Proton and Electron Transfer in the Acceptor Quinone Complex of *Rhodobacter sphaeroides* Reaction Centers: Characterization of Site-Directed Mutants of the Two Ionizable Residues, Glu^{L212} and Asp^{L213}, in the Q_B Binding Site†

Eiji Takahashi and Colin A. Wraight*

Department of Plant Biology and Department of Physiology and Biophysics, University of Illinois, 1201 West Gregory Drive, Urbana, Illinois 61801

Received May 28, 1991; Revised Manuscript Received September 16, 1991

ABSTRACT: Proton and electron transfer events in reaction centers (RCs) from *Rhodobacter sphaeroides* were investigated by site-directed mutagenesis of glutamic acid at position 212 and aspartic acid at 213 in the secondary quinone (Q_B) binding domain of the L subunit. These residues were mutated singly to the corresponding amides (mutants L212EQ and L213DN) and together to give the double mutant (L212EQ/L213DN). In the double mutant RCs, the rate of electron transfer from the primary (Q_A) to the secondary (Q_B) acceptor quinones is fast ($\tau \approx 300$ μs) and is pH independent from pH 5 to 11. The rate of recombination between the oxidized primary donor, P^+, and Q_B^- is also pH independent and much slower ($\tau \approx 10$ s) than in the wild type (Wt), indicating a significant stabilization of the Q_B^- semiquinone. In the double mutant, and in L213DN mutant RCs at low pH, the $P^+Q_B^-$ decay is suggested to occur significantly via a direct recombination rather than by repopulating the $P^+Q_A^-$ state, as in the Wt. Comparison of the behavior of Wt and the three mutant RC types leads to the following conclusions: the pK of Asp^{L213} in the Wt is ≈ 4 for the Q_AQ_B state (pK_{Q_B}) and ≈5 for the $Q_AQ_B^-$ state (p$K_{Q_B^-}$); for Glu^{L212}, p$K_{Q_B} \approx 9.5$ and p$K_{Q_B^-} \approx 11$. In L213DN mutant RCs, pK_{Q_B} of Glu^{L212} is ≤ 7, indicating that the high pK values of Glu^{L212} in the Wt are due largely to electrostatic interaction with the ionized Asp^{L213} which contributes a shift of at least 2.5 pH units. Transfer of the second electron and all associated proton uptake to form Q_BH_2 is drastically inhibited in double mutant and L213DN mutant RCs. At pH ≥ 8, the rates are at least 10^4-fold slower than in Wt RCs. In L212EQ mutant RCs the second electron transfer and proton uptake are biphasic. The fast phase of the electron transfer is similar to that of the Wt, but the extent of rapid transfer is pH dependent, revealing the pH dependence of the equilibrium $Q_A^-Q_B^- \leftrightarrow Q_AQ_BH^-$. The estimated limits on the pK values—p$K_{Q_A^-Q_B^-} \leq 7.3$, p$K_{Q_AQ_B^{2-}} \geq 10.4$—are similar to those derived earlier for Wt RCs [Kleinfeld et al. (1985) *Biochim. Biophys. Acta 809*, 291–310] and may pertain to the quinone head group, per se. The slow phase of electron transfer in L212EQ mutant RCs is accompanied by uptake of the second proton to the quinol. It is suggested that in Wt RCs the first proton is delivered to Q_B^- or Q_B^{2-} directly from Asp^{L213}, and the second is delivered via Asp^{L213} and Glu^{L212}.

The photosynthetic reaction center (RC) of purple bacteria is a membrane-bound pigment–protein complex that catalyzes the conversion of light energy into chemical energy by stabilizing the primary photochemical charge separation. In *Rhodobacter sphaeroides*, the RC is isolatable as a complex of three protein subunits: L, M, and H. L and M are partially homologous and form a quasisymmetrical heterodimer which binds all of the cofactors involved in photochemistry and subsequent charge stabilization. When a RC is activated by light, an electron is transferred from the excited singlet state of the primary donor (P), a dimer of bacteriochlorophyll, via a monomeric bacteriochlorophyll and a bacteriopheophytin, to the primary and secondary acceptor quinones, Q_A and Q_B, which are bound in folds of the M and L subunits, respectively [for review, see Feher et al. (1989)]. The resulting charge separated states, $P^+Q_A^-$ and $P^+Q_B^-$, are relatively stable, with lifetimes of roughly 0.1 and 1 s, respectively. P^+ is normally reduced by a secondary donor (*c*-type cytochromes in vivo), and the RC is ready for another photochemical turnover. The negative charge on the quinones induces pK shifts of nearby amino acid residues (Wraight, 1979), resulting in protonation of the RC and partial shielding and stabilization of the charge on the Q_B^- semiquinone (Maróti & Wraight, 1988a,b; McPherson et al., 1988; Shinkarev et al., 1989). After a second turnover of the RC, Q_B^- is reduced again and acquires two protons from the medium to form a quinol (Q_BH_2), which leaves the RC and is replaced by a quinone.

The protons taken up in the formation of quinol by RCs must pass through regions of both the L and H subunits to reach the Q_B binding site, which is completely occluded from the solvent. This provides a uniquely amenable system for the study of intraprotein proton transfer. Of the many residues that are potentially capable of involvement in proton transfer pathways to the Q_B binding site, two acidic residues have been strongly implicated by recent studies, using site-directed mutagenesis in *Rb. sphaeroides* RCs: glutamic and aspartic acids at positions 212 and 213 of the L subunit (Figure 1). Paddock et al. (1989) altered Glu^{L212} to the nonionizable residue glutamine and found the mutation to essentially eliminate the pH dependences of the one-electron $Q_A^-Q_B \leftrightarrow Q_AQ_B^-$ equilibrium and electron transfer rate at alkaline pH. This identified Glu^{L212}, with an unusually high pK (≈9.6), as the residue that inhibits the electron transfer when ionized. The mutation also exhibited a significantly reduced rate of transfer of the second proton to Q_B^{2-}, resulting in a substantially lower maximum steady-state rate of cytochrome *c* photooxidation when compared to the that of wild type (Wt).

† This work was supported by grants from the National Science Foundation (DMB 86-01744 and DMB 89-04991) and from the United States Department of Agriculture Competitive Grants Office (AG 86-1-CRCR-2149 and AG 89-37262-4462). E.T. gratefully acknowledges receipt of a traineeship from the McKnight Foundation.

0006-2960/92/0431-855$03.00/0

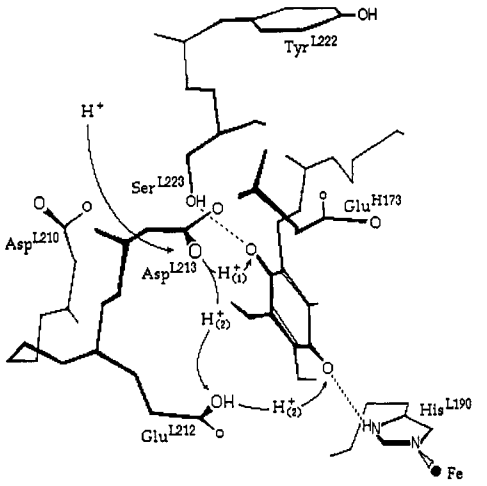


FIGURE 1: Q_B binding site of *Rb. sphaeroides* reaction centers showing some of the polar and ionizable residues. The hydrocarbon side chain of the ubiquinone (Q-10) is truncated after the third isoprene unit. Hydrogen bonds to the quinone carbonyls are shown by dotted lines. The arrowed lines show putative paths for delivery of the first ($H^+_{(1)}$) and second ($H^+_{(2)}$) proton to Q_B, as described in this work. (X-ray crystallographic coordinates kindly provided by J. Allen.)

Alteration of Asp^{L213} to asparagine, by Takahashi and Wraight (1990), resulted in even more dramatic effects, including a large increase in the $Q_A^-Q_B \leftrightarrow Q_AQ_B^-$ equilibrium in favor of Q_B reduction and an altered pH dependence of the equilibrium. Transfer of the second electron to Q_B^- was drastically affected. The mutant exhibited a severe obstruction of the first proton associated with, or necessary for, the double reduction of Q_B after the second flash, leading to a total failure of the transfer of the second electron to Q_B at alkaline pH. In this paper, the behavior of a double mutant of the residues Glu^{L212} and Asp^{L213} (altered to Gln and Asn, respectively) is described and compared to the behaviors of the single mutants of these residues, for which new observations are also reported. A coherent description is presented for the proton and electron transfer events leading to the formation of quinol in the reaction center.

Materials and Methods

Details of the molecular biological techniques involved in generating *Rb. sphaeroides* with mutant RCs have been described previously (Takahashi et al., 1990). The L212 (Glu → Gln) and L213 (Asp → Asn) single mutations and the L212 (Glu → Gln)/L213 (Asp → Asn) double mutation (designated as mutants L212EQ, L213DN, and L212EQ/L213DN, respectively) were obtained by the in vitro mutagenesis method of Kunkel (1986), using oligonucleotides containing alterations of the codons for amino acid residues 212 (GAG → CAG) and 213 (GAT → AAT) of the L-subunit gene. Screening of the desired mutations was performed by DNA sequencing. A kanamycin- (Km-) resistant *Rb. sphaeroides* RC deletion strain, GaKM(+) (Takahashi et al., 1990), was complemented in trans with a tetracycline- (Tc-) resistant broad host range plasmid pRK404 (Ditta et al., 1985; Donohue et al., 1988) carrying the RC genes with the various mutations. Expression of mutant RCs was accomplished by growing the complemented deletion strain under semiaerobic conditions, in the dark, in Sistrom's minimal medium (Sistrom, 1960) supplemented with 0.2% casamino acids, in the presence of antibiotics (25 μg/mL Km and 2 μg/mL Tc).

Reaction centers were isolated by solubilization of chromatophore membranes using lauryldimethylamine *N*-oxide (LDAO), followed by ammonium sulfate precipitation and ion-exchange column chromatography on DEAE-Sephacel, as previously described (Maróti & Wraight, 1988a). RCs were eluted from the DEAE-Sephacel column with 220 mM NaCl/0.1% LDAO/10 mM Tris (pH 8.0). Wild-type RCs were isolated from the strain Ga, parent to GaKM(+). (In this work, wild type (Wt) refers to the electron and proton transfer functions of the reaction center and will not distinguish between variously pigmented strains, such as 2.4.1, Ga, and R26, which differ in their carotenoid content). Purified RCs had about 1.2–1.5 quinones, as indicated by functional assays of endogenous electron acceptor activity; i.e., 20–50% exhibited Q_B activity in the absence of added Q-10.

