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Interruption of the Water Chain in the Reaction Center from *Rhodobacter sphaeroides* Reduces the Rates of the Proton Uptake and of the Second Electron Transfer to Q_B^+

Laura Baciou and Hartmut Michel*

Max-Planck-Institut für Biophysik, Heinrich Hoffmann-Strasse 7, D-60528 Frankfurt/Main, Germany

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ABSTRACT: A chain of bound water molecules was recently identified in the photosynthetic reaction center (RC) from *Rhodobacter sphaeroides* by X-ray crystallography [Ermler et al. (1994) *Structure* 2, 925–936]. The possible role of the chain in proton transfer from the solution to the secondary quinone (Q_B) was investigated by site-directed mutagenesis and flash-induced absorbance spectroscopy. Pro L209, situated along the water chain about 9 Å from Q_B , was changed into the aromatic residues Phe and Tyr in order to interrupt the chain. In the PL209Y (Pro L209 → Tyr) mutant, the very small changes in the $Q_A^-Q_B \leftrightarrow Q_AQ_B^-$ equilibrium constant (K_2) and the first electron-transfer rates ($k_{AB}^{(1)}$) indicate that the mutation does not lead to large structural changes. In the PL209F (Pro L209 → Phe) mutant, a 7-fold decrease of $k_{AB}^{(1)}$ is observed. It follows a pH dependence parallel to that of the wild type. It is consistent with no modification of the pK of the Glu L212 determined from the pH dependence of K_2 . The decreased $k_{AB}^{(1)}$ may reflect some slight structural modification in this mutant and/or rearrangement of the cluster of charged residues close to the L209 position. The major effect of the mutations observed is a concomitant decrease of the rates of the second electron transfer, $k_{AB}^{(2)}$, and of the proton uptake upon the second flash. The relative decrease of the $k_{AB}^{(2)}$ rate values in the mutants is more pronounced above pH 8. Our results indicate that the mutations have specifically altered the pathway of proton transfer to Q_B . We propose that in the wild type the chain of bound water molecules provides the most efficient pathway for the proton transfer from cytoplasm to the close vicinity of Q_B . This pattern might be a common behavior in proton-translocating membrane proteins.

In the photosynthetic membrane from purple bacteria, a pigment–protein complex called reaction center converts light energy into electrochemical energy by performing a sequence of photoinduced electron- and proton-transfer reactions. The reaction center from *Rhodobacter (Rb.) sphaeroides* consists of three polypeptides, L, M, and H, and ten cofactors (four bacteriochlorophylls, two bacteriopheo-

phytins, one carotenoid, two ubiquinone molecules, and a non-heme iron Fe^{2+}) all bound to the L and M subunits. The latter are encoded by the *pufL* and *pufM* genes within the *puf* operon. Two of the bacteriochlorophylls form a non-covalently bound dimer (P)[†] which acts as the primary electron donor close to the periplasmic side of the membrane. Upon the first flash, an electron is transferred to the

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[†] Abbreviations: cyt *c*, horse heart cytochrome *c*; LDAO, *N,N*-dimethyldodecylamine *N*-oxide; P, primary donor; Q_A , primary quinone acceptor; Q_B , secondary quinone acceptor; Q_BH_2 , dihydroquinone; RC, reaction center; WT, wild type.

