Structural and Functional Consequences of a Glu L212 → Lys Mutation in the Q_B Binding Site of the Photosynthetic Reaction Center of *Rhodopseudomonas viridis*†

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ABSTRACT: The properties of the quinone acceptor complex in the photosynthetic reaction center of the atrazine-resistant *Rhodopseudomonas viridis* mutant A2 (Glu L212 → Lys) were studied by EPR spectroscopy and by photoelectric measurements. The EPR signal attributed to the semiquinone–iron ($Q_B^-Fe^{2+}$) was significantly different from wild type and resembled that found in PS II. Essentially normal oscillations of $Q_B^-Fe^{2+}$ were observed upon flash illumination. The kinetics of the first and the second electron transfer from Q_A to Q_B were characterized by a photoelectric double-flash method. Compared to wild type, the rate of the first electron transfer in the large majority of reaction centers was decreased drastically from $k_1 = (18\ \mu s)^{-1}$ in the wild type to $(70\ ms)^{-1}$ in the mutant, whereas the second electron transfer was only slightly slowed down with a rate of $k_2 = (260\ \mu s)^{-1}$ compared to $(65\ \mu s)^{-1}$ in wild type (pH 7). When the pH was raised above 10, in a major fraction of the reaction centers a fast kinetics of the first electron transfer, like that in wild type, reappeared. The experimental results are interpreted as an effect of the positive charge on the lysine causing a significant structural change of the Q_B binding pocket and a strongly diminished affinity for ubiquinone. The slow $Q_A^- \rightarrow Q_B$ electron transfer kinetics are thus attributed to ubiquinone binding, which is rate limiting. The possible role of the residue Glu L212, which is conserved in all purple bacteria, in electron and proton transfer to Q_B is discussed.

In photosynthetic organisms, the conversion of light energy into chemical energy is mediated by transmembrane protein complexes, the photosynthetic reaction centers (RCs).[1] Since the reaction center structures of the two purple bacteria, *Rhodopseudomonas viridis* (*Rps. viridis*) and *Rhodobacter sphaeroides* (*Rb. sphaeroides*), have been determined by X-ray crystallography (Deisenhofer et al., 1984; Allen et al., 1986; Chang et al., 1986), these systems have become the object of numerous studies on structure–function relationships. Photosynthetic RCs use the energy of an absorbed photon for a very fast and efficient process of charge separation, which is followed by electron transfer along a series of cofactors [for a review see Deisenhofer and Michel (1989) and Feher et al. (1989)]. As the reaction sequence can be triggered by a flash of light, photosynthetic RCs are ideal model systems to study biological electron and proton transfer in proteins.

The quinone–iron complex of the acceptor side in the RC of purple bacteria is of special interest because it is the site of the coupling between electron and proton transport [for a review see Okamura and Feher (1992) and Shinkarev and Wraight (1993)]. While the primary acceptor, Q_A, is a one-electron acceptor, the secondary quinone, Q_B, successively accepts two electrons from Q_A together with two protons from the cytoplasmic phase. Since the reduced quinone is located in a binding pocket surrounded by protein, the two protons are supplied to the quinone via adjacent amino acid residues. The two acceptor quinones in the *Rps. viridis* reaction center, Q_A, a menaquinone 9, and Q_B, a ubiquinone 9, are chemically similar. In *Rb. sphaeroides*, the two quinones even are the same species (both are ubiquinone 10). Therefore, the different functions they serve in the electron transport chain are governed by the protein environment.

The availability of site-directed mutants in *Rb. sphaeroides* led to a detailed picture of the role of single amino acid residues in electron and proton transfer to the quinones (Okamura & Feher, 1992). The ring system of the secondary quinone acceptor is bound to a protein pocket formed mainly by the L-subunit of the RC protein. In addition to Ser L223 and Glu L212 which seem to be involved in the transfer of the first and the second protons to Q_B, respectively, Asp L213 was identified to be essential for the transfer of both protons (Paddock et al., 1989, 1990; Takahashi & Wraight, 1990, 1992).

The reaction center structures from both *Rps. viridis* and *Rb. sphaeroides* display a high degree of similarity, and the functional amino acids Ser L223 and Glu L212 are conserved in both systems. In contrast, the residue homologous to asparate L213 in *Rb. sphaeroides* is an asparagine in *Rps. viridis*, indicating that the proton pathway could be different. However, evidence was recently provided that, also in *Rps. viridis*, Ser L223 is involved in the donation of the first proton to Q_B (Leibl et al., 1993). Whether Glu L212 also fulfills the same task in both systems is unknown.

Only recently a system for site-directed mutagenesis has been developed in *Rps. viridis* (Laussermaier & Oesterhelt, 1992) but no site-directed mutagenesis of amino acid residues in the Q_B-binding region has been performed so far. However, a number of herbicide-resistant mutants have been characterized which carry mutations in the Q_B pocket without losing

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[1] Abbreviations: RC, reaction center; Q_A, Q_B, primary and secondary quinone acceptor; DAD, diaminodurene (2,3,5,6-tetramethyl-*p*-phenylenediamine); DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; Wt, wild type.

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their photosynthetic activity (Sinning et al., 1989a,b; Ewald et al., 1990). Herbicides of the triazine type, such as terbutryn or atrazine, are known to block photosynthetic electron transfer by competitive inhibition of Q_B binding.

In this work, we report structural and functional properties of the quinone acceptor complex in whole cells and chromatophores of the atrazine-resistant mutant A2 (L212 Glu → Lys; Ewald et al., 1990) of *Rps. viridis* and compare it to wild type (Wt). In particular, the shape of the $Q_B^-Fe^{2+}$ EPR signal and the kinetics of electron transfer to Q_B have been investigated.

EXPERIMENTAL PROCEDURES

Rps. viridis DMS 133 Wt and the atrazine-resistant mutant A2 were grown as described (Michel et al., 1986; Ewald et al., 1990). The mutant revealed a slightly prolonged generation time that was up to 25% longer than in wild type but comparable to that observed in other atrazine-resistant mutants (Ewald, 1992). For EPR measurements, chromatophores were prepared from cells by a French press treatment (two passes at 1200 psi) with the addition of 0.2 mg/mL DNase after the first passing. After removal of cell debris by 20-min centrifugation at 38000*g*, the chromatophores were sedimented (200000*g*, 50 min), washed once, and resuspended in a small amount of 20 mM MOPS buffer, pH 7.0, and 10 mM EDTA. The chromatophores were stored at 4 °C in the dark for up to 4 days. For the measurement of flash-induced EPR spectra, the chromatophore suspension was adjusted to a BChlb concentration of 375 μM ($\epsilon_{1014} = 10^5\ M^{-1}\ cm^{-1}$).

