In Bacterial Reaction Centers Rapid Delivery of the Second Proton to Q_B Can Be Achieved in the Absence of L212Glu†

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ABSTRACT: In the reaction center (RC) of *Rhodobacter capsulatus*, residue L212Glu is a component of the pathway for proton transfer to the reduced secondary quinone, Q_B. We isolated phenotypic revertants of the photosynthetically incompetent (PS^-) L212Glu→Gln mutant; all of them retain the L212Glu→Gln substitution and carry a second-site mutation: L227Leu→Phe, L228Gly→Asp, L231Arg→Cys, or M231Arg→Cys. We also characterized the L212Ala strain, which is a phenotypic revertant of the PS^- L212Glu-L213Asp→Ala-Ala mutant. The activities of the RCs of these strains—all of which lack L212Glu—were studied by flash-induced absorption spectroscopy. At pH 7.5, the rate of second electron transfer in the L212Q mutant is comparable to the wild-type rate. However, this mutant shows a marked decrease in the rate of cytochrome oxidation under strong continuous illumination and a very slow phase ($0.66\ s^{-1}$) of the proton transfer kinetics following the second flash, indicating that transfer of the second proton to Q_B is slowed more than 1000-fold. The levels of recovery of the functional capabilities in the revertant RCs vary widely; their rates of cytochrome oxidation were intermediate between those of the wild-type and the L212Q mutant. The kinetics of proton transfer following the second flash show a significant recovery in the L212Q + M231C and L212A RCs ($330-540\ s^{-1}$), but the L212Q + L227F RCs recover this function only partially. Compensation for the lack of L212Glu in revertant RCs is discussed in terms of (i) conformational changes that could allow water molecules to approach closer to Q_B and/or (ii) the increase in the negative electrostatic environment and the resultant rise in the free energy level of Q_B^- that is induced by the mutations. The stoichiometries of H^+/Q_B^- proton uptake below pH 7.5 in the L212Q mutant, the L212Q + M231C revertant, and the wild-type strains are essentially equivalent, suggesting that L212Glu is protonated at neutral pH in wild-type RCs. This is also supported by the $P^+Q_B^-$ charge recombination data. Comparison of H^+/Q_B^- proton uptake data with those obtained previously for the stoichiometries of H^+/Q_A^- proton uptake [Miksovska, J., Maróti, P., Tandori, J., Schiffer, M., Hanson, D. K., Sebban, P. (1996) *Biochemistry 35*, 15411−15417] suggests that L212Glu is the key to the electrostatic and perhaps structural interaction between the two quinone sites.

The bacterial reaction center protein complex, whose structure is known to atomic resolution for two species, *Rhodopseudomonas 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., 1995), uses solar energy to catalyze transmembrane charge separation reactions. The core complex is composed of three protein subunits, L, M and H; the L and M subunits bind all of the cofactors involved in the multistep electron transfer reactions. Light initiates charge separation between a dimer of bacteriochlorophyll molecules, situated near the periplasmic side of the reaction center, and the quinone electron acceptor complex, which is located near the cytoplasmic face of the membrane. These events result in the double reduction and double protonation of a quinone molecule, Q_B, producing the dihydroquinone species, Q_BH_2. The exchange of this molecule by an oxidized quinone molecule provides the reducing power that is further used to produce ATP.

Q_B is part of a two-quinone electron acceptor system. The primary quinone acceptor of this system, Q_A, functions as a one-electron carrier and is bound to a relatively hydrophobic part of the reaction center complex. The secondary quinone Q_B acts as a two-electron acceptor and is more closely surrounded by a number of ionizable residues that contribute to its energetic and functional specificity. The first electron transfer to Q_A or Q_B does not involve a net proton transfer to the semiquinones themselves, but rather a substoichiometric proton uptake by ionizable residues whose pK_a values are shifted due to the formation of the Q_A^- and/or Q_B^- species (Maróti & Wraight, 1988; McPherson et al., 1988). In contrast, the second electron transfer from Q_A^- to Q_B^- is kinetically coupled to the uptake of the first proton to Q_B^- to form the $(Q_BH)^-$ species. It has recently been suggested that the delivery of the first proton precedes rate-limiting second electron transfer (Graige et al., 1996). Efficient delivery of the first proton to Q_B is critically dependent on the presence of L213Asp (Takahashi & Wraight, 1990; Paddock et al., 1994), whose carboxyl group is situated 6 Å

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from the O_5 carbonyl of Q_B. Results obtained with the L212Glu→Gln mutant from *Rb. sphaeroides* have implicated L212Glu in the delivery of the second proton to the $(Q_BH)^-$ species (Paddock et al., 1989; Takahashi & Wraight, 1992).

The role of L212Glu is of particular interest because of its unusual electrostatic behavior. Indeed, its apparent pK_a was suggested to be high in *Rb. sphaeroides* (9.8; Paddock et al., 1989) and also in *Rb. capsulatus* (10.1; Hanson et al., 1992a; Maróti et al., 1994). In addition, it plays a role in the electrostatic coupling between the two quinone protein pockets (Brzezinski et al., 1992; Tiede & Hanson, 1992; Maróti et al., 1995; Miksovska et al., 1996).

We previously constructed the L212Glu-L213Asp→Ala-Ala double mutant of *Rb. capsulatus* (Hanson et al., 1992b). This double mutant is incapable of photosynthetic growth, as is the L212Glu→Gln mutant of *Rb. sphaeroides* (Paddock et al., 1989). We isolated several photocompetent phenotypic revertants of the *Rb. capsulatus* L212Ala-L213Ala double mutant strain, and one of them carried a genotypic reversion of the L213Ala codon to Asp while the engineered Ala substitution at L212 remained. We were intrigued that the L212Glu→Ala mutant of *Rb. capsulatus* is photocompetent, while the L212Glu→Gln mutant of *Rb. sphaeroides* is not, especially since the two species share 90% sequence homology in the region surrounding Q_B. To determine whether the phenotypes were due to species-specific differences, we constructed the L212Glu→Gln mutant in *Rb. capsulatus* and found that this mutation, as in *Rb. sphaeroides*, renders this strain incapable of photosynthetic growth.

We isolated several independent spontaneous phenotypic revertants of the L212Glu→Gln mutant of *Rb. capsulatus* (Hanson et al., 1995). None of the revertants carried the single base transversion that would restore the wild-type Glu codon. Each carries a second-site substitution—L227Leu→Phe, L228Gly→Asp, L231Arg→Cys, or M231Arg→Cys—in addition to the original engineered L212Gln mutation. We have measured the stoichiometries of proton uptake by the Q_B^- state in two of the strains that lack L212Glu. The comparison between the wild-type data and those from the strains lacking L212Glu shows that this residue must be essentially protonated at neutral pH in the wild type. Our results show that some of the compensatory mutations alter the electrostatic environment of Q_B^- and others may have restored function by changing the structure of the Q_B binding pocket. Our data show that efficient second proton transfer can proceed in these reaction centers in the absence of L212Glu.

MATERIALS AND METHODS

Isolation and Genetic Characterization of Revertants. The selection and characterization of the L212Ala strain and construction of the L212Gln mutant have been described [respectively, Hanson et al. (1993) and Miksovska et al. (1996)], as have methods used for the selection of independent phenotypic revertants and their genetic characterization by complementation mapping (Hanson et al., 1993). In the text, the names used for revertant strains bearing suppressor mutations will contain both the original site-specific L212Glu→Gln mutation and the second-site mutation, e.g., L212Q + L227F; the strains used in this study are listed in Table 1. Phenotypic growth rates were determined by using dark-grown cultures to inoculate completely filled tubes of RCV medium (Weaver et al., 1975) containing kanamycin. After overnight incubation in the dark, the tubes were transferred to photosynthetic conditions (anaerobic, light intensity of 25 W m^{-2}, 26 °C). Turbidity was monitored with a Klett−Summerson colorimeter. Program "O" (Kleywegt & Jones, 1994) was used for computer modeling of mutant substitutions.