cytoplasmic side of the protein to an electron acceptor complex of two quinone molecules, Q_A and Q_B , respectively, in less than a nanosecond. Q_B^- is not directly protonated. The second reduction of Q_B after the second flash is coupled to the uptake of two protons from the cytoplasm leading to the formation of the dihydroquinone molecule, Q_BH_2 . Q_BH_2 dissociates from the RC and is replaced by an oxidized quinone [for review, see Okamura and Feher (1992) and Shinkarev and Wraight (1993)]. Q_B is buried inside the reaction center protein. According to current models, the uptake, conduction, and delivery of protons to Q_B occurs through the protein matrix along a series of proton donor and acceptor groups (side chain of protonatable residues and/or water molecules) which connect Q_B to the aqueous solvent. These mechanisms were described for several proton translocating proteins [for review, see Meyer (1992)]. Several protonatable amino acids in the vicinity of Q_B have been suggested as participants in the direct proton donation to Q_B . In RCs from *Rb. sphaeroides*, substitution of Asp L213 (Paddock et al., 1990; Takahashi & Wraight, 1990, 1992; Rongey et al., 1993) and Ser L223 (Paddock et al., 1990) by nonprotonatable residues blocks the transfer of the first proton to Q_B . Similarly, Glu L212 was proposed to be involved in the transfer of the second proton to Q_B (Takahashi & Wraight, 1992; Paddock et al., 1989). The participation of water molecules in the proton transfer to Q_B has recently been proposed from studies on revertants from *Rb. sphaeroides* (Okamura et al., 1992) and *Rb. capsulatus* (Hanson et al., 1993; Maroti et al., 1994) and by computational studies (Beroza et al., 1992). The recently determined three-dimensional structure of *Rb. sphaeroides* RCs (Ermler et al., 1994) supports this hypothesis. The crystal structure shows a chain of bound water molecules which might be used for proton transfer from the cytoplasmic surface of RC to Q_B . To test this hypothesis, we have selectively substituted the aromatic residue Phe (PL209F) or Tyr (PL209Y) in the RCs from *Rb. sphaeroides* for the neutral residue Pro L209. Pro L209 is located in the L subunit about 9 Å from Q_B .² The mutants have been designed in order to maintain the electrostatics but to interrupt the continuity of the water chain. We have studied the pH dependence of the first and second electron-transfer rates in these mutants and in native RCs as well as the associated proton uptake rate after the second flash. The $P^+Q_A^-$ and $P^+Q_B^-$ charge recombination rates were also measured. Our results suggest the involvement of the water chain in the proton transfer from external solvent to Q_B .

MATERIALS AND METHODS

The *Rb. sphaeroides* *puf*ΔLMX21 used in these studies was made available by Prof. Oesterhelt and was described previously (Farchaus & Oesterhelt, 1989). The wild-type *Rb. sphaeroides* used was originally the ATCC 17023 strain. *Rb. sphaeroides* cells harboring a *pufL* mutation on pRK404 were grown semiaerobically in Erlenmeyer flasks filled to 50% of the total volume with Sistrom minimal medium

(SMM) supplemented with kanamycin (20 μg/mL) and tetracycline (10 μg/mL). The cultures were grown in the dark at 30 °C on a gyratory shaker (100 rpm). Photoheterotrophic liquid cultures for growth studies were inoculated from late-log-phase chemoheterotrophic cultures in SMM supplemented with kanamycin. The cultures were maintained in darkness for 4–5 h before illumination with white light. Cell growth was monitored with a Klett–Summerson colorimeter equipped with a Corning No66 red filter.

Escherichia (E.) coli JM83 [F^- *ara* Δ(*lac-proAB*) *rpsL* φ80*dlacZ*ΔM15] and DH1 [F^- *recA1* *endA1* *gyrA96* *thi-1* *hsdR17*(r_K^- , m_K^-) *supE44* *relA1*] were used as host strains for all standard cloning steps. The *E. coli* strain S17-I (Simon et al., 1983) [*recA* *pro*[−] *res*[−] *mod*⁺ *Tp*⁺ *Sm*^r pRP4-2-Tc::Mu-Km::Tn7] was used to mobilize derivatives of the plasmid pRK404 into *Rb. sphaeroides* by using the diparental filter-mating procedure described previously (Davis et al., 1988). All *E. coli* strains were grown in LB medium (Maniatis et al., 1982). Strains containing the plasmid pBSIIKS⁺ and pMac/c were grown in the presence of ampicillin (100 μg/mL). Tetracycline was added when the strain harbored derivatives of pRK404.

