Distant Electrostatic Interactions Modulate the Free Energy Level of Q_A^- in the Photosynthetic Reaction Center†

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ABSTRACT: In the reaction centers from the purple photosynthetic bacterium *Rhodobacter capsulatus*, we have determined that residue L212Glu, situated near the secondary quinone acceptor Q_B, modulates the free energy level of the reduced primary quinone molecule Q_A^- at high pH. Even though the distance between L212Glu and Q_A is 17 Å, our results indicate an apparent interaction energy between them of 30 ± 18 meV. This interaction was measured by quantitating the stoichiometry of partial proton uptake upon formation of Q_A^- as a function of pH in four mutant strains which lack L212Glu, in comparison with the wild type. These strains are the photosynthetically incompetent site-specific mutants L212Glu → Gln and L212Glu-L213Asp → Ala-Ala and the photocompetent strains L212Glu → Ala and L212Ala-L213Ala-M43Asn → Ala-Ala-Asp. Below pH 7.5, the stoichiometry of proton uptake from all strains is nearly superimposable with that of the wild type. However, at variance with the wild type, reaction centers from all strains that lack L212Glu fail to take up protons above pH 9. The lack of a change in the free energy level of Q_A^- at high pH in the L212Glu-modified strains is confirmed by the determination of the pH dependence of the rate (k_{AP}) of $P^+Q_A^-$ charge recombination in the reaction centers where the native Q_A is replaced by quinones having low redox potentials. Contrary to the wild-type reaction centers where k_{AP} increases at high pH, almost no pH dependence could be detected in the strains that lack L212Glu. Our data show that the ionization state of L212Glu, either on its own or via interactions with closely associated ionizable groups, is mainly involved in the proton uptake at high pH by reaction centers in the PQ_A^- state. This suggests that the formation of the Q_A^- semiquinone state induces shifts in pK_as of residues in the Q_B proteic environment. This long-distance influence of ionization states is a mechanism which would facilitate electron transfer from Q_A to Q_B on the first and second flashes. The functional communication between the two quinone protein pockets may involve the iron−ligand complex which spans the distance between them.

The bacterial photosynthetic reaction center converts light energy into chemical free energy. This integral membrane complex of proteins and prosthetic groups accomplishes a series of electron transfer reactions leading to a transmembrane charge separation. These processes are intimately coupled to the uptake of protons and their conduction through the protein to a quinone electron acceptor system, situated on the cytoplasmic side of the membrane. The three-dimensional structures are known at atomic resolution for the reaction centers from two purple bacteria, *Rhodopseudomonas viridis* (Deisenhofer et al., 1985, 1995) and *Rhodobacter sphaeroides* (Allen et al., 1988; Chang et al., 1991; Ermler et al., 1994; Arnoux et al., 1995). They constitute models for the study of membrane proteins which carry out electron and proton transfer reactions [reviewed in Okamura and Feher (1992) and Sebban et al. (1995a)].

The core complex of the reaction centers from purple nonsulfur bacteria is composed of three proteins, L, M, and H; the L and M proteins bind all of the reaction center cofactors. The primary electron donor, P, situated near the periplasmic side of the transmembrane complex, is a dimer of bacteriochlorophyll molecules. Its excitation to an electronic singlet excited state results in the fast (less than 4 ps) donation of an electron to a bacteriochlorophyll monomer and then to a bacteriopheophytin molecule, H. In about 200 ps, H^- reduces the primary quinone molecule, Q_A. The electron transfer reaction continues with the reduction of the secondary quinone molecule, Q_B, in 5−200 μs (depending on the species). Both quinones are situated near the cytoplasmic side of the membrane. A second photon generates the $Q_A^-Q_B^-$ state, and the transfer of the second electron from Q_A to Q_B is concomitant with uptake of a proton from the cytoplasm. Q_BH_2, which leaves the reaction center (McPherson et al., 1990), is formed following the uptake of a second proton. Even in reaction centers where their binding sites are occupied by identical ubiquinone$_{10}$ (UQ_{10}) molecules, Q_A and Q_B are not functionally equivalent. The protein environment of the sites dictates the extent to which they are able to accept both electrons and protons.

The formation of the semiquinone anions, Q_A^- and Q_B^-, induces shifts in the pK_as of many ionizable groups located

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in this region of the complex which interact more or less strongly with each other and with Q_A^- and/or Q_B^-. This results in partial proton uptake by the groups in this network, to a level that is proportional to their interaction energy with Q_A^- and/or Q_B^- (McPherson et al., 1988; Maróti & Wraight, 1988). In *Rb. capsulatus*, we have previously shown that the electrostatic effects induced by mutational processes may extend over large distances (Hanson et al., 1992b; Maróti et al., 1994; Sebban et al., 1995b).

