Characterization of Four Herbicide-Resistant Mutants of *Rhodopseudomonas viridis* by Genetic Analysis, Electron Paramagnetic Resonance, and Optical Spectroscopy†

Irmgard Sinning,*,‡ Hartmut Michel,‡ Paul Mathis,§ and A. William Rutherford§

Max-Planck-Institut für Biophysik, Heinrich-Hoffmann-Strasse 7, D-6000 Frankfurt 71, West Germany, and Service de Biophysique, Département de Biologie, Centre d'Etudes Nucléaires de Saclay, 91191 Gif-sur-Yvette Cédex, France

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ABSTRACT: Herbicides of the triazine class block electron transfer in the photosynthetic reaction centers of purple bacteria and PSII of higher plants. They are thought to act by competing with one of the electron acceptors, the secondary quinone, Q_B, for its binding site. Several mutants of the purple bacterium *Rhodopseudomonas viridis* resistant to terbutryn [2-(methylthio)-4-(ethylamino)-6-(*tert*-butylamino)-*s*-triazine] have been isolated by their ability to grow photosynthetically in the presence of the herbicide. Sequence analysis of the genes coding for the L and M subunits of the reaction center showed that four different mutants were obtained, two of them being double mutated: T1 ($Ser^{L223} \rightarrow Ala$ and $Arg^{L217} \rightarrow His$), T3 ($Phe^{L216} \rightarrow Ser$ and $Val^{M263} \rightarrow Phe$), T4 ($Tyr^{L222} \rightarrow Phe$), and T6 ($Phe^{L216} \rightarrow Ser$). The residues L223 and L216 are involved in binding of Q_B, whereas L217 and L222 are not. M263 is part of the binding pocket of the primary quinone, Q_A. The affinity of the reaction centers for terbutryn and the electron transfer inhibitor *o*-phenanthroline, determined via the biphasic charge recombination after one flash, is decreased for all mutants. The affinity for ubiquinone 9 is also decreased, except in T1. Characterization by EPR spectroscopy showed that the $Q_B^{\bullet-}Fe^{2+}$ signal of T4, having a $g = 1.93$ peak, is different from the signals obtained with the wild type and the other mutants but very similar to those of *Rhodospirillum rubrum* and PSII. The results obtained by the combination of these different techniques are discussed with respect to the three-dimensional structure of the wild type and the mode of binding of ubiquinone, terbutryn, and *o*-phenanthroline as determined by X-ray structure analysis.

The absorption of light by the photosynthetic reaction center (RC) leads to a charge separation between the primary electron donor P ("special pair") and the electron acceptor Q_A, forming the state $P^+Q_A^-$ [for review, see Feher and Okamura (1978)]. From Q_A^-, which acts as a one-electron gate, the electron is transferred to the secondary quinone, Q_B, forming $P^+Q_B^-$. When there is no electron donation to P^+, both states can decay by recombination. For *Rhodopseudomonas* (*Rps.*) *viridis* these processes have been studied extensively by optical spectroscopy (Carithers & Parson, 1975; Fleischmann, 1978; Shopes & Wraight, 1985). Two phases can be resolved: the recombination of $P^+Q_A^-$ to PQ_A gives rise to a fast phase ($t_{1/2}$ = 1 ms), whereas the recombination of $P^+Q_B^-$ is much slower ($t_{1/2}$ = 100 ms) (Shopes & Wraight, 1985).

The electron-acceptor complex, which in *Rps. viridis* consists of one menaquinone 9 (Q_A or primary quinone) and one ubiquinone 9 molecule (Q_B or secondary quinone), is located on the cytoplasmic side of the membrane. The quinone ring system of Q_A is exclusively bound by amino acid residues of the M subunit. Q_B could not be identified in the initial electron density maps (Deisenhofer et al., 1984, 1985) because most of it was lost during isolation and crystallization. Therefore, its binding site (Q_B site) has been established by soaking competitive inhibitors and quinones into the crystals and subsequent difference Fourier analysis (Deisenhofer et al., 1985; Michel et al., 1986b). In the refined electron density map at 2.3-Å resolution a weak electron density for the secondary quinone was present (J. Deisenhofer, O. Epp, I. Sinning, and H. Michel, unpublished results). In the three-dimensional structure from *Rhodobacter* (*Rb.*) *sphaeroides* the secondary quinone was present (Chang et al., 1986; Allen et al., 1987).

The reaction center from purple bacteria is structurally and mechanistically similar to the photosystem II (PSII) reaction center of plants and cyanobacteria (Hearst, 1986; Trebst, 1986; Michel & Deisenhofer, 1988). In particular their electron acceptors Q_A and Q_B are chemically very similar and display similar effects of electron transfer inhibitors, specifically the sensitivity toward triazine herbicides. These herbicides are thought to inhibit the photosynthetic electron transport in both systems by displacing the secondary quinone, Q_B (Velthuys, 1981; Wraight, 1981). EPR (electron paramagnetic resonance) spectroscopy has shown that in reaction centers of purple bacteria and in PSII both quinones are magnetically coupled to a non-heme iron, which gives rise to the typical broad semiquinone–iron signals in the $g = 2$ region [reviewed in Feher and Okamura (1978) and Rutherford (1987)]. These signals are affected by the binding of inhibitors which block the electron transfer from Q_A^- to Q_B (Butler et al., 1984; Beijer & Rutherford, 1987; Rutherford et al., 1984a).

In this paper we report the isolation and preliminary characterization of herbicide-resistant mutants from *Rps. viridis*, which have been selected by their ability to grow photosynthetically in the presence of terbutryn. Since the mode of action and the binding of the selecting agent, terbutryn, are known in detail, this can be termed a "site-selected mutagenesis". Site-directed mutagenesis, which would only be used to change residues at very obvious positions in the

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* To whom correspondence should be addressed.
‡ Max-Planck-Institut für Biophysik.
§ Centre d'Études Nucléaires de Saclay.

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three-dimensional structure, is still not applicable for *Rps. viridis*. Our results show clearly that residues, without being directly involved in quinone or inhibitor binding, can also be crucially important by stabilizing the tertiary structure of the protein. The results of these experiments may be helpful not only for the understanding of the mechanisms of electron transfer or herbicide resistance but also for the design of new, powerful herbicides or herbicide-resistant plants by genetic engineering.

Materials and Methods

Biological Materials. *Rps. viridis* DSM 133 was grown as described (Michel et al., 1986a). Herbicide-resistant mutants were obtained by growing the bacteria with 100 μM terbutryn as described in a previous paper (Sinning & Michel, 1987). Chromatophores and reaction centers were isolated as reported by Michel (1982) and Michel et al. (1986a), except that the LDAO buffer was at pH 6.0. For most of the optical and EPR measurements glycerol cultures from the same stock culture as for the genetic characterization were used.

Genomic DNA was isolated from 0.5-L *Rps. viridis* cultures. The cells were sonicated for 1 min in 50 mL of 10 mM Tris-HCl/1 mM EDTA, pH 8.5 at 0 °C, and 3% SDS was added before incubation for 1 h at 40 °C. Proteinase K (20 mg, preincubated) was added, and the suspension was kept at room temperature for 12 h. After the suspension was phenolized three times, the DNA was precipitated by the addition of 2.5 volumes of ethanol in the presence of 1 M LiCl. The DNA was removed with a glass rod, washed with ethanol, and further purified by isopycnic centrifugation on a CsCl gradient.