Recombinant DNA Techniques. Standard molecular biological methods were performed according to Maniatis et al. (1982). The conditions used for restriction endonuclease, T4 DNA ligase, T4 polynucleotide kinase, and *E. coli* T4 polymerase were used as described by the supplier (Boehringer Mannheim). Site-specific mutagenesis was performed using the method of Kunkel (1985). The 37-mer synthetic oligonucleotides 5' G GGC AAG GAA ATG CGC ACG TAC GAT CAC GAG GAT ACG 3' and 5' G GGC AAG GAA ATG CGC ACG TTC GAT CAC GAG GAT ACG 3' were synthesized on a Applied Biosystems 392A-05 DNA synthesizer. The codon corresponding to PL209 (CCG) was changed using *Rb. sphaeroides* codon preferences (Williams et al., 1986) to Y (TAC) and F (TTC) (shown in bold and underlined). An additional silent mutation (in bold print) corresponding to a endonuclease restriction site for *fspI* was used in order to ensure successful mutagenesis. The presence of the desired mutation was confirmed by sequence analysis.

The 5.3 kb *HindIII*–*Bam*HI fragment described previously (Farchaus & Oesterhelt, 1989) contains the *puf* operon from *Rb. sphaeroides* and was cloned into the polylinker region of pBSKSII⁺. Single-strand DNA from the resulting plasmid pBS5.3 was isolated and used as template for site-directed mutagenesis. The entire *Bam*HI–*HindIII* *puf* operon (5.3 kb) containing the mutations was recloned into pRK404, transformed into *E. coli* S17-I, and subsequently transferred into the *Rb. sphaeroides* deletion strain by conjugation. The same procedure was used for the construction of the deletion strain harboring the native *puf* operon.

Protein Purification Techniques. *Rb. sphaeroides* *puf*ΔLMX21 strains harboring wild type or mutations at L209 on the plasmid pRK404 were grown on a large scale, as described above. The cells were harvested by centrifugation (10 000 rpm, for 10 min). The cell pellet was washed with sodium phosphate buffer (0.1 M, pH 7.5), spun down again, and stored at −80 °C. The frozen cell pellet was resuspended in sodium phosphate buffer (0.1 M, pH 7.5) and stirred in the presence of a spatula tip of DNase and PMSF (1 mM) for 20 min. The cells were then passed through a Ribi press (Sorvall) at 20 000 psi and 10 °C twice.

² The structure of the trigonal crystal of the reaction center from *Rb. sphaeroides* (Ermler et al., 1994) was used as a guideline for the distances given in this work. If the discrepancies of the Q_B binding site with the other RC structures from *Rb. sphaeroides* or *Rps. viridis* [for review, see Lancaster et al. (1995)] are taken into account, a small correction of 2 Å must be applied for the shortest distance between the Cα atom of Pro L209 and Q_B .

Cell debris and unbroken cells were removed by two centrifugation steps at 15 000 rpm for 20 min each in a Sorvall SS-34 rotor. The photosynthetic membranes were pelleted at 4 °C and 40 000 rpm in a Ti-70 rotor (Beckman) by centrifugation for 90 min. The pellets were resuspended in sodium phosphate buffer (0.1 M, pH 7.5) and diluted to a final concentration of $A_{850} = 50$. RCs were extracted by slow addition of LDAO (Fluka Chemie, Neu-Ulm, Germany) to a final concentration of 0.35%. The suspension was stirred at 26 °C in total darkness for 15 min. Nonsolubilized membranes were pelleted by centrifugation at 4 °C and 40 000 rpm for 90 min. The extraction with LDAO was repeated using a final concentration of 0.45% under similar conditions. The solubilized RCs were separated from the nonsolubilized material by centrifugation at 40 000 rpm and 4 °C for 90 min and precipitated by addition of ammonium sulfate to a final concentration of 22% (w/v). The RC extract was then dialyzed overnight against 10 mM Tris–0.1% LDAO buffer and loaded onto a DEAE-Sephacrose CL-6B (Pharmacia) column, preequilibrated with the same buffer. The column was washed in steps with the same buffer and increasing NaCl concentrations. The RC was eluted with 300 mM NaCl.