Table 1: Reaction Center Genotypes of *Rb. capsulatus* Strains Used in This Study

strain	genotype					phenotype
	L212E	L227L	L228G	L231R	M231R	
wild type	L212E	L227L	L228G	L231R	M231R	PS^+
L212Gln	Q					PS^-
L212Q + L227F	Q	F				PS^+
L212Q + L228D	Q		D			PS^+
L212Q + L231C	Q			C		PS^+
L212Q + M231C	Q				C	PS^+
L212A	A					PS^+

Absorption Spectroscopy. Cells were grown semi-aerobically in the dark on RPYE medium (Hanson et al., 1992a) containing 30 μg/mL kanamycin to select the plasmid. Reaction centers were isolated as described by Baciou et al. (1993).

Buffers (10 mM) used were as follows: 2-(*N*-morpholino)-ethanesulfonic acid (MES; Sigma) between pH 5.5 and pH 6.5; 1,3-bis[tris(hydroxymethyl)methylamino]propane] (Bis-Tris propane; Sigma) between pH 6.3 and pH 9.5; 3-(cyclohexylamino)propanesulfonic acid (CAPS; Calbiochem) above pH 9.5.

Charge Recombination Kinetics. The $P^+Q_B^-$ charge recombination kinetics were measured at 865 nm on a homemade absorbance-change spectrophotometer as described (Baciou et al., 1993), using a pulsed Yag laser at 532 nm (200 mJ/pulse) for the actinic pulses.

Determination of Kinetics and Stoichiometries of Proton Uptake. The stoichiometries of proton uptake were determined by using pH indicator dyes (bromocresol purple, phenol red, cresol red, or chlorophenol red depending on the pH) at 585 nm (isosbestic point for P^+ absorbance changes) or by using a pH electrode. The assay solution contained 1 μM reaction centers, 50 mM NaCl, 0.03% Triton X100, 100 μM ferrocene, and 60 μM UQ_6. The buffer concentrations were kept below 10 μM by extensive dialysis. The amplitudes of proton uptake by the $PQ_AQ_B^-$ state ($\Delta H^+_{QAQB^-}$) were corrected for the observed proton uptake after the first flash (ΔH^+_{obs}) as previously demonstrated (McPherson et al., 1993):

$$\Delta H^+_{Q_AQ_B^-} = \frac{\Delta H^+_{obs} - [\delta + \alpha(1-\delta)]\Delta H^+_{Q_A^-}}{(1-\alpha)(1-\delta)} \quad (1)$$

where ΔH_{QA^-} represents the proton uptake by the reaction centers in the absence of added quinone, δ is the occupancy of the Q_B site, α is the partition coefficient between the $Q_A^-Q_B$ and $Q_AQ_B^-$ states. Values of α and δ were determined from the charge recombination process (865 nm) and the ratio of the cytochrome *c* oxidation observed with the second vs the first flash, measured at 550 nm.

The kinetics of proton uptake were determined at pH 7.5 by measuring the absorbance change at 585 nm in the presence of dye. The measurements were performed with 1−2 μM reaction centers, 50 mM NaCl, 0.03% Triton, 100 μM ferrocene, 500 μM potassium ferrocyanide, 60 μM UQ_6,

Table 2: Reaction Center Functional Properties of *Rb. capsulatus* Strains Used in This Study

strain	rate constant of proton uptake after the second flash, pH 7.5 (s^{-1})	rate of cytochrome photooxidation pH 7.5 (cyt/RC/s)	rate of second electron transfer k_{AB}(2), pH 7.5 (s^{-1})	doubling time (h)
wild type	950	475	2300	8.5
L212Q[a]	fast phase 780 slow phase 0.66	28	1300	>>30
L212Q + L228D[b]	nd	245	nd	16.1
L212Q + M231C[b]	540	254	800	8.5
L212Q + L227F[b]	fast phase 170 slow phase 1.86	90	700	9.0
L212Q + L231Cys[b]	nd	200	1800	11.9
L212A[b]	330	135	1000	8.3

[a] Site-specific mutant, photosynthetically incompetent. [b] Phenotypic revertant strains. nd, not determined.

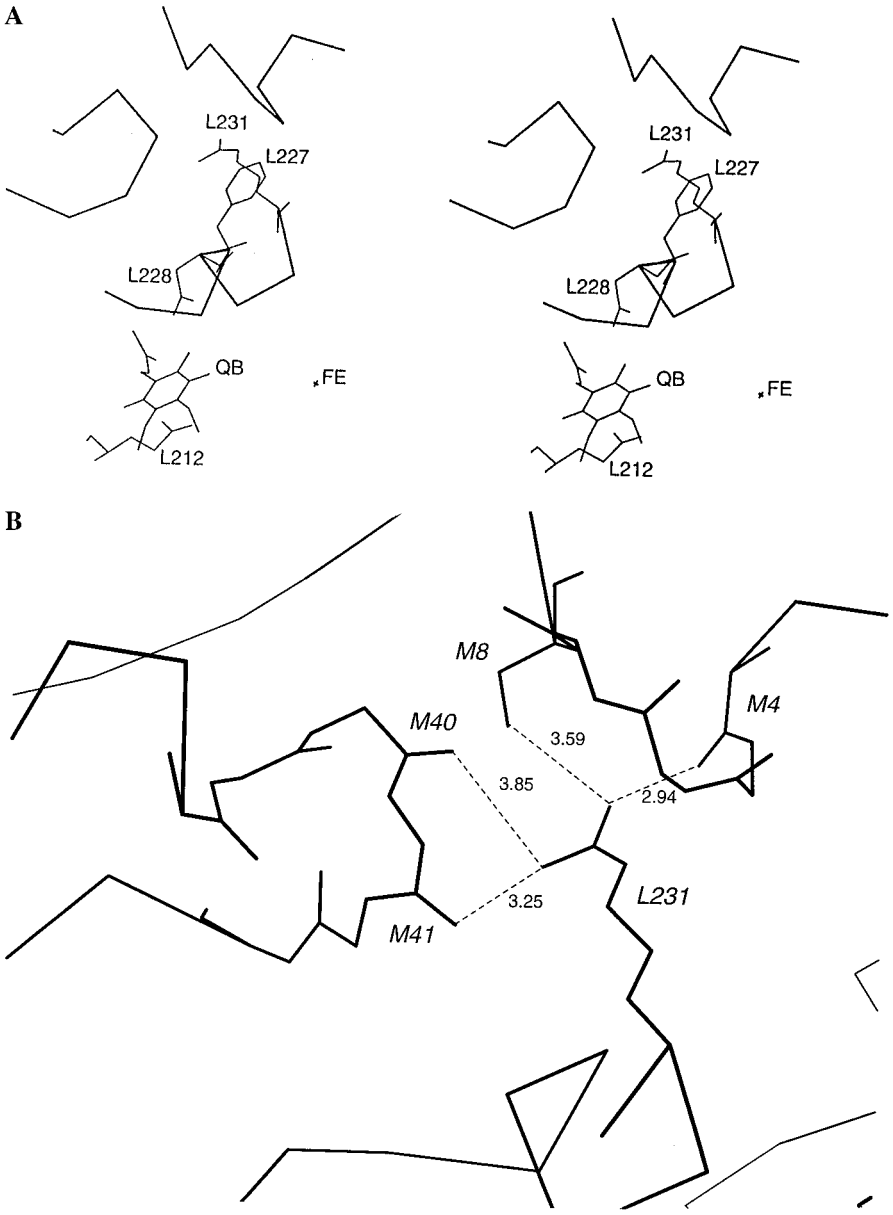

FIGURE 1: (A) Stereoview of a molecular model based on the *Rb. sphaeroides* reaction center structure (Chang et al., 1991) showing the relative locations of L212Glu, Q_B, and various residues that are discussed in the text. Models of substitutions at L227 (Leu→Phe) and L228 (Gly→Asp) that occur in two of the phenotypic revertants are shown; the placement of these residues in this figure does not take into account any potential rearrangment of the peptide backbone (see text for discussion). Wild-type residue L231Arg, which is shown in the figure, is replaced by Cys in another of the phenotypic revertants. [For the relative position of residue M231Arg and the residues with which it forms a salt bridge, the reader is referred to Sebban et al. (1995b).] (B) Molecular model, using the *Rb. sphaeroides* reaction center structure (Chang et al., 1991), showing possible hydrogen bonds formed by residue L231Arg that tether this segment of the L chain to two different segments of the M chain.

and 40 μM *o*-cresol red. The calibrations were performed by adding known amounts of HCl. The net proton uptake was obtained by subtracting the buffered signal from unbuffered traces.

