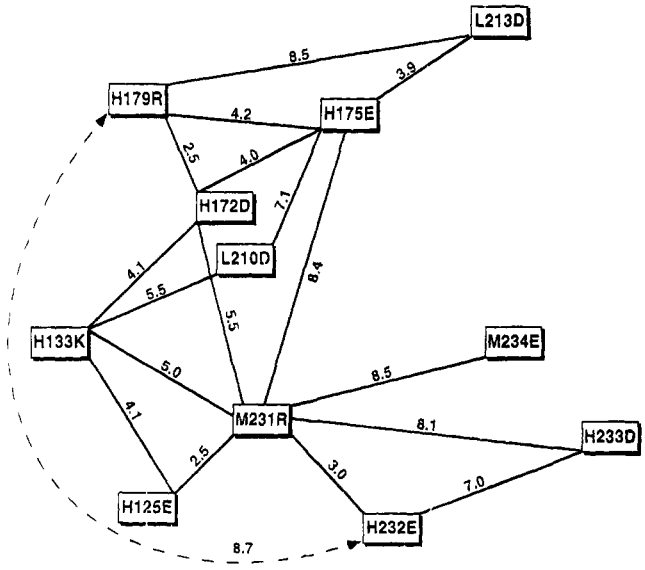

FIGURE 6: Schematic representation of the network of charged residues whose pK_as could be affected by the M231Arg → Leu mutation. Numbers represent the shortest distances, in Å, between side chain functional groups (O and N). Distances greater than 10 Å are not shown.

whose interactions with Q_B^- are observable in our experiments. Since L210Asp and L213Asp are equidistant from M231Arg (≈12 Å), one may expect similar pK_a shifts for these groups in the M231Arg → Leu mutant compared to the wild type. It is therefore reasonable to assume that the pH variations of K_2 above pH 6 in the M231Leu mutant are due to L210Asp and L213Asp and that their pK_as have been shifted by ca. 1.5−2 pH units due to the liberation of H125Glu and H232Glu. The distance between M231Arg and L210Asp and/or L213Asp leads to a rough calculation of a very low value of 10−14 for the local dielectric constant. In contrast, this distance would suggest a value of 43 ± 22 if the formula proposed by Warshel et al. (1984), $\epsilon = [1 + 60(1 - e^{-0.1r})](1 \pm 0.5)$ is applied. This latter value would agree more closely with the value that one may calculate in *Rb. sphaeroides* for the interaction between L213Asp and Q_B^-, ≈40 (Takahashi & Wraight, 1992), or in *Rps. viridis* for the interaction between L217Arg and Q_B^-, ≈47 (Baciou et al., 1991). The apparently very low dielectric constant calculated from the data obtained from the M231Leu mutant reflects the long distance effect of the M231Arg → Leu mutation on the pK_a shift. The group which titrates at very low pH (pK_{aQ_B} = 2.35, p$K_{aQ_B^-}$ = 3.4) in the pH dependence curves of K_2 in the M231Leu mutant has probably also had its pK_a shifted by this mutation and was probably titrating below pH 3 in the wild type. Candidates (Figure 6) for this group include H232Glu (>15 Å away from Q_B), H125Glu (14.0 Å away), H172Asp (12.3 Å away), and H175Glu (8.5 Å away). Although H175Glu is closer to Q_B, we favor the assignment of this pK_a shift to H172Asp since pK_as of aspartic acid residues tend to be lower than those of glutamic acids.

Mechanism for Long Distance Electrostatic Effects. The mechanism by which this interaction is propagated over such distances could possibly be explained by the following arguments. In the wild-type *Rb. sphaeroides* RC structure, this basic residue is involved in a shared salt bridge with H125Glu and H232Glu and is near other charged residues (Figure 6). Thus, it is possible that the disruption of this bridge causes a "domino" effect, modifying the interactions within a network of acidic and basic residues which surround M231Arg. Charged residues which are located within 10 Å of either of the amino groups of M231Arg include H125Glu (2.5 Å), H232Glu (3 Å), H133Lys (5 Å), H172Asp (5.5 Å), H233Asp (8.1 Å), H175Glu (8.4 Å), and M234Glu (8.5 Å) (Figure 6). These residues interact with each other and with other nearby charged residues (Figure 4). For example, H133Lys is equidistant (4.1 Å) from H172Asp and H125Glu; it is also 5.5 Å from L210Asp and 7.3 Å from L212Glu. Since this network is so extensive, a slight (1 Å) rearrangement of salt bridges [which has been observed in other systems (Knossow et al., 1984)] that might be caused by the mutational disruption of the H125Glu-M231Arg-H232Glu interaction could partially relocate the liberated negative charge much closer to the quinone, for example, on residues L210Asp, L213Asp, H172Asp, and/or H175Glu. The removal of the positive charge and the interaction of negative charges would increase the pK_as of these residues, thereby explaining the observed pH dependence of K_2 in the M231Arg → Leu mutant strain.

It is this interactive network of negatively and positively charged residues from the L, M, and H chains that forms the Q_B binding pocket, its environment, and, together with water molecules, channels for proton transfer. This network has a built-in redundancy. Although it is clear that the wild-type RC is the most efficient, these results underscore the flexibility of this finely tuned system in accommodating mutational changes that affect proton conduction and delivery to the buried Q_B site.

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NOTE ADDED IN PROOF

Reorganization of salt bridges, mainly involving side chain conformation changes, was observed in a variant (Arg158→Cys) of apolipoprotein-E (Wilson et al., 1994). Although the mutation is distant from the receptor binding

site (> 12 Å), it affects receptor binding, possibly by changing the electrostatic potential at that site.

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