Among the amino acids whose effects could be of importance in the function of the quinone complex, L212Glu has attracted special interest. Because of its proximity to Q_B (~5 Å), it has been subjected to site-specific replacement (Paddock et al., 1989; Takahashi & Wraight, 1992; Hanson et al., 1992a,b, 1993, 1995). These studies suggested that L212Glu has an important role in the donation of the second proton to Q_B and in triggering the subsequent release of the Q_BH_2 molecule from the reaction center (Shinkarev et al., 1993; McPherson et al., 1994). Mutation of this residue to Gln impairs the photosynthetic growth capacities of *Rb. sphaeroides* (Paddock et al., 1989) and *Rb. capsulatus* (Hanson et al., 1995). However, it was shown that, in *Rb. capsulatus*, the ability to grow under photosynthetic conditions can be recovered by substitution of an alanine at this site (Hanson et al., 1992a, 1993) or by a compensatory mutation at one of several other secondary sites (L227Leu → Phe, L228Gly → Asp, L231Arg → Cys, or M231Arg → Cys; Hanson et al., 1995). L212Glu is one of many charged residues that form an interactive network near Q_B (Sebban et al., 1995b). Electrostatic calculations have shown that, in the *Rb. sphaeroides* reaction center, L212Glu interacts strongly with L210Asp and L213Asp (Beroza et al., 1995). Similar calculations have suggested that, in *Rps. viridis*, L212Glu is a part of a strongly interactive cluster involving three glutamic acids (together with M234 and H177) which might determine the electrostatic potential imposed by the protein near Q_A and Q_B (Lancaster et al., 1996). These clusters have been suggested to be involved in the proton uptake associated with the formation of Q_A^- and Q_B^- at neutral pH. Finally, the protonation state of L212Glu was proposed to be involved in the possible protein relaxation induced by the formation of the Q_A^- and Q_B^- states (Bzrezinski et al., 1992; Tiede & Hanson, 1992; Maróti et al., 1995).

The pH dependence of the ionization state of L212Glu is still a matter of debate. Paddock et al. (1989) initially proposed that this residue could have a high pK_a value of about 9.8 in the reaction centers from *Rb. sphaeroides*. An equivalent pK_a (≈10.1) for L212Glu could be detected in *Rb. capsulatus* (Maróti et al., 1994). The latter studies, however, suggested that this apparent pK_a might not be exclusively attributed to L212Glu. At variance with the hypothesis of Paddock et al. (1989), Hienerwadel et al. (1995) and Nabedryk et al. (1995)—on the basis of kinetic infrared and Fourier transform infrared spectroscopic measurements obtained on the L212Glu → Gln mutant from *Rb. sphaeroides*—have proposed that L212Glu could be partly ionized at neutral pH. This was also suggested by electrostatic calculations (Beroza et al., 1995).

In the present paper we have investigated the interaction between ionized L212Glu and Q_A^-. The pH dependencies of the stoichiometry of H^+/Q_A^- proton uptake and of the $P^+Q_A^-$ charge recombination kinetics with low-potential quinones acting as Q_A have been measured in the reaction centers from the wild type and from four strains lacking L212Glu. These strains are the photosynthetically incompetent (PS^-) site-specific mutants L212Glu → Gln and L212Glu-L213Asp → Ala-Ala and two photocompetent (PS^+) strains, L212Glu → Ala and L212Glu-L213Asp-M43Asn → Ala-Ala-Asp. Our results show that proton uptake by the Q_A^- state is eliminated above pH 9 in the mutant strains and that the lack of L212Glu cancels the change in the free energy level of Q_A^- that is observed with increasing pH in wild-type reaction centers. The results presented here extend and amplify preliminary results (Maróti et al., 1995).

EXPERIMENTAL PROCEDURES

Deletion strain U43 (LHI^-, $LHII^-$, RC^-) serves as the background for all mutants described here (Youvan et al., 1985). The "wild-type" strain U43[pU2922] is LHI^+, $LHII^-$, RC^+ (Bylina et al., 1989); mutant strains carry derivatives of plasmid pU2922. The PS^+ strains are phenotypic revertants of the PS^- L212Ala-L213Ala mutant whose isolation and characterization have been described previously (Hanson et al., 1992a, 1993; Maróti et al., 1994). To construct the L212Gln mutant, an *Eco*RI−*Kpn*I fragment of the pU2922 derivative that encoded the L212Ala mutant was used as a template in the Chameleon double-stranded mutagenesis system (Stratagene). The L212Ala → Gln mutation created an additional *Bst*OI site that was used in preliminary screening of candidate mutant plasmids; the mutation was confirmed by sequencing of double-stranded DNA according to directions from a kit (Sequenase 2.0, Amersham). Subsequent steps to transfer the mutation to *Rb. capsulatus* have been described previously (Bylina et al., 1989; Hanson et al., 1993).

Cells were grown semiaerobically in the dark on RPYE medium (Hanson et al., 1992b) containing 30 μg/mL kanamycin to select the plasmid. Reaction centers were isolated as described by Baciou et al. (1993). The Q_A-depleted reaction centers from wild-type *Rb. capsulatus* and from the mutant strains were prepared as initially described for *Rb. sphaeroides* (Okamura et al., 1975), with some modifications (Sebban, 1988a). For *Rb. capsulatus*, the addition of 10−15% glycerol (v/v) to the reaction center suspension prior to the addition of quinones was of critical importance for the reconstitution of anthraquinones. Occupancy of the Q_A site was restored by adding 50 μM anthraquinones to the Q_A-depleted samples.