EPR spectra of the semiquinone–iron signals were measured essentially as described (Sinning et al., 1989a). Samples of 200 μL in calibrated EPR tubes were dark-adapted for 30 min on ice. Samples of 500 μM TMPD and 250 μM potassium ferricyanide were added to oxidize any Q_B^- present in the dark, and then 5 mM ascorbate was added to the samples as an electron donor system just prior to the flash with thorough mixing after each addition. The samples were stored in liquid nitrogen after freezing in an ethanol/dry ice mixture. For measurements in the presence of inhibitors, atrazine or DCMU were added to the dark-adapted samples from DMSO stock solutions prior to the other additions, and the samples were incubated for 5 min at room temperature. For binding studies with atrazine, stock solutions with different inhibitor concentrations were used in order to keep the DMSO concentration in the samples below 2%. DMSO concentrations up to 2% had no apparent effect on the EPR spectra. The sample preparation was done in complete darkness.

For measuring the $Q_B^-Fe^{2+}$ signal, the samples were thawed, illuminated with a single saturating flash from a Nd-YAG laser (530 nm, fwhm 20 ns) at room temperature and refrozen immediately as described above. For detection of the $Q_A^-Fe^{2+}$ signal, the thawed samples were illuminated for 3 min in a water bath at room temperature with white light from a projector lamp (800 W) that was filtered through 2 cm of water and two Calflex filters. The samples were frozen in the light using an unsilvered dewar.

EPR spectra were recorded with a Bruker ER 200D-SR spectrometer equipped with a liquid helium cryostat (Oxford Instruments).

The quinone content in photosynthetic membranes and in isolated RCs [preparation as described by Michel (1982)] was determined by HPLC analysis. The quinones were quantitatively removed from membranes or reaction centers by extracting them several times with *n*-hexane. The components of the combined extracts were separated by silica gel thin-layer chromatography, and the extracted quinones were analyzed on a reversed-phase HPLC system. The quinone content was calculated from the integrated peaks. The HPLC system consisted of a HP 1090 HPLC instrument equipped with a diode array detector and a reversed-phase column C220 mm (Vydac 218tp54). The data were evaluated with a HP310 PC with the HPLC ChemStation software system.

Table 1

	Wt	A2
membranes		
menaquinone/RC	1.28 ± 0.20	1.68 ± 0.25
ubiquinone/RC	3.76 ± 0.55	3.31 ± 0.50
Isolated RCs		
menaquinone/RC	0.96 ● 0.10	0.12 ± 0.01
ubiquinone/RC	0.33 ± 0.04	0.01 ● 0.01

Photovoltage measurements were performed on whole cells that were treated essentially as described previously (Leibl & Breton, 1991). After a short incubation with 2 μg/mL gramicidin, 200 μM TMPD, and 3 mM potassium ferricyanide in 50 mM buffer, the cells were washed twice in the appropriate buffer to adjust the pH and to remove the ferricyanide. The following buffers were used: MOPS (pH 7), tricine (pH 8), and glycine (pH 9–11). For measurements, the cell suspension was diluted to yield an optical density at 532 nm of about 0.5 (d = 0.01 cm). Oxidized TMPD was added as a redox mediator at a concentration sufficient to ensure reoxidation of Q_B^- between averaging cycles (typically 1 mM).

Time-resolved photovoltage kinetics were detected using the light-gradient method and a microcoaxial measuring cell (Trissl et al., 1987). Saturating submicrosecond preflashes were obtained from a dye laser (Electro-Photonics Ltd. Model 23) operated with rhodamine 6G (λ = 600 nm, energy about 10 mJ/cm², fwhm 200 ns) or from a Q-switched, frequency double Nd-YAG laser (Quantel, France, fwhm 7 ns, λ = 532 nm, energy about 10 mJ/cm²). Picosecond flashes were from a mode locked, frequency doubled Nd-YAG laser (Quantel, France, fwhm 30 ps, λ = 530 nm), the energy of which was attenuated by neutral density filters to about 250 μJ/cm². The delay time between one or more saturating flashes and the picosecond flash was varied, and the photovoltage kinetics induced by the picosecond flash were analyzed for the fraction of RCs with Q_A still reduced at that time. The determination of $[Q_A^-]$ is based on the disapperance of the positive 200-ps phase of Q_A reduction and its replacement by a negative 2.5-ns phase corresponding to the back-reaction of the primary radical pair (Leibl & Breton, 1991).

RESULTS

Determination of the Quinone Content. The quinone contents have been determined for both photosynthetic membranes and isolated reaction centers from Wt and the Glu L212 → Lys mutant A2 (Table 1).

The values of Wt are consistent with those reported by Sinning (Sinning et al., 1990). These values had been confirmed by the results of binding affinity studies of Q_B with optical spectroscopy methods (Sinning et al., 1989a). After isolation of reaction centers of the Wt, only about 30% of the RCs have a ubiquinone bound to its binding site.

In membranes of the mutant, the relative amount of quinones found is very close to that in Wt, with some variation that is still within the error of these experiments. The most striking finding in the mutant is that the contents of both menaquinone (Q_A) and ubiquinone (Q_B) in the isolated RCs are much lower than those of the Wt. A2 is the only atrazine-resistant mutant

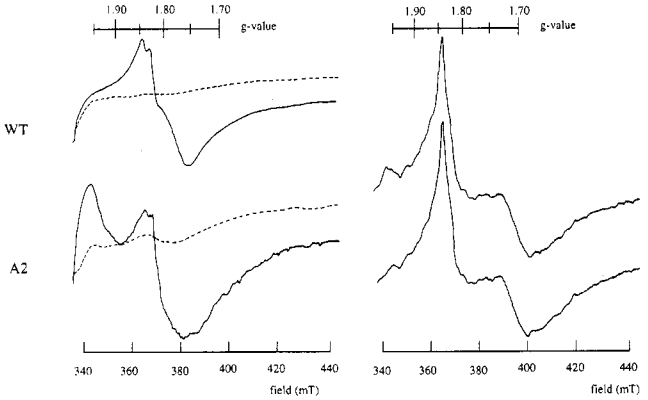

FIGURE 1: EPR spectra of the $Q_B^-Fe^{2+}$ (left panel) and $Q_A^-Fe^{2+}$ (right panel) signals in chromatophores from *Rps. viridis* Wt (upper traces) and the Glu L212 → Lys mutant A2 (lower traces). The spectra were obtained after one flash ($Q_B^-Fe^{2+}$) and 2 min of continuous illumination ($Q_A^-Fe^{2+}$), respectively. The dark spectra are drawn as dashed lines. The spectra shown are the averages of four scans. Instrument settings: microwave frequency, 9.42 GHz; microwave power, 8 dB; modulation amplitude, 20 G; temperature 4.7 (Wt) and 6 K (A2).

showing a significant loss of Q_A during preparation of RCs (Ewald, 1992).

