Evidence That Serine L223 Is Involved in the Proton Transfer Pathway to Q_B in the Photosynthetic Reaction Center of *Rhodopseudomonas viridis*†

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ABSTRACT: In the reaction center of purple photosynthetic bacteria, the reducing equivalents produced by primary charge separation are exported via an ubiquinone molecule working as a two-electron shuttle. This loosely-bound quinone, called Q_B, accepts in successive flashes two electrons from the tightly bound primary quinone acceptor Q_A, along with two protons from the external medium. The surrounding protein plays an important role in stabilizing the semiquinone anion and in providing a pathway for protons from the cytoplasmic phase to Q_B. Herbicides of the triazine type compete with Q_B for the binding pocket and their binding is controlled by nearby amino acid residues. We have studied the kinetics of the first and second electron transfer from Q_A to Q_B in two herbicide-resistant mutants from *Rhodopseudomonas viridis*, T1 (Arg L217 → His, Ser L223 → Ala) and MAV5 (Arg L217 → His, Val L220 → Leu), in order to determine whether these residues are involved in proton transfer to the reduced Q_B. The main effect of the mutant T1 was a drastic (600-fold at pH 7) decrease in the rate of the second electron transfer to Q_B compared to the wild type. In contrast, the rate of the second electron transfer in the mutant MAV5 was decreased only slightly (10-fold) in the pH range from 7 to 11. We attribute the inhibition of the second electron transfer in the Ser L223 → Ala mutation to an essential role of Ser L223 in the donation of the first proton to the reduced Q_B. This further corroborates the thesis that the transfer of the second electron is intimately related to the transfer of the first proton to Q_B and that any inhibition of proton supply will be transformed into an inhibition of the transfer of the second electron. The pH dependence of the electron transfer kinetics may indicate that the second mutated residue in the mutant T1, His L217, plays a role as auxiliary proton donor. We suggest that a cluster of hydrogen bonds involving Ser L223 and the quinone carbonyl oxygen could be important for efficient protonation of Q_B. This cluster is lost in the absence of Ser L223.

In the photosynthetic reaction center (RC)[1] of purple bacteria, the absorption of a photon leads to a transmembrane charge separation with high quantum yield. Within about 200 ps a state is created which consists of an oxidized primary donor P, a special pair of bacteriochlorophylls, and a reduced primary acceptor Q_A, a strongly bound quinone molecule [for a review see Feher et al. (1989)]. The mechanism for exporting the reducing equivalents from the RC and feeding them into the pool of mobile quinones is far from being completely understood. The general features of this mechanism apply to purple bacteria as well as to PS II of higher plants, and they include the control of binding affinities for a quinone at a highly specific site, as well as the consecutive transfer to this loosely bound quinone (Q_B) of two electrons from Q_A and two protons from the cytoplasmic phase. Only the doubly reduced and protonated dihydroquinone can leave the RC, whereas the semiquinone form is about 10^7 times more strongly bound and seems to be unprotonated at pH 7 [for a review of protonation of the RC, see Okamura and Feher (1992)]. For the purple bacterium *Rhodobacter sphaeroides* it is well established that failure to supply the first proton to Q_B^- inhibits the stabilization of a second electron on Q_B, whereas failure to supply the second proton to Q_BH^- inhibits the release of the doubly reduced quinone. The binding site of Q_B is buried in the protein, and no water channels through the protein to Q_B have been found (Allen et al., 1988). Therefore, current research aims at the identification of protonatable amino acid residues which could be involved in proton transfer to Q_B, forming a "bucket brigade".

In the last years, considerable progress has been made to identify the pathway of proton transfer in the RC of *Rb. sphaeroides*, for which a rather consistent picture of the protonation events correlated with electron transfer has emerged, although some details such as the sequence of electron and proton transfer to Q_B are still unknown. By studying site-directed mutants, it was established that in this bacterium Asp L213, Ser L223, and Glu L212 are essential for proton transfer to Q_B (Paddock et al., 1989, 1990; Takahashi & Wraight, 1990, 1992). Replacement of Glu L212 by glutamine still allows for three rereductions of the photooxidized primary electron donor before electron transfer is blocked, leading to the conclusion that this residue is involved in transfer of the second proton to Q_B. The role of Ser L223 is still controversial, although its hydrogen bond to one of the carbonyl oxygens of the quinone makes this residue a likely candidate for a proton donor. Since its replacement by a nonprotonatable residue inhibited the second electron transfer, it was considered to be essential for the transfer of the first proton (Paddock et al., 1990). Alternatively, the effect of serine replacement was suggested to be due to structural changes (Takahashi & Wraight, 1992). A key role was assigned to Asp L213, which was proposed to be involved in the transfer of both protons (Takahashi & Wraight, 1992). Because of its rather low pK of about 4, this residue is expected to be negatively charged

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[1] Abbreviations: RC, reaction center(s); P, primary electron donor; Q_A and Q_B, primary and secondary quinone acceptors; DAD, diaminodurene (2,3,5,6-tetramethyl-*p*-phenylenediamine); TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; Wt, wild type; FWHM, full width at half-maximum.