Buffers (10 mM) used were 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 9.5; and 3-(cyclohexylamino)propanesulfonic acid (CAPS; Calbiochem) above pH 9.5.

The stoichiometries of proton uptake were measured by using a glass pH electrode and pH-sensitive dyes. The conditions were as follows: 2 μM reaction centers, 100 mM NaCl, 0.03% Triton X-100, 50 μM UQ_6, 200 μM ferrocene, and 40 μM dye (bromocresol purple, phenol red, cresol red, or chlorophenol red, depending on the pH). In these measurements, the buffer concentrations were kept below 10 μM by dialysis.

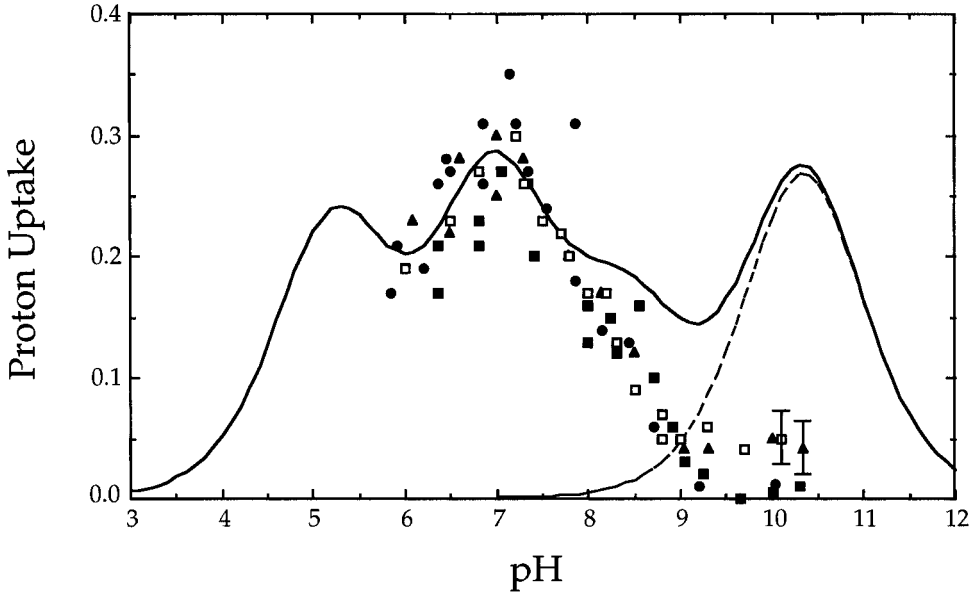

FIGURE 1: pH dependence of the stoichiometries of proton uptake by the PQ_A^- state in the reaction centers from the L212Glu-L213Asp → Ala-Ala double mutant (●), and mutant strains L212Glu-L213Asp-M43Asn → Ala-Ala-Asp (■), L212Glu → Ala (▲), and L212Glu → Gln (□). The solid line represents the fitting of the stoichiometry of proton uptake in wild-type *Rb. capsulatus* reaction centers [data from Sebban et al. (1995a)]. The dotted line represents the fitting corresponding to the group with the highest apparent pK_a, i.e., $pK_{a\,high_{Q_A}} = 10.10 \pm 0.15$ and $pK_{a\,high_{Q_A^-}} = 10.60 \pm 0.25$. Conditions: 2 μM reaction centers, 100 mM NaCl, 0.03% Triton X-100, 100 μM ferrocene, 40 μM dye (bromocresol purple, phenol red, cresol red, or chlorophenol red, depending on the pH). Data obtained with a glass pH electrode and using pH indicator dyes are superimposed. The reaction centers from the wild type were Q_B-depleted according to the method of Okamura et al. (1975). This procedure was not applied to the reaction centers of the L212Glu-modified strains since they contain a negligible amount of Q_B at the end of the preparation.

RESULTS

pH Dependence of the Stoichiometries of H^+/Q_A^- Proton Uptake. The curve drawn in Figure 1 represents the pH dependence of the stoichiometry for proton uptake by the PQ_A^- state of the reaction centers of wild-type *Rb. capsulatus* [previously measured by us (Sebban et al., 1995a)]. The net proton uptake by an ionizable group *i* upon Q_A^- formation may be expressed as (McPherson et al., 1988):

$$\left(\frac{H^+}{Q^-}\right)_i = \frac{1}{1+10^{[pH-pK_{ai_{Q_A^-}}]}} - \frac{1}{1+10^{[pH-pK_{ai_{Q_A}}]}} \quad (1)$$

where $pK_{ai_{Q_A}}$ and $pK_{ai_{Q_A^-}}$ are the pK_a of group *i* in the oxidized and semiquinone form of Q_A, respectively. This equation was used to fit the wild-type data, assuming a minimum of four ionizable groups. The data obtained from measurements achieved with a glass pH electrode and with pH-sensitive dyes are superimposed. A detailed analysis of the apparent pK_as of the four groups involved in the curve for the wild type has been previously described (Sebban et al., 1995a). In this study we shall focus on the high-pH group, which has the following pK_as:

$$pK_{a\,high_{Q_A}} = 10.10 \pm 0.15 \qquad pK_{a\,high_{Q_A^-}} = 10.60 \pm 0.25$$