EPR Spectroscopy. Since both quinones in the electron acceptor complex of bacterial reaction centers are magnetically coupled to the non-heme iron, the singly-reduced quinones give rise to characteristic EPR signals, $Q_A^-Fe^{2+}$ and $Q_B^-Fe^{2+}$, respectively (Feher & Okamura, 1978). The EPR spectra are known to be very sensitive to changes in the environment of the iron and the quinone (Butler et al., 1984). Changes introduced by mutations in the Q_B binding pocket can result in altered EPR spectra (Sinning et al., 1989a).

Semiquinone–Iron Signals of the A2 Mutant. The typical semiquinone–iron signal ($Q_B^-Fe^{2+}$) obtained after one laser flash in *Rps. viridis* Wt chromatophores is characterized by a turning point at $g = 1.82$, a peak-to-trough line width of 18.5 mT, and a split low-field peak at $g = 1.85$ (Rutherford & Evans, 1979a). In Figure 1 (left panel) the flash-induced EPR signals in the $g = 1.82$ region from Wt and the A2 mutant are shown. The EPR signal in the Wt was characteristic of $Q_B^-Fe^{2+}$ as reported earlier.

The flash-induced EPR signal in the mutant was significantly different from that of the Wt and was characterized by two peaks at $g = 1.95$ and $g = 1.85$ with turning points at $g = 1.93$ and $g = 1.82$. The line width of the $g = 1.82$ peak, 13 mT, was significantly narrower compared to the Wt signal. On the other hand, the signal of A2 is very similar to that observed in the terbutryn-resistant *Rps. viridis* mutant T4 (L222 Tyr → Phe) (Sinning et al., 1989a), in *Rhodospirillum rubrum* (Beijer & Rutherford, 1987), and in photosystem II of higher plants (Rutherford et al., 1984; Zimmermann & Rutherford, 1986).

In order to prove the attribution of the observed signal to $Q_B^-Fe^{2+}$, we looked for binary oscillations of the amplitude in a sequence of flashes. This experiment is based on the fact that Q_B is a two-electron acceptor. Each odd flash promotes the formation of a molecule of semiquinone, whereas after each even flash the semiquinone dissappears and a molecule of the fully reduced hydroquinone is formed (Vermeglio, 1977; Wraight, 1977, 1978). Figure 2 shows oscillations in the amplitude of the semiquinone–iron EPR signals depending on the flash number in chromatophores of both the Wt and the A2 mutant. The EPR signal in the mutant is accordingly attributed to $Q_B^-Fe^{2+}$ in a modified environment.

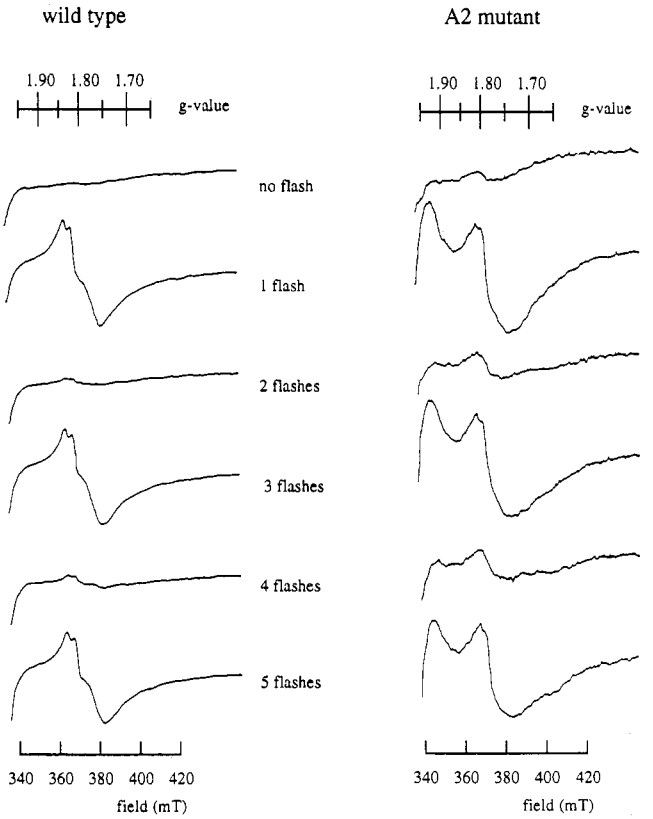

FIGURE 2: EPR spectra from Wt (left) and A2 mutant (right) chromatophores recorded after a series of flashes given to one sample at room temperature. EPR conditions as in Figure 1.

Also in Figure 2, it can be seen that the two parts of the semiquinone–iron signal ($g = 1.82$ and $g = 1.85$) from the mutant showed somewhat different oscillation behavior. The signal at $g = 1.85$ does not oscillate as clearly as does that at $g = 1.93$. A possible explanation is that in a fraction of RCs no Q_A^- to Q_B electron transfer takes place, and thus some $Q_A^-Fe^{2+}$ is present and stable after the first flash. It is of note that the two parts of the $Q_B^-Fe^{2+}$ signal behave differently with temperature. The $g = 1.85$ part declines with increasing temperature; the $g = 1.93$ part reaches its maximum amplitude at about 10 K and then declines with increasing temperature. Above 15 K, the semiquinone signal is obscured by the signal from the Rieske iron–sulfur protein of the cytochrome bc_1 complex. The effects of the temperature on the $g = 1.85$ and $g = 1.93$ EPR signals are similar to those reported for comparable signals in *Rhodospirillum rubrum* and PS II (Beijer & Rutherford, 1987; Rutherford, 1987; Nugent et al., 1992).

The $Q_A^-Fe^{2+}$ signal of the A2 mutant (Figure 1, right panel) obtained after 2 min of continuous illumination at room temperature showed no obvious deviation from the typical Wt signal, which has a turning point at $g = 1.82$ and a 33-mT line width (Prince et al., 1976). This might be taken as an indication that the effect of the mutation is essentially restricted to the Q_B site. The loss of Q_A upon isolation of RCs is probably a secondary effect (see below).