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at physiological pH and seems to interact strongly with other protonatable residues. Interestingly, whereas Glu L212 and Ser L223 are conserved in all purple bacteria, the aspartate is replaced by a nonprotonatable residue (Asn) in *Rhodopseudomonas viridis*. Thus, in the latter species the proton transfer pathway could be quite different.

Unfortunately, site-specific mutagenesis of the RC of *Rps. viridis* is still difficult despite recent progress (Laussermair & Oesterhelt, 1992), and until now no mutants had been available where single amino acid residues in the Q_B-binding region were exchanged. However, photosynthetically-growing mutants of *Rps. viridis* have recently been described which are resistant to the herbicides terbutryn or atrazine (Sinning et al., 1989; Ewald et al., 1990). These inhibitors of photosynthetic electron transport compete with Q_B for the binding site, and resistance is associated with mutation of residues that are involved in binding of the quinone and/or the inhibitor. Because efficient proton transfer can occur only between groups separated by a few angstroms, it is likely that some of these mutations also affect the kinetics and energetics of electron and proton transfer to Q_B. Some of these herbicide-resistant mutants have been characterized by EPR and optical spectroscopy (Sinning et al., 1989; Baciou et al., 1991; Mathis et al., 1992) but no information about the kinetics of the second electron transfer is available which could give insight into the mechanism of proton transfer to Q_B.

In this work, we present a study of the first and second electron transfers from Q_A to Q_B in whole cells of two herbicide-resistant mutants of *Rps. viridis* called T1 (Ser L223 → Ala and Arg L217 → His) and MAV5 (Arg L217 → His and Val L220 → Leu) and compare them to the wild type (Wt). Both are double mutants, but they have one mutation in common. Our results show that in *Rps. viridis* the second electron transfer is strongly correlated with proton transfer to Q_B, and they provide compelling evidence that this proton transfer involves Ser L223.

EXPERIMENTAL PROCEDURES

Cells of herbicide-resistant mutants from *Rps. viridis* were grown as described (Sinning et al., 1989; Ewald et al., 1990). They were stored as pellets at −30 °C until use. For measurements the cells were incubated some minutes with 3 mM ferricyanide and then washed several times in 50 mM of appropriate buffer in the presence of 2 μg/mL gramicidin as described earlier (Leibl & Breton, 1991). Routinely, TMPD or DAD was used as a redox mediator for the reoxidation of Q_B^- between averaging cycles. The concentration of redox compounds was adjusted to yield a reoxidation time for Q_B^- in the time range of seconds. This concentration was typically several millimolar but varied for the different strains and with pH. The optical density of the *Rps. viridis* cell suspension was about 0.5 at 530 nm in a capacitative microcoaxial cell (d = 0.1 mm; Trissl et al., 1987).

Time-resolved photovoltage measurements and analysis of the kinetics were performed using the light-gradient technique, essentially as described (Leibl & Breton, 1991). Saturating preflashes were provided by a Q-switched, frequency-doubled Nd–YAG laser (Quantel, France, FWHM 7 ns, λ = 532 nm, energy about 10 mJ/cm²) and picosecond flashes by a mode-locked, frequency-doubled Nd–YAG laser (Quantel, France, FWHM 30 ps, λ = 530 nm, energy about 250 μJ/cm²). The delay time between one or more saturating flashes and the picosecond flash was varied and the photovoltage kinetics evoked 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 disappearance of the positive 200-ps phase of Q_A reduction and its replacement by a negative 2-ns phase corresponding to the backreaction of the primary radical pair (Trissl et al., 1990; Leibl & Breton, 1991).