Data points for the four different strains lacking L212Glu are also plotted in Figure 1. Below pH 7.5, the pH dependences of proton uptake by the PQ_A^- state in all strains, including the wild type, are nearly superimposable. However, contrary to the wild type, as the pH increases above 7.5, the four strains lacking L212Glu fail to take up protons, so that above pH 9 almost no proton uptake by the PQ_A^- state is observed. Interestingly, this level does not quite reach zero in the two strains that retain L213Asp. The shoulder of proton uptake that occurs in the curve for the wild type between pH 8 and 8.7 is also diminished in the mutant strains.

pH Dependence of the Rate of $P^+Q_A^-$ Charge Recombination in Reaction Centers with Low-Potential Quinones at Q_A. We have further investigated the effect of L212Glu by analyzing, in strains lacking L212Glu, the pH dependence of the $P^+Q_A^-$ charge recombination in reaction centers where the Q_A site has been reconstituted with quinones having low redox potentials. We have replaced the native Q_A by anthraquinone or by 1-amino-5-chloroanthraquinone. According to Woodbury et al. (1986), these quinone analogs possess *in vivo* redox potentials that are 0.16 and 0.21 V more negative than the native UQ_{10}, respectively, in wild-type reaction centers of *Rb. sphaeroides*. Thus, the $P^+Q_A^-$ state should recombine via the thermal pathway in reaction centers in which either of these quinones acts as Q_A. The thermal recombination rate is expected to be sensitive to small perturbations in the free energy of Q_A^- caused by electrostatic interactions with protonatable residues. The native Q_A has been replaced by these quinones in reaction centers from the wild-type *Rb. capsulatus* and from three mutant strains which lack a glutamic acid residue at position L212 (L212Glu → Gln, L212Glu → Ala, and L212Glu-L213Asp → Ala-Ala).

The pH dependences for the rates of $P^+Q_A^-$ charge recombination (k_{AP}) observed in anthraquinone-reconstituted reaction centers of the wild type and the above three mutant strains are displayed in Figure 2A. At pH 7 and at 21 °C, in the wild type, $P^+Q_A^-$ decays in about 3 ms ($k_{AP} = 270 \pm 10\ s^{-1}$). This rate is in good agreement with what was previously observed in *Rb. sphaeroides* reaction centers ($k_{AP} = 230 \pm 30\ s^{-1}$; Sebban, 1988a), suggesting a very similar

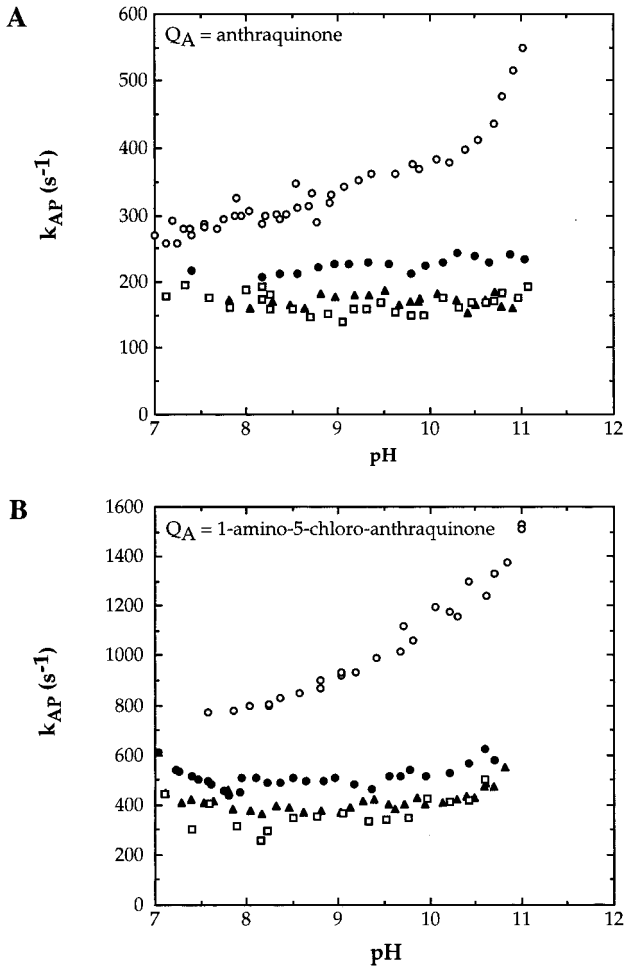

FIGURE 2: pH dependence of the rate of $P^+Q_A^-$ charge recombination in reaction centers from *Rb. capsulatus* where the native Q_A (UQ_{10}) has been replaced by anthraquinone (panel A) or 1-amino-5-chloroanthraquinone (panel B): wild-type (○), and mutant strains L212Glu-L213Asp → Ala-Ala (●), L212Glu → Ala (▲), and L212Glu → Gln (□). Conditions: 0.03% LDAO, buffers depending on the pH, as indicated in the text. The reaction centers from the different strains were depleted of native Q_A using a method derived from that of Okamura et al. (1975). Reconstitutions with anthraquinones were achieved in the presence of 10−15% glycerol (v/v).