Mutant RCs were characterized by optical spectroscopy performed on a kinetic spectrophotometer apparatus of local design. The assay solution was 2.5–50 mM KCl/0.03% Triton X-100/1 mM buffer (succinate, citrate, Mes, Pipes, Tris, glycylglycine, Ches, or Caps, depending on the pH) and about 1 μM RC. For most measurements, secondary quinone activity was supported by the presence of 20 μM ubiquinone (Q-10). For determination of the $P^+Q_A^-$ recombinant kinetics, Q-10 was omitted and 50 μM terbutryn or 1 mM *o*-phenanthroline was added to inhibit residual Q_B activity. The kinetics of $P^+Q_A^-$ and $P^+Q_AQ_B^-$ charge recombination were measured at 430 nm, in the absence of an electron donor. The kinetics of electron transfer from Q_A^- to Q_B were measured at 397 nm, where the spectrum for Q_A^- includes a significant electrochromic response of the nearby bacteriopheophytin (Vermeglio, 1977; Wraight & Stein, 1983). Cytochrome oxidation was monitored at 550 nm, with 20 μM horse heart cytochrome *c*. The semiquinone signals of Q_A^- and Q_B^- were monitored at 450 nm with 5–100 μM ferrocene as the donor to P^+.

Flash-induced proton binding was also followed spectroscopically, at the $P^+Q_A^-$ isosbestic wavelength near 586 nm, using pH-indicator dyes (Maróti & Wraight, 1988a). The assay solution contained 50 mM NaCl, 40 μM pH indicator dye, 20 μM Q-10, 0.03% Triton X-100, 100 μM ferrocene, and 1 μM RC. Calibration was performed with aliquots of standard HCl. Samples were gassed under nitrogen prior to the start of the assay. Similar measurements were made after the addition of 10 mM buffer with appropriate p*K*, and the net proton uptake was derived by subtracting the buffered traces from the unbuffered traces.

We found previously that L213DN mutant RCs can trap Q_B^- very effectively and therefore take a very long time to relax fully to the dark-adapted state, PQ_AQ_B (Takahashi & Wraight, 1990). In this work, therefore, most measurements on RCs with secondary quinone activity were performed with a fresh sample for each measurement. However, continuous pH titrations were employed for the $P^+Q_A^-$ recombination kinetics, with 0.5 mM each succinate and citrate to prevent locally intense acidification during pH adjustment, which was noted to cause denaturation of mutant RCs.

Results

Decay of the Charge Separated States in the Absence of Donors to P^+. At pH 7.0, with no secondary donors present to rereduce the oxidized primary donor, P^+, the charge separated states of isolated RCs from L212EQ/L213DN decayed in a manner very similar to that described for RCs from the single mutant L213DN (Takahashi & Wraight, 1990) (Figure 2). In the absence of added ubiquinone, the decay of P^+ after a flash was fast, with a lifetime, τ, of 80–100 ms, corresponding to charge recombination of the $P^+Q_A^-$ state. When ubiquinone was added the kinetics became very slow, with $\tau \approx 16$ s at pH 7.0, indicative of reconstitution of Q_B activity, but with altered properties since Wt RCs show $\tau \approx 1.4$ s. At pH 5.0, the slow phase titrated in with a half-saturating concentration of ≤2

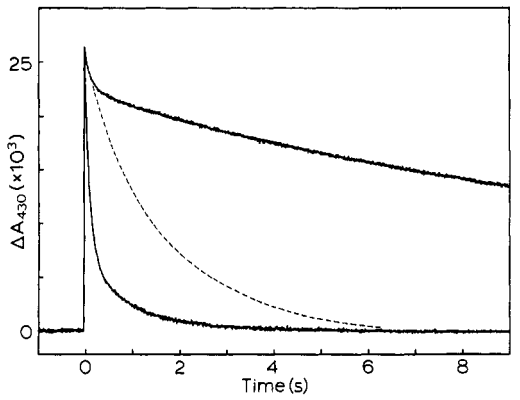


FIGURE 2: Recombination kinetics in isolated RCs from L212EQ/L213DN double mutant of *Rb. sphaeroides*. P^+ decay following a single flash, measured at 430 nm. Conditions: approximately 1 μM RCs in 2.5 mM KCl/1 mM Pipes, pH 7.0/0.03% Triton X-100/20 μM Q-10. Top trace: $P^+Q_B^-$ decay. Bottom trace: $P^+Q_A^-$ decay, plus 420 μM terbutryn. Dashed line: $P^+Q_B^-$ decay in wild-type (Wt) RCs.

μM for Q-10, and in thoroughly dark-adapted samples the maximum amplitude of the slow phase reached 85–90%. At higher pH, however, the level of reconstituted appeared to fall and, in the presence of 20 μM Q-10, the relative amplitude of the slow recovery phase decreased to about 60% at pH 9.0 and above. This implies a more marked pH dependence of Q_B binding than is seen in Wt RCs (Takahashi et al., 1990).

The slow phase of the P^+ decay in mutant RCs, in the presence of ubiquinone, was eliminated by sufficiently high concentrations of the herbicide, terbutryn, known to act by competitive binding at the Q_B site (Stein et al., 1984; Wraight, 1981). This supports the origin of the slow kinetics as arising from the recombination of the state $P^+Q_AQ_B^-$. However, all mutant RCs required somewhat higher (10–30-fold) concentrations of terbutryn than did Wt RCs.

pH Dependence of the $P^+Q_A^-$ Decay Rate. The pH dependences of the $P^+Q_A^-$ recombination kinetics were determined, for Wt and mutant RCs, without added ubiquinone and in the presence of 50 μM terbutryn or 1 mM *o*-phenanthroline to inhibit residual Q_B activity (Figure 3A). At pH < 6, the kinetics for all RCs were essentially monoexpoential, but the mutant RCs exhibited some degree of nonexponentiality at lower pH values. When analyzed as a biexpoential, the fast phase, with τ = 80–120 ms, was strongly dominant (>75%). The biphasicity has previously been attributed to a slow unbinding of the inhibitor, followed by binding of residual quinone during the time course of the recombination and allowing some $P^+Q_B^-$ recombination (Stein et al., 1984). [This is more noticeable at low pH because quinone binding is stronger (Takahashi et al., 1990; C. A. Wraight, unpublished observations).] In all cases, the dominant fast phase is a good approximation of the intrinsic $P^+Q_A^-$ recombination rate, and we have taken this for comparison with the $P^+Q_B^-$ recombination kinetics in the present work.

The $P^+Q_A^-$ recombination rates were weakly pH dependent for all four RC types, increasing steadily at higher pH. This has been previously described for Wt RCs (McPherson et al., 1990), but small quantitative differences existed between the mutants, which were all slightly faster than the Wt. In all cases, the net change in rate over the entire pH range was less than 50%, and the largest difference between any of the RC types was no more than 60%.

Small differences were also observed in the pH dependences of the $P^+Q_A^-$ recombination rate depending on the nature of the occupancy of the Q_B binding site, i.e., with *o*-phenanthroline or terbutryn or in the absence of any agent (quinone-depleted RCs) (not shown). This is currently under further examination.

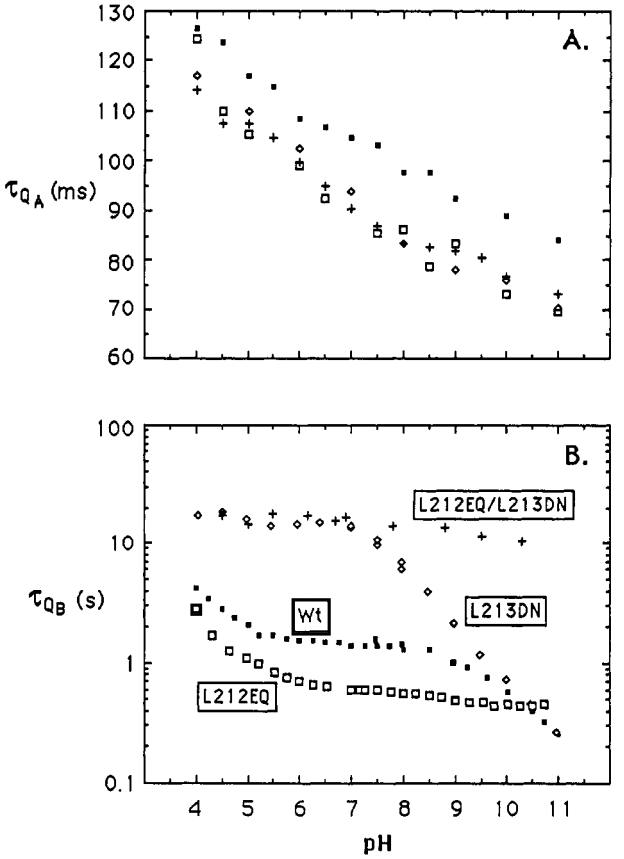


FIGURE 3: pH dependence of the kinetics of charge recombination in wild-type (Wt) and mutant (L212EQ, L213DN and L212EQ/L213DN) RCs of *Rb. sphaeroides*. (A) The lifetime of the fast component of the $P^+Q_A^-$ charge recombination (τ_{Q_A}), determined from a biexponential, nonlinear least-squares (Marquardt) fit to the data. The amplitude of the fast phase was at least 75% of the total. Conditions were as for Figure 2, except without Q-10 and plus 1 mM *o*-phenanthroline for all samples: ■, Wt; □, L212EQ, ◇, L213DN, +, L212EQ/L213DN. Note linear scale for τ_{Q_A}. (B) The lifetime of $P^+Q_AQ_B^-$ charge recombination (τ_{Q_B}), determined from a biexponential, nonlinear least-squares (Marquardt) fit to the data. The ampltiude of the slow phase was 60–90% of the total, decreasing at higher pH. Conditions were as for Figure 2, plus 20 μM Q-10 for all samples. Note logarithmic scale for τ_{Q_B}. Various buffers (1 mM) were used through the pH range, as described under Materials and Methods.

pH Dependence of the $P^+Q_AQ_B^-$ Decay Rate. The pH dependences of the P^+ recovery rates ($P^+Q_AQ_B^- \rightarrow PQ_AQ_B$ charge recombination) for Wt and three mutant RCs are shown in Figure 3B.[1] In the range accessible to study (pH 4–11), Wt RCs exhibited two regions of significant pH dependence—below pH 5.5 and above pH 9. In contrast, the charge recombination rate for L212EQ/L213DN double mutant RCs was essentially pH independent over the entire pH range. The single mutants are shown for comparison, although they have been previously described. In the L212EQ mutant, the rate of $P^+Q_B^-$ recombination was somewhat faster than Wt over most of the pH range ($\tau \approx 0.6$ s, at pH 7.0) and was pH independent at high pH (Paddock et al., 1989). pH dependence was retained at low pH. In L213DN mutant RCs, the decay of $P^+Q_B^-$ was much slower than that of Wt below pH 9 and exhibited pH dependence only at alkaline pH

[1] The data are presented as lifetimes rather than rate constants, because the lifetime (τ_{Q_B}) directly reflects the equilibrium constant between $Q_A^-Q_B$ and $Q_AQ_B^-$, as described by eq 1.