Inhibitor Sensitivity. A convenient and commonly used way of studying inhibitor (and quinone) affinity is by measuring the charge recombination kinetics from either the state $P^+Q_A^-$ or $P^+Q_B^-$ (under conditions where fast rereduction of P^+ cannot take place). In *Rps. viridis*, the decay lifetimes of these states are 1 and 100 ms, respectively, and the fraction of slow phase of the P^+ signal gives directly the fraction of RCs in which the electron is stabilized on Q_B (Shopes & Wraight, 1985). Attempts were made to use this optical

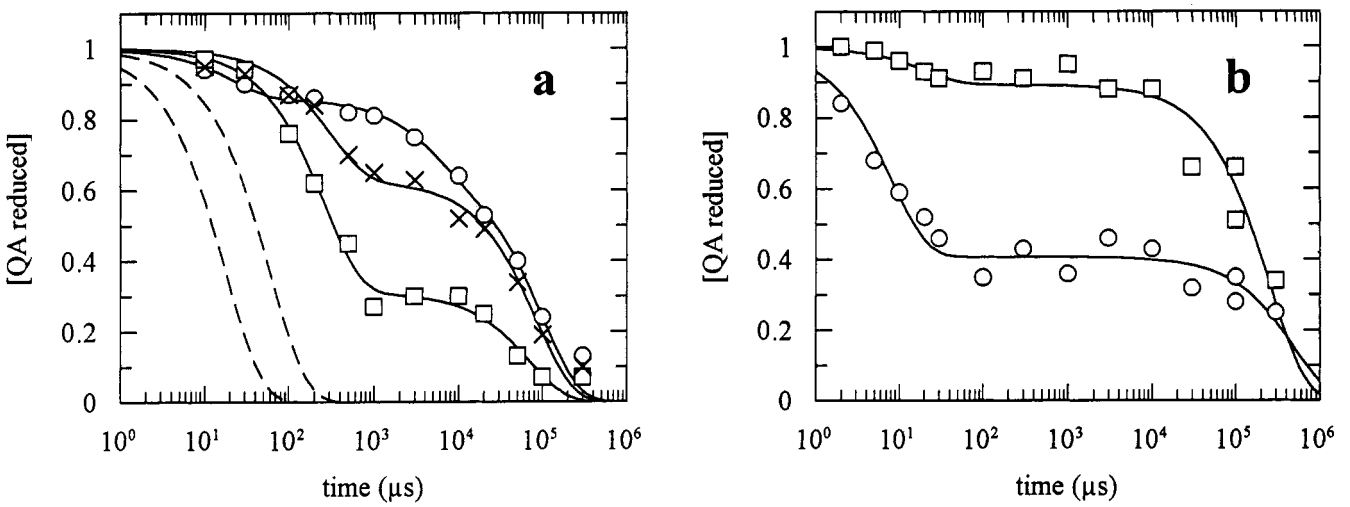

FIGURE 3: (a) Reoxidation kinetics of Q_A^- at pH 7.0 in whole cells of the mutant A2 after one (○), two (□), and three (×) saturating flashes. Solid lines, best fit of two or three exponential phases (see text). Dashed lines, reoxidation kinetics of Q_A^- after one and two saturating flashes in Wt (faster and slower kinetics, respectively). (b) Reoxidation kinetics of Q_A^- at pH 11 in the mutant A2 after one (○) and two (□) saturating flashes. Data points were obtained by analysis of time-resolved photovoltage kinetics induced by a picosecond flash given after the indicated delay time. Time between saturating flashes, 200 ms. Time between averaging cycles, >10 s.

method for inhibitor titration as in an earlier study of herbicide-resistant mutants (Sinning et al., 1989a). However, it turned out that this was not feasible in the mutant A2. As will be shown later, the reason for this is that the electron transfer to Q_B in this mutant is too slow to compete with the $P^+Q_A^-$ back-reaction. Therefore, we used EPR spectroscopy to test the binding of inhibitors.

As the mutants were grown in the presence of atrazine (300 μM), one might expect an increased binding affinity (I_{50}) for this herbicide. In order to determine the binding affinity for atrazine in Wt and mutant chromatophores, the decrease in the amplitude of the $g = 1.85$ peak (and of the $g = 1.95$ peak in the mutant) was monitored as a function of the inhibitor concentration. Because the solubility of atrazine was limiting at higher concentrations no accurate value for I_{50} could be determined. But the data obtained show that the binding affinity of atrazine to the Q_B site is in the range of 100 μM for the wild type and 1–5 mM for the mutant chromatophores at a BChlb concentration of 375 μM. The value for Wt is in agreement with the results of Sinning and co-workers, who reported a decrease of about 3-fold in the $Q_B^-Fe^{2+}$ EPR signal after the addition of 500 μM atrazine. It is worth noting that the binding of atrazine leads to a largely diminished $Q_A^-Fe^{2+}$ signal in Wt (Sinning et al., 1989a), and therefore only the reduced size of the $Q_B^-Fe^{2+}$ signal (at $g = 1.93$) could be used for monitoring inhibitor binding. Also further effects of the inhibitors at the very high concentrations necessary for the mutants cannot be excluded. Therefore the given values should be considered as approximate.

No sensitivity toward DCMU (0.5 mM) was found in the mutant.

Kinetics of the $Q_A \rightarrow Q_B$ Electron Transfer. To characterize the effect of the mutation further, we studied the kinetics of the electron transfer from Q_A^- to Q_B and to Q_B^- in the mutant. For these measurements, we took advantage of the sensitivity of time-resolved photovoltage kinetics to the redox state of Q_A as described earlier (Leibl & Breton, 1991). In Figure 3a, the reoxidation kinetics of Q_A^- after one and two saturating flashes (circles and squares, respectively) at pH 7 in whole cells of the Glu L212 → Lys mutant A2 are shown and compared to the kinetics observed in Wt (dashed lines). We verified that these kinetics correspond to the first and second electron transfer to Q_B, by monitoring the binary oscillation of the concentration of Q_A^- at a certain delay time after a number of preflashes (not shown). An example of this oscillation is shown by the fact that the kinetics after three flashes are similar to that after one flash (compare crosses and circles in Figure 3a). The most striking result was the finding that the first electron transfer $Q_A^- \rightarrow Q_B$ was drastically inhibited in the mutant. The rate constant was $k_1 = (70\ \text{ms})^{-1}$ compared to $(18\ \mu s)^{-1}$ in Wt. The transfer of the second electron is only slightly slowed down ($k_2 = (260\ \mu s)^{-1}$ compared to $(65\ \mu s)^{-1}$ in Wt). This mutant is probably the first example where the transfer of the second electron to Q_B is much faster than the transfer of the first electron.