in vivo redox potential for the Q_A species in reaction centers from both species. The effect of the mutations on k_{AP} is small at neutral pH, where the k_{AP} values are slightly smaller than in the wild type: at pH 7, $k_{AP} = 225 \pm 15$ s^{-1} in the L212Ala-L213Ala double mutant, while $k_{AP} = 175 \pm 20$ s^{-1} in the L212Ala mutant and the L212Gln mutant. In the modified strains these values are pH-independent over the whole pH range studied (7−11). The behavior of the wild type is different. A continuous increase of k_{AP} is observed above pH 7, reaching a value of about 380 s^{-1} at pH 10, with a more substantial acceleration above pH 10, reaching a value of 550 s^{-1} at pH 11.

The pH-dependence patterns for the recombination rates that are measured when 1-amino-5-chloroanthraquinone acts as Q_A are shown in Figure 2B. The k_{AP} value measured at pH 7 in the wild type (750 ± 10 s^{-1}) is notably higher than that obtained for anthraquinone. This is consistent with previous measurements carried out with *Rb. sphaeroides* reaction centers (675 ± 25 s^{-1}; Sebban, 1988b) and with the lower estimated *in vivo* redox potential of the 1-amino-5-chloroanthraquinone compared to anthraquinone (Woodbury et al., 1986). Similarly to the case with anthraquinone, at pH 7 in the L212Ala-L213Ala double mutant, the L212Ala strain, and the L212Gln mutant, k_{AP} is somewhat smaller than in the wild type. At pH 7, $k_{AP} = 620 \pm 20$ s^{-1} for the double mutant, while $k_{AP} = 450 \pm 15$ s^{-1} for both the L212Ala and the L212Gln strains. In the modified strains, k_{AP} is relatively pH independent, increasing slightly above pH 10.5, and also below pH 8, in the double mutant. This behavior for k_{AP} is different from that of the wild type, where a continuous and much more substantial acceleration of the $P^+Q_A^-$ recombination process is measured above pH 8, such that at pH 11, k_{AP} equals 1520 ± 20 s^{-1}.

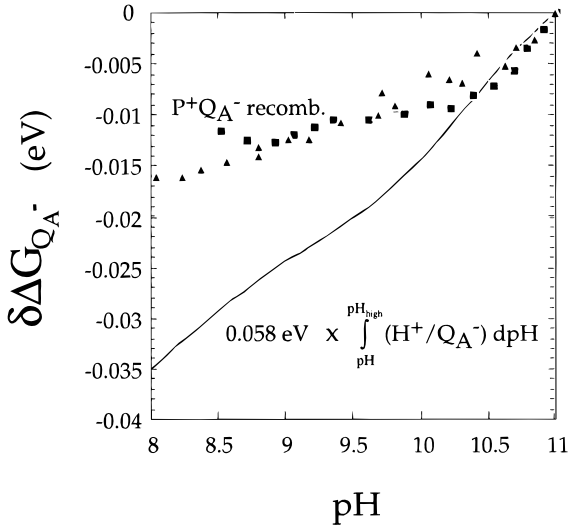

FIGURE 3: Comparison between the change in the free energy of the Q_A^- species in the wild-type reaction centers of *Rb. capsulatus*, calculated from integration of the stoichiometry of proton uptake by the PQ_A^- state (data from Figure 1) and from $P^+Q_A^-$ charge recombination where anthraquinone (▲) or 1-amino-5-chloroanthraquinone (■) acts as Q_A.

The data displayed in both panels of Figure 2 agree, showing a substantive difference in the differential free energy level of Q_A^- between the wild-type and L212Glu-modified strains within the region of neutral to high pH.

Change in the Free Energy Level of Q_A^-. The pH dependence of the variation in the free energy level of Q_A^- can be calculated from both the proton uptake and charge recombination measurements. The change in the free energy level can be derived from the integration of the stoichiometry of proton uptake induced by the formation of Q_A^- (McPherson et al., 1988):

$$\delta\Delta G^\circ_{Q_A^-}(\mathrm{pH}) = kT \ln 10 \int_{\mathrm{pH}}^{\mathrm{pH_{high}}} (\mathrm{H^+}/\mathrm{Q_A^-})\ \mathrm{dpH} \quad (2)$$

where kT represents the Maxwell−Boltzmann term and pH_{high} is an arbitrary high value where all groups interacting with Q_A^- are deprotonated. Integration of the proton uptake curve of Figure 1 for the wild type leads to the line drawn in Figure 3.