Cloning was done as described by Sinning and Michel (1987) except that the complete 1.9-kb *Eco*RI/*Sal*I fragment coding for the L and M subunits of the RC was sequenced. The M13/mp9 system was used for sequence analysis by the dideoxy method of Sanger as described (Michel et al., 1986a). Seven heptadecamer oligonucleotides were synthesized with an Applied Biosystems 380A oligonucleotide synthesiser. All procedures used were essentially as described by Maniatis (1982).

EPR Measurements. Chromatophores were resuspended in 20 mM Hepes buffer, pH 7.5, by a short sonication procedure and adjusted to an absorption at 830 nm of 4.2, which corresponds to a reaction center concentration of 15 μM (ϵ = 280000; Clayton & Clayton, 1978; Weyer, 1987). Samples of 250 μL in calibrated EPR tubes were dark adapted for 15 min at 20 °C before addition of 2.5 μL of DAD (=diaminodurene, 0.1 M in DMSO) and 2.5 μL of ascorbate (0.5 M in water). After 15 min of further dark adaptation or after illumination (see below), the samples were frozen in an ethanol/solid CO_2 bath and immediately transferred to liquid nitrogen. For the measurements with inhibitors, 100 μM terbutryn, 5 mM *o*-phenanthroline, or 500 μM atrazine [2-chloro-4-(ethylamino)-6-(isopropylamino)-*s*-triazine], all in DMSO, was added after dark adaptation, before DAD and ascorbate. The binding studies were done with stock solutions of different inhibitor concentrations to keep the DMSO in the samples below 2%. DMSO up to 2% had no apparent effect on the EPR spectra nor on the photochemistry. All sample handling (addition of inhibitors, incubation, mixing, freezing) was performed in darkness.

The samples were illuminated with a single flash from a Nd-YAG laser (530 nm, 20 ns) or with continuous white light (filtered through 2 cm of water and two Calflex filters) from an 800-W projector lamp (5 min in a water bath at room temperature).

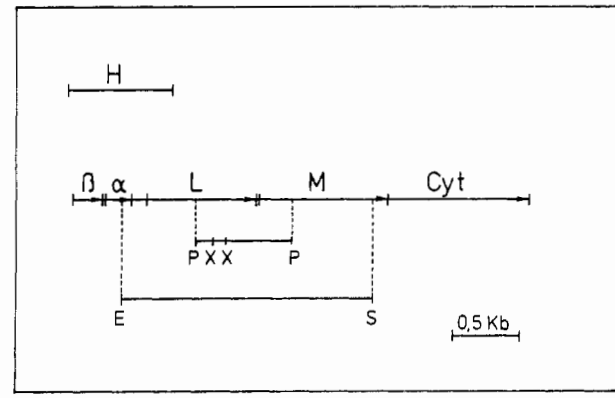

FIGURE 1: Arrangement of the genes coding for the α and β polypeptides of the light harvesting complex and the H, L, M, and cytochrome subunits of the RC from *Rps. viridis*. The restriction sites of the endonucleases used for cloning of the L and M subunit genes of the herbicide-resistant mutants are indicated: E (*Eco*RI), S (*Sal*I), P (*Pst*I), and X (*Sma*I). The *Eco*RI/*Sal*I fragment of 1.9-kb length was isolated and sequenced.

EPR spectra were recorded at liquid helium temperatures with a Bruker ER 200D-SR spectrometer equipped with an Oxford instruments cryostat. The spectroscopic conditions were microwave frequency 9.44 GHz, modulation amplitude 12.5 G, microwave power 8 dB, and temperature 4.5 K.

Optical Spectroscopy. The reaction center concentration was 5 μM. The samples were in sodium phosphate buffer (20 mM, pH 6.0) containing 0.1% LDAO (lauryldimethylamine *N*-oxide). The measurements were performed essentially according to the procedure of Shopes and Wraight (1985, 1987) except that flash-induced absorbance changes were measured at 1300 nm with a germanium diode. P_{960}^+ has a broad absorption peak at 1312 nm (Netzel et al., 1977; Trosper et al., 1977). A 10-ns flash from a dye laser (595 nm, 5–10 mJ) was used for excitation of the sample contained in a 10 × 10 mm cuvette at 21 °C. The output was via a Tracor TN 1710A. A total of 20 flashes was averaged, and the absorbance amplitude was recorded for 0.5 s following the flash. Terbutryn was a gift from Ciba-Geigy, Basel (Dr. K. Pfister). Ubiquinone 9 and menaquinone 9 were a gift from Hoffmann-La Roche, Basel (Dr. Vetter). Atrazine was from Riedel-de-Häen. The quinones were added in 30% LDAO and the inhibitors in DMSO.

Results

Seven independent terbutryn-resistant mutants of *Rps. viridis* were isolated, selection being for photsynthetic growth in the presence of the herbicide as described (Sinning & Michel, 1987). They were named T1 to T7 (the T stands for terbutryn). A prolonged lag phase was observed for the mutants T5, T6, and T7, indicating a higher sensitivity to illumination. In the log phase all mutants grew at approximately the same rate as the wild type.

Cloning and Nucleotide Sequence Analysis

The genes coding for the four subunits of the *Rps. viridis* wild-type RC have been isolated (Michel et al., 1985, 1986a; Weyer et al., 1987). They are thought to form a polycistronic operon together with the genes coding for the α and β polypeptide chains of the light harvesting complex (Figure 1). At 184 bp upstream of the start codon of the L subunit gene there is an *Eco*RI site and 104 bp upstream of the stop codon of the M subunit gene a *Sal*I site. This *Eco*RI/*Sal*I fragment of 1890-bp length carries the complete L subunit gene and 91% of the M subunit gene.

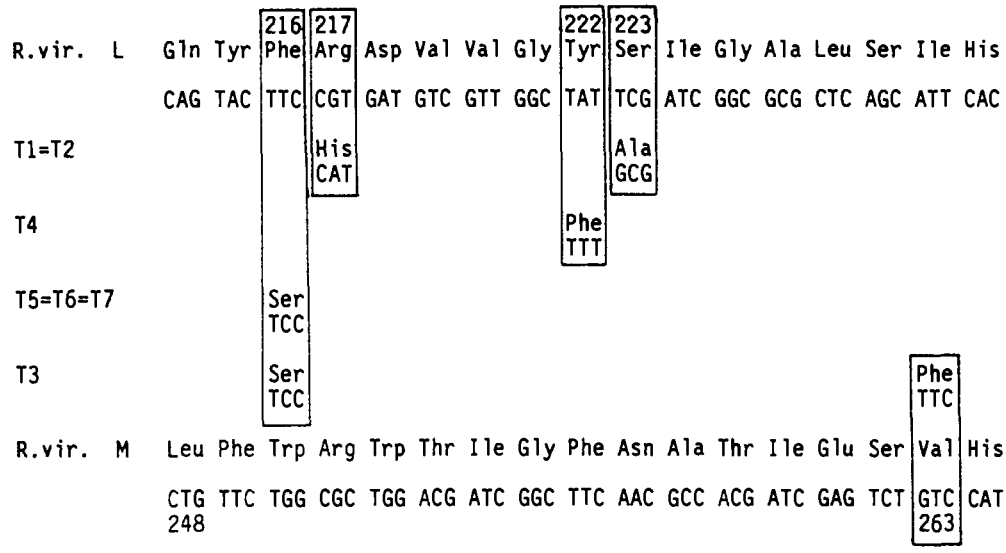

FIGURE 2: Alignment of the nucleotide sequences of the region of the L and M subunit genes of *Rps. viridis* wild type [from Michel et al. (1986a)] coding for the connecting loop between the fourth and fifth transmembrane helices which form the Q_B and Q_A sites. The amino acid sequence is derived from the DNA sequence. The changes which occur in the herbicide-resistant mutants T1 to T7 are shown.