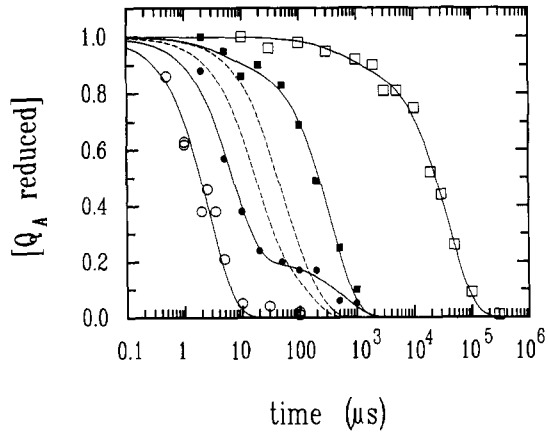

FIGURE 1: Reoxidation kinetics of Q_A^- at pH 7 after one (circles) and two (squares) saturating flashes. Open symbols, T1 mutant (Arg L217 → His, Ser L223 → Ala); closed symbols, MAV5 mutant (Arg L217 → His, Val L220 → Leu); dashed lines, Wt (faster and slower kinetics after one and two saturating flashes, respectively). Conditions: 50 mM Hepes (pH 7) and 1 mM TMPD. Data points were obtained by analysis of time-resolved photovoltage kinetics induced by a picosecond flash given after a variable delay time. Time between two saturating flashes (squares): 100 ms. Time between averaging cycles: >10 s. Solid lines give the best fit of one or two exponentials (see text).

RESULTS

We measured the kinetics of reoxidation of Q_A^- by detecting the fraction of Q_A still reduced at a variable delay time after a saturating flash. The fraction of Q_A^- was tested by measuring the time-resolved photovoltage induced by a picosecond flash. It shows characteristic differences whether forward electron transfer to Q_A can take place or not (Deprez et al., 1986; Leibl & Breton, 1991). The advantage of photoelectric measurements is that the RCs can be observed in situ (detergent isolation of RCs risks to modify the Q_B site). In *Rps. viridis* the application of a double-flash method is facilitated by the fast reduction of the photooxidized primary donor by the bound cytochrome subunit.

Figure 1 shows on a logarithmic time scale the kinetics of Q_A^- reoxidation after one and two saturating flashes (pH 7). Under normal conditions this reoxidation can be ascribed to the transfer of the electron from Q_A to Q_B. The kinetics of Q_A^- reoxidation after one flash is found to be accelerated in both mutants compared to Wt. This acceleration is more pronounced in the mutant T1, where the time constant of about 3 μs (7 μs for MAV5) is close to the limit of timing accuracy between the preflash and the picosecond test flash in our experiment. Taking into account an exponential time constant for this reaction of 18 μs in Wt, acceleration factors of 6 and 2.5 are calculated for the mutants T1 and MAV5, respectively. The kinetics could be fitted by a single exponential phase. The minor phase present in the kinetics of MAV5 in Figure 1 can clearly be attributed to some Q_B^- being present in the dark due to suboptimal redox conditions. This attribution is based on the out-of-phase oscillation of this minor phase (Leibl & Breton, 1991). The kinetics of Q_A^- reoxidation after two flashes is slower in the mutants than in the Wt (Figure 1). Whereas the rate decreases from $(65\ \mu s)^{-1}$ in Wt to $(370\ \mu s)^{-1}$ in the mutant MAV5, a rate constant of $(40\ ms)^{-1}$ was found for the mutant T1. This corresponds to a deceleration at pH 7 by a factor of 6 for the mutant

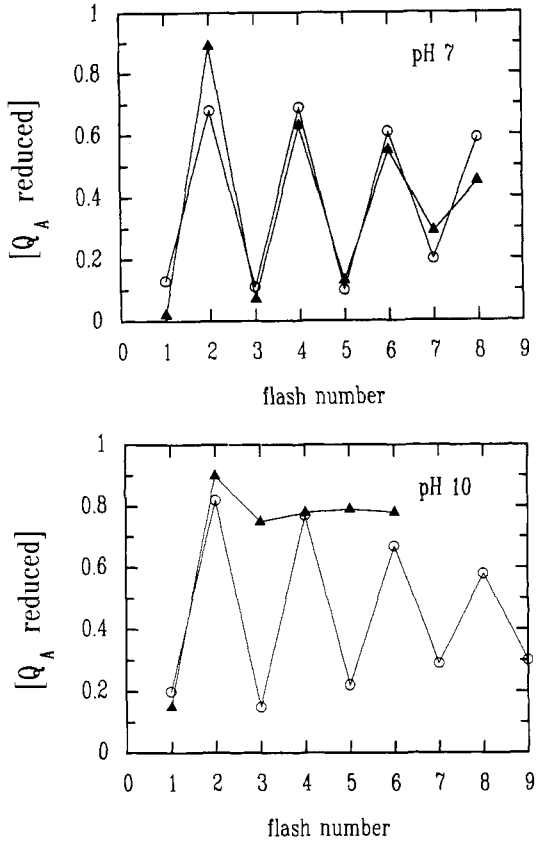

FIGURE 2: Binary oscillations of the fraction of Q_A still reduced at a certain delay time after the last of a variable number of saturating flashes at pH 7 (upper panel) and pH 10 (lower panel). (▲) T1, (○) MAV5. Time between saturating flashes: 100 ms (MAV5), 300 ms (T1, pH 7), and 1 s (T1, pH 10). Delay time for picosecond flash: 500 μs (MAV5, pH 10), 100 μs (MAV5, pH 7; T1). Other experimental conditions were as in Figure 1 except for the buffer, which was 50 mM glycine (pH 10) in the lower panel.