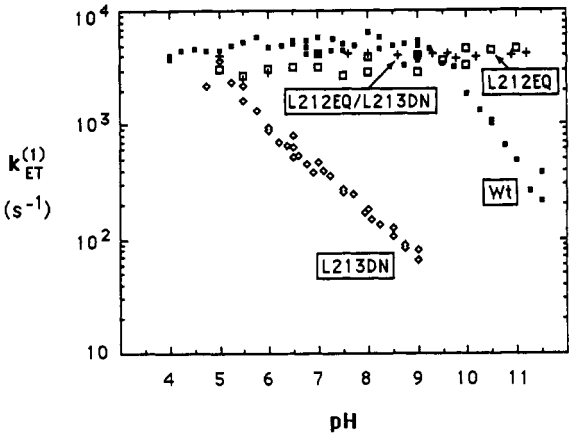


FIGURE 4: pH dependence of the observed rate constant for $Q_A^-Q_B \rightarrow Q_AQ_B^-$ electron transfer ($k_{ET}^{(1)}$) in RCs from wild type (Wt) and mutant (L212EQ, L213DN and L212EQ/L213DN) RCs of *Rb. sphaeroides*, measured at 397 nm. Conditions were as for Figure 3B.

(Takahashi & Wraight, 1990).

Kinetics of $Q_A^-Q_B$ to $Q_AQ_B^-$ Electron Transfer. In Wt RCs, the electron transfer kinetics showed some biphasicity between pH 7 and 9, with rate constants for the two components differing by up to 5-fold. This has been described previously by us (Marôti & Wraight, 1989; Takahashi et al., 1991; and unpublished observations), but the phenomenon is not fully understood at the present time. L213DN mutant RCs also showed some biphasicity at alkaline pH, but the L212EQ and double mutants did not reveal any detectable or consistent biphasic behavior. This is currently under further investigation. In the current work, where two components were obtained from the exponential analyses, only the dominant (fast) phase is considered.

The pH dependence of the $Q_A^-Q_B \rightarrow Q_AQ_B^-$ electron transfer rate[2] for Wt and mutant RCs is shown in Figure 4. In Wt RCs, the observed rate constant, $k_{ET}^{(1)}$, was 5.10^3 s^{-1} up to pH 9 but became slower at higher pH with an apparent p*K* of about 9.5 (Kleinfeld et al., 1984a; Stein, 1985). As reported by Paddock et al. (1989), the rate in the L212EQ mutant was essentially constant from pH 5 to pH 11 and was similar to the maximum Wt value. The behavior of the L212EQ/L213DN double mutant was very similar to that of the L212EQ single mutant. In marked contrast, the rate in the L213DN mutant was similar to that in the Wt only at pH < 6 and declined steadily at higher pH. At pH > 8 the electron transfer rate was comparable to the $P^+Q_A^-$ recombination rate, but the data analysis took this into account by fitting both the rising ($Q_A^-Q_B \rightarrow Q_AQ_B^-$ electron transfer) and decaying (P^+ decay) portions of the transient.

Reaction Center Turnover in Multiple Flashes: Cytochrome Oxidation. The ability of isolated RCs to perform multiple turnovers in the presence of adequate electron donation to P^+ is a measure of the functional integrity of the quinone electron acceptor activities. The turnover capabilities of Wt and mutant RCs was monitored via the oxidation of cytochrome *c* in a series of saturating flashes (Figure 5). Very strong inhibition was seen in the L213DN and L212EQ/L213DN mutant RCs, and moderate inhibition was seen in L212EQ. In L213DN

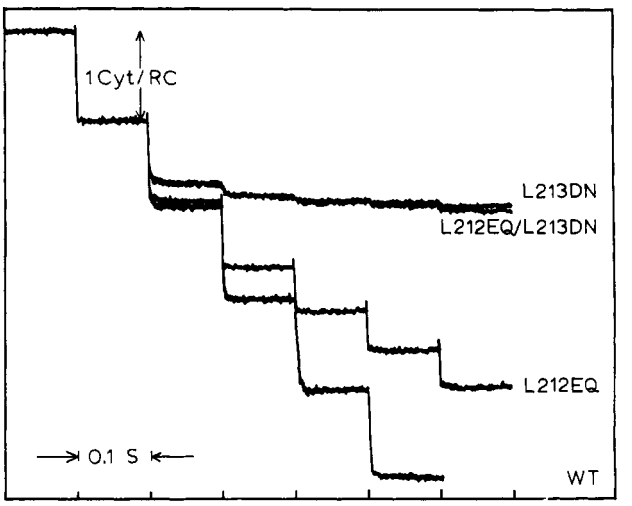


FIGURE 5: Flash-induced cytochrome *c* oxidation by wild-type (Wt) and mutant (L212EQ, L213DN and L212EQ/L213DN) RCs of *Rb. sphaeroides*. Cytochrome *c* oxidation measured at 550 nm. Conditions were as for Figure 3B with 1 mM Pipes, pH 7.0, plus 20 μM horse heart cytochrome *c*. Time between flashes was 0.1 s.

and L212EQ/L213DN mutant RCs, at pH > 6.5, significant cytochrome oxidation was observed only on the first two flashes and very little was observed after subsequent flashes, even at low repetition rates (<1 Hz). Activity was partially restored at lower pH.

Impairment of the turnover capabilities of the L212EQ mutant was also readily detectable in this assay but was strong only with high flash repetition rates (> 20 Hz). Variation of the flash repetition rate showed that the turnover time of L212EQ RCs, determined in the steady state, was about 100 ms at pH 8.0 (not shown). This is 50–100 times slower than the Wt.

Reaction Center Turnover in Multiple Flashes: Semiquinone Behavior. Turnover of the RCs was also inferred from the semiquinone behavior in multiple flashes in the presence of exogenous donor (ferrocene). The typical oscillatory behavior seen for the Wt RC (Vermeglio, 1977; Wraight, 1977, 1979) was not observed with L212EQ/L213DN double mutant RCs above pH 7.5 (Figure 6A, bottom) or in L213DN RCs at pH values above 6.5 (Takahashi & Wraight, 1990). Above these pH values, a stable semiquinone absorbance signal was observed after the first flash, but on subsequent flashes the additional absorbance change decayed slowly, approximately to the first flash level.[3] The lifetime of the decay of the semiquinone signal after the second flash was about 16 s at pH > 8, indicating a drastically inhibited rate for the second electron transfer to Q_B when compared to that in Wt RCs [$\tau \approx 1$ ms, at pH 8.0 (Kleinfeld et al., 1985; Vermeglio & Clayton, 1977; Wraight, 1979)]. Below an apparent p*K* value of about 8 for both L213DN and L212EQ/L213DN mutant RCs, the rate of the second electron transfer increased almost linearly with increasing H^+ ion concentration (Figure 6B), and strong oscillations in the formation and disappearance of the semiquinone signal were seen at acidic pH for L213DN and L212EQ/L213DN RCs (Figure 6A, top).

The semiquinone behavior of L212EQ mutant RCs was also examined (Figure 7A). At pH 8.0, the decrease in absorbance

[2] The observed rate constant, $k_{ET}^{(1)}$, is equal to $k_{AB}^{(1)} + k_{BA}^{(1)}$, the sum of the forward and backward rate constants for the first electron transfer. For Wt, L213DN, and L212EQ/L213DN RCs, the one-electron transfer equilibrium ($K_{ET}^{(1)}$) is sufficiently large over most of the pH range that $k_{BA}^{(1)}$ is small compared to $k_{AB}^{(1)}$. However, for L212EQ RCs above pH 6, and for Wt and L213DN RCs above pH 10, a small correction must be applied according to $k_{AB}^{(1)} = k_{ET}^{(1)}[1 + 1/K_{ET}^{(1)}]^{-1}$.

[3] The small amount of decaying semiquinone signal seen on the first flash, at pH 6.0, is due to the presence of some Q_B^- in the dark adapted state. This is a reflection of the much greater stability of the semiquinone in this mutant, compared to in Wt RCs (see Discussion). Very similar behavior was reported previously for L213DN mutant RCs (Takahashi & Wraight, 1990).

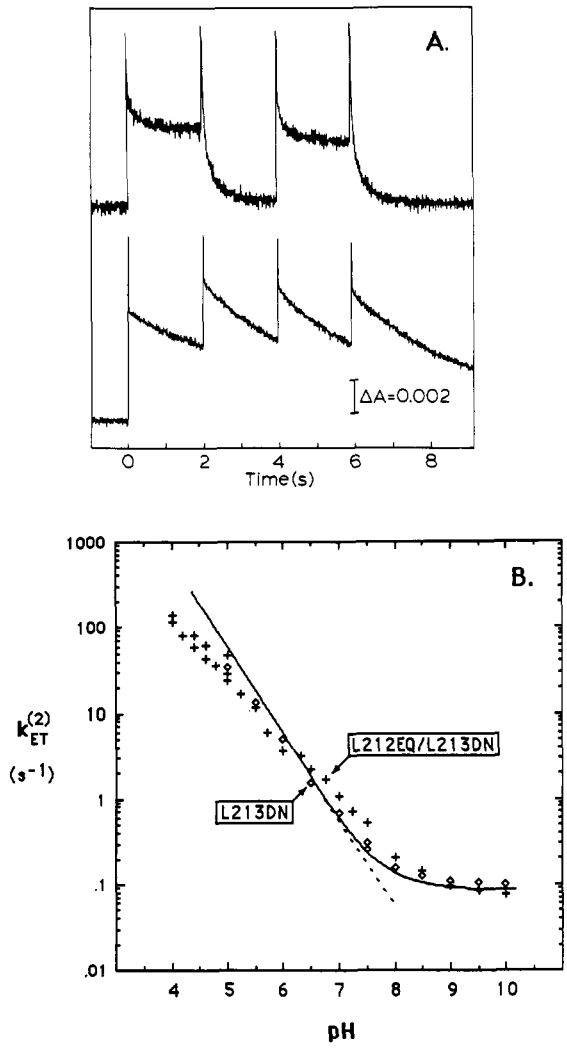
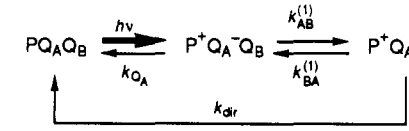


FIGURE 6: Semiquinone behavior in L212EQ/L213DN double mutant RCs. (A) Absorbance changes at 450 nm in a series of four flashes given at 2-s intervals. Conditions were as for Figure 2B, plus 100 μM ferrocene. Top: 1 mM Mes, pH 6.0. Bottom: 1 mM glycylglycine pH 8.0. (B) pH dependence of the rate constant of $Q_A^-Q_B^-$ decay after the second flash, for L212EQ/L213DN double mutant (+) and L213DN single mutant (◇) RCs. The dashed line shows a linear dependence of the rate on H^+ concentration; the solid curve indicates the effect of an alternative decay route becoming dominant at high pH, giving an apparent pK of 8.0.

at 450 nm after the second flash was distinctly biphasic: a fast phase ($\tau \approx 1$ ms) comprised about 60% of the amplitude, followed by a slow phase with $\tau \approx 1.3$ s. The rates and amplitudes of both phases were strongly pH dependent (Figure 7B,C). The slow phase decreased in amplitude at lower pH and was also much accelerated. It could not be reliably detected below pH 7.0. At pH 10.5, the decay of the A_{450} signal was all slow and the rate of electron transfer from Q_A^- to Q_B^- could not be distinguished from adventitious reoxidation of Q_A^- by exogenous agents.