The degree of the change in free energy of Q_A^- may also be extracted from the charge recombination data, presented in Figure 2, that were acquired from anthraquinone-reconstituted reaction centers (Shopes & Wraight, 1987; Sebban & Wraight, 1989):

$$k_{AP} = k_d e^{(-\Delta G^\circ_{AH}/kT)} + k_T \quad (3)$$

where $\Delta G°_{AH}$ represents the free energy gap between $P^+Q_A^-$ and P^+H^- and k_d is the rate constant of charge recombination from P^+H^- or from a relaxed state of P^+H^-. By analogy with *Rb. sphaeroides*, we have used a value of 2×10^7 s^{-1} for k_d (Shopes & Wraight, 1987). The *kT* term represents the Maxwell−Boltzmann constant, and k_T is the rate constant of $P^+Q_A^-$ charge recombination at low temperature (10 s^{-1}; Sebban et al., 1991), i.e., when the mechanism of electron tunneling through the protein dominates. Assuming that the free energy level of the P^+H^- state is not significantly affected by interactions with ionizable groups when compared to that of the $P^+Q_A^-$ state and that k_d and k_T are pH-independent, one may derive the free energy level, $\Delta G°$, of the $P^+Q_A^-$ state at each pH. The points derived in this manner from the k_{AP} data are presented in Figure 3 for the wild-type reaction centers with anthraquinone and 1-amino-5-chloroanthraquinone acting as Q_A.

The variations in the differential free energy level of Q_A^- calculated from proton uptake and charge recombination were arbitrarily normalized to zero at pH 11. Clearly, the two evaluations differ. They are superimposable in the pH range 10.4−11, but below pH 10.4 the integration of the proton uptake data leads to a greater change in the free energy level of Q_A^-, a 35-meV change from pH 8 to 11. The value derived from the recombination data yields a change of 15 meV in the free energy level from pH 8 to 11.

DISCUSSION

We have previously demonstrated that, in *Rb. capsulatus* reaction centers, residue L212Glu is involved in proton uptake that occurs with the formation of Q_A^-. The electrostatic interaction between these two groups, which is responsible for proton uptake above pH 9 upon the formation of Q_A^- is manifested over a distance of 17 Å and was found to be absent in the L212Glu → Ala mutant strain (Maróti et al., 1995). In the experiments presented here, we report the same effect, as expected, in other mutant strains—both PS^+ and PS^-—which lack Glu at L212. In addition, we directly demonstrate that the lack of L212Glu nullifies the change in the free energy level of Q_A^- that is observed as a function of increasing pH in wild-type reaction centers.

Effect of L212Glu on Proton Uptake with Increasing pH. Proton uptake occurs in a pH-dependent manner with the formation of the Q_A^- and Q_B^- states. We have previously fitted the H^+/Q_A^- and H^+/Q_B^- proton uptake stoichiometries obtained over a broad pH range with eq 1, assuming a minimum of four and five interacting groups, respectively (Maróti et al., 1995; Sebban et al., 1995a). Similar studies have been reported for wild-type reaction centers of *Rb. sphaeroides* (McPherson et al., 1988; Maróti & Wraight, 1988) which show a slightly higher amplitude of H^+/Q_A^- proton uptake (0.3−0.45) than found here in wild-type reaction centers of *Rb. capsulatus* (0.2−0.3) in the range pH 6−10.5. In both species, a group with a high pK_a is needed to account for the proton uptake above pH 9.0. The apparent pK_a of this group is slightly higher in *Rb. capsulatus* ($pK_{a_{Q_A}} = 10.1$ and $pK_{a_{Q_A^-}} = 10.6$) than in *Rb. sphaeroides* [$pK_{a_{Q_A}} = 9.2$ and $pK_{a_{Q_A^-}} = 9.65$ (Maróti & Wraight, 1988)]. The $pK_{a\,high_{Q_A}}$ value (10.1) is the same as that previously measured for the group with the highest pK_a in the PQ_B^- proton uptake stoichiometry curve in *Rb. capsulatus* reaction centers (Sebban et al., 1995a). This pK_a value also matches that derived for the oxidized form of Q_B, obtained from the pH titration curve of the $Q_A^-Q_B \leftrightarrow Q_AQ_B^-$ equilibrium constant, K_2 (Hanson et al., 1992b). In *Rb. sphaeroides*, the equivalent pK_a value (≈9.8; Paddock et al., 1989) for the Q_B species is again slightly lower than in *Rb. capsulatus*. In *Rb. sphaeroides*, this apparent pK_a was assigned to L212Glu (Paddock et al., 1989). By analogy with *Rb. sphaeroides*, and because we have observed the disappearance of this pK_a in the pH-dependence curves of K_2 and the stoichiometry of PQ_B^- proton uptake for the L212Glu → Gln and the L212Glu → Ala strains of *Rb. capsulatus* (J. Miksovska, L. Kálmán, M. Schiffer, P. Maróti, P. Sebban, and D. K. Hanson, unpublished data), it is likely that L212Glu contributes to the apparent pK_a of 10.1 (Q_B oxidized) that is observed in wild-type *Rb. capsulatus* reaction centers.