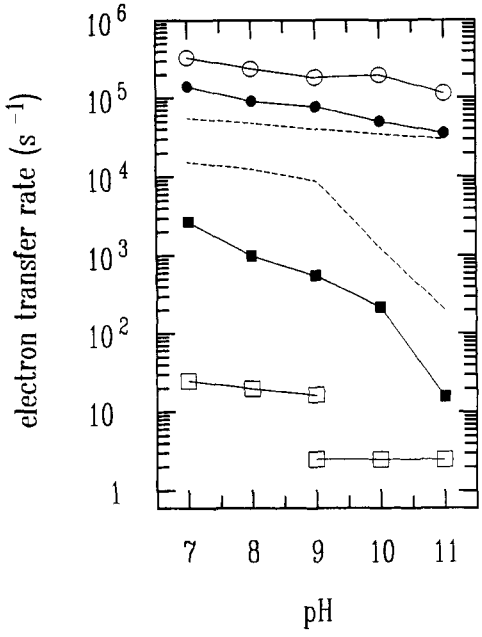

FIGURE 3: pH dependence of the rate of the first (circles) and second (squares) electron transfer to Q_B. Open symbols, T1 mutant; closed symbols, MAV5 mutant; dashed lines, Wt (faster and slower kinetics represent first and second electron transfer, respectively). Data points were obtained from a fit of an exponential function to the corresponding Q_A^- reoxidation kinetics (see Figure 1). In cases where a minor second phase was present, the exponential rate constant of the dominant phase was taken.

MAV5 and by a factor of about 600 for T1. The drastic decrease of the rate of the second electron transfer in the mutant T1 is the most important result (see Discussion).

To prove the assignment of these reoxidation kinetics to electron transfer to Q_B, we verified that there is a binary oscillation of the reoxidation kinetics of Q_A^- as a function of the number of flashes, i.e., electron transfer to Q_B is followed by an electron transfer to Q_B^-—which in general shows a slower kinetics—and that this one again is followed by a transfer to Q_B. For this reason, we varied the number of saturating preflashes and tested the fraction of Q_A still reduced at a certain delay time after the last preflash. As shown in Figure 2, this results in a binary oscillation pattern, in which a low value of $[Q_A^-]$ indicates a fast reoxidation and a high value a slow reoxidation. If the kinetics of the first and second electron transfer to Q_B are well separated, it is possible to select a delay time between the last saturating preflash and the test flash for which the faster transfer is completed while the slower one has not yet started (compared Figure 1). Under these conditions the measured fraction of Q_A^- corresponds directly to the fraction of Q_B which had been in the semiquinone state before the last saturating preflash and therefore it shows the same oscillation pattern as Q_B^- (Figure 2). Oscillations associated with the quinone/semiquinone transitions are always more or less damped for different reasons (Verméglio, 1977; De Grooth et al., 1978). They are especially difficult to observe when the kinetics of electron transfer to Q_B are rather slow, because side reactions (such as a slow reoxidation of Q_A^- and/or Q_B^- by artificial acceptors, or charge recombination reactions) start to become competitive. Binary oscillations were easily observed in Wt and in the mutant MAV5 in the whole pH range (7–11), whereas this was more difficult in the case of T1 due to the slow transfer of the second electron. They are, however, clearly seen at pH 7 (Figure 2, upper panel) and pH 8 (not shown).

Another piece of evidence supporting the idea that the observed Q_A^- reoxidation kinetics are due to electron transfer to Q_B comes from corresponding measurements in the presence of inhibitors of the $Q_A \rightarrow Q_B$ electron transfer. Although the mutants T1 and MAV5 are resistant to terbutryn and atrazine, respectively, they are sensitive to the inhibitor *o*-phenanthroline (Sinning et al., 1989; Ewald, 1992). After addition of 10 mM *o*-phenanthroline in both mutants, the lifetime of Q_A^- is increased to about 1 s as in Wt (not shown). These kinetics are independent of the number of preflashes and can be attributed to a charge recombination with oxidized cytochrome at the donor side of the RC.