Proton Binding in a Flash Series. Flash-induced proton binding by L212EQ/L213DN double mutant RCs was fully consistent with the observed semiquinone behavior. At pH > 7.5, a small rapid uptake of H^+ occurred after the first flash, but very little occurred after subsequent flashes (Figure 8, bottom). Some slow proton uptake was seen after the second and subsequent flashes, accompanying the slow decay of the semiquinone signal. The extent of net proton binding after the first flash was substantially less in the double mutant RCs, at high pH, than in Wt RCs. In the presence of exogenous electron donors to P^+, the latter show substantial H^+ binding

Scheme I



throughout the pH range from 5 to 10 and ≥0.7 H^+/RC between pH 8 and 10 (Marôti & Wraight, 1988b; McPherson et al., 1988), but the stoichiometry of H^+ binding in double mutant RCs was very small at pH 8.0 (0.25 H^+/RC) and fell to zero at higher pH (not shown).

At pH < 7.5, where oscillations in the semiquinone signal were observed, the stoichiometry of H^+ binding was very similar to that of the Wt—approximately 0.6 H^+ on the first flash and 1.4 H^+ on the second (Marôti & Wraight, 1988b; McPherson et al., 1988), with low-amplitude oscillations apparent in a series of flashes (Figure 8, top). The major component of the kinetics of H^+ binding on the second flash was very similar to that of the $Q_A^-Q_B^- \rightarrow Q_AQ_B^{2-}$ ($Q_AQ_BH_2$) electron transfer ($\tau \approx 100$ ms at pH 6.0).

In L212EQ mutant RCs, the proton binding observed in a series of flashes also accurately reflected the semiquinone behavior (Figure 9). At pH 8, on the first flash, about 0.4 H^+/RC was taken up rapidly. On the second flash, a further 0.8 H^+/RC was taken up rapidly, followed by about 0.7 H^+/RC with kinetics that matched the slow phase of the semiquinone decay.

Discussion

$Q_A^-Q_B \leftrightarrow Q_AQ_B^-$ Electron Transfer Equilibrium. The decay of $P^+Q_AQ_B^-$ can be described by two parallel routes, one via the equilibrium population of $P^+Q_A^-Q_B$ and one as a direct recombination between P^+ and Q_B^- (Blankenship & Parson, 1979; Kleinfeld et al., 1984a; Wraight, 1979), as shown in Scheme I. In *Rb. sphaeroides* Wt RCs, it is established that k_{dir} is negligible and that the decay of $P^+Q_AQ_B^-$ is dominated by charge recombination via $P^+Q_A^-Q_B$, over a wide range of pH (Kleinfeld et al., 1984a; Wraight & Stein, 1980). In such a case, the observed rates of P^+ decay in the absence (k_{Q_A}) and presence (k_{Q_B}) of functional Q_B provide a simple assay of the one-electron transfer equilibrium ($K_{ET}^{(1)} = k_{AB}^{(1)}/k_{BA}^{(1)}$) as given by

$$K_{ET}^{(1)} = k_{Q_A}/k_{Q_B} - 1 = \tau_{Q_B}/\tau_{Q_A} - 1 \qquad (1)$$

In general—and this is true for all RC types examined in this work—the $P^+Q_A^-$ decay is only mildly pH dependent so that the pH dependence of τ_{Q_B} quite accurately reflects the pH dependence of $K_{ET}^{(1)}$. Similarly, the variability in τ_{Q_A}, depending on the occupation state of the Q_B site, has little influence on the magnitude of $K_{ET}^{(1)}$.

The observed decay of $P^+Q_B^-$ in L212EQ/L213DN mutant RCs and in L213DN mutant RCs at low pH is sufficiently slow that the direct charge recombination between P^+ and Q_B^- cannot be considered negligible a priori and could contribute to the decay. Thus, the limiting small value of this rate may not reflect a limiting (large) value for the electron transfer equilibrium constant, as implied by the relationship of eq 1, and caution must be exercised in interpreting these data.[4]

[4] In a herbicide-resistant mutant of *Rps. viridis*, where $K_{ET}^{(1)} \approx 5 \times 10^3$, Baciou et al. (1991) have recently shown that the slow $P^+Q_B^-$ decay still proceeds via the $P^+Q_A^-$ state and not by the direct route. However, the halftime of the decay is only about 1 s. By contrast, in *Chromatium minutissimum*, Shinkarev et al. (1991) find that $K_{ET}^{(1)} \approx 300$, at pH < 7, while the halftime of $P^+Q_B^-$ decay is more than 10 s, and they suggest that the recombination may proceed substantially via the direct route at low pH.

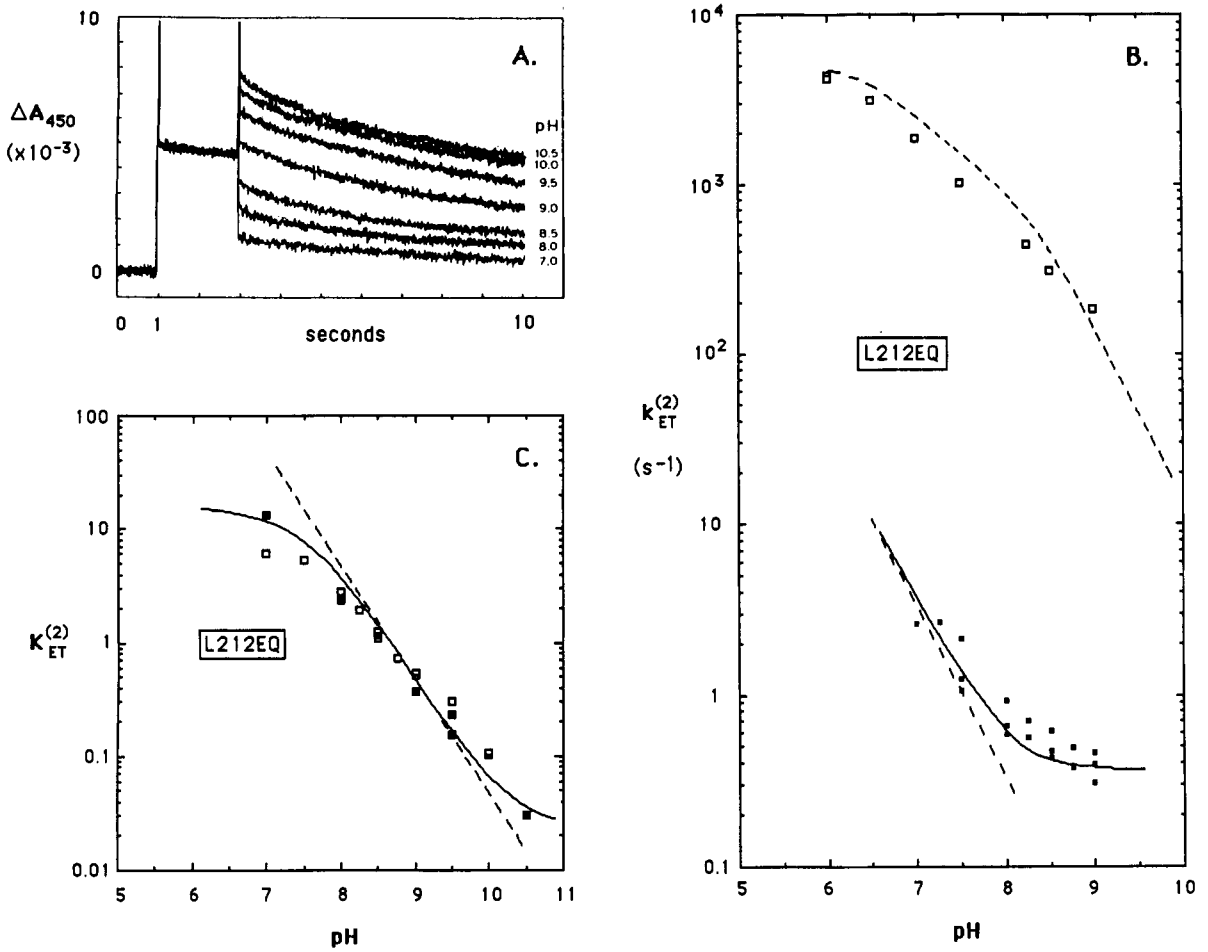


FIGURE 7: Semiquinone behavior in L212EQ mutant RCs. (A) Absorbance changes at 450 nm in a pair of flashes. Conditions were as for Figure 3B, plus 100 μM ferrocene; pH was as indicated. (B) pH dependence of the rate constants of the two phases of decay of $Q_A^-Q_B^-$ after the second flash. The ferrocene concentration was varied in the range 1–200 μM to optimize resolution of the fast and slow phases. The dashed line shows the pH dependence of the rate in Wt RCs [redrawn from Wraight (1979)]; the solid line shows a pH dependence of the same type as drawn for double mutant RCs (Figure 6), with a linear dependence on H^+ concentration at low pH and a slow, limiting process at high pH. (C) pH dependence of the equilibrium constant for the $Q_A^-Q_B^- \leftrightarrow Q_AQ_BH^-$ electron transfer, $K_{ET}^{(2)}$, calculated from the relative amplitude of the fast phase of decay, according to the equation $K_{ET}^{(2)} = 1.6\Delta A_{450}^{(1)}/(\Delta A_{450}^{(1)} + \Delta A_{450}^{(2)}) - 1$ where $\Delta A_{450}^{(1)}$ and $\Delta A_{450}^{(2)}$ are the changes in absorbance at 450 nm immediately after the first and second flashes, respectively, associated with the fast formation or disappearance of semiquinone. The factor 1.6 arises from the ratio of extinction coefficients for the states Q_B^- and $Q_A^-Q_B^-$ (Kleinfeld et al., 1985). The equation does not take into account the first electron equilibrium, $K_{ET}^{(1)}$, the effect of which is small at pH < 10.5. The dashed line shows the expected behavior, according to log $K_{ET}^{(2)} = 2.303(8.7 - pH)$; the solid curve indicates the effects of pKs (see Scheme III) near the experimental range: $pK_{Q_A^-Q_B^-} = 7.4$, $pK_{Q_AQ_B^{2-}} = 10.3$.

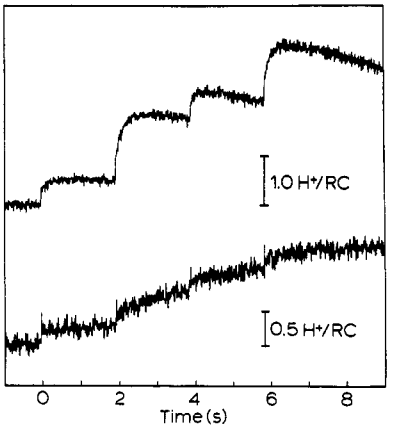


FIGURE 8: Proton binding by L212EQ/L213DN mutant RCs. Conditions: approximately 1 μM RCs. 50 mM NaCl, 0.03% Triton X-100, 20 μM Q-10, 40 μM indicator dye; the measuring wavelength 586 nm. Four flashes were given at 2-s intervals. Top: pH 6.0; chlorophenol red was the indicator. Bottom: pH 8.0; cresol red was the indicator.