The apparent $pK_{a\,high_{Q_A}}$ also involves the ionization state of L212Glu, as shown by the data of Figure 1. Indeed, proton uptake by the PQ_A^- state above pH 8.5 is extremely small or absent in all of the strains we examined that lacked a glutamic acid residue at position L212. Data obtained at selected pH values with the L212Glu → Gln mutant of *Rb. sphaeroides* (Paddock et al., 1994) are in agreement with our data. The dramatic reduction in the level of proton uptake due to Q_A^- formation in the L212Glu-less mutants above pH 8.5−9 is consistent with the hypothesis that, in the wild type, at high pH, L212Glu interacts either directly or indirectly with Q_A^- with an apparent interaction energy of at least 30 ± 18 meV [58 meV($pK_{a\,high_{Q_A^-}} - pK_{a\,high_{Q_A}}$)]. A value of 65 meV was calculated by Beroza et al. (1995) for *Rb. sphaeroides*.

Theoretical electrostatic calculations have been recently carried out for the reaction centers of *Rb. sphaeroides* (Beroza et al., 1995) and of *Rps. viridis* (Lancaster et al., 1996). In *Rb. sphaeroides*, these calculations have suggested that the bulk of the proton uptake upon formation of Q_A^- below pH 7.5 was due primarily to the influence of L212Glu and that M247Arg is responsible for proton uptake in the Q_A^- state of the reaction centers above pH 8. In *Rps. viridis*, calculations have suggested that, below pH 8, L212Glu is involved in proton uptake by the Q_A^- state. Above pH 8, these authors assigned the bulk of the proton uptake to H66Lys.

These aspects of the calculations do not match our experimental data. The data of Figures 1 and 2 indicate that L212Glu is the major group interacting with Q_A^- at high pH. In addition, below pH 7.5, the Q_A^- proton uptake data—which are nearly superimposable in the wild type and in the strains lacking L212Glu—are not consistent with a substantial ionization of L212Glu in this pH range.

If proton uptake with the formation of the Q_A^- state is necessary to prime the reaction center for subsequent electron transfer to Q_B, a deficiency in this function has the potential to adversely affect the photosynthetic growth phenotype. Proton uptake by the Q_A^- state above pH 8.5 is therefore not critically involved in determining phenotype, since this level is very low and equivalent in both PS^- (e.g., L212Gln) and PS^+ (e.g., L212Ala) strains. The Ala substitution could allow the entry of a water molecule which might assume the role played by L212Glu in proton delivery to Q_B in the wild-type reaction center, similar to observations with the L223Ser → Ala (PS^-) and L223Ser → Gly (PS^+) mutants (Bylina & Wong, 1992; Paddock et al., 1990, 1995).

However, it is clear from these results that the water molecule cannot duplicate the electrostatic effect of L212Glu on proton uptake due to the formation of Q_A^-.

In reaction centers from the two strains lacking L212Glu but retaining L213Asp, a slight amplitude of proton uptake remains at high pH, but the level is much lower than when L212Glu is present. This shows that L213Asp alone, or even together with other residues, cannot duplicate the system of interactions that is present in the wild type, i.e., when L212Glu is present.

L212Glu Affects the Energy Level of Q_A^-. In *Rb. sphaeroides* reaction centers, it has been shown previously that the $P^+Q_A^-$ charge-separated state recombines directly to the PQ_A ground state via an activationless electron tunneling process that is not very sensitive to the energy level of Q_A^- (Kleinfeld et al., 1985). This mechanism has also been suggested for *Rb. capsulatus* reaction centers (Baciou et al., 1993). In *Rb. sphaeroides*, it was shown that when the free energy gap between the $P^+Q_A^-$ and P^+H^- states is decreased by replacing the native UQ_{10} by a quinone having a low *in vivo* redox potential, the $P^+Q_A^-$ state recombines through a thermally activated energy level (Gunner et al., 1982, 1986; Woodbury et al., 1986; Sebban, 1988b). This state is likely to involve a relaxed state of P^+H^- (Sebban & Barbet, 1984; Woodbury & Parson, 1984). Woodbury et al. (1986) have shown that in *Rb. sphaeroides*, the thermally activated recombination process becomes predominant when the free energy barrier between $P^+Q_A^-$ and P^* is smaller than about 0.8 eV. The thermal pathway is expected to be sensitive to conditions which modulate the relative energy levels of the P^+H^- and $P^+Q_A^-$ states. Thus, the pH dependence of charge recombination in anthraquinone-reconstituted reaction centers reflects the differential interaction energies of these two states with ionizable residues.

The data presented here show definitive evidence that L212Glu, from a distance of 17 Å, participates in determining the free energy level of Q_A^-. The absence of acceleration of the $P^+Q_A^-$ charge recombination process at high pH in the anthraquinone- and 1-amino-5-chloroanthraquinone-reconstituted reaction centers from the L212Glu-less mutants confirms this hypothesis. The similar recombination curves measured for the PS^+ L212Ala strain and the PS^- L212Gln mutant show, first, that no important structural change occurs in reaction centers where the smaller residue Ala replaces Glu, and second, that water molecules which might fill the void left by the substitution of Ala for Glu are not responsible for screening the interaction between Q_A^- and an amino acid(s) other than L212Glu.

