A single mutation in the M-subunit of *Rhodospirillum rubrum* confers herbicide resistance

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Abstract Cells of the photosynthetic bacterium *Rhodospirillum rubrum* were rendered resistant against the inhibitor 2-(1-phenyl)ethylamino-3-propionylamino-4-cyano-thiazole (PPCTH). Electron transport in reaction centers prepared from one of the mutants (M6) was neither inhibited by PPCTH and other NH-thiazoles nor terbutryn. These inhibitors are known to bind at the Q_B site of the L-subunit. Compared to the wild type, chromatophores from M6 exhibited strongly altered $Q_B^-Fe^{2+}$ and $Q_A^-Fe^{2+}$ EPR signals. Inhibitor resistance is due to a mutation in the bacterial reaction center M-subunit, where Glu^{234} is exchanged against Lys. This is the first example of an inhibitor resistance in the Q_B site caused by a mutation in the M-subunit.

Key words: Bacterial reaction center; Amino-thiazole; Glu M^{234} > Lys

1. Introduction

In the primary charge separation of the reaction center of photosynthetic bacteria, the electron discharged from the bacteriochlorophyll dimer molecule is transferred via a bacteriochlorophyll monomer and a bacteriopheophytin to the primary and secondary electron acceptors Q_A and Q_B [1]. Q_A and Q_B are located at the bacterial M- or L-subunit, respectively. Due to X-ray crystallography, the structure of the reaction centers from *Rhodopseudomonas viridis* and *Rhodobacter sphaeroides* is well known [1–3]. Inhibitors can disrupt electron flow between Q_A and Q_B by binding to the Q_B site and displacing the native ubiquinone molecule, which accepts electrons from Q_A. From X-ray crystallography, the position of two inhibitors, the *s*-triazine terbutryn and *o*-phenanthroline within the Q_B binding pocket is well established [4].

Previously, mutants of photosynthetic bacteria have been isolated and characterized which are resistant against certain inhibitors and commercial herbicides which are also active in the photosystem II reaction center of higher plants [5]. The mutations, responsible for inhibitor resistance, are due to amino acid exchanges and have been localized in the L-subunit of the bacterial reaction center in transmembrane helices D and E and the parallel helix DE (for review, see [5]). These mutants include positions Glu^{212}, Phe^{216}, Tyr^{222}, Ser^{223}, Thr^{226}, Gly^{228} and Leu^{229} [5]. In addition, a mutant has been described, where a mutation in the L-subunit (Phe^{216} > Ser) is simultaneously accompanied by a mutation in the M-subunit (Val^{263} > Phe) [6].

We wish to report here that a single amino acid mutation in the bacterial reaction center M-subunit, the substitution $MGlu^{234}$ > Lys causes resistance against NH-thiazoles, which have recently been reported to be efficient inhibitors of photosystem II and the bacterial reaction center as well [7]. To our knowledge this is the first case of inhibitor resistance which is solely conferred by a mutation in the M-subunit.

2. Materials and methods

Cultures of *Rhodosprillum rubrum* FR1 (DSM 1068) were grown under anaerobic photoheterotrophic conditions according to Ormerod et al. [8]. Inhibitor-resistant mutants were obtained by growing the cells in the presence of 100 μM PPCTH in liquid medium. The mutants were then selected on agar plates containing the same concentration of PPCTH.

Chromatophores were prepared by a modified method of Jochline and Reiss-Husson [9]. French-press cell extracts were centrifuged at 13 000 × *g* for 20 min, followed by centrifugation of the supernatant at 180 000 × *g* for 90 min. The pellet was resuspended in a small volume of 100 mM NaPP, pH 7.5. The RCs were isolated according to Vadeboncoeur [10] with slight modifications. The chromatophores were diluted with 50 mM NaPP, pH 8.0, to $OD_{880} = 50$. 50 mM NaPP, pH 8.0 with 0.4–0.8% LDAO was added to the membranes in the ratio of 1:1. The mixture was stirred at 0°C for 30 min and then diluted again with 50 mM NaPP, pH 8.0, in the ratio of 1:1. After a centrifugation at 144 000 × *g* for 90 min the RCs in the supernatant were precipitated with $(NH_4)_2SO_4$ (40%) and centrifuged for 20 min at 7000 × *g*. The resulting oily swimming-pellet was resuspended in a few millilitres of H_2O. The RC containing solution was loaded on a DEAE–cellulose 52 (10 ml). The column was washed and eluted with 50 mM NaPP, pH 8.0, 0.1% LDAO. For further purification the RCs were loaded on a sucrose-density gradient (0.2–0.8 M sucrose in 50 mM NaPP, pH 8.0, 0.08% LDAO) and centrifuged for 20 h at 240 000 × *g*. The RCs were then found in a red-brown band in the middle of the gradient. The activity of isolated RCs was measured as reported in [12].