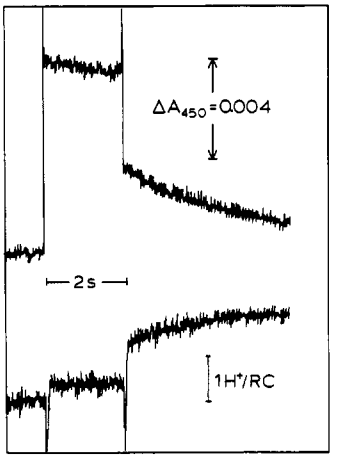
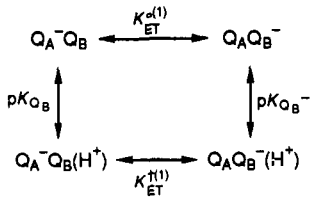


FIGURE 9: Proton binding and semiquinone kinetics in L212EQ mutant RCs. Top: Semiquinone absorbance changes at 450 nm. Conditions were as for Figure 7 with 1 mM glycylglycine, pH 8.0. Bottom: Proton binding, measured at 586 nm. Conditions were as for Figure 8, pH 8.0, with cresol red as the indicator.

With this proviso, the pH dependence of the electron transfer equilibrium can be qualitatively understood in terms of protonation equilibria of residues that interact with Q_B. The semiquinones, Q_A^- and Q_B^-, do not bind protons directly

Scheme II



(Wraight, 1979), but the electrostatic influence and associated charge compensation of the anions cause pK shifts in a few or many residues of the protein (Marôti & Wraight, 1988b; McPherson et al., 1988). The interaction with ionizable residues is stronger for Q_B^- than for Q_A^-, leading to net stabilization of $Q_AQ_B^-$ by proton uptake. For a single protonation site, the interaction between protonation and electron transfer can be represented as shown in Scheme II (Kleinfeld et al., 1984a; Marôti & Wraight, 1988b). pK_{Q_B} and $pK_{Q_B^-}$ are pK values for a group interacting with Q_B, i.e., near it, when Q_B is in its oxidized and semireduced states, respectively. $K_{ET}^{o(1)}$ and $K_{ET}^{+(1)}$ are the equilibrium constants for the electron transfer, at the high and low pH limits when the protonatable group is fully ionized and fully protonated, respectively. The pH dependence of the net electron transfer equilibrium is then given by

$$K_{ET}^{(1)} = [Q_AQ_B^- + Q_AQ_B^-(H^+)]/[Q_A^-Q_B^- + Q_A^-Q_B(H^+)] = K_{ET}^{+(1)} \frac{1 + 10^{pH-pK_{Q_B^-}}}{1 + 10^{pH-pK_{Q_B}}} \quad (2)$$

More complicated expressions can be derived for multiple protonation equilibria by involving interactions with both Q_A and Q_B. However, for simplicity, in the following discussions all influences will be ascribed to interactions with the various redox states of Q_B. Thus, any pKs associated with the $Q_A^-Q_B$ state will be defined as pK_{Q_B} rather than $pK_{Q_A^-}$.[5]

Below pH 7.0, the charge recombination rate (k_{Q_B}) in L213DN mutant RCs was essentially pH independent, in contrast to that in Wt RCs which displayed a region of pH dependence below pH 5.5 (Takahashi & Wraight, 1990). Thus, Asp^{L213} could be responsible for the pH-dependent behavior in the Wt RC, with an apparent pK value with Q_B^- present ($pK_{Q_B^-}$) of 5.5. Although no low-pH plateau is apparent from the available data, the slope of the pH dependence for Wt RCs at low pH suggests that the pK for Asp^{L213} with Q_B oxidized is $pK_{Q_B} \approx 4$, but it could be lower if the limiting rate of $P^+Q_B^-$ recombination represents the direct route.

The region of pH dependence of the charge recombination rate in L213DN RCs seems to correspond with the high-pH region of the Wt, but with the pK shifted down from 9.5 to 7.5. Again, this could be lower if other P^+ decay routes, such as direct charge recombination, are significant and it is noteworthy that the onset of pH dependence is rather sharp at pH 7–7.5. The decay in L213DN and in double mutant RCs is sufficiently slow that adventitious donors to P^+ could compete with recombination, and this does occur to some extent, as evidenced by the accumulation of Q_B^- after several flashes in the absence of added donors (Takahashi & Wraight, 1990). It is not possible to eliminate such adventitious donation altogether—even clean glassware has some activity—but the extent of donation is small (a few percent after each flash) and is a minor contributor to the decay of P^+. Another possibility is that Q_B^- escapes from the mutant RCs, leaving a long-lived P^+Q_A state. The decay would then also be via exogenous donation, presumably from QH_2 formed by disproportionation of the semiquinones in solution. However, this is expected to be strongly pH dependent due to the rate-determining influence of the formation of QH^-, the reactive species (McComb, 1987; Rich, 1981), in contrast to the unequivocal pH independence of the P^+ decay in the double mutant RCs.

We are left with the direct recombination of $P^+Q_B^-$ as the most likely path and the essential identity of the limiting rates in L213DN and double mutant RCs, and the fact that this appears to be slower than the low-pH limit value indicated by the Wt data lends some circumstantial support to this notion. This would place a value on k_{dir} of about 0.06 s^{-1}, consistent with the upper limit of 0.1 s^{-1} obtained by Kleinfeld et al. (1984a). A separate study by Kleinfeld et al. (1984b) showed that $P^+Q_B^-$, trapped by illumination during freezing, decayed with a complex time course over a period of days at 77 K. If this represents the same phenomenon as that seen in the mutant RCs, the implication is that the "direct" recombination of $P^+Q_B^-$ is thermally activated to a significant extent, in contrast to the well-studied tunneling recombination of $P^+Q_A^-$ (Gunner et al., 1986). This is currently under investigation.

The slope of the pH dependence of τ_{Q_B} in L213DN mutant RCs, above pH 7, is −0.5 over 3 pH units. The origin of this extended range and nonunity slope probably includes the progressive change in surface charge and surface pH with increasing bulk pH, but it may also implicate novel, ionizable groups in the charge compensation of Q_B^-. Nevertheless, if we do identify the onset of pH dependence with pK_{Q_B} of Glu^{L212}, we conclude that at least 2–2.5 pH units of the anomalous pK for Glu^{L212} in the Wt results from interaction with Asp^{L213} and the high pK of Glu^{L212} is evidently not due simply to a significantly hydrophobic environment in the Q_B pocket, suggested by Paddock et al. (1989).

The behavior of the L212EQ/L213DN double mutant is consistent with these conclusions concerning the pH dependences in Wt and L213DN mutant RCs. It exhibits very slow, almost pH-independent, $P^+Q_B^-$ recombination kinetics over a wide pH range, as expected for the absence of any ionizable residues in the Q_B pocket.

The recombination rates for L213DN and L212EQ/L213DN are substantially slower than the Wt over most of the measured pH range, and especially between pH 5 and 8 where the rates differ by an order of magnitude or more. This indicates a significant additional stabilization of the electron on Q_B^-. When combined with the slightly faster $P^+Q_A^-$ recombination rates of the mutants, according to eq 1, we find $K_{ET}^{(1)} \approx 200$, at $5 < pH < 8$, with the understanding that this is a lower limit if the direct recombination route contributes significantly to the $P^+Q_B^-$ decay. Assuming that the redox midpoint potential (E_m) of Q_A/Q_A^- is unchanged in the mutant, application of the relationship $\Delta E_m = RT \ln K$ yields an E_m for Q_B/Q_B^- that is at least 70–80 mV higher than in the Wt. This is consistent with the difficulty encountered in depleting Q_B^- from L213DN and double mutant RCs by dark adaptation. For the Wt, $E_m(Q_B/Q_B^-) \approx 40$ mV at pH 8, in chromatophores (Rutherford & Evans, 1980), so, for the mutants, $E_m(Q_B/Q_B^-) \geq 120$ mV. It is likely that the average potential, $E_m(Q_B/Q_BH_2)$, is little changed in these mutants, in which case the E_m for the second electron (Q_B^-/Q_BH_2) is expected to be lowered by an equivalent amount, relative to the Wt, according to

[5] In fact, pK_{Q_B} and $pK_{Q_B^-}$ would be better represented as $pK_{Q_A^-Q_B}$ and $pK_{Q_AQ_B^-}$ to indicate that they are resultant pK values reflecting the mutual influences of Q_A/Q_A^- and Q_B/Q_B^-. In Kleinfeld et al. (1984a), they are denoted $pK_{Q_A^-}$ and $pK_{Q_B^-}$, respectively.

$$E_m(Q_B/Q_BH_2) = \tfrac{1}{2}[E_m(Q_B/Q_B^-) + E_m(Q_B^-/Q_BH_2)]$$

For the Wt, $E_m(Q_B^-/Q_BH_2) \approx -40$ mV at pH 8, in chromatophores (Rutherford & Evans, 1980), so, for the mutants, $E_m(Q_B^-/Q_BH_2) \leq -120$ mV. This may place $E_m(Q_B^-/Q_BH_2)$ below $E_m(Q_A/Q_A^-)$, possibly contributing to the failure of transfer of the second electron in these mutants.

$Q_A^-Q_B \leftrightarrow Q_AQ_B^-$ Electron Transfer Rate. In L212EQ/L213DN double mutant RCs, the $Q_A^-Q_B \rightarrow Q_AQ_B^-$ electron transfer rate, $k_{AB}^{(1)}$, is identical to that previously reported for L212EQ (Paddock et al., 1989) and is similarly pH independent from pH 4 to 11, indicating that reprotonation of Asp^- (L213) is not rate limiting. In L213DN mutant RCs, however, $k_{AB}^{(1)}$ exhibits pH dependence at much lower pH than in Wt RCs, further indicating the influence of this mutation on the p*K*s of other ionizable residues near Q_B. Interestingly, the pH dependence of $k_{AB}^{(1)}$ extends to much lower pH than does that of k_{Q_B} (τ_{Q_B}), and with a similar, nonunity slope. Qualitatively the behavior of $k_{AB}^{(1)}$ in L213DN is consistent with the ionization of Glu^{L212} at lower pH in the mutant than in the Wt. The onset of pH dependence at about pH 5 may represent the new pK_{Q_B} for Glu^{L212}, which would then be expected to be reflected in pH dependence of $K_{ET}^{(1)}$ in this region. Since this is not observed, it supports the notion that eq 1 does not yield $K_{ET}^{(1)}$ at low pH and that the direct recombination of P^+ and Q_B^- is significant when the net recombination time is in excess of a few seconds. The corresponding value of $K_{ET}^{(1)}$ ($\approx$200) is then, indeed, only a lower limit.