With either of the anthraquinones acting as Q_A, the $P^+Q_A^-$ charge recombination rates observed in the strains lacking L212Glu are systematically slightly smaller than in the wild type (Figure 2). This is consistent with partial ionization of L212Glu at neutral pH, at least in the $P^+Q_A^-$ state. This observation agrees with previous experimental results (Hienerwadel et al., 1995; Nabedryk et al., 1995). It also agrees in part with theoretical calculations (Beroza et al., 1995) which predict the gradual titration of L212Glu over a wide pH range. This result is consistent with the H^+/Q_A^- proton uptake data of Figure 1, where the difference between the amplitudes of the proton uptake measured in the four strains lacking L212Glu and that observed in the wild type begins at pH 7.5 and gradually increases to a maximum above pH 9.0. These data suggest that the deprotonation of L212Glu extends over quite a large pH range starting around pH 7.5, while its maximum effect on proton uptake occurs above pH 9.5.

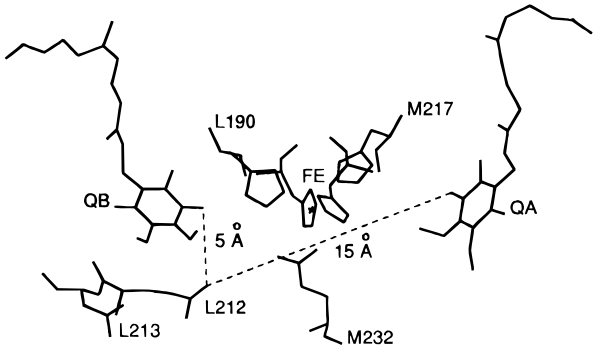

FIGURE 4: Structure of the reaction center from *Rb. sphaeroides* (Chang et al., 1991) showing the iron−ligand complex that spans the region between the quinones and the respective L212Glu-Q_A^- and L212Glu-Q_B distances. Conserved residue M232Glu is located directly between Q_A and the side chain of L212Glu. The distances are measured from the carbonyl oxygen of L212Glu to the nearest carbonyl oxygen of each quinone. The 17-Å value stated in the text refers to the distance from L212Glu to the center of the Q_A ring.

Estimation of the Change in Energy Level of Q_A^- Due to Influence of L212Glu. As shown in Figure 3, the changes in the free energy level of Q_A^- ($\delta\Delta G^\circ_{Q_A^-}$) calculated from integration of the proton uptake measurements and from the charge recombination data agree between pH 10.4 and 11. However, below pH 10.4 a much higher $\delta\Delta G^\circ_{Q_A^-}$ value is calculated when the proton uptake method is used compared to the recombination data. As mentioned above, the $\delta\Delta G^\circ_{Q_A^-}$ value derived from the recombination data is modulated by the differential interaction of some residues with the H^- and Q_A^- states, respectively. Therefore this latter estimation of $\delta\Delta G^\circ_{Q_A^-}$ necessarily leads to a smaller value than that derived from the PQ_A^- proton uptake data, where only the Q_A^- species is stabilized. The only case where both evaluations of $\delta\Delta G^\circ_{Q_A^-}$ may match is when the interaction of the ionizable group(s) with H^- is negligible compared to that with Q_A^-. In this regard, the region of pH 10.4−11 of Figure 3, where both estimations match, falls in the same pH region as that proposed by us for the involvement of L212Glu. This result suggests that L212Glu develops a preferential interaction with Q_A^-, or in any case one that is much more effective than with H^-. Below pH 10.4, any mechanisms for charge stabilization must exert an equal effect on both Q_A^- and H^-.

Several studies have indicated that the quinone proteic pockets functionally interact with each other (Tiede & Hanson, 1992; Bzrezinski et al., 1992; Baciou & Sebban, 1995; Maróti et al., 1995). However, the structure shows that the network of interactive charged residues that is contiguous to the Q_B site (Maróti et al., 1995) does not extend to either the Q_A or pheophytin binding sites. Therefore, we suggest that the the iron−ligand complex may provide the means to connect the two quinone binding sites, facilitating electrostatic communication between them (Baciou & Sebban, 1995; Maróti et al., 1995; Sebban et al., 1995a); this connection would not, then, extend to include H^-. This complex has been proposed to provide the functional link between Q_A and Q_B in reaction centers of *Rps. viridis* (Baciou & Sebban, 1995). As shown in Figure 4, conserved ligand M232Glu is located midway between Q_A and L212Glu and

could be involved, possibly as part of the iron complex, in transmitting the effect of the charge on Q_A to L212Glu. The mechanism by which the Q_A and Q_B sites communicate is not yet clear.

The interaction shown here suggests that the formation of Q_A^- does influence the electrostatic potential near Q_B, and therefore the ionization state of protonatable groups which interact with Q_B. The electrostatic and/or structural rearrangements associated with the formation of Q_A^- could facilitate electron and proton transfers to Q_B following the first and the second flash, respectively.

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