To get further information about protonation events, we also studied the pH dependence of the Q_A^- reoxidation in the mutants. In Figure 3 the rate constant of this reaction is shown as a function of pH. As in Wt (dashed lines in Figure 3), the rate constant of the first electron transfer shows only a weak pH dependence in both mutants, and it is on the *second* electron transfer that a clear effect of the proton concentration on the rate of the electron transfer is observed. Generally, with increasing pH a region where there is a modest decrease in the rate changes into a region where the rate of electron transfer decreases linearly with the bulk proton concentration. The pH dependence of the transfer rate of the second electron in the mutant MAV5 is not very different from the one in Wt, except for a deceleration by about a factor of 10 and a shift of the onset of the strong pH dependence to somewhat higher pH (solid squares in Figure 3).

The rate of transfer of the second electron in the mutant T1, which was strongly diminished compared to Wt, showed

only a weak pH dependence below about pH 9. At higher pH the measured reoxidation kinetics of Q_A^- were further decreased. At the same time, however, the binary oscillations visible at lower pH were lost even when the time between the saturating flashes was increased to 1 s in order to allow even a very slow second electron transfer to take place (Figure 2, lower panel). For these experiments it was crucial to use a concentration of oxidized mediators low enough not to reoxidize Q_B^- in the time between the first and second flash and thereby interfere with a slow electron transfer. We therefore checked that the lifetime of Q_B^- created by the first flash was longer than the measured reoxidation time of Q_A^-. Despite these efforts, we were not able to determine the exact rate of the second electron transfer in this mutant at high pH because, in the absence of oscillations, we could not prove that the observed reoxidation kinetics were indeed due to electron transfer to Q_B^-. The fact that virtually identical kinetics of Q_A^- reoxidation were obtained after the second and third flash makes it more probable that electron transfer to Q_B^- cannot compete with charge recombination between Q_A^- and a cytochrome. For these reasons, a value of about $(400\ ms)^{-1}$ represents only an upper limit for the rate of the second electron transfer in the mutant T1 at pH > 9 (Figure 3). Around pH 9 heterogeneous kinetics of Q_A^- reoxidation were found, which are a combination of the kinetics at lower and higher pH. Therefore the two pH regions were not connected by a line in Figure 3 (open squares).

DISCUSSION

The most important difference in the electron transfer kinetics between the mutants and Wt is the drastic inhibition of the second electron transfer to Q_B in the mutant T1. Although this reaction is also somewhat slower in MAV5, the decrease of the rate in T1 by nearly 3 orders of magnitude points to a specific effect, different from the one seen in the mutant MAV5. We therefore assign this decrease of the rate to the replacement of Ser L223 by alanine in T1. X-ray crystallography shows no significant structural change in the mutant T1 compared to Wt, which could explain the inhibition of electron transfer (Sinning et al., 1990; see also Figure 4). We conclude that an essential feature for efficient transfer of the second electron is the presence at position L223 of a protonatable residue, which is somehow necessary for efficient proton donation to Q_B. In the mutant T1, the unprotonatable alanine blocks the transfer of the first proton to Q_B and thereby destabilizes the second electron. This link between electron and proton transfer is well established in studies of other RCs and was already concluded from observations of the pH dependence of the second electron transfer in Wt (Wraight, 1979; Leibl & Breton, 1991).

Our observation of inhibition of the second electron transfer to Q_B upon replacement of Ser L223 by an unprotonatable residue is very similar to the effect of a corresponding mutation in *Rb. sphaeroides*, where the rate of the second electron transfer was found to be reduced 350-fold at pH 7.5 (Paddock et al., 1990). This supports our conclusion that the main effect observed in the mutant T1 is not related to the second mutation Arg L217 → His. In MAV5, the other mutation (Val L220 → Leu) can be assumed to be neutral with respect to electron and proton transfer. Therefore the slight decrease of the rate of the second electron transfer seen in this mutant might be attributed to the exchange of arginine by histidine. Compared to the consequences of the Ser → Ala replacement this effect is of second order.

As stated above, Ser L223, which is conserved in all purple bacteria, was found to form a hydrogen bond to one of the carbonyl oxygens of the quinone in *Rps. viridis* (Michel & Deisenhofer, 1988) as well as in *Rb. sphaeroides* (Allen et al., 1988; El-Kabbani et al., 1991). This makes Ser L223 a likely candidate for a direct proton donor by internal proton transfer along the hydrogen bond to Q_B, although its high p*K* suggests that it can presumably only be deprotonated and reprotonated in a concerted manner (Paddock et al., 1990). Alternatively, it was discussed, that the (transiently) *protonated* hydroxyl group of Ser L223 could be the actual proton donor (Okamura & Feher, 1992). A similar mechanism seems to be an attractive hypothesis in the case of *Rps. viridis*. As shown in Figure 4, the presence of Ser L223 leads to the formation of a cluster of hydrogen bonds between the hydroxyl group of serine and the side chain of Asn 213 and the carbonyl oxygen of Q_B, respectively, and between the quinone carbonyl oxygen and the backbone N–H of Gly L225 as the third member (Michel & Deisenhofer, 1988; Sinning et al., 1990). In the mutant T1, only the last hydrogen bond is conserved (and this one is not able to act in a hydrogen donor chain), whereas in MAV5 all three bonds seem to be present (Ewald, 1992). The protonation of Q_B could be initiated and facilitated by the presence of these hydrogen bonds.