2.1. EPR measurements

EPR measurements were performed according to Beijer and Rutherford [11]. Chromatophores were diluted with 0.1 M HEPES, pH 7.5, to an $OD_{865} = 90$ and frozen at −80°C. To samples of 250 μl in calibrated EPR tubes, 1 μl of 0.1 M EDTA was added. After dark adaptation for 10 min at 0°C, 4 μl of 0.1 M DAD in DMSO and 2 μl of 0.5 M aqueous ascorbate were added. After 1 min of dark adaptation or after illumination (see below) the samples were frozen in darkness to −80°C in a cooled ethanol bath and immediately transferred to liquid nitrogen. The samples were illuminated with a single saturating flash from a Na-YAG laser (530 nm, 20 ns) in order to generate Q_B^- or with continuous white light (800 W, filtered through 2 cm of water) for 5 min at room temperature in order to generate Q_A^-. EPR spectra were recorded at liquid helium temperature with a Bruker ER 200 spectrometer equipped with an Oxford instrument cryostat. The conditions were: microwave frequency, 9.43 GHz; modulation amplitude, 22 G; microwave power, 8 dB; and temperature, 4.5–15°K.

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Abbreviations: DAD, diaminodurene; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethyl-urea; PPCTH, 2-(1-phenyl)ethylamino-3-propionylamino-4-cyano-thiazole; RC, reaction center

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2.2. Cloning and sequencing of the mutant pufL and pufM genes

Genomic DNA from *Rhodospirillum rubrum* was isolated according to Silhavy et al. [13]. After restriction with *Bam*HI the fragments were 'shot gun' ligated into pBluescript KS$^+$ and cloned into *E. coli* TG1. Positive clones were identified by colony filter hybridisation according to Sambrook et al. [14] with a part of the wild-type *pufL* gene as homologous hybridisation probe. Labeling of the probe was done with the polymerase chain reaction (PCR) amplification with digoxygenin (Dig)-dUTP. The labeled DNA was detected using the DIG Nucleic Acid Detection kit. DNA sequencing was performed by the dideoxy chain-termination procedure of Sanger et al. [15] using [α-^{35}S]dATPαS (600 Ci/mmol) with a T7 DNA Polymerase Sequencing kit.

The following primers, used for PCR and DNA sequencing, are listed in 5′–3′ orientation. Numbering is according to [18]: I, 428–451; II, 889–802; III, 451–428; IV, 1330–1308; V, 1745–1728; VI, 1435–1453.

3. Results and discussion

Rhodospirillum rubrum was grown anaerobically under photo-organotrophic conditions with malate as the single carbon source. Strains resistant against NH-thiazoles were selected on agar plates in the presence of 100 μM PPCTH. Interestingly, PPCTH (compound IV in [7]) had the lowest pI_{50} value of 4.5 of all NH-thiazoles tested [7]. However, only PPCTH was able to elicit resistance, whereas the other more potent NH-thiazoles were not, probably due to their higher lipophilicity.