It turned out that it was difficult to obtain pure redox states of the secondary quinone acceptor in the mutant. This is the reason for the 20–30% contamination of the kinetics with a second phase corresponding to the wrong (out-of-phase) redox state of Q_B as can be clearly seen in Figure 3a. The reoxidation of the semiquinone by the added redox mediator seemed to be less efficient in the mutant than in Wt. Similar observations were made during the EPR measurements, where a significant $Q_B^-Fe^{2+}$ signal present in the dark had to be removed by the addition of ferricyanide prior to the measurements. The attribution of the slow kinetics of Q_A^- reoxidation to the state Q_B was confirmed by measurements at lower redox potential. In the presence of ascorbate, a faster reoxidation of Q_A^- after one flash and a slower reoxidation after two flashes were observed (data not shown). This effect was reversible when oxidizing conditions were reestablished by the addition of ferricyanide. As under more reducing conditions, a large part of the RCs can be assumed to be in the state Q_B^- in the dark, these findings support the above attribution.

The kinetics of electron transfer were studied as a function of pH (Figure 4). The rate of the first electron transfer showed no significant variation between pH 7 and pH 10. At higher pH however, a drastic change of the kinetics was observed. As can be seen in Figure 3b at pH 11 a major part of the reaction centers (≈60%) showed fast kinetics for the first electron transfer, comparable to those observed in Wt ($k_1 \approx (20\ \mu s)^{-1}$). In the remaining fraction of RCs, the reoxidation kinetics of Q_A^- were very slow (>100 ms). A closer look at the kinetics at different values of pH shows clearly and highly reproducibly that a small contribution of the 20-μs phase is present at all pH values (see Figure 3a). When the relative

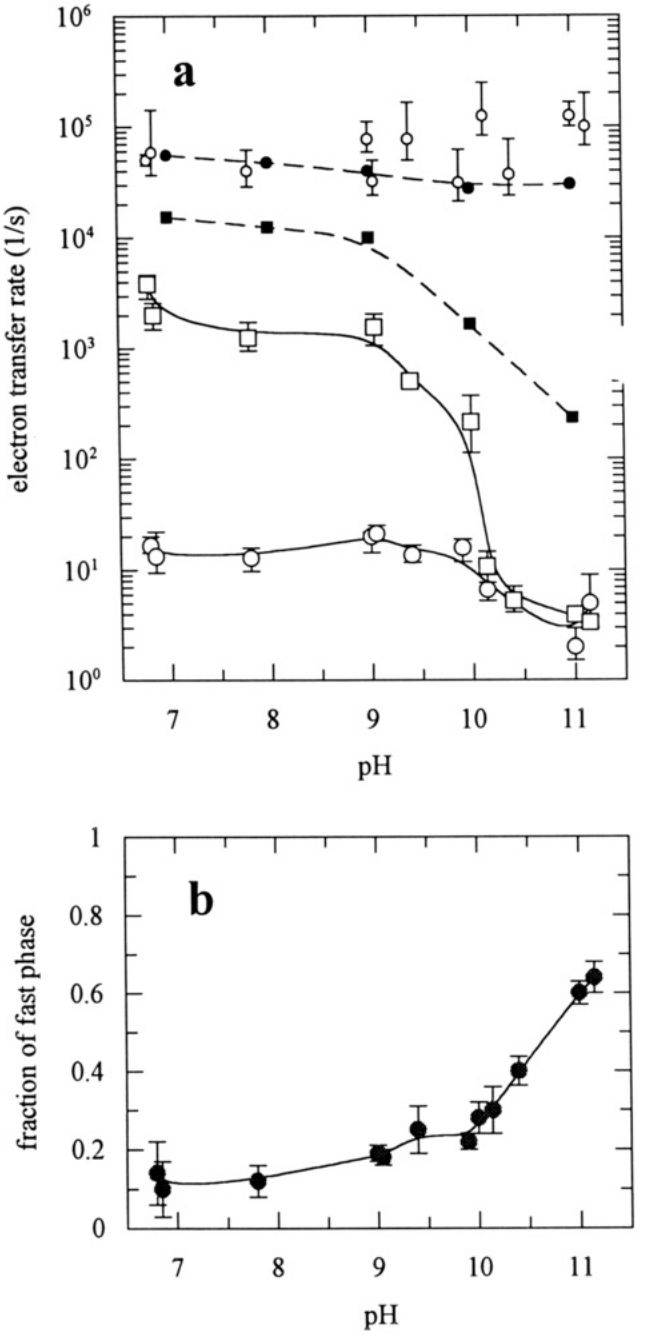

FIGURE 4: (a) pH dependence of the rate of the first (○) and second (□) electron transfer to Q_B. Open symbols and solid lines, mutant A2; filled symbols and dashed lines, Wt. In the mutant, the first electron transfer is generally biphasic (small and large open circles, respectively). (b) pH dependence of the fraction of fast phase of the biphasic first electron transfer in the mutant A2.

amplitude of this phase is plotted as a function of pH (Figure 4b), an apparent p*K* value of about 11 shows up. The rate constant of this fast phase is rather pH independent (see small open cycles in Figure 4a). The effect induced by high pH was reversible when the pH was lowered below 10. Measurements at pH values higher than 11 were not possible since the redox mediator TMPD decomposes irreversibly under the necessary oxidizing conditions.

The second electron transfer showed a similar pH dependence as in Wt. The rate is not significantly pH dependent between pH 7 and pH 9 but starts to decrease by a factor of about 10 per pH unit above pH 9. At pH > 10, however, it decreases sharply to a very slow value, close to the one of the slow phase of first electron transfer at this pH (Figure 4a). It should be noted that the values for these very slow (>100 ms) kinetics are not very precise as in this time range competing reactions (including the reoxidation of Q_B by external acceptors) start to interfere. They might therefore be regarded as an upper limit for the electron transfer rates. At pH > 10, the binary oscillations of the electron transfer kinetics described above could no longer be observed.

DISCUSSION

EPR Signal. The $Q_B^-Fe^{2+}$ EPR signal found in this study (Figure 1) was very different from the corresponding Wt spectrum and resembled closely that found in PS II of higher plants, *Rhodospirillum rubrum*, and the terbutryn-resistant mutant T4 (L222Tyr → Phe) of *Rps. viridis* (Rutherford et al., 1984; Beijer & Rutherford, 1987; Sinning et al., 1989a). A comparison of the amino acid sequences of these different systems reveals that the similarity in the EPR signal is not reflected by homologies of the primary structure. The mutation in the atrazine-resistant mutant A2 seems to influence the interaction between the secondary quinone and the ferrous iron in the same way as the mutation in the terbutryn-resistant mutant T4, causing a similar EPR spectrum.