Interestingly, in the mutant T1 where Asn L213 can no longer form a hydrogen bond with Ser L223, the former residue forms a hydrogen bond with the second residue exchanged in the mutant, His L217 (Figure 4; Sinning et al., 1989). Asn L213 thereby switches into a position comparable to the one found for Asp L213 in *Rb. sphaeroides*, which presumably forms a salt bridge to Arg L217 in this species (Allen et al., 1988). These facts, and the pH dependence of the electron transfer kinetics, might help to provide a rationale for the statistically very unlikely second mutation in the mutant T1. We found, for example, that in this mutant at high pH (pH > 9) the rate of electron transfer on the second flash was slowed down even further. We were not able to determine the exact rate of the second electron transfer, but it is decreased further by at least 1 order of magnitude compared to that at pH 7–8, and the electron transfer to Q_B can apparently no longer compete with backreactions. We propose that this inhibition is due to deprotonation of His L217, on the basis of a recent assignment of a p*K*(Q_B^-) of 8.3 for this residue (Baciou et al., 1991). It has already been argued that the second mutation Arg L217 → His is compensating for detrimental effects of the primary mutation Ser L223 → Ala, which itself is responsible for herbicide resistance (Michel & Deisenhofer, 1988; Sinning et al., 1989). We suggest that in the mutant T1 the second mutation His L217 is necessary for photosynthetic growth and that at normal pH it provides at least a minimal rate of proton supply. Arginine might not be able to participate in proton donation because of its high p*K*. The distance betwen histidine and the quinone carbonyl oxygen seems too large for a direct transfer of protons, even if some movement of the quinone and the rather slow rate are taken into account. To overcome this problem, one might speculate about a mechanism which is based on the hydrogen acceptor properties of Asn L213. This residue might participate in proton transfer from histidine to Q_B by a piggyback mechanism (Williams, 1988). Moving into a more Wt-like orientation, it could transfer the proton of the hydrogen bond.

Our attribution to His L217 of a role as an auxiliary proton donor is based on its relative proximity to the quinone carbonyl oxygen, which in Wt forms a hydrogen bond to Ser L223, and on the fact that, at a pH higher than its p*K*, no other proton