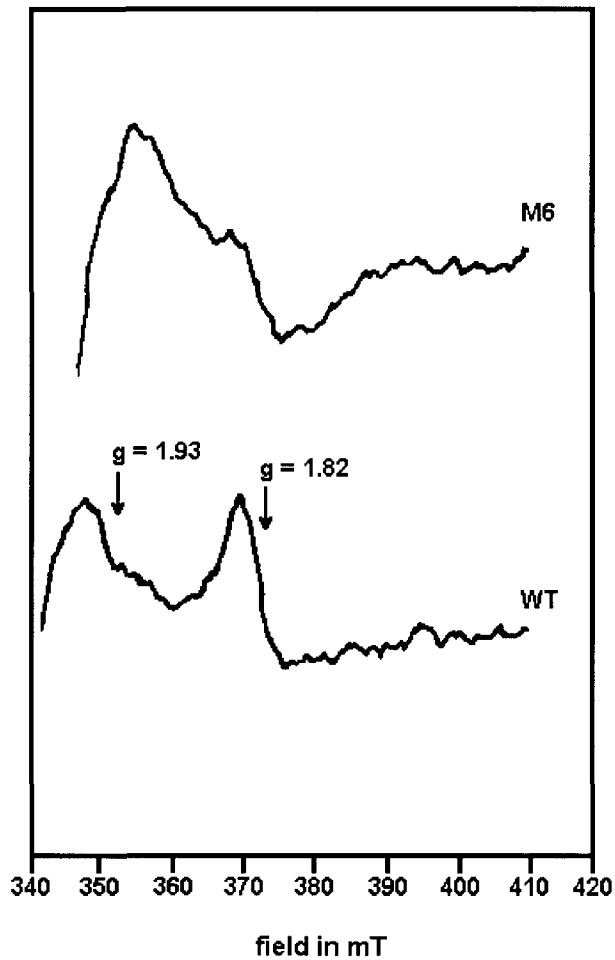

Fig. 1. $Q_B^-Fe^{2+}$ EPR signal at 4.8°K of wild-type *Rs. rubrum* (WT) and mutant M6 induced by a 20 ns laser flash (530 nm). For conditions, see Section 2.

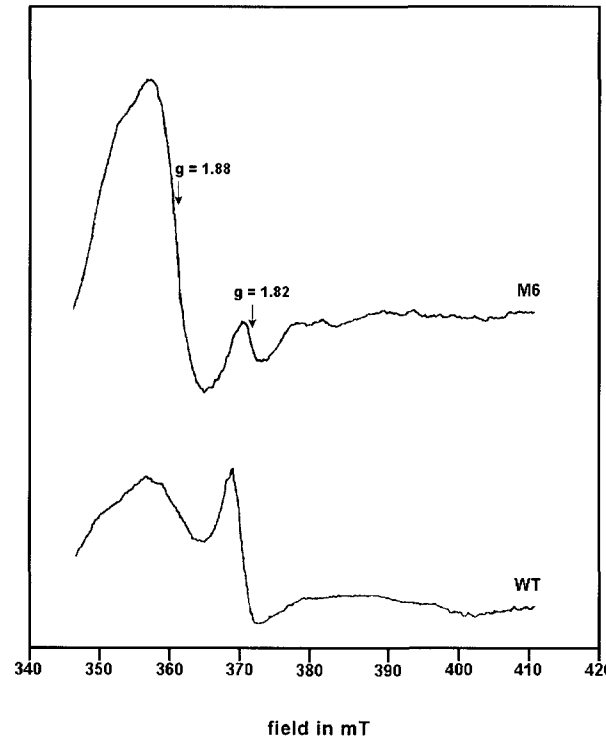

Fig. 2. $Q_A^-Fe^{2+}$ EPR signal at 4.8°K of wild-type *Rs. rubrum* (WT) and mutant M6 induced by 5 min illumination with actinic light at room temperature. For conditions, see Section 2.

High lipophilicity prevents uptake of the compound through the outer membrane of the bacterium.

In addition to PPCTH, some agar plates were also treated with the mutagens 5′-fluoro-2′-deoxyuridine and ethyl methane sulfonate. In total eight mutant strains (M1–M8) were obtained, of which three were generated without addition of mutagen (M6–M8).

The eight mutant strains were all examined by EPR. The EPR signals arising from $Q_A^-Fe^{2+}$ and $Q_B^-Fe^{2+}$ were generated in chromatophores of the resistant strains and were compared with those of the wild type. The semiquinone–iron EPR spectra of strain M6 were markedly different from those of the wild type. Fig. 1 shows the EPR spectrum of wild-type *Rs. rubrum* at 4.8°K after illumination with a single flash. It shows two major features at g values of 1.93 and 1.82 and is characteristic of the $Q_B^-Fe^{2+}$ in this species [11]. The EPR spectrum formed under the same conditions in chromatophores from the mutant M6 is different from that in the wild type in two respects: the intensity of the $g = 1.82$ feature is relatively small and the other main feature is shifted to higher field $g \cong 1.88$ (Fig. 1).

The EPR spectrum of $Q_A^-Fe^{2+}$ in chromatophores from wild-type *Rs. rubrum* at 4.8°K after continuous illumination is shown in Fig. 2. It is characterized by features at $g \cong 1.89$ and at $g = 1.82$ [11,16]. In the M6 mutant the first signal is enhanced in size, while the second is diminished (Fig. 2).