If we presume that the true electron transfer equilibrium in L213DN is pH dependent down to pH 5, with the same average slope as observed above pH 7, then the limiting decay time approaches 300 s, equivalent to $K_{ET}^{(1)} \approx 5000$. Although a low pH plateau for $K_{ET}^{(1)}$ is not reached in the wild type, the slope of the data indicates a value not larger than 100. If such a large difference does exist between Wt and mutant RCs, it would imply greatly different solvation of the Q_B^- anion by the neutral aspartic acid of the Wt and the amide of the mutant RCs. This could be consistent with the significantly greater dipole moment of amides compared to those of carboxylic acids (Hansch & Leo, 1979; J. Keske and P. L. Dutton, personal communication).

In view of the much larger one-electron equilibrium constant favoring Q_B^- formation in L213DN RCs, it is perhaps surprising that the forward electron transfer rate is much slower than in the Wt—by a factor of 10 at pH 6.5 and a factor of 100 at pH 9.0. The fact that it is not fast, together with $K_{ET}^{(1)} \geq 100$, implies that the reverse rate ($k_{BA}^{(1)}$) is greatly slowed in this mutant (by 100–1000-fold near neutral pH). If $K_{ET}^{(1)}$ is taken to be pH independent at low pH, i.e., direct recombination of $P^+Q_B^-$ is not invoked, then calculation from $K_{ET}^{(1)}$ and $k_{AB}^{(1)}$ requires $k_{BA}^{(1)}$ to accelerate at low pH. This is counterintuitive behavior for a process that is accompanied by proton release.

Considerations of the surface charge density, which changes with the bulk pH due to ionization of surface groups, may account for the slope of the pH dependences of $k_{AB}^{(1)}$ and k_{Q_B} in L213DN RCs. Detailed electrostatic calculations may be able to establish this, but further studies at high ionic strength are currently underway to test this more directly. An alternative explanation of the extended range of the pH dependences might be the involvement of more than one ionizable group influencing the kinetics and equilibria, invoking novel effects in the mutant that are not seen in the Wt. Since significant alterations in the screening of internal charges could occur as a result of the mutational substitution, this is certainly a possibility. However, the fact that no such novel pH dependences are seen in L212EQ/L213DN double mutant RCs argues strongly against it.

As previously reported (Paddock et al., 1989), the first electron transfer in L212EQ mutant RCs occurs at essentially Wt rates, except at pH > 9.5 where the mutant remains fast while the Wt decelerates. This is entirely consistent with inhibition of the Wt electron transfer equilibrium by ionized Glu^{L212} above its $pK_{Q_B^-}$ of about 9.5; under these conditions, significant electron transfer requires a rate limiting reprotonation (Kleinfeld et al., 1984a; Wraight, 1979).

The L212EQ/L213DN double mutant exhibits very slow, pH-independent $P^+Q_B^-$ recombination kinetics similar to the low pH limit of the L213DN single mutant but rapid and pH independent Q_A^- to Q_B electron transfer kinetics similar to those of the L212EQ single mutant. This is understandable in terms of the neutral nature of the substitutions in the Q_B pocket in the double mutant, providing strong stabilization of the Q_B^- species and lacking any electrostatic restrictions to the rate of electron transfer. As discussed above for L213DN, the very slow $P^+Q_B^-$ decay in the double mutant probably reflects a direct recombination and provides only a lower limit to $K_{ET}^{(1)}$.

It is noteworthy that Asp^{L213} is indicated to have $pK_{Q_B} =$ 4–4.5 in Wt and L212EQ mutant RCs. This is a low value for a sequestered residue that is probably paying a significant desolvation penalty (Bashford & Karplus, 1990; Warshel & Russell, 1984) and suggests that significant charge compensation is occurring (Honig et al., 1986). Although significant electrostatic energies in proteins are often due to the summation of many small and distant contributions, more local and specific influences may come from nearby positive groups, such as Arg^{L207}, His^{L211}, or Arg^{L217}. Detailed calculations of the electrostatic energies will be needed to address this. Similar considerations apply to Glu^{L212} if its p*K* in L213DN is taken from the pH dependence of the electron transfer rate, $k_{AB}^{(1)}$ ($pK_{Q_B} \approx 5.5$). Conversely, the p*K* taken from the pH dependence of τ_{Q_B} (pK_{Q_B} 7–7.5) would be consistent with a desolvation penalty associated with its buried nature and might even be expected to be higher. If the true p*K* is not lower (masked by the involvement of the direct recombination pathway), this elevated value, compared to Asp^{L213}, may imply a relatively more negative electrostatic energy at this residue, including possible interactions with Glu^{H173} or Asp^{L210} which are quite close to Glu^{L212} (7–10 Å). Nevertheless, we favor the lower p*K* value for this residue in L213DN mutant RCs, associated with a significant contribution from a direct recombination pathway in this mutant.

Structural perturbations accompanying these mutations appear to be quite small as the general properties of the RCs, including quinone binding, the $P^+Q_A^-$ kinetics, and the limiting value of the Q_A^- to Q_B electron transfer rate at low pH, are very similar for the Wt and all three mutants. However, the $P^+Q_B^-$ charge recombination rate for L212EQ is slightly faster (indicating a smaller $K_{ET}^{(1)}$) than the plateau value for Wt between pH 6 and 9. This suggests that glutamine is less accommodating of the Q_B^- anion than protonated glutamic acid, in contrast to the effect discussed above for the aspartic acid/asparagine substitution at L213. Both effects could derive from the greater dipole moment of the amide if the orientations of the two residues were opposite. Alternatively, protonated Glu^{L212} in the Wt may partially alleviate the inhibitory effect of the negative charge of Asp^{L213} in a way that glutamine cannot. This may reflect the greater H-bond donor potential of the carboxylic acid (Hansch & Leo, 1979; J. Keske and P. L. Dutton, personal communication).

Summary of the One-Electron Transfer Equilibrium and Rate Processes. From this and previous work, the pH dependence of the charge recombination in Wt RCs can be described by eq 1 in five regions, three of which are readily accessible.

(1) At low pH (<4), both Glu^{L212} and Asp^{L213} are protonated and neutral in the dark adapted state, and the one-electron transfer equilibrium strongly favors Q_B reduction ($K_{ET}^{(1)} \geq 50$).

(2) Above pK_{Q_B} of Asp^{L213} ($pK_{Q_B} \approx 4$), ionization of Asp^{L213} provides an electrostatic inhibition to the electron transfer from Q_A^- to Q_B, but interaction with Q_B^- raises the pK of Asp^{L213} ($pK_{Q_B^-} \approx 5$) so that it is reprotonated as electron transfer to Q_B occurs. This gives rise to pH dependence of the electron transfer equilibrium and might also be expected to cause a slowing down of the electron transfer rate ($k_{AB}^{(1)}$). The latter effect is small, however, and only the equilibrium effect is clearly seen. Since the equilibrium constant is quite large (≈10; see below) even when Asp^{L213} remains fully ionized (i.e., above pH 6), this may simply be due to the small amplitude of any H^+-limited component. Ionization of Asp^{L213} also prevents Glu^{L212} from ionizing; i.e., the pK of Glu^{L212} is raised.

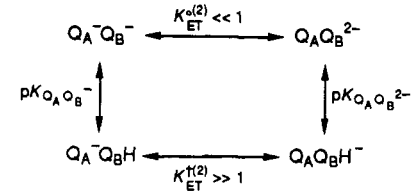
(3) Above $pK_{Q_B^-}$ of Asp^{L213} ($pK_{Q_B^-} \approx 5.5$), Asp^- remains ionized even after electron transfer. The electron transfer equilibrium thus becomes essentially pH independent ($K_{ET}^{(1)} \approx 10$).

(4) At about pH 9, pK_{Q_B} of Glu^{L212} is approached ($pK_{Q_B} \approx 9.5$); the electron transfer equilibrium begins to decrease again and the rate also becomes strongly pH dependent. Both effects are due to the electrostatic interaction between Glu^- and Q_B^-. This raises the pK of Glu^{L212} to about 11, causing the carboxylate to reprotonate and giving rise to a pH-dependent electron transfer equilibrium. The fact that the electron transfer rate is pH dependent shows that reprotonation involves rapid equilibrium with H^+ ions from the medium (Gutman & Nachliel, 1990).

(5) Above the pK of Glu^{L212} in the presence of Q_B^- ($pK_{Q_B^-} \approx 11$), the electron transfer equilibrium and rate, which are now small ($K_{ET}^{(1)} \approx 1$, $k_{ET}^{(1)} \approx 10\ s^{-1}$), become pH independent again. Both ionizable residues, Asp^- and Glu^-, remain negatively charged.

The magnitude of the shift in pK of Glu^{L212} due to Q_B^- is 1.5–2 pH units while that due to ionization of Asp^{L213} is at least 2 pH units and may be in excess of 4.5. These values may be considered in the light of the distances between Glu^{L212} and Asp^{L213} (7.4 Å between carboxyl centers) and Glu^{L212} and Q_B^- (5.9 Å from carboxyl center to carbonyl oxygen, O2) obtained from the static X-ray structure. Clearly, a simple, homogeneous dielectric picture does not adequately account for these interactions—either some movement must be considered or the atomic interactions must be taken into account explicitly. In a similar vein, $K_{ET}^{(1)}$ in L213DN mutant RCs exhibits a significantly broader region of pH dependence than Wt RCs—from pH ≤7.5 to >11 compared with pH 9.5 to ≈ 11. If other groups are not brought into consideration in this range, this implies that Q_B^- causes a larger pK shift in Glu^{L212} in the mutant than in Wt. This, in turn, suggests a change in the effective dielectric or a structural perturbation in the mutant allowing Glu^{L212} to approach closer to Q_B^- than in the Wt. Using a distance-dependent dielectric, such as that suggested by Warshel et al. (1984),[6] to provide a crude estimate of the required magnitude of such a movement, we find that for a distance between Glu^{L212} and Q_B^- of 6 Å in Wt RCs the mutant value would be no more than 4.5 Å, a significant but possible movement.