Cloning was done essentially as described in Sinning and Michel (1987). The complete 1.9-kb fragments from all seven mutants and the wild type as a control were sequenced at least twice by the dideoxy method of Sanger (Sanger et al., 1977a). In contrast to a previous paper (Sinning & Michel, 1987), we turned to this procedure to check whether there are mutations out of the Q_B site. Overlapping sequences were obtained without further digestion or subcloning by use of seven synthesized heptadecamer oligonucleotides as primers. These oligonucleotides were chosen by running the computer programs FOLD and MATCH from the UWGCG (University of Wisconsin Genetic Computer Group) system (Zuker & Stigler, 1981) on the sequences of the 1.9-kb *Eco*RI/*Sal*I fragment from *Rps. viridis* wild type and the M13mp9 vector.

Figure 2 shows the nucleotide sequence and the deduced amino acid sequence of the quinone binding region of the L and M subunits of *Rps. viridis* wild type and the changes that lead to herbicide resistance in the mutants. Three transversions and three transitions took place in the genes of the mutants, indicating that there is no preference for a particular nucleotide. The remaining sequence of the L and M subunit genes is completely identical with that of the wild type.

Four different mutants were found: In T1 (=T2) the change of two base pairs lead to the alteration of serine L223 to alanine and arginine L217 to histidine, $Ser^{L223} \rightarrow$ Ala and $Arg^{L217} \rightarrow$ His (Sinning & Michel, 1987). Only one base pair is altered in T4, the tyrosine at position L222 being replaced by a phenylalanine, $Tyr^{L222} \rightarrow$ Phe. In T5 (=T6=T7) a single base pair is changed resulting in the replacement of phenylalanine L216 by a serine, $Phe^{L216} \rightarrow$ Ser, as described in a preliminary note (Sinning & Michel, 1987). In T3 the same mutation as that in the T5 (=T6=T7) mutant is found; however, a second mutation occurs. Surprisingly, the second change in T3 was not located in the L subunit. T3 is the first mutant resistant toward a triazine herbicide which carries a mutation also in the Q_A site. Valine M263 is changed to phenylalanine, $Val^{M263} \rightarrow$ Phe. This residue is part of the connecting loop of the fourth and fifth transmembrane helices of the M subunit, which form the Q_A site.

In contrast to the mutants from *Rb. sphaeroides* (Paddock et al., 1988) every change of a nucleotide resulted in the alteration of one amino acid. No replacement of one amino acid was caused by the change of two nucleotides as it was in the mutant YG222 ($Tyr^{L222} \rightarrow$ Gly) in *Rb. spaeroides*. It should also be noted that *Rps. viridis* obviously has a high tendency to give double mutants. This may be due to the selecting conditions (Sinning & Michel, 1987). The mutants were always grown in the presence of 100 μM terbutryn in order to prevent reversion. They were grown in the presence of the herbicide for more than 1 year, before the experiments described here were performed.

Characterization by EPR Spectroscopy

The EPR signals of the electron-acceptor complex from the photosynthetic reaction centers of purple bacteria and from PSII preparations are well characterized [reviewed in Feher and Okamura (1978) and Rutherford (1987)]. They are known to be very sensitive to changes in the environment of the iron and the quinones. We therefore looked for effects of the mutations on the characteristics of the semiquinone–iron signals and for effects of inhibitor binding.

$Q_B^{\bullet-}Fe^{2+}$ and $Q_A^{\bullet-}Fe^{2+}$ Signals. Dark-adapted chromatophores showed no EPR signal in the $g = 1.82$ region. However, by monitoring their EPR signals, we found that under these conditions the two low-potential cytochrome hemes were largely oxidized and the two high-potential hemes were reduced [not shown, but see Nitschke and Rutherford (1989)].

Different illumination procedures allowed the formation of either Q_B^- or Q_A^-. First, upon excitation by a single flash, charge separation takes place in the reaction center leading to the eventual reduction of the secondary quinone Q_B and the oxidation of one of the high-potential cytochrome hemes. The exogeneous electron-donor system DAD/ascorbate rereduces the photooxidized cytochrome, preventing charge recombination and thus stabilizing Q_B^-. Second, continuous illumination at room temperature results in the complete reduction of the Q pool and the trapping of Q_A^- at the expense of the exogeneous electron donor.

After one flash, the characteristic $Q_B^{\bullet-}Fe^{2+}$ signal occurred in wild-type chromatophores (Figure 3, left) having a turning point at $g = 1.82$, a peak to trough line width of 18.5 mT, and a split $g = 1.85$ peak (Rutherford & Evans, 1979). For all mutants flash-induced signals occurred in the $g = 1.82$ region, indicating that electron transport is still functional in the mutants. These signals can be attributed to the $Q_B^{\bullet-}Fe^{2+}$ state (see also below) although they are modified (Figure 3, left).

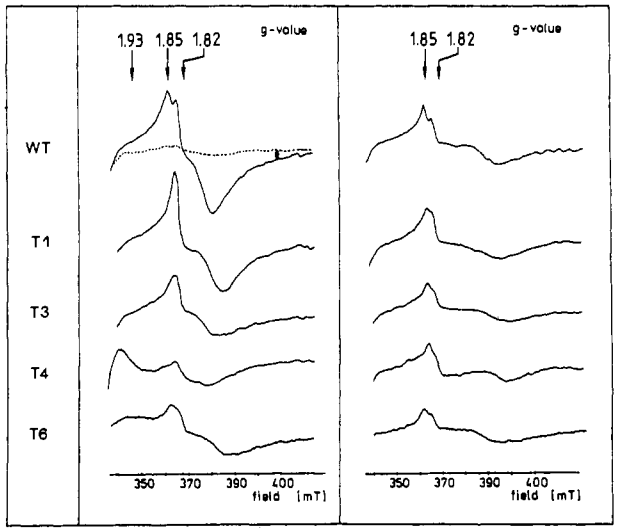

FIGURE 3: EPR spectra of chromatophores from *Rps. viridis* wild type and the mutants T1, T3, T4, and T6: (Left) $Q_B^{\bullet-}Fe^{2+}$ signals (after one flash). The dark spectrum of the wild type is drawn as a dashed line. (Right) $Q_A^{\bullet-}Fe^{2+}$ signals (after 5-min illumination at room temperature). Conditions: sample concentration OD_{830} = 4.2; Hepes buffer (20 mM, pH 7.5); sample volume 250 μL; DAD 1 mM; ascorbate 5 mM; frequency 9.44 GHz; modulation amplitude 12.5 G; microwave power 8 dB; temperature 4.5 K.

The signal of T1 ($Ser^{L223} \rightarrow Ala$, $Arg^{L217} \rightarrow His$) looked more like that reported from *Rb. sphaeroides*: the peak at $g = 1.85$ is not obviously split, although the line width is still small compared to that of $Q_B^{\bullet-}Fe^{2+}$ in *Rb. sphaeroides* (Wraight, 1978; Rutherford & Evans, 1980; Butler et al., 1984). T3 ($Phe^{L216} \rightarrow Ser$, $Val^{M263} \rightarrow Phe$) and T6 (=T5=T7) ($Phe^{L216} \rightarrow Ser$) always showed a weaker signal. We have not determined whether this loss of signal intensity is due to a change in the magnetic interaction or due to a systematic overestimation of the concentration of reaction centers in the chromatophores of this mutant. In T4 ($Tyr^{L222} \rightarrow Phe$) the $Q_B^{\bullet-}Fe^{2+}$ signal was drastically altered: it was apparently much narrower, line width 13 mT, and had a second turning point at $g = 1.93$. This is very similar to the $Q_B^{\bullet-}Fe^{2+}$ signals observed in *Rhodospirillum (R.) rubrum* (Beijer & Rutherford, 1987) and in PSII reaction centers (Rutherford et al., 1984b; Zimmermann & Rutherford, 1986). Furthermore, the T4 signal showed similar effects of temperature and power saturation as reported in the two other systems; i.e., unlike the $g = 1.82$ signal, the $g = 1.93$ signal is not optimized at very low temperatures and high powers (data not shown).