Cytochrome Photooxidation. These measurements were made under conditions of strong continuous light (1 W/cm^2) in the presence of 5 mM UQ_0. A solution of 4 mM cytochrome *c* was vortexed few seconds with 40 mM sodium ascorbate to reduce it prior to the experiments. This solution was loaded onto a PD-10 column containing Sephadex G-25M gel (Pharmacia). Reduced ascorbate-free cytochrome *c* was collected; free sodium ascorbate was retained by the column. The absence of sodium ascorbate during the measurements prevents the reduction of part of the exogeneous quinone pool (UQ_0), which could artifactually decrease the rates of cytochrome oxidation.

RESULTS

Photosynthetic Growth Rates of Revertant Strains. Doubling times for the wild-type, L212Q mutant, and family of phenotypic revertants are shown in Table 2. Under the conditions of this experiment, revertant strains L212A and L212Q + M231C have essentially wild-type rates of growth. The L212Q + L227F revertant strain has only a slightly slower rate of growth even though the functional capabilities of its reaction centers are significantly different from those of the wild type (see below). Doubling times for the L212Q + L231C and L212Q + L228D revertant strains are much longer than that of the wild type. In the case of the latter strain, this may arise from a very low occupation of the Q_B pocket due to steric hindrance induced by the presence of Asp in position L228 (Gly in the wild type). No growth of the L212Q mutant strain was observed during the course of the experiment (>7 days).

pH Dependence of the Rate of $P^+Q_B^-$ Charge Recombination. The pH dependencies of the rate of $P^+Q_B^-$ charge recombination kinetics (k_{BP}) measured in the L212Gln mutant, the L212Ala revertant, and the L212Q + M231C, L212Q + L227F and L212Q + L231C phenotypic revertants are compared in Figure 2 with k_{BP} for the wild-type *Rb. capsulatus* reaction centers [previously measured; Baciou et al. (1993)]. At neutral pH, all the strains display higher values for k_{BP} than the wild type. Since the rates of the $P^+Q_A^-$ charge recombination (k_{AP}) are essentially the same (within 10%) in all revertant strains, the relative k_{BP} values correspond to the variations in the free energy gap between the Q_A^- and Q_B^- states in the different mutant strains.

In general, the rates of $P^+Q_B^-$ charge recombination in reaction centers of the revertant strains are greater than that of the L212Q mutant, with the greatest rate observed in the L212A strain. The pH-dependent curves for k_{BP} of the L212Q + L227F, L212Q + M231C, and L212Q + L231C reaction centers are intermediate between those of the L212Q mutant and the L212A strain. The curve for the L212Q + L227F strain shows no pH dependence in the pH range 5−11; its greatest rate (~20 s^{-1} at pH 7.7) is equivalent to that of the wild type at pH 10.2. The rate of charge recombination for the L212Q + M231C reaction center increases from pH 5.2 to pH 8 with a pH dependence that is parallel to that of the wild type. Between pH 8 and pH 9, k_{BP} decreases slightly. The highest value for k_{BP} (~30 s^{-1} at pH 7.8) is equivalent to that of the wild type at pH 10.7. The k_{BP} curve for the L212Q + L231C reaction center is independent of pH in the pH range 4.8−6, it increases from pH 6 to pH 7.5 and then decreases from pH 7.5 to pH 9.5. Its maximum value of ~40 s^{-1} is reached at pH 7.4.

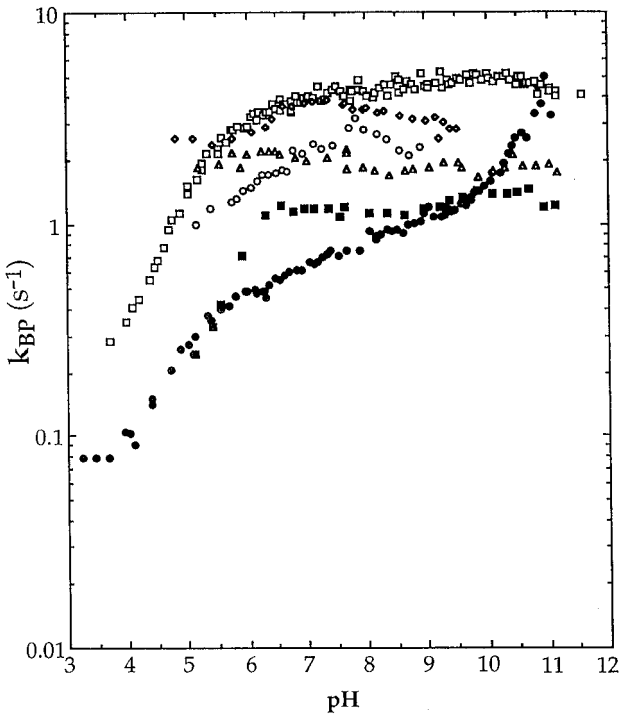

FIGURE 2: pH dependence of the rates of $P^+Q_B^-$ charge recombination in reaction centers from *Rb. capsulatus*: wild type (●), the PS$^-$ L212Q mutant (■), and phenotypic revertant strains L212Q + M231C (○), L212Q + L227F (△), L212Q + L231C (◊), and L212A (□). Conditions: 0.05% LDAO or 0.03% Triton X-100 buffers depending on the pH, as indicated in the text; 50−75 μM UQ_6 added.

In the L212Q + M231C and L212Q + L231C revertant reaction centers, the pH titrations of k_{BP} could not be extended above pH 9 and pH 9.5, respectively, because of the weak binding of Q_B at high pH. Also, with the exception of the L212A strain, the reaction centers from all strains lacking L212Glu were very unstable below pH 6, thus the curves do not extend to low pH.

In the wild type, k_{BP} increases substantially above pH 10. In contrast, no pH dependence for k_{BP} above pH 10 is observed for reaction centers of the L212Q mutant, the L212Ala strain, or the L212Q + L227F strain. This observation supports the hypothesis that the deprotonation of L212Glu contributes to the destabilization of Q_B^- above pH 10 in the wild-type *Rb. capsulatus* reaction centers (Sebban et al., 1995a; Miksovska et al., 1996). Similar observations have been reported by Paddock et al. (1989) for the L212Glu→Gln mutant of *Rb. sphaeroides*. It is of interest to note that a lower free energy gap between Q_A^- and Q_B^- is detected in the L212Ala strain as compared to the L212Q mutant. This result suggests a greater stabilization of Q_B^- (a more positive environment) when position L212 is occupied by Glu or Gln as opposed to Ala.

Proton Uptake upon Formation of Q_B^-. We have previously reported the pH-dependent curve of the proton uptake due to the formation of Q_B^- in the wild-type reaction centers of *Rb. capsulatus* (Figure 3) (Sebban et al., 1995a). We had shown that this curve can be fitted reasonably well assuming the existence of a minimum of five groups. The line drawn in Figure 3 represents this fit according to:

$$H^+/Q_B^- = \sum_i \left(\frac{1}{1 + 10^{(pH - pK_{aiQB^-})}} - \frac{1}{1 + 10^{(pH - pK_{aiQB})}} \right) \quad (2)$$

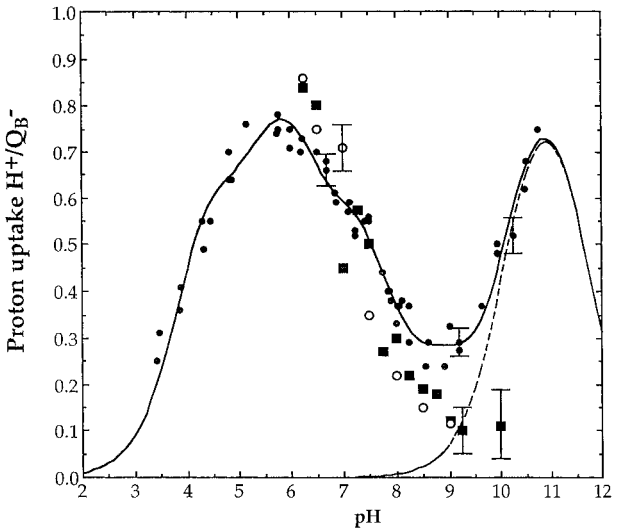

FIGURE 3: pH dependence of the stoichiometries of proton uptake by the PQ_B^- state in reaction centers from the wild-type *Rb. capsulatus* (●), the PS^- L212Q mutant strain (■), and the L212Q + M231C revertant strain (○).The line drawn represents the fitting of the stoichiometry of proton uptake in wild-type reaction centers [data from Sebban et al. (1995a)]. The dotted line represents the fit corresponding to the group with the highest apparent pK_a, i.e., p$K_{ahighQB}$ = 10.10 ± 0.15 and p$K_{ahighQB^-}$ = 11.60 ± 0.4. Conditions: 2 μM reaction centers, 50 mM NaCl, 0.03% Triton X-100, 100 μM ferrocene, 50−75 μM UQ_6, 40 μM dye (bromocresol purple, phenol red, cresol red, or chlorophenol red depending on the pH); 585 nm. Data obtained with a glass pH electrode and using pH indicator dyes are superimposed.