The PS II-like $Q_B^-Fe^{2+}$ signal of the mutant T4 was interpreted as indicating that the mutation makes this reaction center PS II-like. This suggestion was supported by the observation that T4 is sensitive to DCMU, a urea-type herbicide that affects PS II reaction centers of higher plants but usually does not affect bacterial reaction centers (Sinning et al., 1989b). As the $Q_B^-Fe^{2+}$ EPR signal of the A2 mutant was remarkably similar to the signal of the T4 mutant and PS II, the influence of DCMU in chromatophores of the mutant A2 was investigated. No sensitivity toward this inhibitor was found in the A2 mutants, indicating that there is no direct relation between the EPR signal of the semiquinone and inhibitor sensitivity.

It was suggested that the DCMU sensitivity of the mutant T4 is mainly due to a structural rearrangement in the Q_B pocket rather than to a change in the charge distribution in this part of the protein, as the pH dependence of the $P^+Q_B^-$ charge recombination kinetics is very similar to that observed in the wild type (Baciou et al., 1991). Indeed, it was shown by X-ray crystallography that the mutation L222 Tyr → Phe in the mutant T4 induces large structural changes of the RC protein that are not restricted to the microenvironment of the residue L222 (Sinning et al., 1990; Sinning, 1992). It is not known whether a similar structural change is responsible for the PS II-like EPR signal in the mutant A2. Such a potential structural change must, however, be different from the one in the mutant T4 as it does not lead to DCMU resistance. The result of this study seems to support earlier observations that there exist generally two forms of EPR signals characteristic for two different semiquinone–iron interactions. The comparison of the three-dimensional structures of the Q_B site from the A2 and T4 mutants could provide further information that could help to explain the similar EPR spectra. Unfortunately, all attempts to crystallize the RC of the mutant A2 have failed (Ewald, 1992).

The binary oscillation of the $Q_B^-Fe^{2+}$ signal in a series of flashes was demonstrated for both Wt and the A2 mutant chromatophores (Figure 2). Oscillations measured by the EPR signal have not previously been reported in this species. They clearly show that in chromatophores of the A2 mutant Q_B still functions as a two-electron gate. This observation is important for A2 since the usual optical methods for demonstrating Q_B function were not possible in this mutant.

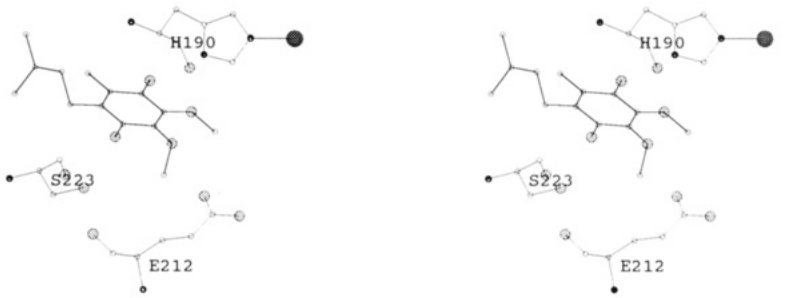

FIGURE 5: Stereo view of part of the Q_B site of *Rps. viridis* wild type. Ser L223 and His L190 form hydrogen bonds to the carbonyl oxygens of the quinone. The nearest oxygen of Glu L212 is at 2.9 and 3.3 Å distance from the carbon atoms of the two methoxy groups of the quinone. The figure was produced using the program pdViewer (Crofts, 1992).

The analysis of the quinone content revealed that after isolation of RCs not only Q_B but also most of Q_A was lost. In our view, this loss of Q_A in the RCs of the mutants is another example of indirect structural consequences of mutations and is presumably due to the fact that the mutant RC is less stable to the detergent concentrations which have been optimized for Wt RCs.

The study of the effect of different inhibitors on the $Q_B^-Fe^{2+}$ EPR signal showed, as expected, a strongly decreased sensitivity to atrazine. Whereas the mode of binding of terbutryn and *o*-phenanthroline to the RC has been established by X-ray crystallography (Michel et al., 1986), the interactions of atrazine with the protein are not known. Glu L212, the residue that is mutated to lysine in A2, forms a large part of the quinone-binding pocket but is probably not directly involved either in quinone binding or in inhibitor binding. Hence the inhibitor resistance in this mutant might be due to some more secondary effect of the mutation, such as electrostatic repulsion between the side chain of L212-Lys and the potentially positively charged nitrogens of the aminoethyl group of the herbicide (Trebst, 1984).

Electron Transfer Kinetics. It is well established that the first electron transfer from Q_A to Q_B is not accompanied by a protonation of Q_B. In contrast, the second electron transfer is closely correlated with the delivery of the first proton to Q_B. In Wt, the first electron transfer is rather fast ($k_1 = (18\ \mu s)^{-1}$). To discuss the decrease in the rate of electron transfer from Q_A to Q_B, we will use the empirical approximation for intraprotein electron transfer of Moser and Dutton (1992):

$$\log k = 15 - 0.6R - 3.1(-\Delta G° - \lambda)^2/\lambda$$

From the parameters in this equation, the values for the edge-to-edge distance between Q_A and Q_B (R = 13.5 Å) and for the free energy of the reaction ($\Delta G°$ = –0.131 eV; Baciou et al., 1991) are known, whereas the value for the reorganization energy of the reaction, λ, is unknown. Using the value for the rate constant measured in the Wt, the above equation gives λ = 0.95 eV as an estimate for the reorganization energy. An independent estimation of this value can be obtained following the treatment of Krishtalik (1989). The formula for the dominant part, the medium reorganization energy

$$\lambda_S = e^2 C_{eff}(1/2r_{eff1} + 1/2r_{eff2} - 1/R)$$

allows us to calculate λ_s = 0.66 eV. This value is obtained with R = 17.6 Å for the center-to-center distance and using the values for the effective radii of menaquinone and ubiquinone, as well as for C_{eff} (C_{eff} = 0.15) given in this reference. Taking into account that the contribution to λ from the intramolecular reorganization energy was neglected and that the region between the two quinones might be more polar than in an average protein (suggesting a somewhat higher value for C_{eff}), both values are in reasonable agreement. In the Glu L212 → Lys mutant in the majority of RCs, the rate of the first electron transfer is decreased by more than 3 orders of magnitude. As an approximation, we shall discuss in the following the isolated effect of each one of the three parameters that determine the electron transfer rate.