Continuous illumination (5 min) at room temperature results in the formation of the typical semiquinone–iron signal of the primary acceptor in wild-type chromatophores (Figure 3, right): turning point is at $g = 1.82$; line width = 33 mT; the $g = 1.85$ peak is less split (Prince et al., 1976). For the mutants similar signals were obtained (Figure 3, right). Nevertheless the small effects on the line width and signal shape proved to be repeatable.

Effect of Inhibitor Treatments on $Q_A^{\bullet-}Fe^{2+}$ and $Q_B^{\bullet-}Fe^{2+}$. The effect of inhibitors on the EPR signals in the wild type and the mutants was studied with terbutryn (100 μM), atrazine (500 μM), and *o*-phenanthroline (5 mM).

Effects on the Wild Type. All three inhibitors are known to block the electron transport between Q_A and Q_B in the wild type (Stein et al., 1984). Therefore, the EPR signals formed by one flash (Figure 4, left) and continuous illumination (Figure 4, right) should both arise from $Q_A^{\bullet-}Fe^{2+}$. In the wild type the $Q_A^{\bullet-}Fe^{2+}$ signal is markedly affected by inhibitor binding to the Q_B site (Figure 4). Binding of terbutryn greatly

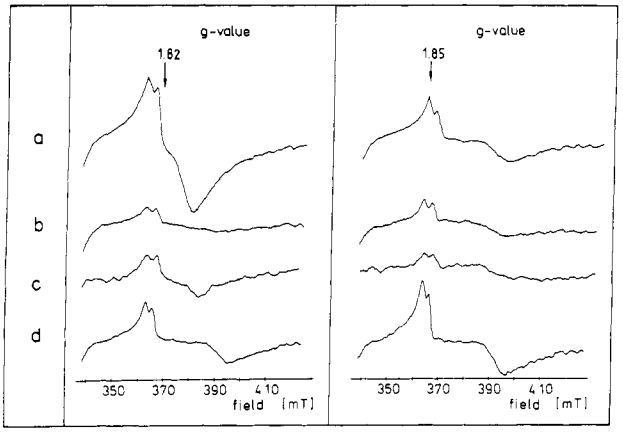

FIGURE 4: Influence of inhibitor treatment on the semiquinone–iron signals of *Rps. viridis* wild-type chromatophores: (a) sample without inhibitor; (b) with 100 μM terbutryn; (c) with 500 μM atrazine; (d) with 5 mM *o*-phenanthroline. (Left) Signals obtained after a single flash; (right) signals obtained after 5-min illumination. Conditions were as in Figure 3.

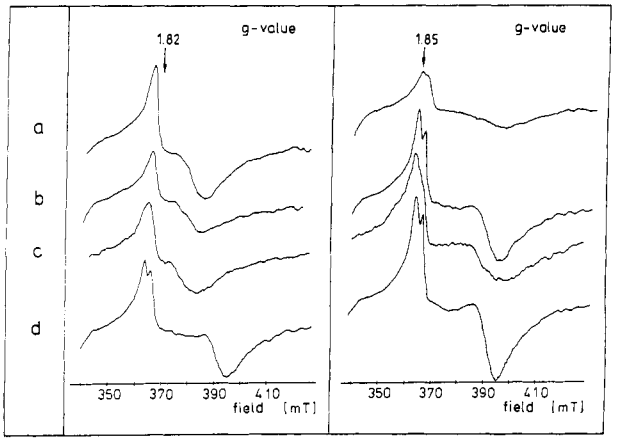

FIGURE 5: Influence of inhibitor treatment on the semiquinone–iron signals of the T1 mutant ($Ser^{L223} \rightarrow Ala$, $Arg^{L217} \rightarrow His$): (left) signals after one flash; (right) after 5-min illumination. Conditions were as in Figure 3. Spectra a–d are for the same inhibitors as in Figure 4.

decreased the nonsaturated amplitude of the signal and modified the shape of the $g = 1.85$ peak. Atrazine had virtually the same spectroscopic effect although less complete at this concentration due to its lower binding affinity. There is still some $Q_B^{\bullet-}Fe^{2+}$ formed by a flash in the presence of 500 μM atrazine (Figure 4c, left). The presence of *o*-phenanthroline led to a $Q_A^{\bullet-}Fe^{2+}$ signal with a greater amplitude and with a $g = 1.85$ peak which is slightly more split. A similar effect has also been seen in *Rb. sphaeroides* (Butler et al., 1984).

Effects of the Mutations on the Semiquinone–Iron Signals. In all mutants electron transfer from Q_A^- to Q_B was still functional in the presence of terbutryn and atrazine. Differential sensitivity toward *o*-phenanthroline was observed. The results are only shown for T1 (Figure 5) and T4 (Figure 6). It can be seen from Figure 5 that T1 is resistant toward terbutryn and atrazine since the $Q_B^{\bullet-}Fe^{2+}$ signal is formed after one flash. However, the T1 mutant is still sensitive toward *o*-phenanthroline since in the presence of this inhibitor the $Q_A^{\bullet-}Fe^{2+}$ signal is formed after one flash. T4 is resistant toward all three inhibitors (Figure 6). The T3 and T5 (=T6=T7) mutants were also resistant to all three herbicides at the concentrations used. The spectroscopic effect of inhibitor binding on the $Q_A^{\bullet-}Fe^{2+}$ signal in the mutants is quite different from that seen in the wild type. The increased intensity and the appearance of split $g = 1.85$ signals in the

Table I: Summary of the Q_{50}'s and I_{50}'s of *Rps. viridis* Wild Type and Herbicide-Resistant Mutants[a]

Rps. viridis	changed residue	ubiquinone 9 Q_{50} (μM)	terbutryn I_{50} (μM)	*o*-phenanthroline I_{50} (μM)
WT		4.5	1.8	30
T1	$Arg^{L217} \rightarrow His$, $Ser^{L223} \rightarrow Ala$	0.5	400	150
T3	$Phe^{L216} \rightarrow Ser$, $Val^{M263} \rightarrow Phe$	80	300	2000
T4	$Tyr^{L222} \rightarrow Phe$	100	$\gg$10000	600
T6	$Phe^{L216} \rightarrow Ser$	60	$\gg$5000	$\gg$10000
WT + MQ-9		22		
WT + LDAO		13		

[a] The values were obtained as described in the text and in Figure 7; the values for T3 are preliminary only; see text. WT = wild type; WT + MQ-9 = wild type in the presence of 33 μM menaquinone 9; WT + LDAO = wild type in the presence of 0.5% LDAO.