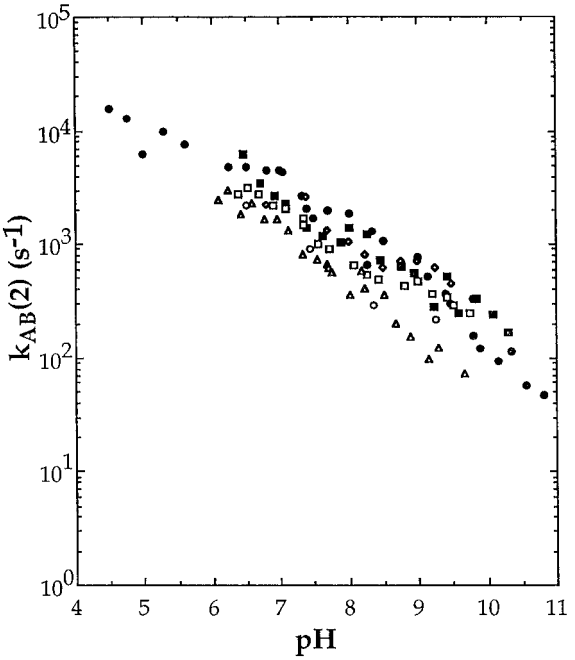

FIGURE 4: pH dependence of the rates of second electron transfer from Q_A^- to Q_B^- in reaction centers from *Rb. capsulatus*: wild type (●), the PS^- L212Q mutant (■), and phenotypic revertant strains L212Q + M231C (○), L212Q + L227F (△), L212Q + L231C (◇), and L212A (□). Conditions: 2 μM reaction centers, 0.05% LDAO or 0.03% Triton X-100 buffers depending on the pH, as indicated in the text, 20 μM cytochrome *c*, 200 μM sodium ascorbate; 75 μM UQ_6 added; 450 nm.

where pK_{aiQB^-} and pK_{aiQ_B}, respectively, represent the pK_a of group *i* interacting with Q_B and Q_B^-. The associated pK_a shift, ΔpK_{ai}, relates to the coulombic interaction between this group and the semiquinone species by the following formula:

$$\Delta G^\circ = -\ln(10)\, kT(pK_{aiQ_B^-} - pK_{aiQ_B}) = -0.058 \text{ eV } \Delta pK_{ai} \quad (3)$$

The group with the highest pK_a ($group_{high}$) has apparent values of pK_{aQB} = 10.3 ± 0.3 and pK_{aQB^-} = 11.6 ± 0.5 (dashed line in Figure 3). We associated $group_{high}$ with L212Glu, either directly or indirectly. As shown in Figure 3, the H^+/Q_B^- amplitudes measured in two reaction centers lacking L212Glu (the L212Q mutant and revertant strain L212Q + M231C) fall well below the wild-type values above pH 8.5. These results show that the interaction between Q_B^- and $group_{high}$ described above is lost above pH 8.5 in the strains that lack L212Glu. Our results for H^+/Q_B^- proton uptake parallel results that we have previously reported for the H^+/Q_A^- amplitude of proton uptake when the wild type is compared to four different strains lacking L212Glu (Miksovska et al., 1996).

Interestingly, there is no significant difference between the proton uptake data for the L212Q, the L212Q + M231C, and the wild-type strains at pH 7.5. Below pH 7−7.5, however, it seems that the amplitude of the H^+/Q_B^- proton uptake in the L212-modified strains may be slightly higher than in the wild type (if further experiments show this difference to be significant beyond the experimental error).

Rates of Second Electron Transfer from Q_A^- to Q_B^-, $k_{AB}(2)$. The pH dependencies of the rates [$k_{AB}(2)$] of transfer of the second electron from Q_A^- to Q_B^-, measured at 450 nm, are presented in Figure 4 for all the strains. The wild-type curve, which was measured previously (Maróti et al., 1994), is also shown. The data for the L212Q and L212Q + L231C strains are the most similar to that of the wild type. The slopes of the data points for the L212Q + M231C and L212Q + L227F revertant strains parallel that of the wild type, but the overall rates for these two strains are 4-fold slower. The rate of second electron transfer is somewhat smaller for the L212A strain as compared to that of the wild type, and its slope is the same as that of the wild-type curve in the pH range 6−8. The slope decreases and $k_{AB}(2)$ for the L212A reaction center is the same as that of the wild type at pH 9. The rates for all strains vary between 2000 and 6000 s^{-1} at pH 6.5 to 100 to 500 s^{-1} at pH 9.5. The rates determined at pH 7.5, which show a 3-fold variability among the members of this set of strains, are summarized in Table 2.

Rates of Proton Transfer, k_{H^+}. In order to probe the functional recovery of the proton transfer process in the revertant strains, we have studied the kinetics of proton transfer after the second flash (k_{H^+}). The kinetics measured on slow (Figure 5) and fast (Figure 6) time scales were obtained by following the absorbance changes of the pH-sensitive dye cresol red at 585 nm, at pH 7.5. The calibrations of the stoichiometries of proton uptake and the measurements of the kinetics for the wild-type, the L212Q mutant, and the L212Q + M231C revertant strains were achieved as described in the methods. In the case of the L212A and L212Q + L227F revertant strains, the H^+/Q_B^- amplitudes were normalized to two protons after the second flash.

In the wild type, the second flash induces a fast (millisecond time range) uptake of about 1.4 protons. In the L212Q mutant, however, only about two-thirds of the amplitude of proton uptake on the second flash appears to