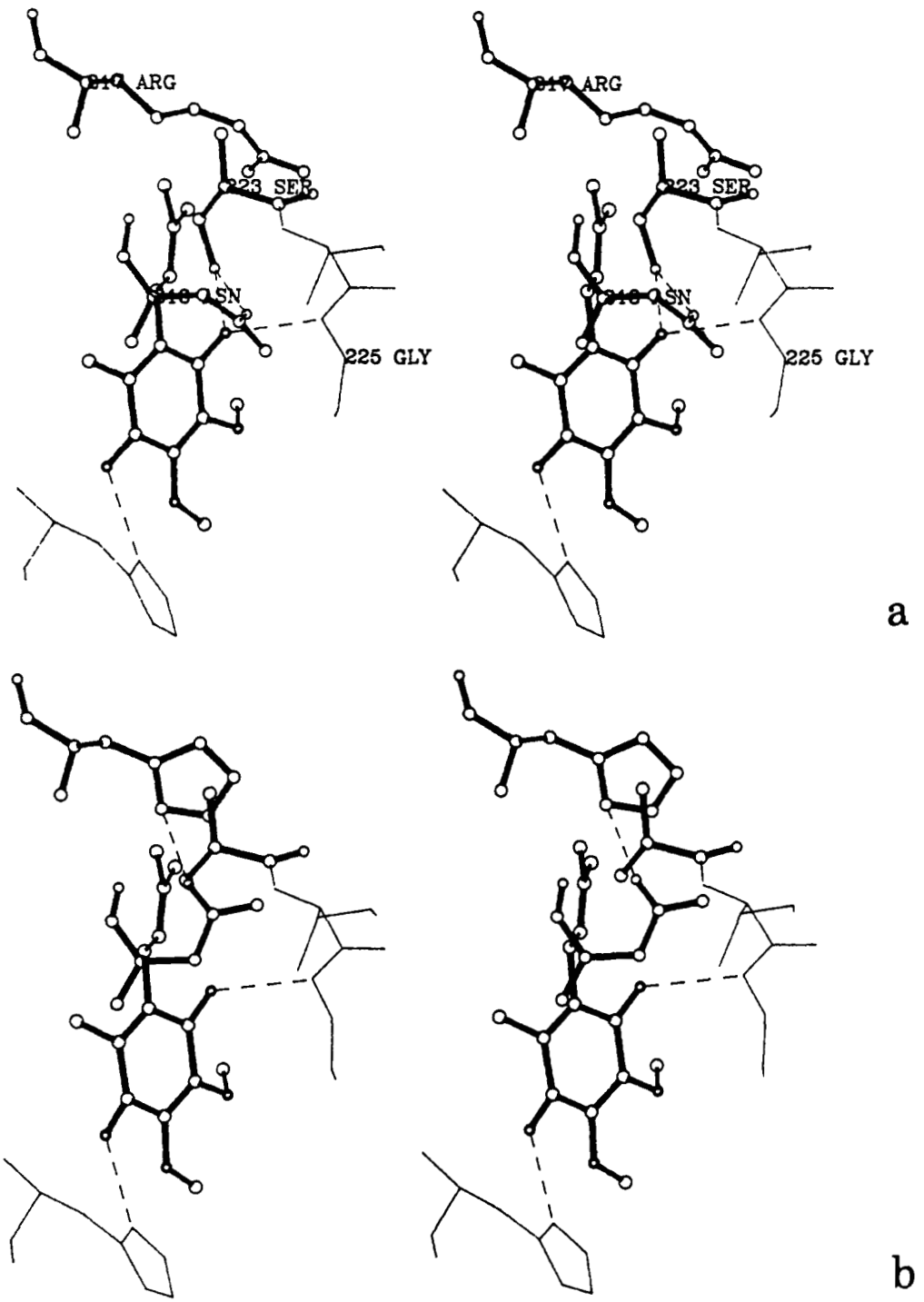

FIGURE 4: Stereo plots of part of the Q_B sites of (a) *Rps. viridis* wild type and (b) mutant T1 (Ser L223 → Ala, Arg L217 → His). Possible hydrogen bonds are drawn as dashed lines. Figure redrawn from Sinning et al. (1990).

donor seems to be available. However, this interpretation does not explain why there is also a second mutation of Arg L217 to His in the mutant MAV5. In this mutant, Ser L223 and the cluster of hydrogen bonds discussed above are present, and Asn L213 displays an orientation as in Wt (Ewald, 1992). According to the interpretation above, serine also functions as primary proton donor in the mutant MAV5, and in line with this, no inhibition of protonation was observed. We tentatively suggest that there is no common cause for the Arg → His mutation in both mutants. Being of functional importance to reestablish proton transfer in the mutant T1 (which therefore could be regarded as a suppressor mutant), this second mutation might be necessary for atrazine resistance, along with the other mutation Val L220 → Leu in the mutant MAV5. Unlike in the case of terbutryn, the mode of binding of atrazine is not known, although it is thought that the binding regions of both inhibitors overlap (Sinning et al., 1989; Ewald et al., 1990).

The comparison of the rate and pH dependence of the second electron transfer in the two mutants and in Wt (Figure 3) invites one to speculate about a possible indirect role for Arg L217 in proton transfer. The positive charge on this residue could induce a negative pK shift of a group directly involved in proton transfer, giving rise to the apparent pK in the kinetics of about 9.3 in Wt. When Arg L217 is replaced by histidine, the positive charge is probably absent at pH > 8.3, thereby shifting the apparent pK of the above-mentioned protonatable residue to higher values. This might correspond to the

observation that in MAV5 the onset of the strong pH dependence of the electron transfer rate is shifted to about pH 10 (Figure 3).

In both mutants studied in this work, the first electron transfer at pH 7 was found to be accelerated compared to the Wt. The acceleration is more pronounced in T1 than in MAV5. Recently, the kinetics of the first electron transfer were measured in the mutant T1 and in Wt by optical spectroscopy, and half-times of about 6 μs for T1 compared to 25 μs for Wt were reported (Mathis et al., 1992). Our results, obtained in whole cells, are in good agreement with these values obtained on chromatophores and isolated RCs.