Electron-Transfer Measurements. All experiments were carried out at 21 °C on a homemade apparatus as described previously (Baciu et al., 1990) using 1–3 μ M RC solution. $P^+Q_A^-$ and $P^+Q_B^-$ recombination kinetics were monitored at 865 nm. The $P^+Q_A^-$ charge recombination rate (k_{AP}) was determined in the presence of terbutryn (100 μ M). The $P^+Q_B^-$ charge recombination rate (k_{BP}) was determined from the slow phase of recovery of the oxidized donor P^+ . Occupancy of the Q_B site was routinely restored in RCs by addition of 50–100 μ M UQ_6 . The rate of the first electron transfer $Q_A^-Q_B \rightarrow Q_AQ_B^-$ ($k_{AB}^{(1)}$) was measured at 750 nm in the electrochromic band-shift region of the bacteriopheophytin. The rate of the second electron transfer $Q_AQ_B^- \rightarrow Q_AQ_B^{2-}$ ($k_{AB}^{(2)}$) was determined at 450 nm after a second flash in the presence of 40 μ M cytochrome *c* and 1 mM sodium ascorbate. Depending on the pH range, the pH buffers used were MES [2-(*N*-morpholino) ethanesulfonic acid], Bistris-propane [1,3-bis[[tris(hydroxymethyl)methyl]amino]propane], or CAPS [3-(cyclohexylamino)-1-propanesulfonic acid].

Proton Uptake Kinetics. The proton uptake kinetics after the second flash were determined at pH 7.5 and 8.5 by measuring the optical absorbance change at 582 nm of the pH indicator dye cresol red (40 μ M) (Maroti & Wraight, 1988; Paddock et al., 1990; Wraight, 1979). The buffer concentration was kept below 5 μ M by extensive dialysis. Excess quinone (50–100 μ M) and 100 μ M ferrocene were added to the samples.

RESULTS

Construction and Growth of the L209 Mutants

The native or modified *puf* operons were constructed and transferred for transcomplementation into the deletion strain *puf* Δ LMX21 from *Rb. sphaeroides* as described in Materials and Methods. The doubling times of the native and the modified strains are similar under semiaerobic growth conditions (5–6 h). Growth studies using plates or liquid cultures show that all complemented deletion strains carrying the mutated *puf* operon were photosynthetically competent.

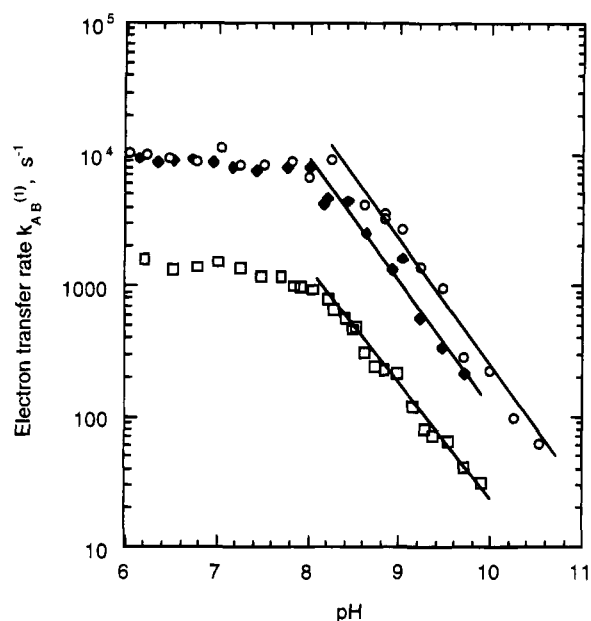


FIGURE 1: pH dependence of $k_{AB}^{(1)}$, the rate constant for the $Q_A^-Q_B^- \rightarrow Q_AQ_B^-$ electron transfer in the RCs from WT (\blacklozenge) and the PL209F (\square) and PL209Y (\circ) mutants of *Rb. sphaeroides*. Conditions: 3 μ M RCs, 10 mM MES, 10 mM Bistris-propane, 10 mM CAPS, 0.1% LDAO, and 100 mM NaCl.

However, generation times of the mutant strains when grown under photosynthetic conditions in liquid culture are slightly longer than in the native strain. For the PL209F and PL209Y mutants 25% and 15% longer generation times, respectively, were observed when compared to WT.