It is known that the two main features of the semiquinone–iron spectra in *Rs. rubrum* show different dependencies on temperature, the $g = 1.82$ feature being somewhat favoured at lower temperature [11]. To verify that the spectral differences reported above were not due to slight temperature differences from sample to sample, comparisons of the spectra

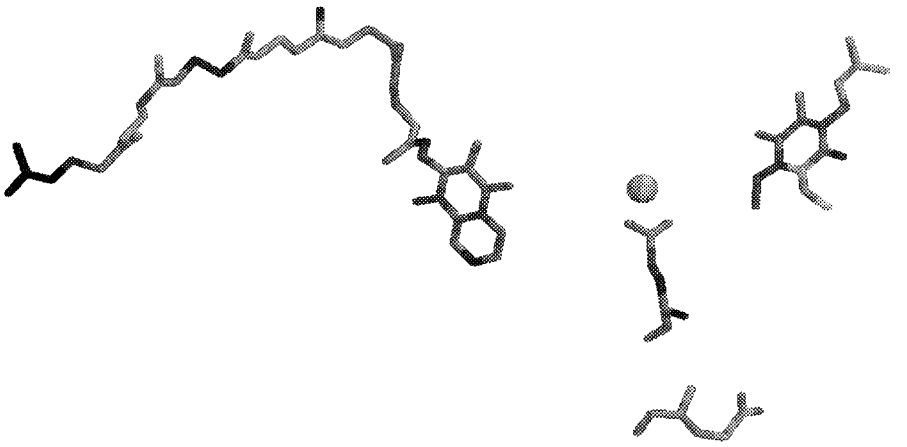

Fig. 3. Location of M Glu_{234} (bottom) relative to M Glu_{232} (above), the non-heme iron, Q_A (left) and Q_B (right), taking the reaction center of *R. viridis* [1] as a model.

were done up to 15°K. It was found that the main spectral differences of the strains as described above were present throughout this temperature range (data not shown). Since the overall shape of the signal formed with a flash in M6 is different from that formed by continuous illumination (i.e., $Q_A^-Fe^{2+}$) in the same strain, it seems clear that the flash-induced signal can be attributed to $Q_B^-Fe^{2+}$. However, given the similar positions of the *g* values between this flash-induced signal and the $Q_A^-Fe^{2+}$ signal, we cannot rule out that the flash-induced spectrum contains a contribution from $Q_A^-Fe^{2+}$. Indeed, as judged from the presence of some light-induced bacteriochlorophyll special pair triplet signal as generated by continuous illumination at 4.5°K (see ref. [11]) of the flash-induced sample, it seems likely that a fraction of $Q_A^-Fe^{2+}$ is indeed present under these conditions. This indicates that electron transfer between Q_A and Q_B is partially impaired in the M6 mutant. This is indeed the case, from the electron transport activities reported in Table 1 it is evident that the rate of electron transport in the mutant amounts to only 62% of the wild type.

The differences in the EPR spectra in the M6 strain and the indication of impaired electron transfer between Q_A and Q_B presumably reflect structural changes around the quinone sites which are responsible for the herbicide resistance and which perturb the magnetic interactions between the semiquinones and the iron.

Mutant M6 in the presence of 100 μM PPCTH exhibited a growth rate identical to that of the wild-type strain. Growth of the wild type was inhibited by 90% in the presence of 100 μM PPCTH (data not shown). RCs were made from the mutant M6 and various NH-thiazoles and other known inhibitors of the bacterial photosynthetic electron transport chain were assayed for their inhibitory activity (Table 1). As is evident from Table 1, the RCs were not only resistant against PPCTH (1-phenyl), which has been used to generate the mutants, but also against NH-thiazoles substituted by either (1-4′-ethylphenyl) or (1-3′,4′-dichlorophenyl) residues, which are much more potent inhibitors as compared to PPCTH. In addition, RCs from mutant M6 are also resistant against terbutryn and *o*-phenanthroline, whose orientation within the Q_B-binding niche of the bacterial RC is known from the X-ray structure [4]. Furthermore, RCs from the mutant were less sensitive against thiazolylidene–ketonitriles [12]. Electron transport in the M6 mutant was not inhibited by DCMU (Table 1). This observation is of interest since terbutryn resistance in the $LTyr^{222} > Phe$ mutant induced DCMU sensitivity [17]. These results indicate that the inhibitors have a reduced binding affinity towards the Q_B-binding site, and, hence, the RC structure of the mutant M6 must be altered around the Q_B site as compared to the wild type.