If the decreased rate of $Q_A^- \rightarrow Q_B$ electron transfer was due to a change of the free energy of the reaction, this would imply $\Delta G° \approx +0.37$ eV in the mutant compared to $\Delta G° = -0.131$ eV in Wt, i.e., $\Delta\Delta G° \approx 500$ meV. As one of the effects of the mutation, it could be expected that the redox midpoint potential of the Q_B/Q_B^- couple is changed. The electrostatic interaction with the positively charged lysine side chain, which probably is only at a few Ångstrom units distance from the quinone (Figure 5), could shift the potential toward more positive values stabilizing the Q_B^- state. The assumption that Lys L212 is positively charged is based on the fact that lysine generally has a high pK and, as will be shown later, a change in electron transfer kinetics occurs around pH 10.8, which is interpreted as deprotonation of the Lys L212.

The effect of the extra charge on the E_m (in millivolts) as a function of the distance (in Ångstroms) can be estimated using Coulomb's law, $\Delta E_m = 14387/(\epsilon r)$. Using r = 5 Å and $\epsilon \approx 30$ (Shinkarev et al., 1992; Baciou et al., 1991), a ΔE_m of about +95 mV is calculated.[2] This value could be smaller if deprotonation of another amino acid residue partly compensates for the charge on the Lys L212. Evidence for electrostatically strongly interacting residues has been provided for *Rb. sphaeroides* where the unusually high pK of Glu L212 was shown to be due to interaction with Asp L213 and other residues [Takahashi & Wraight, 1992; see also Gunner and Honig (1992)]. Although in *Rps. viridis* the corresponding residue L213 is asparagine, it is likely that other residues play a comparable role (e.g., Asp M43; Hanson et al., 1992; Rongey et al., 1993). Independent of the absolute value, the electrostatic effect should stabilize the charge on Q_B and therefore accelerate the electron transfer.

However, specific interactions between the protein and the quinone could well lead to a considerable destabilization of Q_B^-. The carboxyl group of the side chain of Glu L212 in Wt is within 2.9 and 3.26 Å distance from the carbon atoms of the two methoxy groups on Q_B (see Figure 5). Upon replacement of Glu L212 by a lysine in the mutant, the methoxy torsion angle could be modified and, as a consequence, the electron affinity of Q_B could be changed significantly (Robinson & Kahn, 1990). Therefore the possibility of a change in the redox midpoint potential of Q_B/Q_B^- by as much as –500

[2] Note that this value implies that in the Wt Glu L212 is protonated/neutral, otherwise ΔE_m would be twice as large.

meV, although very unlikely, cannot be totally excluded on structural grounds. However, such a large change in the E_m of Q_B/Q_B^- would result in the equilibrium between the redox states Q_A^-/Q_B and Q_A/Q_B^- being greatly shifted toward the former state and electron transfer would no longer occur. This is in contradiction with our observation of functional electron transfer to Q_B in the mutant (see, e.g., the oscillation of the $Q_B^-Fe^{2+}$ EPR signal); therefore, we can exclude a large change of $\Delta G°$ as being responsible for the decreased kinetics.

If the reorganization energy were changed in the mutant, it would have to be increased by $\delta\lambda \approx 1$ eV from $\lambda \approx 1$ eV in Wt (see above) to about $\lambda = 2$ eV in the mutant. An increase of the reorganization energy could in principle be detected by a measurement of the activation energy of the reaction, which correspondingly would have to be about twice as large in the mutant A2 as in Wt. It appears unreasonable that the replacement of a glutamate by a lysine could lead to such a drastic increase in the reorganization energy of electron transfer, and thus this possibility also seems unlikely.

The third parameter in the above equation, the distance between electron donor and acceptor, is usually the most important one in controlling the electron transfer rate in bioenergetic systems. The ratio of the rates in the mutant A2 and Wt would imply an increase of the distance between Q_A and Q_B of $\delta R = 5.8$ Å independent of the actual values of ΔG and λ. A perturbation of the protein resulting in such a large distance change can be excluded because the second $Q_A^- \rightarrow Q_B^-$ electron transfer is not impaired and such a large reversible movement of the protein depending on the redox state of Q_B is unreasonable.

From the forgoing discussion, it appears that the much slower reoxidation kinetics of Q_A^- in the mutant can probably not be explained by a change in the parameters of electron transfer. Therefore, one has to think about alternative explanations. In the following, the implications of a possible decreased quinone binding in the Glu L212 → Lys mutant shall be discussed.

Lowered ubiquinone affinity would result in a bigger fraction of centers in which the Q_B site was unoccupied at the time of the flash. Fast Q_A^- reoxidation kinetics would be observed in centers in which Q_B was present while in the other centers electron transfer would be limited by quinone binding and thus be much slower. This situation could well correspond to that seen in the mutant since, at pH 7, a small fraction of centers show a fast electron transfer rate like that in the Wt (Figure 3). At high pH, the Q_A^- to Q_B electron transfer rate becomes fast in the majority of the centers (Figure 4b). This suggests that Lys L212 deprotonation ($pK \approx 10.8$) allows increased occupancy of the Q_B site. At pH 7, before electron transfer can occur a ubiquinone molecule has to bind in the majority of the mutant RCs, but not in the Wt.

The low occupancy of the Q_B site in the dark would not be detected by the $Q_B^-Fe^{2+}$ EPR experiment since the EPR measurements are made using a procedure that prevents any back-reaction and thus stabilizes Q_A^- until ubiquinone binding occurs. Therefore, the EPR experiment would be less sensitive to the binding constant as it is essentially the final equilibrium state that is observed.

An explanation of how the mutation in A2 affects quinone binding might be based on a direct interaction of the residue on position L212 with the quinone. Nonella and Schulten (1991) have calculated the interaction of Q_B in its different redox states with the protein matrix in *Rps. viridis*. They found that the strongest interaction of the protein with the neutral ubiquinone is an electrostatic attraction between glutamate L212 and the positively polarized methoxy methyl groups and the ring system of Q_B (see Figure 5). In these calculations, Glu L212 was assumed to be in its anionic form, which is not necessarily the case at neutral pH. Independent of this, it is probable that in the mutant a similarly strong, but repulsive interaction with the lysine exists. This might well be the dominant interaction, which means that in the mutant the binding of the quinone would be drastically disturbed. However, a decreased affinity could also result from relatively subtle structural changes induced (directly or indirectly) by the positive charge on the lysine.