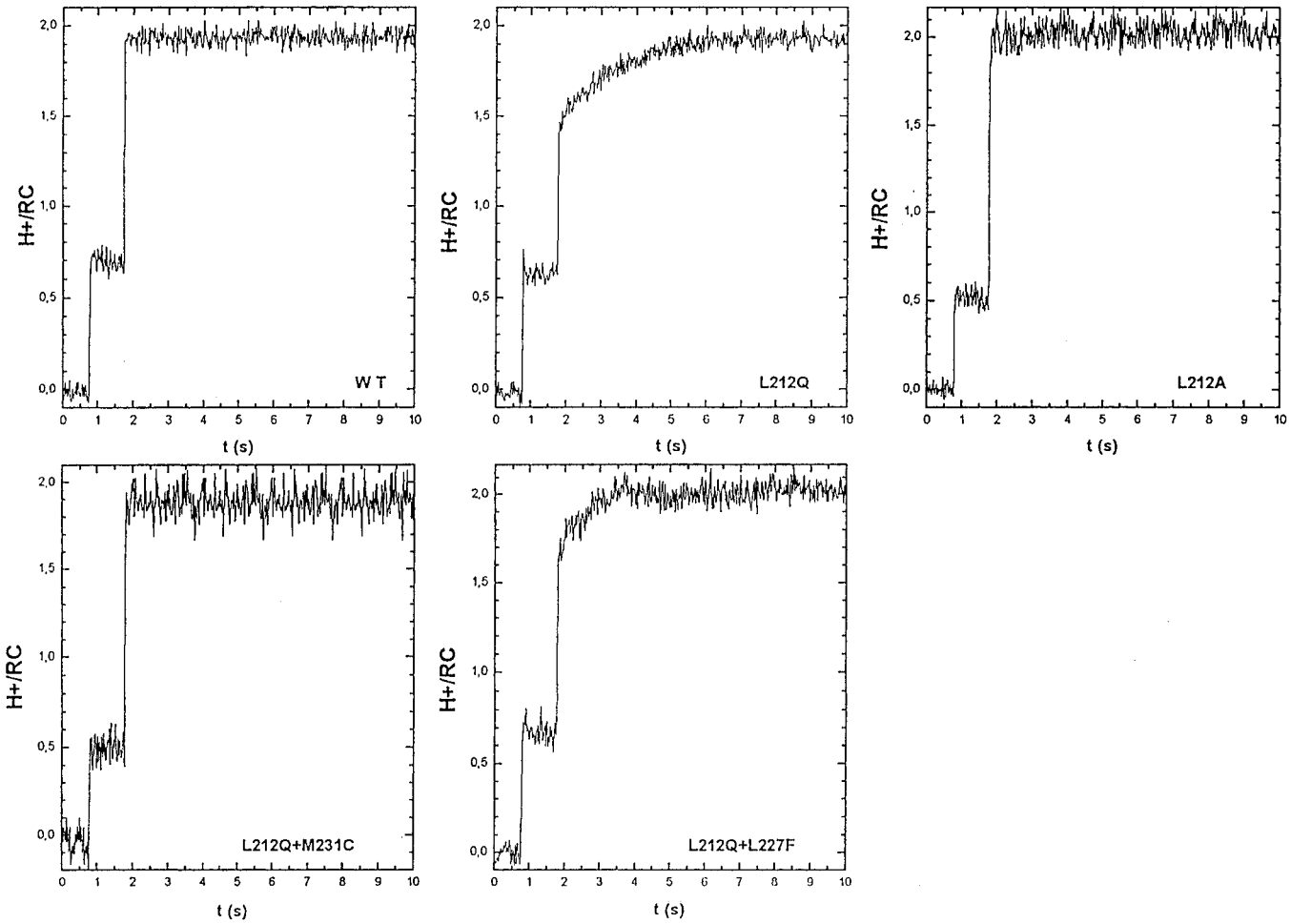

FIGURE 5: Stoichiometries and kinetics of proton uptake after the first and the second flash in the reaction centers from the wild-type *Rb. capsulatus*, the PS^- L212Q mutant, and the L212A, L212Q + M231C, and L212Q + L227F revertant strains. Conditions as for Figure 3 + 500 μM potassium ferrocyanide. Dye: cresol red. The data from the L212A and from the L212Q + L227F strains are normalized to $2H^+$ taken up per reaction center after the second flash.

occur on the fast time scale, with the remaining portion occurring on a very slow time scale with a rate of 0.66 ± 0.1 s^{-1}. This behavior has already been observed in the same mutant from *Rb. sphaeroides* (Takahashi & Wraight, 1992; Paddock et al., 1994). Since we have observed similar k_{AB}-(2) values in the L212Q mutant and the wild type, it is likely that the slow proton uptake measured on the second flash in the L212Q mutant reflects slow delivery of the second proton. Interestingly, only the fast phase of proton uptake was observed in the L212A and the L212Q + M231C strains. However, in the L212Q + L227F reaction center, an intermediate situation is observed. The amplitude of the slow phase of proton uptake on the second flash is smaller (about one-fourth of the total uptake occurring with the second flash), and the rate is accelerated 3-fold (≅1.86 ± 0.2 s^{-1}) in comparison to the L212Q strain. This suggests only a partial recovery of the proton transfer capabilities in the L212Q + L227F reaction center.

The kinetics of the fast portion of proton transfer observed on the second flash (Figure 6) were found to be biphasic in all of the strains studied. The values presented in Table 2 represent the average rates. In the wild type, the rate is 950 ± 50 s^{-1}; this rate was not substantially diminished in the L212Q mutant (780 ± 100 s^{-1}). In the L212Q + M231C revertant strain, the rate is 540 ± 100 s^{-1}, that of the L212A revertant is 330 ± 75 s^{-1}, and a somewhat slower rate of 170 ± 50 s^{-1} is observed in reaction centers of the the L212Q + L227F revertant strain.

Effect of Flash Repetition on the Cytochrome c Oxidation. Rate-limiting processes in the photocycle of the reaction centers can be tracked by determining—as a function of the rate of flash repetition—the extent of damping of the amount of oxidized cytochrome *c* formed after on the third saturating flash relative to the second one (Δ_3/Δ_2). We have chosen to plot the Δ_3/Δ_2 ratio instead of the Δ_3/Δ_1 ratio, since the latter ratio is much less sensitive to the occupancy of the Q_B site, which varies greatly in reaction centers of the L212Q revertant family. The results of the experiment conducted at pH 7.8 are shown in Figure 7. The saturating laser flash repetition was varied from 1 to 25 Hz (1000 vs 40 ms between flashes, respectively). Clearly, no damping of the Δ_3/Δ_2 ratio is observed, with the exception of the L212Q mutant strain. The Δ_3/Δ_2 ratio determined at 25 Hz in this mutant drops to about half of the ratio measured at low flash repetition.

Rates of Cytochrome Oxidation under Strong Continuous Illumination. The rates of photooxidation of added cytochrome *c* under continuous illumination measured at 550 nm, at pH 7.8, are presented in Figure 8. In the wild type, the measured rate is >500 cyt_{ox}/RC/s, similarly to what was previously measured in the reaction centers from *Rb. sphaeroides* (Paddock et al., 1994; Tandori et al., 1995). The rate of cytochrome oxidation is strongly diminished in the L212Q mutant (28 ± 5 cyt_{ox}/RC/s). This rate is measured