Scheme III



$Q_A^-Q_B^- \leftrightarrow Q_AQ_B^{2-}$ ($Q_AQ_BH_2$) Electron and Proton Transfer Equilibria. In all three mutants, major effects are observed in the transfer of the second electron to Q_B^- and the associated proton uptake. Although RCs take up protons from the medium on the first flash, it is well established that this uptake is not to the quinone itself but presumably to a residue, or residues, of the protein (Marôti & Wraight, 1988a,b; McPherson et al., 1988; Wraight, 1979). In L213DN and L212EQ/L213DN mutant RCs, the second electron transfer is drastically inhibited compared to Wt.[7] At pH values above 8, the halftime for decay of the semiquinone signal, after the second flash, approached 10 s. Under these conditions the rate of electron transfer to exogenous acceptors is significant compared to the slow disproportionation of $Q_A^-Q_B^- \rightarrow Q_AQ_BH_2$. The slightly faster decay of the semiquinone after the second flash, at pH 8.0, compared to the first, may be due to the greater reactivity of Q_A^- with exogenous oxidants (Shopes & Wraight, 1985) and to the accumulation of additional oxidized exogenous donor—ferrecenium, in this case, which is known to oxidize the semiquinones directly (Marôti & Wraight, 1988b). Similarly, the faster decay of the semiquinone after the first flash at pH 8.0 compared to pH 6.0 may reflect a pH dependence of the exogenous acceptor activity of positively charged ferrecenium, complementary to that described for negatively charged ferricyanide (Shopes & Wraight, 1985). Thus, the observed rate of decay of the semiquinone at high pH is only an upper limit for the rate of transfer of the second electron to Q_B^-, and the intersection of pH-dependent and pH-independent behavior may not reflect a true pK. Acceleration of the transfer rate at lower pH allows strong oscillations in the formation and disappearance of the semiquinone signal to be seen. However, the rate of transfer is still very slow compared to the Wt and is strongly pH dependent. It appears, therefore, that Asp^{L213} has a specific and primary role in the terminal steps of the proton transfer path to Q_B^- or Q_B^{2-}, i.e., the provision of the first proton in the ultimate formation of QH_2.

It was concluded above, for the first electron transfer equilibrium, $Q_A^-Q_B \leftrightarrow Q_AQ_B^-$, that the ionization of Asp^{L213} determines the Wt behavior between pH 4 and 6. This implies that this residue is fully ionized in the $Q_AQ_B^-$ state above pH 6. In order to participate in proton donation on the second transfer, therefore, it must be rapidly and transiently protonated, by internal transfer, in the $Q_A^-Q_B^-$ or $Q_AQ_B^{2-}$ states. We have previously considered two kinetic possibilities in which either electron or proton transfer is the first event, forming Q_B^{2-} or Q_BH^- as intermediate states, respectively (Wraight, 1979). These correspond to the two routes, from $Q_A^-Q_B^-$ to

[6] Warshel's equation is $e = 1 + 60(1 - e^{-0.1r})$. As a continuum approximation it is only quantitatively valid at distances greater than 5 Å. We use it here because we are only interested in a crude estimate of a relative change in distance.

[7] The amplitude of the second flash semiquinone signal in double mutant RCs, at high pH (Figure 6A), is smaller than expected from the extinction coefficients given by Kleinfeld et al. (1985). This is due to a loss of Q_B binding at high pH, and the signal size is in reasonable agreement with the 60–65% Q_B activity implied by the relative amplitude of the slow phase of P^+ decay seen at this pH.

$Q_AQ_BH^-$, around the equilibrium given in Scheme III. Limiting values for the four constants were provided by the elegant experiments of Kleinfeld at al. (1985) on non-mutant (strain R26) RCs: $pK_{Q_A^-Q_B^-} < 8$, $pK_{Q_AQ_B^{2-}} \geq 10.7$. The two routes around Scheme III are both characterized by an unfavorable initial equilibrium followed by a potentially favorable one, pulling the net electron transfer toward $Q_AQ_BH^-$. In the upper route, the high pK of the dianion Q_B^{2-} [$pK \geq 13$, in vitro (Chambers, 1974; Morrison et al., 1982)] will allow this species to obtain a proton readily from neutral Asp^{L213}. In the lower route, the electron transfer to the neutral semiquinone is expected to be facile.

Earlier studies on L212EQ mutant RCs suggested that Glu^{L212} is involved in donation of the second proton to the quinol, at the O2 atom (Paddock et al., 1989). With Glu^{L212} replaced by the nonionizable residue glutamine, uptake of the second proton was slowed down and only three rapid turnovers of the RC could occur. This presumably leads to accumulation of the state $PQ_A^-Q_BH^-$ and implies that Q_BH^- is tightly bound, like the anionic semiquinone, and that only the QH_2 species is readily released. We report, here, that the second electron transfer in this mutant is strongly biphasic. At pH 8.0, a rapid transfer accounts for about 60% of the total and the process is completed with slower kinetics. This pattern is also reflected in the proton uptake. The rapid electron transfer occurs with kinetics very similar to the Wt, but the extent is limited by the constraints of the equilibrium of Scheme III, which includes proton transfer via the Asp^{L213} pathway. Subsequently, the equilibrium is pulled over further with the uptake of a second proton from the medium, shown by

$$Q_AQ_BH^- \xrightarrow[H^+]{} Q_AQ_BH_2$$

which is slow in the L212EQ mutant. This implicates Glu^{L212} in the donation of the second H^+, as suggested by Paddock et al. (1989).

Scheme III implies that the relative amplitude of the fast phase yields the value of the initial electron transfer equilibrium constant, $K_{ET}^{f(2)} \geq 15$ below pH 7.0, decreasing to $K_{ET}^{o(2)} \leq 0.02$ at higher pH. This allows estimation of possible pK values, as shown by the curve in Figure 7B: $pK_{Q_A^-Q_B^-} \leq 7.4$, $pK_{Q_AQ_B^{2-}} \geq 10.3$. It should be noted, however, that the small amplitude of one or the other phase of the electron transfer at low and high pH renders this assay unreliable outside the pH range 7.5–10. Thus, the pK values, which are derived from the curvature, are open to question and should be considered as limits similar to those given for Wt RCs (Kleinfeld et al., 1985). A more robust parameter for comparison with the Wt is the pH at which $K_{ET}^{(2)} = 1$. In the Wt, this occurs at pH 9.5, while in L212EQ mutant RCs it is lowered to pH 8.7. This indicates that the $K_{ET}^{(2)}$/pH curve is shifted down in the mutant, equivalent to a decrease of 50 mV in the free energy drop from $Q_A^-Q_B^-/Q_A^-Q_BH$ to $Q_AQ_B^{2-}/Q_AQ_BH^-$. This presumably reflects a lesser ability of glutamine, compared to glutamic acid, to charge compensate Q_BH^- and/or Q_B^{2-}. This is consistent with its weaker stabilization of Q_B^- and could arise from specific shifts in the pKs, for example, via hydrogen bonding, or from electrostatic influences on the charged species (Wraight, 1982).

$Q_A^-Q_B^- \leftrightarrow Q_AQ_B^{2-}$ ($Q_AQ_BH_2$) Electron and Proton Transfer Kinetics. In Wt RCs, the second electron transfer occurs with $\tau \approx 1$ ms, at pH 8.0. The rate decreases at higher pH, but the precise slope has been variously reported from −0.3 to −1.0 (Kleinfeld et al., 1985; Vermeglio, 1982; Wraight, 1979). We suggest that in the Wt, proton transfer from the medium keeps pace with the internal events so that the second proton does not significantly limit the formation of Q_BH_2.[8] In L212EQ mutant RCs, however, only the first proton is provided via an intact delivery pathway. The kinetics of the associated fast phase of the electron transfer are very similar to those in Wt RCs over a wide range of pH (the slightly slower rates of the mutant are within the variations experienced with different RC preparations). The rate is pH independent at pH < 6, perhaps because Asp^{L213} is readily available in its protonated state, having been reprotonated after the first flash ($pK_{Q_B^-} \approx 5.5$). At higher pH the rate slows down and appears to be rate limited by the aqueous proton concentration. However, for a single turnover event, this does not translate into a bimolecular rate constant, which would be 10^{10}–10^{11} M^{-1} s^{-1}—greater than that for diffusion-limited protonation events in solution (Eigen et al., 1964; Gutman & Nachliel, 1990). A more probably interpretation is that H^+ delivery is determined by the equilibrium—or quasiequilibrium—protonated state of some residue(s) whose concentration, above its pK, decreases directly with the prevailing H^+ ion concentration. The isoelectric point of RCs is about 6 (Prince et al., 1974), and the amino acid composition indicates that they bear a large net negative charge at alkaline pH, resulting in a surface pH substantially lower than in the bulk phase. Accordingly, the slope of the pH dependence (in terms of bulk pH) may deviate from −1.0 (Takahashi et al., 1991; Marôti and Wraight, submitted for publication).

The slow phase of the second electron transfer seen in L212EQ mutant RCs accelerates at low pH in a manner similar to the L213DN and double mutant RCs, although it is somewhat faster throughout. Furthermore, this phase is accelerated by small weak acids, like azide and nitrite, acting as protonophores, just as in L213DN and double mutant RCs (Takahashi & Wraight, 1991), and we consider the rate limitation in all cases to be penetration of protons through the protein matrix. Similarly, in all three mutants the pH independence at high pH may reflect domination of the semiquinone decay via exogenous oxidants, although this has not been directly demonstrated in any of them.

Pathways of Proton Transfer to the Secondary Quinone. The behavior of L213DN and L212EQ/L213DN mutant RCs demonstrates a primary lesion in the normal uptake of the first proton associated with, or necessary for, the double reduction of Q_B after the second flash, leading to a failure of the transfer of the second electron to Q_B. The process is completely blocked at pH ≥ 8.0 but is facilitated at lower pH. Proton delivery to the Q_B site is likely to involve a number of protonatable residues whch cooperate to transfer protons from the aqueous phase to the protein interior, and some residues which may be involved in such a scheme have been indicated by Allen et al. (1988). The side chain of Asp^{L213} is close (≈5 Å) to one of the Q_B carbonyl oxygens (O5) and to the hydroxyl group of Ser^{L223} (≈3.5 Å), which is thought to hydrogen bond to the nearby quinone carbonyl (2.7 Å).

Recent work on a mutant with Ser^{L223} altered to alanine (L223SA) showed this mutant also to be impaired in its second electron delivery (Paddock et al., 1990), although it is not as severely inhibited as the L213DN mutant. Because of its close proximity to Q_B, Ser^{L223} may seem a likely candidate for the immediate proton donor, but it may be too basic ($pK_a \approx 15$, in solution) to permit net H^+ transfer, although this might be

[8] In fact, biphasic kinetics *are* observable for both first and second electron transfers in Wt (Ga or R26) RCs, although the slow phase is much faster than in L212EQ mutant RCs (Marôti & Wraight, 1989; and unpublished observations). This behavior is currently under further investigation.

facilitated by a concerted transfer from Asp^{L213}. Alternatively, the role of Ser^{L223}, implied by the effect of the $Ser^{L223} \rightarrow Ala$ mutation, may be to influence the pK or the orientation of Asp^{L213} as the actual donor. An important role for Glu^{L212} is also well established but, as with Asp^{L213}, the nature of the primary donor species is equivocal. The X-ray structure shows that the second carbonyl of Q_B is hydrogen bonded to His^{L190}, and the pK of the histidine N_δ may be sufficiently lowered by the proximity and liganding of the iron (Fe^{2+}) atom for His^{L190} to act as the primary proton donor, with immediate or concerted transfer from Glu^{L212} to the histidine anion. In fact, the resolution of these points is not important to our discussion at the present time.