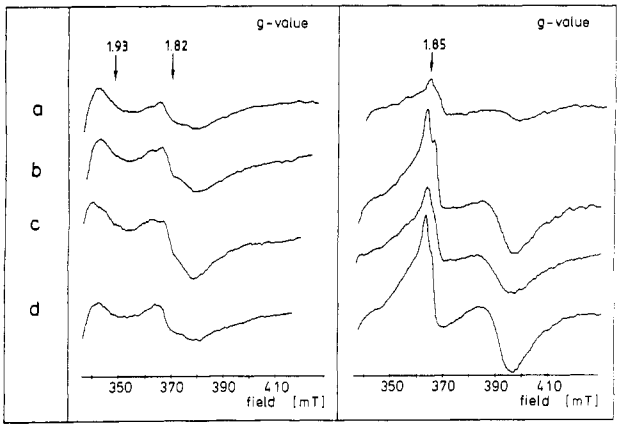

FIGURE 6: Influence of the inhibitor treatment on the semiquinone–iron signals of the T4 mutant ($Tyr^{L222} \rightarrow Phe$): (left) signals after one flash; (right) after 5-min illumination. Conditions and inhibitors are as in Figures 3–5.

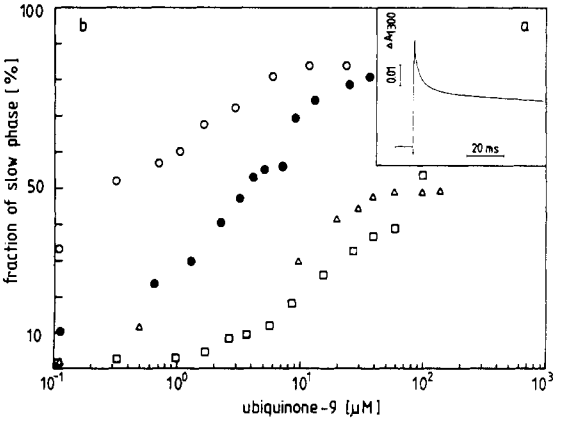

FIGURE 7: (a) Flash-induced absorption change observed in RCs from *Rps. viridis* wild type monitored at 1300 nm at [UQ9] = Q_{50}. Conditions: 5 μM RC; 20 mM sodium phosphate buffer, pH 6.0; 0.1% LDAO. The exciting flash is indicated by an arrow. (b) Effect of ubiquinone 9 on the reconstitution of slow back-reaction monitored at 1300 nm. Conditions: 5 μM RC; 20 mM sodium phosphate buffer, pH 6.0; 0.1% LDAO. (●) *Rps. viridis* wild type; (○) T1; (□) T4; (△) T6.

$Q_A^{\bullet-}Fe^{2+}$ spectra formed by continuous illumination of the mutants indicate inhibitor binding under these conditions. This behavior is to be expected to some extent since the UQ pool is fully reduced in these samples and UQH_2 is thought not to bind to the Q_B site (Wraight, 1982).

We also used EPR spectroscopy for binding studies on chromatophores with the inhibitors. Chromatophores contain an unknown high but probably constant concentration of ubiquinone competing for the Q_B site. In the binding study with terbutryn on the wild type, the decreasing amplitude of the $g = 1.85$ peak was monitored while the inhibitor concentration was increased (not shown). The inhibitor concentration at which half-maximum amplitude (I_{50} about 4 μM) was obtained is very close to the I_{50} from optical spectroscopy (see below). For the mutants the solubility of the inhibitors is limiting because the affinity of the inhibitors is greatly decreased. In most cases it was not possible to get high enough herbicide concentrations.

Characterization by Optical Spectroscopy

Binding Affinity for Ubiquinone 9. In isolated reaction centers the cytochrome *c*-558 was largely oxidized. Ferricyanide was avoided since it can rapidly oxidize Q_A^- in *Rps. viridis* wild type (Shopes & Wraight, 1985). Following the flash, the absorbance at 1300 nm increased due to the formation of P_{960}^+. It then decays in a biphasic manner with $t_{1/2}$ of 1 and 100 ms. The two phases are attributed to a recombination between P_{960}^+ and Q_A^- and Q_B^-, respectively. Both phases were well resolved for all the mutants studied here. The binding affinity for ubiquinone 9 at the Q_B site was determined by monitoring the fraction of slow recombination from the $P^+Q_B^-$ state (Figure 7a) as a function of the quinone concentration. The quinone concentration at which 50% slow recombination is obtained (Q_{50} or apparent binding constant) reflects the binding of the quinone at the Q_B site. The binding curves are shown in Figure 7b. Table I summarizes the results obtained for the wild type and the mutants T1 ($Ser^{L223} \rightarrow Ala$, $Arg^{L217} \rightarrow His$), T3 ($Phe^{L216} \rightarrow Ser$, $Val^{M263} \rightarrow Phe$), T4 ($Tyr^{L222} \rightarrow Phe$), and T6 ($Phe^{L216} \rightarrow Ser$). The data given there for T3 are preliminary values only, because in the most recent measurements made on this mutant the kinetics for the $P^+Q_A^-$ and $P^+Q_B^-$ recombination were both much slower and the binding constants were different. This could indicate an instability in the mutation, and further work is required to understand this change.

A high Q_{50} value indicates a decreased binding affinity for the secondary quinone, which is found for all mutants except T1. This particular mutant showed a lower Q_{50} than the wild type; the observation that 30–40% slow phase was already obtained without addition of ubiquinone indicates that a third of the centers retained Q_B in the reaction centers isolated by the standard procedure. Without addition of ubiquinone the fraction of centers with Q_B was below 10% for the wild type and below 3% for the other mutants. These values are in agreement with the determination of the ubiquinone 9 content from HPLC experiments (not shown). In T4 and T6 the Q_{50} is 15–25-fold higher than that of the wild type, indicating that in these mutants quinone binding is also affected by the changes in the amino acid sequence.

Influence of the Detergent on the Reconstitution. The detergent is known to influence the binding of the secondary quinone (Okamura et al., 1975; Wraight & Stein, 1983). UQ9 is highly hydrophobic and practically insoluble in water. The quinone activity in an aqueous detergent solution is lowered by increasing the detergent at constant total quinone concentration. Table I shows that increasing the LDAO concentration to 0.5% resulted in a 3-fold increase of the Q_{50} in

the wild type. Therefore, the additions of the quinones were standardized to equalize the detergent concentration in all measurements (0.1%). Although the data obtained in the binding experiments here are probably influenced by the detergent, they do allow a direct comparison of the wild type with the mutants at this given detergent concentration.

Selectivity of the Q_B Site for Ubiquinone 9. In order to test the selectivity of the Q_B site for the secondary quinone, we added menaquinone 9 (33 μM) to reaction centers from the wild type. The addition of this quinone did not result in an increase of the fraction of slow recombination in the absence of added ubiquinone 9. However, when we determined the Q_{50} of ubiquinone 9 in the presence of menaquinone 9, a much higher Q_{50} for UQ9 was obtained (Table I). This result may indicate that menaquinone competes with ubiquinone for binding to the Q_B site but does not act as an electron acceptor, presumably due to its lower redox potential.

Binding Affinity for Terbutryn and o-Phenanthroline. The binding affinities for inhibitors were determined by monitoring the decreasing fraction of slow phase as a function of the inhibitor concentration. The I_{50} was measured in samples which contained 5 μM RC, 0.1% LDAO, and ubiquinone 9 at a concentration equivalent to the Q_{50} value (Shopes & Wraight, 1987). The fraction of slow phase obtained at this quinone concentration was normalized to 100%. The I_{50} is the inhibitor concentration at which the amount of slow recombination, $P_{960}^{+}Q_B^{-}$, was further diminished by 50%. In T6 and T4 very high concentrations of terbutryn had to be added before an effect became significant; for these measurements the solubility of the inhibitor was limiting. Table I summarizes the results. In T1 the affinity of terbutryn is 200-fold lower than that in the wild type whereas in T4 and T6 it is more than 5500- and 2700-fold lower, respectively. The affinity of *o*-phenanthroline is most drastically reduced in T6, whereas in T1 and T4 it is reduced only by 5- and 20-fold, respectively. In T6 the single change of the phenylalanine L216 to a serine affects the binding of both inhibitors.