Electron- and Proton-Transfer Rates

Semiquinone Oscillations. In order to test the cyclic function of the RCs, the formation and disappearance of the semiquinone species after successive saturating flashes were followed at 450 nm, at pH 7.5, in the presence of an exogenous electron donor to P^+ (ferrocene 100 μ M) and excess UQ_6 (100 μ M) (data not shown). The semiquinone oscillation pattern observed in the PL209Y RCs, using actinic flashes separated by 1 s, was essentially identical to that observed in WT. In the PL209F mutant, a slight decrease of the rate of the Q_BH_2 formation was observed. This substantial slowing down of the second electron and/or proton transfer in the PL209F mutant was confirmed by direct measurements at 450 nm (see below).

First Electron Transfer. The pH dependence of the rate constant of the first electron transfer measured at 750 nm is shown in Figure 1 for PL209Y, PL209F, and native RCs. $k_{AB}^{(1)}$ depends only slightly on the pH in the range 6–8 and decreases more steeply above pH 8. The turning point near pH 8 has previously been attributed to the titration of Glu L212 (Paddock et al., 1989; Takahashi & Wraight, 1992). The PL209Y mutant shows a pH dependence very similar to that of the WT. However, a slight shift of the turning point toward a higher pH value (8.2 \rightarrow 8.5) is observed. The pH dependence pattern of PL209F RCs is very close to that of the wild type, but the $k_{AB}^{(1)}$ value is decreased about 7-fold in the whole pH range studied. The similar values of the apparent pK value in both mutants suggest that the mutations did not affect the electrostatic environment of Glu L212.

Second Electron Transfer. The pH dependence of $k_{AB}^{(2)}$ for the L209 mutants and for native RCs is shown in Figure

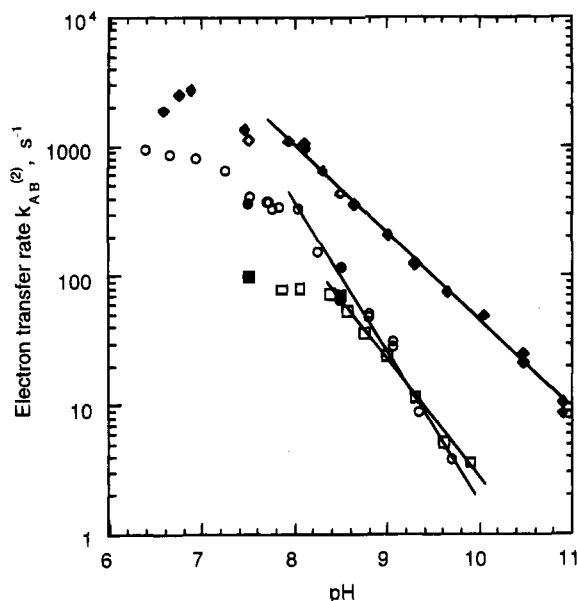


FIGURE 2: pH dependence of $k_{AB}^{(2)}$, the second electron-transfer rate in the RCs from the WT (◆) and the PL209F (□) and PL209Y (○) mutants of *Rb. sphaeroides*. The rates of proton uptake kinetics are plotted for the WT (◇) and the PL209F (■) and PL209Y (●) mutants. Conditions: for the electron-transfer rate kinetics, same as in Figure 1 except 40 μ M cyt *c* and 1 mM sodium ascorbate, 450 nm; for the proton uptake measurements, same as in Figure 1 except extensively dialyzed RCs (buffer concentration <5 μ M), 40 μ M cresol red, and 100 μ M ferrocene, 582 nm.

2. The second electron transfer in the mutants is slowed down over the whole pH range. Below pH 8, the PL209Y and WT RCs have similar slopes and turning points around pH 8. Above pH 8, the second electron transfer of the PL209Y mutant decreases faster than in the WT: 3 times slower at pH 8 and up to 10 times at pH 9. In the PL209F mutant, the rate is nearly pH independent below pH 8.5 and substantially smaller (\approx 15-fold) than in the wild type. In this mutant, the apparent pK is slightly shifted to \approx 8.5 compared to WT, with again a steeper slope than the WT above pH 8.5. It is of interest to note that, above the turning points, $k_{AB}^{(2)}$ is proportional to $[H^+]^1$ in PL209Y RCs and to $[H^+]^{0.9}$ in the PL209F RCs. These slopes are higher than those measured in the WT RCs above pH 8, where $k_{AB}^{(2)}$ is proportional to $[H^+]^{0.6}$.