The totality of the lesion in proton delivery in L213DN mutant RCs shows that Glu^{L212} cannot substitute for Asp^{L213} in any degree and suggests the possibility that Asp^{L213} delivers both the first proton (to O5 of Q_B), directly, and the second proton (to O2 of Q_B), via Glu^{L212}.

The fact that the slow, second electron transfers seen in L212EQ, L213DN, and double mutant RCs are all accelerated by small weak acids (Takahashi & Wraight, 1991) suggests the possibility that aqueous protons might penetrate the protein in a partially diffusive manner, finally being gated by Asp^{L213} at one end of the quinone, and by Glu^{L212} at the other. Conceivably, small variations in RC conformation could modulate the contributions of diffusion and H-bonded conduction through the protein, to account for the variations in the pH dependence of the second electron transfer rate reported for the Wt kinetics (Kleinfeld et al., 1985; Vermeglio, 1982; Wraight, 1979). However, by comparison with their effect in protonation mutants of bacteriorhodopsin, these small protonophores work very poorly in RCs (Tittor et al., 1989). This suggests that the relevant protein matrix of the RC, i.e., the H subunit, is not very accommodating of small molecules. This could be considered consistent with its very polar nature and the extensive hydrogen bonding that would limit its flexibility, in contrast to the proton channel of bacteriorhodopsin which is quite hydrophobic (Henderson et al., 1990).

Our description of events implies two different terminal steps in the donation of the first and second protons in the formation of Q_BH_2 in RCs of *Rb. sphaeroides*, perhaps with Asp^{L213} and Glu^{L212} acting in sequence for donating the second proton. Certainly the overall pathways need not be as extensively separated as has been suggested by Allen et al. (1988). (In *Rhodopseudomonas viridis* there does not appear to be a second chain of hydrogen-bonded residues extending to the aqueous medium.) As we have previously noted, in *Rps. viridis* (Michel et al., 1986) and *R. rubrum* (Belanger et al., 1988) the residue L213 (or equivalent) is naturally asparagine. It is especially remarkable that the low pH behavior of $K_{ET}^{(1)}$ in *Rhodospirillum rubrum* is very similar to that of *Rb. sphaeroides* (V.P. Shinkarev, personal communication). Presumably compensating differences allow other residues, with similar properties, to function in this role. M.Y. Okamura (personal communication) has drawn our attention to the complementarity of the nearby residue M44/43, which is Asn in *Rb. sphaeroides* (M44; $\approx$ 7.5 Å from the Q_B carbonyl oxygen, O5) but Asp in *Rps. viridis* (M43) and *R. rubrum* (M43).

Electrostatic Interactions and H^+ Binding Models. The H^+ binding that accompanies reduction of the acceptor quinones has been described in terms of two somewhat different models. Marôti and Wraight (1988b) suggested that a small number of groups (approximately 4) were responsible for proton binding in the $P^+Q_B^-$ state, over the range pH 5–11. McPherson et al. (1988) considered a similar description but suggested that the proton binding should be distributed over a large number of residues due to the long-range nature of the electrostatic interactions with Q_A^- and Q_B^-. These are not mutually exclusive views, but the present work and our previous studies on the L213DN mutant (Takahashi & Wraight, 1990; Takahashi et al., 1991) do not support a general electrostatic effect on the pK values of many distant ionizable groups. Thus, in those mutants where the second electron transfer is severely blocked, the formation of the state $Q_A^-Q_B^-$ does not induce significant proton uptake except accompanying the limited electron transfer to form Q_BH^-. Furthermore, in the Glu^{L212} and Asp^{L213} mutants, specific loss of H^+ uptake occurs in pH regions corresponding to the mutated residues (Figure 8; V. P. Shinkarev, E. Takahashi, and C. A. Wraight, unpublished observations). This implies that the charges of the semiquinone anions are effectively compensated by local interactions, resulting in the specific protonation of certain groups in response to Q_B^- and, probably, Q_A^-.

Registry No. Asp, 56-84-8; Glu, 56-86-0; H^+, 12408-02-5.

References

Allen, J. P., Feher, G., Yeates, T. O., Komiya, H., & Rees, D. C. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 6162–6166.

Allen, J. P., Feher, G., Yeates, T. O., Komiya, H., & Rees, D. C. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 8487–8491.

Baciou, L., Rivas, E., & Sebban, P. (1990) *Biochemistry 29*, 2966–2976.

Baciou, L., Sinning, I., & Sebban, P. (1991) *Biochemistry* (in press).

Bashford, D., & Karplus, M. (1990) *Biochemistry 29*, 10219–10225.

Belanger, G., Berard, J., Corriveau, P., & Gingras, G. (1988) *J. Biol. Chem. 263*, 7632–7638.

Blankenship, R. E., & Parson, W. W. (1979) *Biochim. Biophys. Acta 545*, 429–444.

Chambers, J. Q. (1974) in *The Chemistry of the Quinonoid Compounds* (Patai, S., Ed.) pp 737–792, Wiley Interscience, New York.

Crofts, A. R., & Wraight, C. A. (1983) *Biochim. Biophys. Acta 726*, 149–185.

Ditta, G., Schmidhauser, T., Yakobson, E., Lu, P., Liang, X.-W., Finlay, D. R., Guiney, D., & Helinski, D. R. (1985) *Plasmid 13*, 149–153.

Davis, J., Donohue, T. J., & Kaplan, S. (1988) *J. Bacteriol. 170*, 320–329.

Eigen, M., Kruse, W., & Maasse, G. (1964) *Prog. React. Kinet. 2*, 286–318.

Feher, G., Allen, J. P., Okamura, M. Y., & Rees, D. C. (1989) *Nature 339*, 111–116.

Gunner, M. R., Robertson, D. E., & Dutton, P. L. (1986) *J. Phys. Chem. 90*, 3783–3795.

Gutman, M., & Nachliel, E. (1990) *Biochim. Biophys. Acta 1015*, 391–414.

Hansch, C., & Leo, A. (1979) *Substituent Constants for Correlation Analysis in Chemistry and Biology*, John Wiley & Sons, New York.

Henderson, R., Baldwin, J. M., Ceska, T. A., Zemlin, F., Beckmann, E., & Downing, K. H. (1990) *J. Mol. Biol. 213*, 899–929.

Honig, B., Hubbell, W. L., & Flewelling, R. F. (1986) *Annu. Rev. Biophys. Biophys. Chem. 15*, 163–193.

Kleinfeld, D., Okamura, M. Y., & Feher, G. (1984a) *Biochim. Biophys. Acta 766*, 126–140.

Kleinfeld, D., Okamura, M. Y., & Feher, G. (1984b) *Biochemistry 23*, 5780–5786.

Kleinfeld, D., Okamura, M. Y., & Feher, G. (1985) *Biochim. Biophys. Acta 809*, 291–310.
Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488–492.
Marôti, P., & Wraight, C. A. (1988a) *Biochim. Biophys. Acta 934*, 314–328.
Marôti, P., & Wraight, C. A. (1988b) *Biochim. Biophys. Acta 934*, 329–347.
McComb, J. C. (1987) Ph.D. Thesis, University of Illinois, Urbana–Champaign.
McComb, J. C., Stein, R. R., & Wraight, C. A. (1990) *Biochim. Biophys. Acta 1015*, 156–171.
McPherson, P. A., Okamura, M. Y., & Feher, G. (1988) *Biochim. Biophys. Acta 934*, 348–368.
McPherson, P. H., Okamura, M. Y., & Feher, G. (1990) *Biochim. Biophys. Acta 1016*, 289–292.
Michel, H., Weyer, K. A., Gruenberg, H., Dunger, I., Oesterhelt, D., & Lottspeich, F. (1986) *EMBO J. 5*, 1149–1158.
Morrison, L. E., Schelhorn, J. E., Cotton, T. M., Bering, C. L., & Loach, P. A. (1982) in *Function of Quinones in Energy Conserving Systems* (Trumpower, B. L., Ed.) pp 35–58, Academic Press, New York.
Paddock, M. L., Rongey, S. H., Feher, G., & Okamura, M. Y. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 6602–6606.
Paddock, M. L., McPherson, P. H., Feher, G., & Okamura, M. Y. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 6803–6807.
Prince, R. C., Cogdell, R. J., & Crofts, A. R. (1974) *Biochim. Biophys. Acta 347*, 1–13.
Rich, P. (1981) *Biochim. Biophys. Acta 637*, 28–33.
Rutherford, A. W., & Evans, M. C. W. (1980) *FEBS Lett. 110*, 257–261.
Shinkarev, V. P., Verhovskii, M. I., & Zakharova, N. I. (1989) *Biokhimiya 54*, 256–264.
Shinkarev, V. P., Verhovskii, M. I., Sabo, J., Zakharova, N. I., & Kononenko, A. A. (1991) *Biochim. Biophys. Acta* (in press).
Sistrom, W. R. (1960) *J. Gen. Microbiol. 22*, 778–785.
Takahashi, E., & Wraight, C. A. (1990) *Biochim. Biophys. Acta 1020*, 107–111.
Takahashi, E., & Wraight, C. A. (1991) *FEBS Lett. 283*, 140–144.
Takahashi, E., Marôti, P., & Wraight, C. A. (1990) in *Current Research in Photosynthesis* (Baltscheffsky, M., Ed.) pp 169–172, Kluwer Academic Publishers, Dordrecht.
Takahashi, E., Marôti, P., & Wraight, C. A. (1991) in *Electron and Proton Transfer in Chemistry and Biology* (Diemann, E., Junge, W., Müller, A., & Ratazczak, H., Eds.) Elsevier Publishers, Amsterdam (in press).
Tittor, J., Soell, C., Oesterhelt, D., Butt, H. J., & Bamberg, E. (1989) *EMBO J. 8*, 3477–3482.
Vermeglio, A. (1977) *Biochim. Biophys. Acta 459*, 516–524.
Vermeglio, A. (1982) in *Function of Quinones in Energy Conserving Systems* (Trumpower, B. L., Ed.) pp 169–180, Academic Press, New York.
Vermeglio, A., & Clayton, R. K. (1977) *Biochim. Biophys. Acta 461*, 159–165.
Warshel, A., & Russell, S. T. (1984) *Q. Rev. Biophys. 17*, 283–422.
Warshel, A., Russell, S. T., & Churg, A. K. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4785–4789.
Williams, J. C., Steiner, L. A., & Feher, G. (1986) *Proteins: Struct., Funct., Genet. 1*, 312–325.
Wraight, C. A. (1979) *Biochim. Biophys. Acta 548*, 309–327.
Wraight, C. A. (1981) *Isr. J. Chem. 21*, 348–356.
Wraight, C. A. (1982) in *Function of Quinones in Energy Conserving Systems* (Trumpower, B. L., Ed.) pp 182–192, Academic Press, New York.
Wraight, C. A., & Stein, R. R. (1980) *FEBS Lett. 113*, 73–77.
Yeates, T. O., Komiya, H., Chirino, A., Rees, D. C., Allen, J. P., & Feher, G. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 7993–7997.