Stability of the Reaction Centers of the Mutants. Without any quinone additions the absorbance change which was obtained with the mutants was much smaller than that with the wild type (30% in T1, 20% in T3, 8% in T4, 10% in T6). The signal size increased upon quinone addition (to 70% in T3; to 20% in T4, to 30% in T6; no increase in T1). It was thus obvious that Q_A was partially lost. The activity could not be fully restored by the addition of ubiquinone 9 or menaquinone 9, indicating a certain amount of damaged quinone binding sites. The loss of Q_A during preparation of the reaction centers was confirmed by HPLC analysis (not shown) and by the observation of the P_{960} triplet signal in RC samples of the mutants as checked by EPR spectroscopy under illumination at 5 K. The triplet was also seen when Q_A was oxidized. In addition to this, an EPR signal at $g = 2.9$ that has been attributed to damaged low-spin cytochrome heme (Nitschke & Rutherford, 1989) was observed in the mutants (data not shown). This indicates a far-reaching effect of the mutations in the Q_B site which seems to decrease the stability of the whole complex when isolated from the membrane by the procedures developed for the wild type.

Discussion

The binding of terbutryn and *o*-phenanthroline to the reaction center of *Rps. viridis* has been established by X-ray crystallography (Michel et al., 1986b). It was shown that their binding sites overlap only partially. Terbutryn binds close to the entrance of the Q_B site (Figure 8a), whereas *o*-phenanthroline binds at the bottom of the pocket, close to the iron (Figure 8b). *o*-Phenanthroline is hydrogen bonded to histidine L190—which is liganded to the iron—and is in close contact with leucine L193 and isoleucine L229.

The binding of terbutryn occurs by numerous van der Waals interactions and two hydrogen bonds with the protein. One hydrogen bond is likely between the hydroxyl group of serine L223 as acceptor and the aminoethyl side chain of terbutryn as donor and the other between a nitrogen of the *s*-triazine ring system as acceptor and the peptide nitrogen of isoleucine L224 as donor (Figure 8a). The hydroxyl group of serine L223 also acts as a hydrogen bond donor to the side-chain oxygen of asparagine L213. Phenylalanine L216 forms a major part of the terbutryn binding pocket (Michel et al., 1986b).

In the refined electron density at 2.3-Å resolution the secondary quinone could be detected (J. Deisenhofer, O. Epp, I. Sinning, and H. Michel, unpublished results). Its binding site overlaps partially with those of terbutryn and *o*-phenanthroline. The aromatic ring systems of *o*-phenanthroline and the ubiquinone have the same orientation. One carbonyl group of Q_B is hydrogen bonded to histidine L190 which is liganded to the iron and which is also involved in the binding of *o*-phenanthroline (Figure 9). A second hydrogen bond is likely between the second carbonyl group of the quinone and the hydroxyl group of serine L223 and an additional hydrogen bond to the backbone N–H of glycine L225.

We will try to relate this information about inhibitor and quinone binding to the mutations which lead to herbicide resistance and to the effects observed by EPR and optical spectroscopy on the mutants.

As shown in Figure 4 we observed quite different effects of terbutryn and *o*-phenanthroline on the EPR signals of the wild type. The $Q_A^{\bullet-}$-Fe^{2+} signal is markedly affected by inhibitor binding. Therefore, we looked for conformational changes in the crystal structure due to inhibitor binding, which should result in extra peaks in the difference Fourier maps; no changes in the iron environment were observed (J. Deisenhofer and H. Michel, unpublished data). This could indicate effects which are too small to be detected by X-ray crystallography or that a structural difference is present only when Q_A is reduced. In *Rps. viridis* wild type terbutryn binds much better than atrazine, indicating that the protein is able to discriminate between different substitutions of the basic atrazine ring system [see also Shopes and Wraight (1987)]. The large *tert*-butyl group of terbutryn can make more van der Waals contacts with the protein than the smaller isopropyl group of atrazine. The same holds for the methylthio or chloro substituents in terbutryn and atrazine, respectively.

From the mode of binding of terbutryn it is easy to understand why the replacement of serine L223 by an alanine leads to herbicide resistance in T1: this mutation abolishes one of the two hydrogen bonds which are possible between terbutryn and the protein and, therefore, should decrease the binding of the inhibitor significantly, although the effect on *o*-phenanthroline binding is expected to be less marked. The binding of terbutryn in T1 is indeed decreased by a factor of 200 while that for *o*-phenanthroline is only decreased by a factor of 5 (Table I). EPR studies with inhibitors also indicate that *o*-phenanthroline can still bind to the Q_B site in T1.

Since serine L223 in *Rps. viridis* is also involved in binding of the ubiquinone, one would expect the binding of Q_B in this mutant also to be decreased. However, quinone binding is increased in T1. This is the only herbicide-resistant mutant reported so far which binds Q_B better than the wild type. There are several possible reasons for this surprising observation. First, the replacement of serine L223 by alanine re-