Since it seemed likely that the inhibitor resistance of the M6 mutant was due to an amino acid mutation in the RC proteins, the *puf*L and *puf*M genes, which code for the L- and M-subunit, respectively [18], in the M6 mutant were sequenced. For this purpose, a 2.1 kb *Bam*HI fragment, containing the *puf*L and *puf*M genes, was used. The sequences of the *puf*L genes in the wild-type and mutant M6 were completely identical. However, in the *puf*M gene of the mutant M6 a base exchange from G to A in position 1563 (numbering according to [18]) took place. Consequently, the triplet GAG coding for

Table 1
pI_{50} values of various inhibitors of electron transport in wild-type and mutant M6 RCs of *R. rubrum*

Inhibitor	pI_{50} value (wild type)	(mutant M6)
... ethylamino-3-propionylamino-4-cyano-thiazole		
2-(1-phenyl)-	4.9	3.5
2-(1-4′-ethylphenyl)-	5.5	<4
2-(1-3′,4′-dichlorophenyl)-	6.7	<4
terbutryn	6.1	<4
o-phenanthroline	5.4	<4
2-[4-(3′,4′-dichlorphenyl)-2,3-dihydrothiazol-2-yliden]-3-oxo-4-phenyl-butyronitrile	4.9	4.3
DCMU	<4	<4

The electron transport rates for the wild type were 2.40 and for the M6 mutant 1.48 mmol cytochrome $c_{ox.}$·mg $Bchl^{-1}·h^{-1}$.

$GluM^{234}$ is replaced by the triplet GAA coding for $LysM^{234}$ (data not shown).

Until now, all amino acid exchanges, leading to herbicide resistance, have been localized in the L-subunit [5]. It has also been found that a mutation in the L-subunit may be accompanied by a mutation in the M-subunit, for example $LPhe^{216} > Ser$ and $MVal^{263} > Phe$. However, the $MGlu^{234} > Lys$ mutant reported here is the only one described where a mutation in the M-subunit alone causes inhibitor resistance.

$GluM^{234}$ is a conserved amino acid among all known sequences of purple photosynthetic bacteria [4,18–20]. It is located at the cytoplasmatic side of the membrane between helices D and the parallel helix DE in the region of the Q_A-binding niche. In the model of the bacterial reaction center [1] Glu^{234} is directly adjacent to Glu^{232} which has been recognized as a bidentate ligand to the non-heme iron (Fig. 3). Taking the reaction center of *R. viridis* as a model [1], the backbone carbon atom of Glu^{234} has a distance to O–1 of Q_A of 15.2 Å and to O–4 of Q_B of 14.9 Å, i.e. it is located in the middle between the two quinone acceptors. The backbone carbon atom of Glu^{234} is located 11.9 Å from the non-heme Fe (Fig. 3). The change from Glu which is likely to be negatively charged to Lys which is probably positively charged is liable to have significant effects on the structure and the function of the quinone sites. The electrostatic change itself, which are predicted to be present in the M6 mutant, may well perturb the redox potentials of the quinones and the protonation events associated with Q_B reduction. Such effects could be partially responsible for the apparent impairment of electron transfer from Q_A to Q_B. Further characterization of the electron transfer and the related protonation events in this mutant may be of interest.

In addition, the electrostatic difference alone could conceivably have a direct influence on the magnetic properties of the semiquinone iron states and thus perturb the EPR signals. Indeed, it was shown earlier that the position of the low field feature of $Q_B^-Fe^{2+}$ in *Rs. rubrum* was particularly sensitive to the pH and this was attributed to the deprotonation of an amino acid close to the quinone site [11]. In the earlier work the low field peak shifted to lower fields at pH values higher than pH 9, i.e. upon the net gain of negative charges [11]. The present study shows an apparent shift to higher field values due to the potential net gain of two positive charges. Future work might determine if the *g* value of the semiquinone–iron signal can be related to the electrostatic environment.

Perhaps more important than the direct effect of the electrostatic change in the M6 mutant are the indirect effects of such a change. A change from a negatively charged group to a positively charged group is likely to have triggered changes in the protein to accommodate the new electrostatic environment. Such rearrangements could well be responsible for the modification of the Q_B site which confers resistance to herbicides. Such structural changes could also be responsible for the modified EPR spectra and the impairment of electron transfer.

To properly assess the structural repercussions of the Glu to Lys change reported here a comparison of the crystal structure of the mutant and wild type is required. Since the *Rs. rubrum* structure has not been solved the most appropriate approach is to introduce the mutation reported here into a species which is amenable to crystallography.

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