T1 is the only herbicide-resistant mutant showing a binding affinity for the secondary quinone which is higher than in Wt (Sinning et al., 1989). It could be argued that this might lead to a higher occupancy of the Q_B site in T1 and thereby to an acceleration of the first electron transfer. This interpretation would imply that the binding of Q_B is rate limiting for the transfer of the first electron. However, the high rate of electron transfer [$(20\ \mu s)^{-1}$ in Wt] makes Q_B binding unlikely as a rate-limiting step. A more reasonable hypothesis is that Q_B or the protein must move into an active, more tightly bound conformation before electron transfer can take place and that this conformation is favored in T1. Evidence for structural changes following the reduction of Q_A and preceding electron transfer to Q_B in *Rb. sphaeroides* has been reported (Kleinfeld et al., 1984; Brzezinski et al., 1992). The molecular process underlying this structural change is unknown, but it seems likely that a similar mechanism exists in *Rps. viridis*.

The very weak pH dependence of the kinetics shows that there is no specific effect of the proton concentration on the first electron transfer, in line with the idea that Q_B^- is unprotonated and that protonation of the protein is not the rate-limiting step for the stabilization of the first charge on Q_B. Under these conditions, the electron transfer is nonadiabatic, and the rate depends mainly on the distance R between the quinones, the free energy ΔG, and the reorganization energy λ of the reaction (Marcus & Sutin, 1985; Moser et al., 1992). The empirical approximation for the electron transfer rate

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

was given by Moser and Dutton, and it was shown that it applies to a wide variety of electron transfer reactions in proteins (Moser & Dutton, 1992). Although these authors state that a factor of 10 is "in the noise" in their relationship, it can be useful for a qualitative estimation of the influence of the three parameters involved.

The increase in the electron transfer rate by a factor of 6 in the mutant T1 compared to Wt (pH 7) would necessitate a decrease in the distance between the quinones by 1.3 Å, if the other parameters were held constant. Such a large structural change is unlikely, even if one takes into account that the structure of the Q_B site is less well defined than that of other parts of the RC (Sinning et al., 1990). A significant change in the free energy of the reaction via a change of the redox midpoint potential of Q_B/Q_B^- induced by the mutation can also be excluded. The increase in the rate would imply a considerable stabilization of the semiquinone state of Q_B. However, this stabilization is not seen either in equilibrium redox titration of Q_B/Q_B^- (Albert, 1992; Rutherford et al., 1979; Rutherford & Evans, 1979) or in the comparison of the kinetics of $P^+Q_B^-$ charge recombination in the mutant T1 and in Wt, respectively (Baciou et al., 1991). On the contrary, Baciou et al. concluded from the faster $P^+Q_B^-$ charge recombination in T1 that the semiquinone anion is slightly destablized (δG = +3 mV) in T1 at neutral pH. Neglecting this minor change and using the value of ΔG = −130 mV (Baciou et al., 1991), a model calculation gives a change in the reorganization energy of $\delta\lambda \approx -270$ mV. This value depends only slightly on the absolute value of the reorganization energy, which has not been determined in *Rps. viridis* but which can be estimated to be in the range of $\lambda \approx 0.7$–2 eV from data obtained in *Rb. sphaeroides* or other purple bacteria (Paddock et al., 1991; Chamorovsky et al., 1976; Moser et al., 1992). In summary, under the assumption that the equation given above is valid at least as a first approximation, it appears that the mutation has a minor effect on the free energy of the electron transfer reaction but has a relatively strong effect on the reorganization energy. The decrease in the reorganization energy by $\delta\lambda \approx -270$ mV in the mutant corresponds to a decrease in the activation energy of the electron transfer reaction by $\delta E^* \approx -65$ mV.

It is worth noting that from the large number of Q_B site mutations studied in *Rb. sphaeroides*, the Ser L223 → Ala mutant is the only one that shows an acceleration of the first electron transfer similar to the one found in this work for the mutant T1 (Paddock et al., 1990; Okamura & Feher, 1992). We therefore suggest that the replacement of serine by alanine is mainly responsible for the increase of the rate. This mutation removes not only the hydroxyl group of serine but also the hydrogen bond to the quinone carbonyl oxygen. Both losses result in a less polar environment. This is expected to lead to a decrease of the reorganization energy for the quinone reduction, in accordance with the above estimation. The value of $\delta\lambda$ is on the upper limit of the energy range for a hydrogen bond. On the other hand, it could have been expected that the changes in the Q_B environment observed for the mutant would have led to detectable changes also in the redox midpoint potential of the quinone.

In conclusion, the similarity of the results obtained for the Ser L223 → Ala mutation in *Rb. sphaeroides* (Paddock et al., 1990) and *Rps. viridis* (this work) strengthens the attribution of a proton donor role to Ser L223 in both systems. Although alternative proton pathways from the cytoplasmic phase into the Q_B pocket may exist (Beroza et al., 1992; Hanson et al., 1992), this similarity also indicates that the different pathways might converge in the proximity of the quinone and that the direct proton donors might be conserved in all purple bacteria.

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