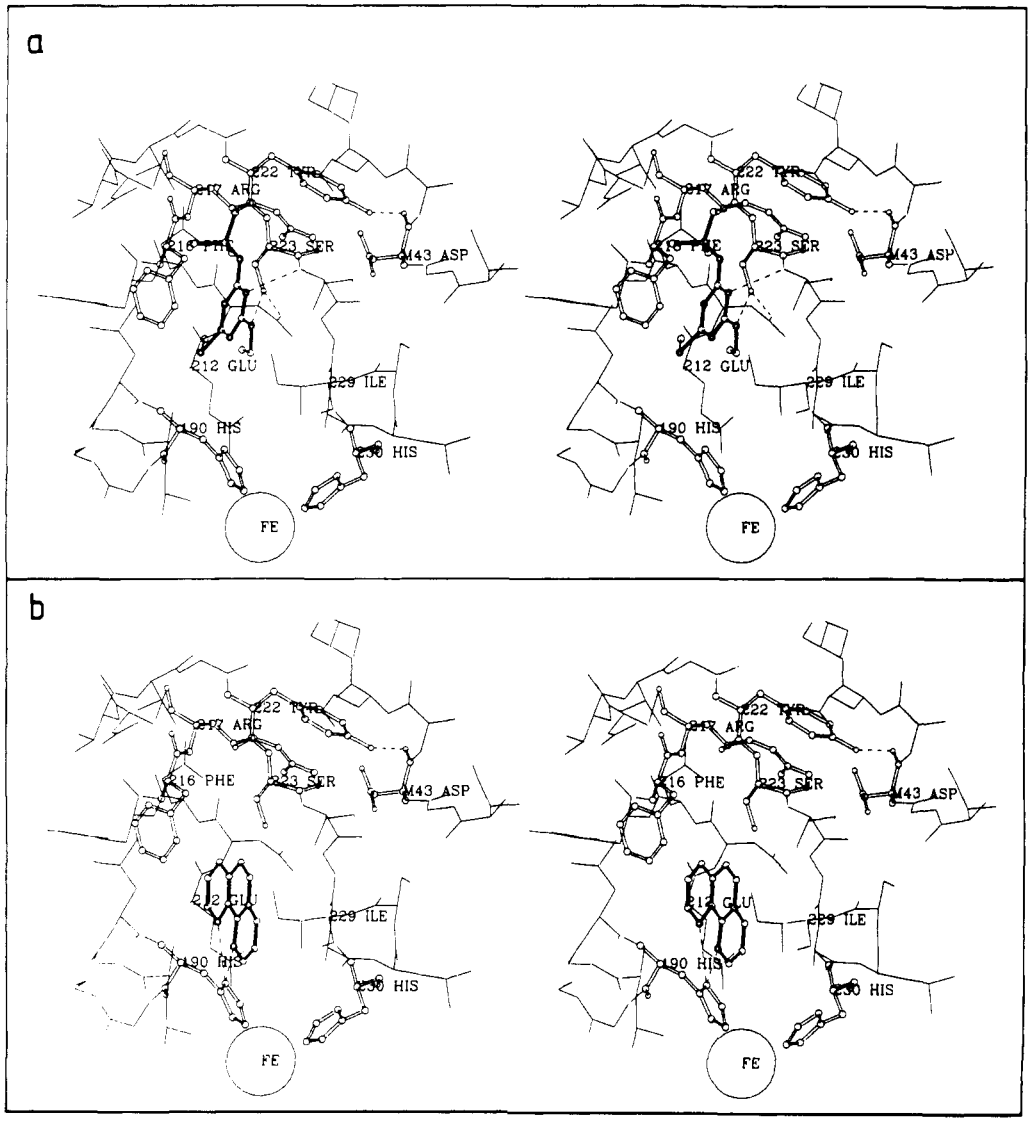

FIGURE 8: Stereo plots of the Q_B binding site of the RC of *Rps. viridis* wild type (a) with terbutryn (Michel et al., 1986b) and (b) with *o*-phenanthroline (Michel et al., 1986b). Possible hydrogen bonds are drawn as dashed lines. The residues Phe L216, Arg L217, Tyr L222, and Ser L223, which are altered in the herbicide-resistant mutants, and the histidines L190 and L230 are shown as atomic models; the residues which line up the Q_B site are drawn in a skeletal mode. Figures 8 and 9 were produced by a computer program written by Lesk and Hardman (1985).

moves only one of the two hydrogen bonds between the carbonyl group of the quinone and the protein (see Figure 9), whereas terbutryn seems not to have two hydrogen bonds between the ethylamino group and the protein. However, in *Rb. sphaeroides* the SP223 mutant ($Ser^{L223} \rightarrow Pro$) showed the most drastically reduced affinity for UQ_0 of all the herbicide-resistant mutants from this organism (Paddock et al., 1988). Second, there is a second mutation in T1 which replaces arginine L217 by a histidine. The same double mutation was found in T2, and no mutant with a single alteration of the serine L223 was obtained from *Rps. viridis*. This double mutation cannot be explained by statistical reasons. We suggest that the increase in quinone binding in T1 might be the result of a structural rearrangement caused by the secondary mutation of the arginine L217 into a histidine. This would compensate for a detrimental effect of the primary mutation. In the case of the hemoglobin β chain, from serine proteases and cystein proteases for comparison it is known that some amino acid residues linked by a specific role mutate in a coordinated manner (Nagai et al., 1987; Altschuh et al., 1988). These coordinated mutations may play important roles in stabilizing protein structure or determining functional differences. We suggest a similar reason for the double mutation in T1 and T2.

The replacement of phenylalanine L216 by a serine in T5 (=T6=T7) may change the polarity of this region. Phenylalanine L216 is in van der Waals contact with terbutryn. The hydrophobic interactions which are possible between the herbicide and the protein and which seem to contribute most to the binding energy would be greatly decreased by this mutation. On the other hand, serine with its high tendency to form hydrogen bonds may cause structural rearrangements. This latter explanation is favored by the finding of much higher I_{50} values for terbutryn and *o*-phenanthroline in T6 than in the other mutants (see Table I). From *Rb. sphaeroides* no equivalent mutant has been reported.

It was surprising to find that the single alteration of tyrosine L222 to phenylalanine in T4 leads to herbicide resistance, because this residue does not participate in inhibitor binding directly. The aromatic ring system of this residue is about 7 Å away from the ubiquinone (Figure 9). Therefore, if we had been using "site-specific mutagenesis" this residue would have been far from the obvious choice as a target. In a mutant from *Rb. sphaeroides*, tyrosine L222 is replaced by glycine, a change

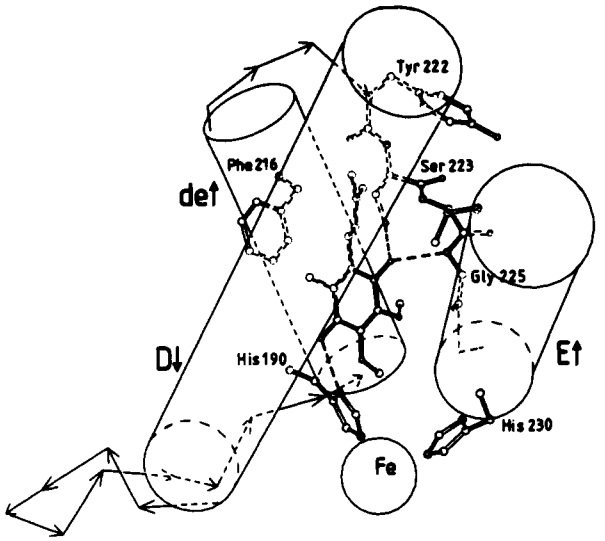

FIGURE 9: Schematic drawing of the Q_B binding site of the RC of *Rps. viridis* wild type with the ubiquinone (from the same point of view as in Figure 8). The L subunit is shown from the transmembrane helix D to the end of helix E. The helices (D, E, de) are represented by barrels. Possible hydrogen bonds between the two carbonyl groups of the ubiquinone and the protein are indicated as dashed lines. Amino acids which are involved in quinone binding, histidine L230 and tyrosine L222, are shown as atomic models. A complete picture will be given in J. Deisenhofer, O. Epp, I. Sinning, and H. Michel (unpublished results).

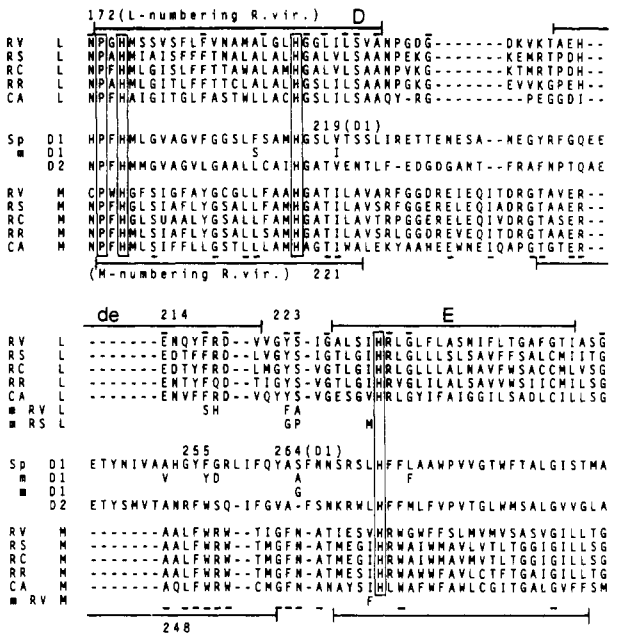

FIGURE 10: Amino acid sequences of the quinone binding sites in the L and M subunits from *Rps. viridis* wild type (RV; Michel et al., 1986a) are compared with those of the purple bacteria *Rb. sphaeroides* (RB; Williams et al., 1983, 1984), *Rb. capsulatus* (RC; Youvan et al., 1984), and *R. rubrum* (RR; Belanger et al., 1988), with those of the D1 and D2 proteins from spinach chloroplasts (Sp; Zurawski et al., 1982; Alt et al., 1984; Holschuh et al., 1984), and with those of the L and M subunits of the aerobic green bacterium *Cf. aurantiacus* (CA; Ovchinnikov et al., 1988a,b). The mutations leading to herbicide resistance in the different systems are indicated in the lines marked with m (for references, see text). Amino acids common to all six organisms are boxed. Residues common to the L or M subunits of all five bacteria are marked by a bar. The transmembrane helices D and E and the short helix in the connecting loop (de) are indicated by bars.

which also results in herbicide resistance (Paddock et al., 1987).