Proton Uptake Kinetics. We have measured the rates of proton uptake (k_{H^+}) upon the second flash at 582 nm using cresol red as the pH indicator dye at pH 7.5 and 8.5. The values obtained for both WT and mutants are plotted in Figure 2, together with the second electron-transfer rates. It is noticeable that, within experimental errors, the k_{H^+} values are essentially the same as the $k_{AB}^{(2)}$ values at the same pH. This agrees with the idea of coupling of the second electron-transfer and first proton-transfer processes and supports the idea of decreased rates of proton conduction in the mutants.

$P^+Q_A^-$ and $P^+Q_B^-$ Charge Recombination Rates and the $Q_A^-Q_B \leftrightarrow Q_AQ_B^-$ Equilibrium Constant. In RCs from WT *Rb. sphaeroides*, it is established that the decay of $P^+Q_B^-$ occurs by charge recombination via $P^+Q_A^-$ and that both states are in thermal equilibrium (Kleinfeld et al., 1984). The equilibrium constant K_2 between the $Q_A^-Q_B$ and $Q_AQ_B^-$ states is given by (Mancino et al., 1984)

$$K_2 = k_{AP}/k_{BP} - 1 \quad (1)$$

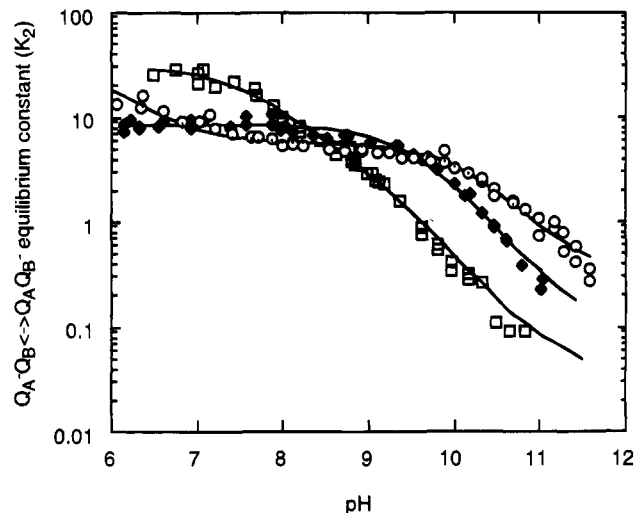


FIGURE 3: pH dependence of the equilibrium constant for the $Q_A^-Q_B \leftrightarrow Q_AQ_B^-$ electron transfer, K_2 , in RCs from WT (◆) and the PL209F (□) and PL209Y (○) mutants from *Rb. sphaeroides*. The solid curves indicate the fitting of the experimental data using eq 2 (see text).

where k_{AP} is the $P^+Q_A^-$ charge recombination rate constant [which was found to be similar in the WT and in the L209 mutants (data not shown)] and k_{BP} is the $P^+Q_B^-$ charge recombination rate constant. The pH dependence of K_2 , determined by using eq 1, is shown in Figure 3 for the WT and the mutants PL209Y and PL209F. Within experimental error, the pH dependence of K_2 displays a similar profile in native and in PL209Y RCs, except for a slight shift at high pH. In the PL209F mutant, K_2 exhibits a more marked dependence on pH than in WT RCs but on a wider pH range.

The fitting procedure used for the pH dependence of K_2 follows the equation:

$$K_2(pH) = K_2^{H^+} \frac{1 + 10^{(pH - pK_{iQ_B^-})}}{1 + 10^{(pH - pK_{iQ_B})}} \quad (2)$$