The weak binding of the quinone suggested by the results of the kinetic measurements does not imply a loose binding of the semiquinone form Q_B^-. In *Rb. sphaeroides*, the semiquinone was proposed to be 10^5 times more strongly bound than Q_B (Crofts & Wraight, 1983). This probably applies also to *Rps. viridis* Wt, although the calculations of Nonella and Schulten (1991) reveal a repulsive interaction between Glu L212 (assumed to be negatively charged) and Q_B^-. In contrast to the case of the neutral quinone, the negative charge on the semiquinone will dominate the interaction with the positively charged lysine in the Glu L212 → Lys mutant. Thus, the destabilizing effect of the protonated lysine in the state Q_B will not be relevant in the state Q_B^-, suggesting that also in the mutant the semiquinone is strongly bound. The latter fact can explain the surprising result of a much faster kinetics for the second electron transfer than for the first (at pH < 10). Once the quinone is in the semiquinone form after the first flash, the second electron can be transferred to the now firmly bound Q_B^- with a kinetics similar to those in Wt. Our results indicate that the kinetics of the second electron transfer are not limited by quinone binding.

Although the phenomena observed in the mutant can be satisfactorily explained by lowered Q_B occupancy (as discussed above), other explanations of these effects are possible. In particular, it is possible to imagine a situation in which protonation reactions may limit the electron transfer rate from Q_A^- to Q_B in the mutant. However, given the complexities of the protonation reactions in this system and the lack of specific information concerning the *Rps. viridis* and its mutants, specific discussion does not seem justified.

In the case of *Rb. sphaeroides* there is some evidence for structural rearrangements accompanying $Q_A \rightarrow Q_B$ electron transfer and for the implication of Glu L212 in these rearrangements. The presence of distinct conformational states of the RC was first shown by Kleinfeld et al. (1984a). These authors showed that in RCs frozen in the light, the rate of electron transfer from Q_A to Q_B was several orders of magnitude faster than in RCs frozen in the dark. The $Q_A \rightarrow Q_B$ electron transfer has a high activation energy and is blocked at low temperature (Kleinfeld et al., 1984b). Furthermore, there is evidence that in *Rb. sphaeroides* the protonation state of Glu L212 determines the electron transfer kinetics (Paddock et al., 1989). Recently, it was reported that Glu L212 is involved in an electrogenic (conformational) transition of the RC following photoreduction of Q_A with kinetics similar to those for electron transfer to Q_B (Brzezinski et al., 1992). Combining these pieces of information with the results of this work, the hypothesis can be put forward that the conserved acid residue Glu L212 plays an important role in the electron transfer process and that a drastic change, like its replacement by the basic residue lysine, traps the RC or the quinone in a conformation which is unfavorable for efficient electron transfer to Q_B.

Independent of the exact mechanism which inhibits the first electron transfer at normal pH, the process seems to be reversible at basic pH, as indicated by the reconstitution of a major part of fast electron transfer kinetics at pH 11. We suggest that the reason for this is a loss of the positive charge after deprotonation of Lys L212. The deprotonation might reestablish electrostatic conditions that resemble those in Wt and allow either a structural relaxation or a tighter binding of Q_B.

Glu L212 and Protonation of Q_B. Another interesting aspect of the mutant studied in this work is that Glu L212 might be involved in the donation of protons to the reduced ubiquinone. The residue L212 is conserved in all reaction centers from purple bacteria (Williams et al., 1984; Youvan et al., 1984; Bélanger et al., 1988) and *Chloroflexus aurantiacus* (Ovchinnikov et al., 1988). Its side chain forms a large part of the bottom of the Q_B-binding pocket. In *Rb. sphaeroides*, evidence for the participation of this residue in the proton transport to the fully reduced Q_B^{2-} has been provided (Paddock et al., 1989; Takahashi & Wraight, 1992). In a recent report, Shinkarev et al. (1993) showed that in a Glu L212 → Gln mutant, Q_BH_2 is not released from the RC because the quinone is blocked in the state Q_BH^-.

In the mutant studied in this work, at normal pH, neither the first nor the second proton transfer to Q_B seems to be impaired as can be concluded from the essentially normal kinetics of transfer of the second electron (coupled to the first proton) and the presence of binary oscillations. The latter observation shows that the reduced quinone is exchanged by an oxidized quinone within the time between two flashes (200 ms in the photoelectric measurements, 1 s in EPR experiments), and this is only possible if two protons have been supplied within this time (Shinkarev et al., 1993). The first proton transfer presumably involves Ser L223 as in Wt (Leibl et al., 1993). The result of an unimpaired second proton transfer is also not very surprising as the glutamic acid has been replaced by another protonatable residue which has a rather high p*K* and eventually might take over the role of proton donor (the ability to grow photosynthetically might be considered as further evidence that the proton supply is not too greatly perturbed). In addition, the absence of oscillations at pH 11 observed in this work would be compatible with a failure of the supply of protons to the reduced Q_B as a consequence of the deprotonation of Lys L212. This would indicate that, at position L212, an amino acid residue capable of proton donation is necessary. Thus, in the Wt, Glu L212 could be involved in the donation of the second proton as has been shown in *Rb. sphaeroides* (Paddock et al., 1989; Shinkarev et al., 1993). However, from the present data, it is possible that at pH 11 the transfer of the second electron to Q_B is already blocked. In this case, no conclusion about subsequent reactions could be drawn. This possibility cannot be excluded because of the observation of nearly identical reoxidation kinetics of Q_A^- after the second and third flash at pH 11. It seems clear that the kinetics of the second electron transfer at pH 11 in the mutant are slower than in Wt by at least a factor of 50. The measured rate of about 3 s^{-1} is however on the lower limit of the time resolution of our method. In the seconds time range, secondary reactions start to become competitive, as are, for example, the back-reaction of Q_A^- with an oxidized cytochrome or the reoxidation of Q_B^- by the added redox chemicals. At present, we have no explanation for the sharp decrease of the transfer rate of the second electron at pH > 10. Therefore, the definitive assignment of a proton donor role to Glu L212 in *Rps. viridis* has to await further experiments with a mutant where Glu L212 is replaced by a nonprotonatable amino acid residue.

The mutant studied in this work is characterized by a single but potentially detrimental mutation, replacing an acidic side chain by a longer, basic one. To achieve resistance against a herbicide, in this case, the mutation has resulted in a slowing down of electron transfer to Q_B by 3 orders of magnitude. The *in vivo* significance is a growth rate which is decreased only by 20%. This seems not much, but in the natural environment with strong competition for limited resources, the optimized photosynthetic efficiency of the wild type might be essential.

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