The $Q_B^{\bullet-}$-Fe^{2+} signals of the T4 mutant are totally different from those of all other mutants but similar to the $Q_B^{\bullet-}$-Fe^{2+} signals observed in PSII preparations (Rutherford et al., 1984b; Zimmermann & Rutherford, 1986) and in *R. rubrum* (Beijer & Rutherford, 1987). Because of this striking similarity in terms of the EPR signal, we compared the amino acid sequences of PSII (Zurawski et al., 1982), *R. rubrum* (Belanger et al., 1988), and the T4 mutant (Figure 10) in the region of tyrosine L222. Tyrosine L222 is conserved in the L subunits of all the purple bacteria and the green bacterium *Chloroflexus* (*Cf.*) *aurantiacus* (Ovchinnikov et al., 1988a,b), and therefore, the unusual EPR signal does not directly correlate with the amino acid sequence. The different characteristics of the two parts of the $Q_B^{\bullet-}$-Fe^{2+} signal in *R. rubrum* led to the suggestion of two EPR forms due to two populations of centers with different semiquinone–iron interactions (Beijer & Rutherford, 1987). The presence of the $g = 1.93$ peak and the $g = 1.82$ peak in T4 probably reflects similar heterogeneity. The presence of this alternative spectral form at close to $g = 1.93$ in PSII, *R. rubrum*, and now in the T4 mutant of *Rps. viridis* indicates that this signal reflects a second specific conformation for the semiquinone–iron complex different from that which gives the well-known $g = 1.82$ form. The $g = 1.93$ signal, being closer to the uninteracted semiquinone position ($g = 2.004$) than the $g = 1.82$ form, may represent a weaker influence of the iron on the semiquinone. This could be due to a minor structural change resulting in a greater distance between the semiquinone and the iron. An equivalent second spectral form has been reported for $Q_A^{\bullet-}$-Fe^{2+} in PSII [Rutherford & Zimmermann, 1984; see also Nugent et al. (1981)] and in *R. rubrum* (Prince & Thornber, 1977). The binding of herbicides and changes in pH influence the distribution of the two states.

Binding of *o*-phenanthroline is also affected in T4, indicating that the binding pocket may have completely changed. The hydroxyl group of tyrosine L222 is hydrogen bonded to the backbone carbonyl group of aspartic acid M43 (distance 2.7 Å, Figure 8). We suggest this interaction is crucially important for the structure of the Q_B site so that the removal of this hydrogen bond is enough to cause a structural rearrangement. An X-ray structure analysis is under way to clarify this question. T4 seems to be an example of the importance of amino acid residues stabilizing the tertiary structure of the protein without being directly involved in pigment or inhibitor binding.

The analysis of mutants obtained by site-directed mutagenesis will provide detailed information on structure–function relationships. However, from the three-dimensional structure of the RC it is not always obvious which amino acid residues are really critical for the stability of the protein, for electron transfer, or for inhibitor binding. In PSII, where no three-dimensional structure is available so far, the use of a great variety of inhibitors of electron transfer led to a model for the quinone binding site which is similar to the Q_B site of purple bacteria (Trebst, 1987). Figure 10 shows the aligned sequences of the regions involved in quinone binding in the D1 and D2 proteins from spinach (Zurawski et al., 1982; Alt et al., 1984; Holschuh et al., 1984) and the L and M subunits from four species of purple bacteria and one aerobic green bacterium. In D1 and D2 the connections of the D and E helices possess nearly the same length, whereas the L subunit is seven amino acids shorter than the M subunit. The connecting loop in the D1 protein is 17 residues longer than that in the L subunit. These residues have been proposed to form an additional loop into the intrathylakoidal space (Michel & Deisenhofer, 1988); otherwise, this region would hardly fit into a model based on the homology to purple bacteria. The proposed additional loop

is favored by the finding that none of the herbicide-resistant mutants known from higher plants, algae, or cyanobacteria so far have shown mutations in this inserted sequence. Serine D1 264 was proposed to be hydrogen-bonded to Q_B in PSII (Hirschberg et al., 1984). It seems to be equivalent to serine 223 in the L subunit of the RC of *Rps. viridis*. Changes of serine D1 264 to alanine in *Chlamydomonas* (*Chl.*) *reinhardtii* (Erickson et al., 1984) or to glycine in *Solanum nigrum*, *Amaranthus hybridus* (Hirschberg & McIntosh, 1983; Hirschberg et al., 1984), and *Anacystis nidulans* (Golden & Haselkorn, 1985) leading to herbicide resistance have been reported. The replacement of serine D2 264 slows down the electron transport from Q_A to Q_B (Arntzen et al., 1982). The same was observed in the SP223 *Rb. sphaeroides* mutant ($Ser^{L223} \rightarrow Pro$) (Paddock et al., 1988). In a herbicide-resistant mutant from *Chl. reinhardtii*, phenylalanine D1 255 is replaced by tyrosine (Galloway & Mets, 1984). Phenylalanine D1 255 is equivalent to Phe L216, which is highly conserved in the L subunit of purple bacteria. In the M subunit a tryptophan is in the corresponding position, which is also conserved in the various bacteria and in the D2 proteins. In addition to these mutants of higher plants, other mutations leading to herbicide resistance which have no equivalent in the photosynthetic bacteria have been reported. In *Chl. reinhardtii* the change of valine 219 in the D1 protein to an isoleucine causes resistance to DCMU (Erickson et al., 1985), which is not effective in purple bacteria. Alanine 251 mutates to valine in *Chlamydomonas*, a change which decreases the binding of metribuzin (Johannigmeier et al., 1987), a triazinone herbicide. The replacement of leucine 275 by phenylalanine (Rochaix & Erickson, 1988) and phenylalanine 211 by serine in *Synechococcus* PCC 7002 (Gingrich et al., 1988) also leads to resistance.

Figure 10 also includes the amino acid sequence of the quinone binding region from *Cf. aurantiacus*. This aerobic green photosynthetic bacterium is only distantly related to the purple photosynthetic bacteria (Stackebrandt & Woese, 1981). Its RC is the simplest known at present because it consists only of two subunits, which are homologous to the L and M subunits of purple bacteria (Ovchinnikov et al., 1988a,b). The quinone binding sites are highly conserved in the green bacterium *Cf. aurantiacus*, which contains two menaquinones as electron acceptors. Despite the high sequence homology, electron transport from Q_A to Q_B in *Cf. aurantiacus* is blocked by *o*-phenanthroline but not by the triazine herbicides terbutryn and atrazine [Hansen and Blankenship, unpublished results quoted in Blankenship and Fuller (1986)], and no herbicide-resistant mutants have been reported so far.

Obviously the decreased stability of the RCs, the altered quinone, and inhibitor binding properties of the mutants described in this paper cannot be predicted or explained in detail from the three-dimensional structure of *Rps. viridis* wild type. X-ray structure analyses of the RCs of the mutants will provide a more complete picture of the changes in the electron-acceptor region. It will contribute to our understanding of structure–function relationships in one of the best-characterized proteins involved in photosynthesis.

Registry No. Ubiquinone, 303-97-9; terbutryn, 886-50-0; *o*-phenanthroline, 66-71-7.

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