where $K_2^{H^+}$ is the value of K_2 at low pH and $pK_{i(Q_B)}$ and $pK_{i(Q_B^-)}$ represent the pK s of one or two titratable groups interacting with the charged semiquinone states $Q_A^-Q_B$ and $Q_AQ_B^-$, respectively. In native RCs, above pH 8, K_2 displays a pH dependence indicating interaction with at least one titratable residue. Fitting it with one residue yields values for $pK(Q_B)$ and $pK(Q_B^-)$ of 9.5 ± 0.3 and >11 , respectively. These are consistent with previous work (Kleinfeld et al., 1984). In WT RCs, the group responsible for the pH dependence of K_2 at high pH was assigned to Glu L212 by studying RCs modified by site-directed mutagenesis (Paddock et al., 1989; Takahashi & Wraight, 1992). For the PL209Y mutant, values for $pK(Q_B)$ and $pK(Q_B^-)$ are 10.1 ± 0.3 and 11.4 ± 0.3 for the high pH region. In PL209F RCs, these pK values are 9.0 ± 0.3 and 11.1 ± 0.3 , respectively. In order to fit the data, an additional group which interacts more weakly with Q_B is required to be postulated for this mutant: $pK(Q_B) = 7.6 \pm 0.3$ and $pK(Q_B^-) = 8.4 \pm 0.3$.

DISCUSSION

In this work we have constructed and characterized RCs from *Rb. sphaeroides*, in which Pro L209 situated close to

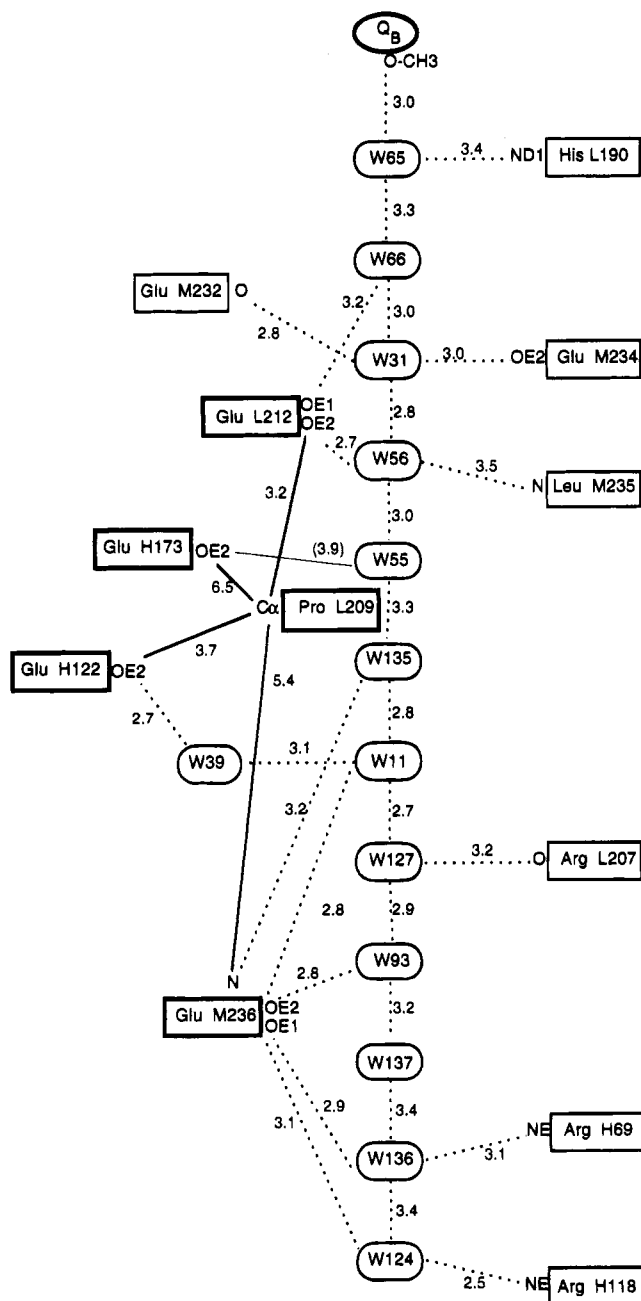


FIGURE 4: Position of the Pro L209 relative to the water chain and the interacting residues in the *Rb. sphaeroides* RC. The water molecules are in oval boxes. Distances are given in angstroms.