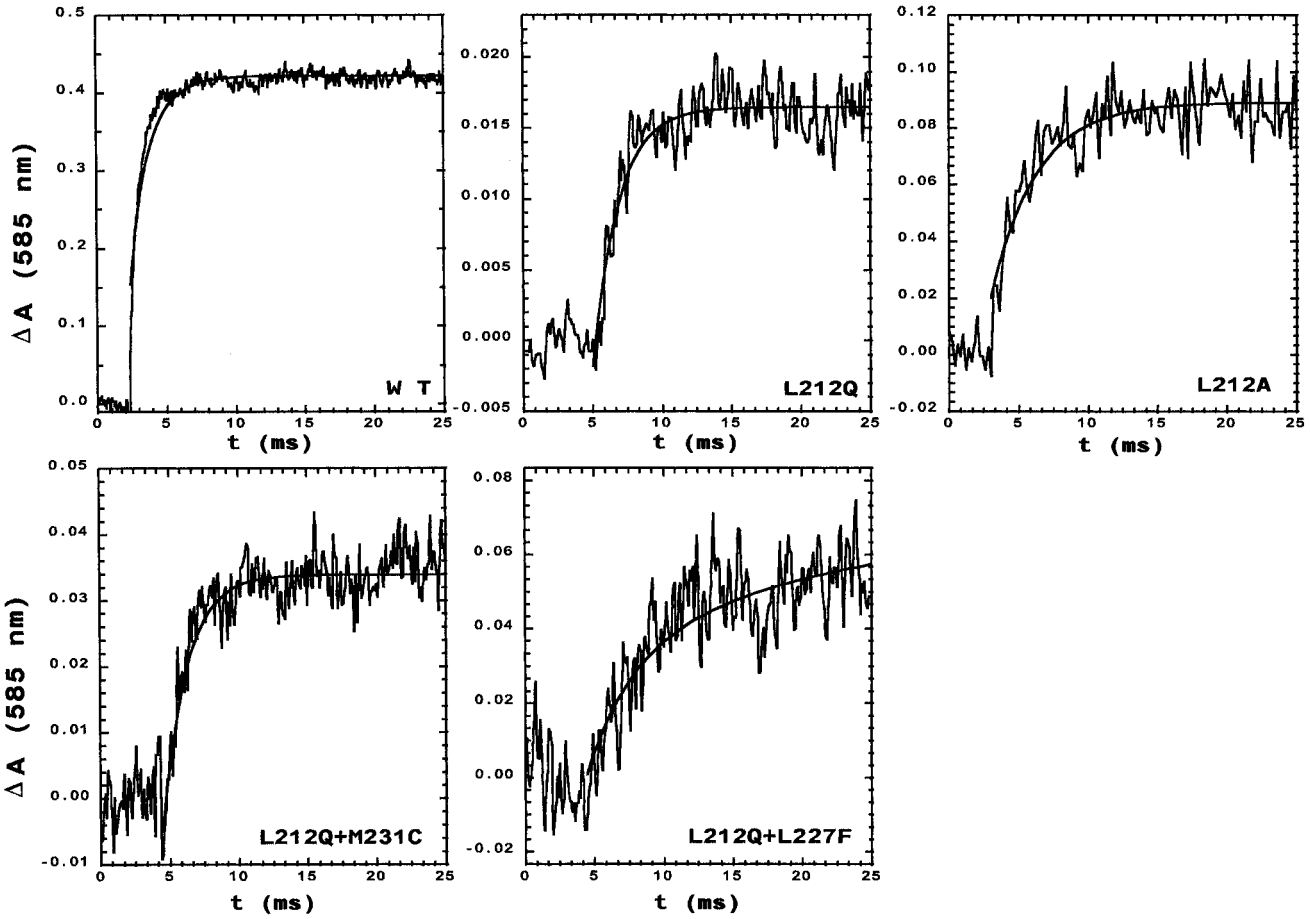

FIGURE 6: Kinetics of fast proton uptake on the second flash in the reaction centers from the wild-type *Rb. capsulatus*, the PS$^-$ L212Q mutant, and the L212A, L212Q + M231C, and L212Q + L227F revertant strains. Conditions as for Figure 3. The kinetic rate constants derived from fitting of these curves are presented in Table 2. The lines through the curves represent these fits.

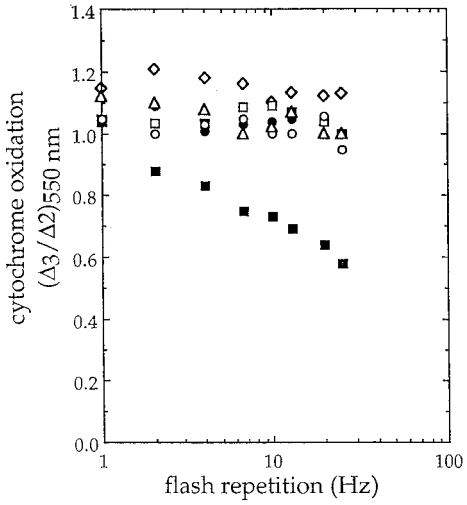

FIGURE 7: Effect of flash repetition on the cytochrome *c* oxidation turnover. The ratio Δ_3/Δ_2 represents the amount of cytochrome oxidized on the third flash relative to that of the second one: wild type (●), the PS$^-$ L212Q mutant (■), and phenotypic revertant strains L212Q + M231C) (○), L212Q + L227F (△), L212Q + L231C (◇), and L212A (□). Conditions as in Figure 4; 1 μM reaction centers.

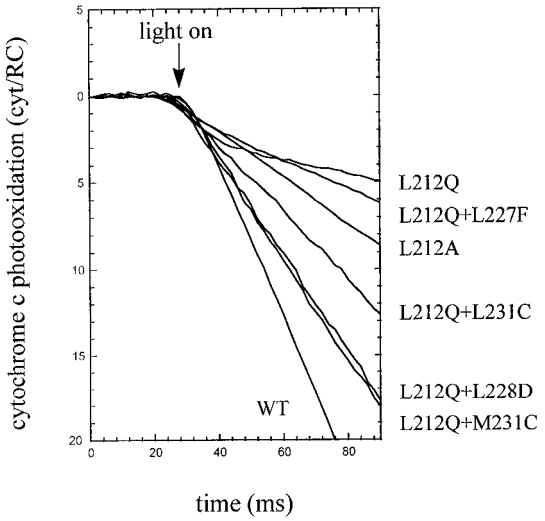

FIGURE 8: Rates of photooxidation of exogenous cytochrome *c* under continuous illumination in the reaction centers from the wild-type *Rb. capsulatus*, the PS$^-$ L212Q mutant, and the family of phenotypic revertant strains. The measured rate values are presented in Table 2. The values in parentheses represent the oxidation rates, i.e., the amount of cytochrome oxidized per reaction center per second. Conditions: 1 μM reaction centers, 10 mM Tris (pH 7.8), 20 μM reduced cytochrome *c*, 5 mM UQ_0; 550 nm.

after 2.5−3 cytochromes have been rapidly oxidized. Interestingly, a whole range of rates is measured in the various revertant reaction centers; in decreasing order, those rates are L212Q + M231C (254 ± 20 s^{-1}) > L212Q + L228D (245 ± 20 s^{-1}) > L212Q + L231C (200 ± 15 s^{-1}) > L212A (135 ± 10 s^{-1}) > L212Q + L227F (90 ± 5 s^{-1}). The Q_B binding sites of the strains lacking L212Glu were not fully occupied, even with the addition of 5 mM UQ_0. We have therefore corrected the cytochrome oxidation rates for the reduction in the amount of quinone binding.

DISCUSSION

Overview of Results. In *Rb. sphaeroides*, it has previously been shown that L212Glu participates in the delivery of the second proton to Q_B (Paddock et al., 1989; Takahashi & Wraight, 1992; Shinkarev et al., 1993; McPherson et al., 1994). We show here that the situation is similar in the *Rb. capsulatus* reaction center, but that other residues can substitute for L212Glu to restore a quasi-normal function. These compensatory mutations appeared in spontaneous photocompetent phenotypic revertant strains isolated from the PS^- L212Glu→Gln mutant. Rates of transfer of the second electron are not dramatically affected by the absence of L212Glu in the L212Q mutant or any of the revertant strains, which is the expected result if L212Glu is not involved in the delivery of the first proton to Q_B. Our data show that the extent of recovery of the other functional capabilities of the reaction centers from the revertant strains appears to vary quite widely. The mechanisms by which the delivery of the second proton is accelerated are probably different in the various revertant strains; however, they have some features in common. We begin with a discussion of the effects of the engineered L212Glu→Gln substitution.

Site-Specific Mutant L212Glu→Gln. The involvement of L212Glu in the delivery of the second proton to Q_B is readily apparent from the measurements of the kinetics of proton uptake (Figures 5 and 6), which yield a fast rate for uptake of the first proton but a much slower rate for uptake of the second proton. At pH 7.5, the latter step occurs on a time scale of a few seconds, i.e., more than 1000 times slower than in the wild type. The rate of cytochrome oxidation under strong illumination is substantially reduced in this mutant. In the case of the wild type, the kinetic bottleneck for cytochrome turnover probably occurs at the step of the replacement of Q_BH_2 by an oxidized quinone and/or is dependent on the light intensity (S. Osvath and P. Maróti, unpublished data). Since the second electron transfer rate of the L212Q mutant is similar (within a factor of 2) to that of the wild type, it is likely that the slow cytochrome turnover rate observed in the L212Q mutant arises from rate-limiting delivery of the second proton leading to slow formation of the quinol. This kinetic limitation in the cycling of the L212Q reaction centers is confirmed by observation of the damping of the cytochrome oxidation that occurs as the flash repetition rate is increased (Figure 7). The rate of cytochrome turnover that is measured for the L212Q mutant under strong continuous illumination (~28 cyt_{ox}/RC/s) may seem high as compared to the very slow rate observed for transfer of the second proton that was measured in Figure 5 (~0.66 s^{-1}). However, it was previously pointed out (Paddock et al., 1989; Shinkarev et al., 1993) that the apparent disparity between the rates of cytochrome turnover and the second proton transfer in the L212Q mutant arises from the ability to rapidly form the $PQ_A^-(Q_BH)^-$ state, even in reaction centers where the delivery of the second proton to Q_B is impaired. The transfer of the second proton with the third flash [where reaction centers are in the $Q_A^-(Q_BH)^-$ state] may be substantially faster than proton uptake following the second flash since formation of Q_A^- itself accelerates the proton uptake kinetics by the $(Q_BH)^-$ state (McPherson et al., 1994). One may therefore expect that under strong illumination the apparent cycling rate may be much higher than expected in light of the kinetic bottleneck of the delivery of the second proton to Q_B^- after two flashes. In *Rb. sphaeroides* a value of ~12 cyt_{ox}/RC/s was measured for the same mutant (Okamura & Feher, 1992).