waters W55 and W135 (Figure 4) was changed to Phe and Tyr. These mutant RCs were designed in order to interrupt the chain of firmly bound water molecules, which was recently identified in the trigonal crystal form of *Rb. sphaeroides* RCs (Ermler et al., 1994). Pro L209 was selected, since it is far enough from Q_B (9 Å from Q_B in the trigonal structure) to avoid direct structural or electrostatic influence on the quinone binding site. The fact that Pro L209 possesses a secondary amino group is not important since in *Rps. viridis* RCs the homologous residue is an alanine. The inspection of the RC structure indicates that the L209 position is well suited for introduction of an aromatic residue. Assuming that the main-chain conformation and, consequently, the direction of the $C\alpha$ – $C\beta$ bond remain constant, the aromatic side chain at position L209 is pointing toward the water chain. A tyrosine or a phenylalanine is large enough to interrupt the water chain but small enough to avoid

severe clashes with surrounding amino acids. In addition, neutral residues were purposely chosen in contrast to earlier site-directed mutants where acidic residues were replaced by neutral ones. These changes close to Q_B have dramatic effects on the measured proton- and electron-transfer rates (Paddock et al., 1989, 1990; Rongey et al., 1993; Takahashi & Wraight, 1990, 1992). Site-directed mutagenesis of protonatable residues that are further away from Q_B also has significant effects (Okamura & Feher, 1992; Paddock et al., 1992). Alterations of the electron and proton transfer in our L209 mutants should therefore not arise from electrostatic changes caused by removal or addition of charges.

In the PL209Y mutant, the turning point of the pH dependence of $k_{AB}^{(1)}$ is weakly shifted to higher pH, which represents the new pK value for Glu L212. This observation is consistent with the pH dependence of K_2 at high pH. However, the lack of important changes in the pH dependence of the first electron-transfer rate suggests that no structural modifications have occurred in the mutant. The mutation has only slightly shifted the pK of the Glu L212, situated about 3.2 Å from the $C\alpha$ atom of the L209 residue, to higher pH. In the PL209F mutant, $k_{AB}^{(1)}$ is decreased 7-fold compared to the WT. The effect of the mutation in the PL209F mutant on $k_{AB}^{(1)}$ is surprising compared to the PL209Y mutant. In addition, the K_2 equilibrium constant displays a broader region of pH dependence than that of native RCs (from 7.5 to 11 compared with 9 to 11). L209 is surrounded by a cluster of strongly interacting charged amino acid residues (Glu H173, Glu H122, Glu M236) which are all located within about 4–6 Å as minimal distance. The introduction of a hydrophobic phenylalanine in this region might have disturbed the interaction and/or induced a slight structural reorganization of this cluster, causing the observed reduced rate of the first electron transfer and the affected pH dependence of K_2 . However, the turning point of the pH dependence curve of $k_{AB}^{(1)}$ in the PL209F mutant is unchanged compared to the WT, suggesting that the pK of Glu L212, situated at ≈ 5 Å from Q_B , is probably not much affected by the mutation.

In both mutants, the most striking effect observed is the decrease of the second electron-transfer rates together with that of the proton uptake kinetics upon the second flash. The substantial slowing down of the second electron transfer and of the proton transfer in the mutants suggests that the transfer of at least the first proton coupled to the double reduction of Q_B has been affected by the mutations. Above pH 8, the pH dependence of $k_{AB}^{(2)}$ has a slope of ≈ 1 decade/pH unit in the PL209Y and PL209F mutants, which is significantly higher than in the WT (0.6 decade/pH unit). This result suggests for the mutants a higher nonspecific diffusion process for proton transfer, the rate of which is limited by the aqueous proton concentration. The presence of an aromatic residue leads to a lack of specificity of the proton transfer probably due to the partial interruption of the pathway used in the WT. It is possible that aqueous protons might penetrate the protein matrix by diffusion using other possible parallel proton-transfer pathways. This hypothesis is consistent with the smaller effect of the mutations on the proton-transfer rate than of the mutations of Ser L223, Asp L213, or Glu L212, which may serve as final gates for the protons. We therefore propose that, in the PL209Y and PL209F mutants, the reduction of the proton-transfer rate through the protein matrix is due to a partial blockage of

the proton pathway to Q_B. In the mutants, proton diffusion by alternative pathways can compensate for the partial blockage introduced by the mutations. In the WT, the chain of firmly bound water molecules may be the most efficient pathway for proton conduction from the cytoplasm to Q_B. Whether the chain of water molecules has indeed been interrupted in the mutants is currently being investigated by X-ray crystallography.

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