Revertant L212Ala. At position L212, the native glutamic acid can be substituted effectively by a nonpolar alanine residue. The mutation of Gln to Ala results in a reaction center in which cytochrome oxidation (135 cyt_{ox}/RC/s) and second proton transfer kinetics are greatly accelerated relative to the L212Gln reaction center. If we assume that L212Glu transfers a proton, perhaps obtained from L213Asp (Okamura & Feher, 1992; Takahashi & Wraight, 1992), to the neighborhood of the O2 atom of Q_B, then Gln at L212 cannot bind the proton and actually blocks its pathway. On the other hand, the smaller Ala side chain could permit a water molecule to occupy the position of the carboxyl group of the normal glutamic acid side chain and thus may substitute for it. While the Gln replacement prevents rapid delivery of the second proton to Q_B^-, the Ala substitution allows for a functional reaction center. This role of L212Glu is, to some extent, analogous to the function of L223Ser in the delivery of the first proton. It was shown that the L223Ser→Ala mutant of *Rb. sphaeroides* is photosynthetically incompetent, but if the smaller residue Gly replaces L223Ser, the functionality of the reaction centers is restored (Paddock et al., 1995). Thus, in contrast to its ability to efficiently replace L212Glu, Ala cannot replace L223Ser. This may result from the already smaller side chain size of Ser as compared to Glu. An alanine substitution at L212 leaves room for a water molecule whereas, at L223, an even smaller residue with no side chain, Gly, is required.

Second-Site Compensatory Mutation L227Leu→Phe. In reaction centers where the L212Gln mutation is present, a second mutation—L227Leu→Phe—is one type of substitution that can restore the photocompetent phenotype. This mutation does not change the net charge of the system. The reaction center of the L212Q + L227F strain is the least efficient revertant of the family that is characterized here; recovery of the proton transfer capabilities in this revertant reaction center is incomplete. The rate of cytochrome oxidation in the L212Q + L227F reaction center (≅90 cyt_{ox}/RC/s) is increased only 3-fold as compared to the L212Q mutant. The kinetics of proton uptake are biphasic; the rate for the fast phase is reduced 5-fold in comparison to the wild-type value. Similar to the observations with the L212Q mutant, a portion of the proton uptake in this strain (25% of the amplitude) occurs with a rate that is approximately 500-fold slower than that of the wild type. The rate for this fraction of the proton uptake in the L212Q + L227F strain is accelerated from that of the L212Q mutant, but by only a factor of 3.

The most likely hypothesis for the acceleration of the proton transfer process in the reaction center of the L212Q + L227F revertant as compared to the L212Q mutant is a conformational change affecting the Q_B site. Modeling of the replacement of residue L227Leu with Phe suggests that this large ring can be accommodated in the structure (Figure 1A). This residue could lie within 3.5 Å of L231Arg. It is possible that π-orbital interactions between them could pull L227Phe toward L231Arg, therefore displacing that segment of the L chain away from Q_B. This movement of the protein backbone could allow a water molecule to be accommodated near Q_B. Also, the $\pi-\pi$ interactions between Phe and Arg may partially neutralize the positive charge of the arginine

(Flocco & Mowbray, 1994; Dougherty, 1996), yielding the 30 meV increase in the free energy level of Q_B (pH 7.5) and the accompanying acceleration of the second proton transfer to Q_B that is reminiscent of other strains where suppressor mutations substitute charged residues.

In *Rhodopseudomonas viridis*, changes in the free energy level of semiquinones (± 30 meV) have been observed in strains where mutations were suggested to either establish (with Q_A^-; Baciou et al., 1991) or interrupt (with Q_B^-; Baciou et al., 1991) planar $\pi-\pi$ interactions between the rings of a phenylalanine residue and the quinone. For a similar interaction to take place in the in L212Q + L227F revertant reaction center, a significant structural rearrangement would have to occur in order to bring L227Phe to a position that is parallel to and within 3 Å of the Q_B ring. The nature of our results, however, does not suggest a substantial conformational rearrangement for the L212Q + L227F reaction center, primarily because its properties are fairly similar to those of the L212Q reaction center.

Compensatory Mutations L228Gly→Asp and L231Arg→Cys. Two other second-site mutations, L228Gly→Asp and L231Arg→Cys, yield functional reaction centers when they occur in tandem with the engineered L212Q mutation. These mutations may affect both the charge distribution near Q_B and the conformation of the Q_B binding site. The reaction centers from these two strains display relatively fast cytochrome oxidation rates (≅245 and ≅200 cyt_{ox}/RC/s, respectively). The free energy level of Q_B^- in both of these revertant reaction centers is higher than that of the wild type. Unfortunately, because of the low occupancy of the Q_B site and because of the bad dye signal in the reaction centers from these strains, we were not able to measure the kinetics of proton transfer with reasonably good ratios of signal to noise.

The L228Gly→Asp mutation introduces a potential negative charge close to the Q_B pocket. Further modeling of this change, using the crystal structure of the *Rb. sphaeroides* reaction center (Chang et al., 1991), shows that any orientation of the long side chain of Asp at position L228 crowds neighboring atoms of the main chain, Q_B, or other side chains. Thus, a conformational change of this chain segment that forms a part of Q_B pocket would be required to accommodate the Asp substitution. This may provide room for water molecules to approach Q_B.

The L231Arg→Cys mutation removes an Arg residue and replaces it with a neutral one, as seen in the cases of the M231Arg→Leu (Hanson et al., 1992a, 1993) and M231Arg→Cys (see below) suppressor mutations. Unlike the situation for M231Arg, conserved residue L231Arg acts as a structural arginine (e.g., Borders et al., 1994), forming possible hydrogen bonds with carbonyl oxygen atoms of the protein backbone of the M chain (M4, M40, and M41) and with the side chain of M8Ser (Figure 1B). These hydrogen bonds tether this segment of the L chain near Q_B to two different loops of the M subunit that are part of the second sphere of residues that isolate Q_B from the aqueous environment. In addition to the loss of the positive charge with the Arg→Cys mutation, the loss of these hydrogen bonds may lead to a major rearrangement in this region of the protein that could change the orientations of protonatable groups and water molecules, thereby opening up a substitute proton delivery pathway to the quinone. A large conformational change has been observed in the L222Tyr→Phe mutant of *Rps. viridis*, which was induced by the loss of a structural interchain hydrogen bond between L222Tyr and M43Asp (Sinning et al., 1989).

Intergenic Suppressor Mutation M231Arg→Cys. The final revertant of this series is the L212Q + M231C strain. Its functional capabilities are the most efficient of all the revertants studied here. As in other revertants, the free energy level of Q_B^- is higher than in the wild type. Its rate cytochrome oxidation is the fastest of the modified strains (≅254 cyt_{ox}/RC/s), and the proton transfer kinetics following the second flash are only approximately 2-fold slower than those of the wild type. Unlike the above strains in which we expect some local structural changes near Q_B, recovery from the loss of L212Glu is effected in this strain by a change in the electrostatic potential near Q_B that is induced by the distant M231Arg→Cys mutation. The M231Arg→Leu mutation has already been reported by us as a suppressor mutation that helps to compensate for the loss of L213Asp (Hanson et al., 1992a,1993; Maróti et al., 1994; Sebban et al., 1995b). M231Arg (M233 in *Rb. sphaeroides*; Okamura et al., 1992) forms salt bridges with two acidic residues—H125Glu and H232Glu—at a distance of about 15−20 Å from Q_B in the wild-type *Rb. sphaeroides* RC structure (Chang et al., 1991). Breakage of the salt bridges by mutation of M231Arg to Cys was suggested to cause a realignment of charge−charge interactions within a network of polar residues in this region of the reaction center, which could partially relocate the liberated negative charge much closer to Q_B (Sebban et al., 1995b). The effects of this mutation produce the driving force for protons to be pushed toward Q_B. It is remarkable that this distant electrostatic mutation is so efficient in restoring the reaction center capabilities.

Role of L212Glu in Proton Uptake—Interaction with the Semiquinone Species. Several lines of evidence have previously suggested that L212Glu has an unsual electrostatic behavior. The pH dependences of the rates for first electron transfer, ($k_{AB}(1)$) and $P^+Q_B^-$ charge recombination (k_{BP}) indicate a group having an apparent pK_a of 9.8 (in the oxidized form of Q_B) in reaction centers of *Rb. sphaeroides* (Paddock et al., 1989); this observation was confirmed for reaction centers of *Rb. capsulatus* where the apparent pK_a of this group is slightly higher (10.1; Hanson et al., 1992a; Maróti et al., 1994). The titration behavior of this group (designated $group_{high}$) is dependent, either directly or indirectly, on residue L212Glu (Paddock et al., 1989; Hanson et al., 1992a; Takahashi & Wraight, 1992; Maróti et al., 1994; McPherson et al., 1994). The observation that k_{BP} is pH-independent above pH 10 in the L212Q mutant, the L212A revertant, and the L212Q + L227F revertant strains lends further support to the hypothesis that L212Glu is involved in the high pH region of pH titration of the wild-type electron transfer rates. It also shows that the mutations restoring some of the proton transfer capabilities in the revertant strains do not mimic the electrostatic effect of L212Glu. Additional evidence which suggests that $group_{high}$ in the wild type involves L212Glu, either directly or indirectly, comes from measurements of the pH dependence of proton uptake in the reaction centers that lack Glu at L212. The data of the L212Q mutant and L212Q + M231C revertant strains (Figure 3) show progressive decreases in the amplitudes of H^+/Q_B^- proton uptake above pH 8 (compared to the wild type), resulting in a quasi-absence of proton uptake above

pH 9, again supporting the hypothesis of a high pK_a for L212Glu. Assuming a classic amino acid titrations (which may not be strictly true), the proton uptake data suggest an apparent coulombic interaction energy between L212Glu and Q_B^- of about 1.5 ΔpK_a units; these calculations assume classical titration behavior, which can be an oversimplification in the case of strongly interacting residues. The measurements presented in Figure 3 for the stoichiometries of proton uptake by the $P^+Q_B^-$ state are suggestive of those previously obtained for formation of $P^+Q_A^-$ in strains lacking L212Glu (Maróti et al., 1995; Miksovska et al., 1996). Instead of a ΔpK_a of 1.5 pH unit, an apparent interaction energy of 0.5 ΔpK_a unit was found between L212Glu and Q_A^-, at high pH, reflecting the greater distance between L212Glu and Q_A^- ($\cong$17 Å) versus that between L212Glu and Q_B^- ($\cong$5 Å). Interestingly, in both cases the pK_{ahigh} measured for the oxidized form of the quinone is the same, in agreement with the idea that a common group interacts with both Q_A and Q_B. It must be noted that what we detect here could be a direct interaction of Q_B^- with L212Glu, or alternatively, the electrostatic coupling of the semiquinone with a cluster of strongly interactive residues that includes L212Glu. In the absence of L212Glu (e.g., in the L212Q and L212Q + M231C strains), the apparent pK_a of the cluster may decrease resulting in uptake of protons at lower pH, as could be suggested by the slightly higher amplitude of H^+/Q_B^- proton uptake detected below pH 7 in the modified strains (if further measurements establish that the difference is significant beyond the experimental error). Taken as a whole, the proton uptake data for the formation of both Q_A^- and Q_B^- suggest that ionized L212Glu does interact with both semiquinones and could be a key residue involved in the electrostatic connection between both cofactors and both binding pockets.

It is interesting to compare the H^+/Q_B^- proton uptake data measured at pH 7.5 or below between the wild type and the strains lacking L212Glu. In the pH range 7−7.5, the stoichiometries of proton uptake measured in the wild type, in the L212Q, and in the L212Q + M231C revertant are the same within the experimental error, and below this pH, the values may be even slightly lower in the wild type than in the two strains lacking L212Glu. Since the loss of Glu does not significantly change the amount of proton uptake, this evidence strongly suggests that L212Glu is essentially protonated at neutral pH. This hypothesis is also supported by the comparison of the k_{BP} values, at neutral pH, of the L212Q mutant, the wild type, and the L212A revertant strains. Indeed, these data show that the effect of L212Gln on the energy level of Q_B^- is about the same as L212Glu (protonated), while the energy level of Q_B^- in the L212Ala revertant strain is much higher. Our present data are consistent with what we have found for proton uptake with formation of Q_A^- where the data from the wild type were superimposable at neutral pH to those from the strains lacking L212Glu (Maroti et al., 1995; Miksovska et al., 1996). Data points for proton uptake with the formation of Q_A^- and Q_B^- by the L212Q mutant of *Rb. sphaeroides* (measured at pH 6.5, 7.5, and 8.5) also fit well with our data (McPherson et al., 1994). FTIR measurements achieved in *Rb. sphaeroides* reaction centers have suggested a partly ionized state for L212Glu at neutral pH (Nabedryk et al., 1995). Since the data of McPherson et al. are obtained in *Rb. sphaeroides*, it is unlikely that the difference observed between FTIR and proton uptake measurements by flash-induced absorbance spectroscopy arises from differences in the two species. The slight disagreement between the data measured in aqueous samples containing dyes (direct proton uptake measurements), and the data measured by FTIR in very concentrated reaction center suspensions remain obscure.

Compensating for L212Glu versus L213Asp. In *Rb. capsulatus*, we have previously studied phenotypic revertants of the PS^- L212Glu-L213Asp→Ala-Ala double mutant (Hanson et al., 1992a, 1993; Maróti et al., 1994; Sebban et al., 1995b), and more recently we have characterized a large set of revertants isolated from the PS^- quadruple mutant L212Glu-L213Asp-M246Ala-M247Ala→Ala-Ala-Glu-Asp (Valerio-Lepiniec et al., 1997). All of the second-site substitutions that compensate for the engineered L213Asp→Ala mutation raise the free energy level of Q_B and are of an electrostatic nature: M231Arg→Leu, M5Asn→Asp, M43Asn→Asp, L217Arg→Cys, and L225Gly→Asp. Although they do not necessarily restore function to the reaction center by the same mechanism, the mutations described in this paper that occur in the family of L212Q revertants are in fact similar to those that compensate for loss of L213Asp in that they too decrease the energy gap between Q_A^- and Q_B^- by roughly 30−60 meV.

The loss of L212Glu, even though it results in a PS^- phenotype, is less destructive for reaction center function than is the loss of L213Asp. The absence of L212Glu can be sufficiently offset in the revertants by substitutions which change the electrostatic and/or structural environment of Q_B. The loss of L213Asp, however, causes a 10^3-10^4-fold reduction in the rate for transfer of the first proton (Takahashi & Wraight, 1990; Maróti et al., 1994; Paddock et al., 1994). In *Rb. capsulatus*, the second-site suppressor mutations listed above accelerate this process by only about 10−20 times; thus, the rate of transfer of the first proton in these phenotypic revertant strains remains more than 100 times slower than that of the wild type (Maróti et al., 1994; Delcroix et al., 1995). Similar results have been obtained for a revertant of the L213Asp→Asn mutant of *Rb. sphaeroides* (Senft et al., 1995). The only example of a reaction center where the role of L213Asp could be effectively substituted by another residue is the L213Asp-M44Asn→Asn-Asp double mutant of *Rb. sphaeroides* (Rongey et al., 1993).

The difference in the level of functional recovery that is achieved in revertant reaction centers which lack L212Glu versus those which lack L213Asp emphasizes the idea that the electrostatic environment of the quinone acceptors is not the only important parameter that is required to drive protons from the cytoplasm to Q_B. L213Asp is involved in delivery of both the first and second protons and has a large role in the connectivity of a web of charge−charge interactions that occurs in this region of the reaction center complex. The continuity of this network, which is composed of ionizable residues as well as structured and mobile water molecules, is very important for ensuring fast rates of proton transfer (Hanson et al. 1992a; Sebban et al., 1995b; Baciou & Michel, 1995; Beroza et al., 1995; Lancaster et al., 1996). The relative inefficiency of the L213Asp-less revertant reaction centers suggests that L213Asp is involved in many facets of proton transfer such that it cannot be effectively replaced—as L212Glu can be—by single substitutions of a water molecule(s), a residue that causes an increase in the electrostatic potential nearby Q_B or at a distant site, or a residue that

causes conformational changes allowing water molecules to penetrate the Q_B binding pocket.

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