Sequence Analysis of Four Atrazine-Resistant Mutants from *Rhodopseudomonas viridis*

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Four atrazine-resistant mutants from the purple bacterium *Rhodopseudomonas viridis* were isolated. Sequence analysis revealed three different mutant strains carrying mutations in the herbicide-binding pocket: i) MAV 2: L212-Glu → Lys, ii) MAV 3: L216-Phe → Ser and iii) MAV 4 = MAV 5: L217-Arg → His, L220-Val → Leu. Except MAV 3 all *Rps. viridis* mutants are different from those selected by their resistance towards the closely related triazine terbutryn.

Introduction

In photosynthetic reaction centers light energy is utilized to catalyze the primary charge separation and to transfer an electron across the thylakoid membrane. The reaction center from the photosynthetic purple bacterium *Rhodopseudomonas (Rps.) viridis* consists of four protein subunits (cytochrome *c*, L, M and H subunit) and nine pigment molecules (four bacteriochlorophyll (Bchl) *b*s, two bacteriopheophytin (Bpheo) *b*s, one menaquinone (Q_A), one ubiquinone (Q_B) and one carotenoid molecule) [1]. The primary electron donor P (two Bchl *b* molecules forming the so-called "special pair") releases a first electron *via* an "accessory Bchl *b*" and a Bpheo *b* (intermediate I) to the tightly bound primary electron acceptor Q_A, followed by electron transfer to the secondary acceptor Q_B. A second charge separation results in the twofold reduction of Q_B [2]. After protonation the quinol Q_BH_2 is released into the membrane and reoxidized by the cytochrome b/c_1 complex.

The crystallization of the reaction center from *Rps. viridis* and subsequent X-ray structure analysis have provided a complete picture of the pigment arrangement, the protein structure and pigment-binding sites [3–5]. The commercially available triazine herbicides atrazine (2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine) and terbutryn (2-thiomethyl-4-ethylamino-6-*t*-butylamino-*s*-triazine) are inhibitors of electron transport from Q_A to Q_B in PS II as well as in the reaction centers from purple bacteria [6]. Both atrazine and terbutryn are considered to be competitive inhibitors of the secondary quinone Q_B. The action of the triazine herbicides on both PS II and purple bacterial reaction centers is due to the structural similarities between both reaction centers as discussed [7, 8].

The X-ray crystallographic analysis of the reaction center from *Rps. viridis* [5] provides structural details of the pigment and inhibitor-binding sites. Terbutryn binding is characterized by van-der-Waals contacts to L220-Val, L229-Ile and L216-Phe as well as two hydrogen bonds i) between N3 of the triazine ring and the peptide nitrogen of L224-Ile and ii) between the ethylamino nitrogen of terbutryn and the hydroxyl group of L223-Ser. Mutation of L223-Ser to alanine in *Rps. viridis* [9] or to glycine in *Rhodobacter sphaeroides* [10] results in terbutryn resistance. Unexpectedly, the L223-Ser → Ala mutation in *Rps. viridis* is always accompanied by a L217-Arg → His mutation [11].

L217-Arginine is not involved in terbutryn binding. The simultaneous occurrence of the L223-Ser → Ala and L217-Arg → His [9] led to the conclusion that the change of L223-Ser to Ala is responsible for the herbicide resistance and the change of L217-Arg to His is required to compensate for detrimental effects of the L223-Ser → Ala change [9]. A mutant strain carrying only the L223-Ser → Ala mutation cannot grow photosynthetically in *Rb. capsulatus* [12] and does not occur in *Rps. viridis*.

The mutation L216-Phe → Ser in *Rps. viridis* leads to terbutryn resistance [9]. Other mutants resistant towards terbutryn (likely caused by structural rearrangement) carry the mutation L222-

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Tyr → Phe in *Rps. viridis* [10] or L222-Tyr → Gly in *Rb. sphaeroides* [10].

In addition, site-directed mutagenesis has been used to produce mutants of *Rb. capsulatus* [13]: out of 17 changes of L229-Ile 8 lead to varying atrazine resistance. Alteration of L228-Gly (to Val or Arg) and L222-Tyr may disarrange L229-Ile and L223-Ser respectively and therefore lead to resistance [12]. In this communication we describe the results of the DNA-sequence analysis of four independent mutants resistant towards atrazine. To our surprise we find three different mutants, only one being identical to a previously isolated terbutryn-resistant mutant [9].

Materials and Methods

Rhodopseudomonas viridis (DSM 133) was grown anaerobically in sodium succinate medium 27 [14]) in screw-capped bottles under stirring at 30 °C in white light (5–10 W/m^2). For selection of herbicide-resistant mutants medium 27 containing 0.3 mM atrazine per liter was inoculated with 5% of total volume from an exponentially growing wild-type culture. Growth of atrazine-resistant mutants was observed after 6 to 8 weeks. In order to obtain single clones aliquots of liquid cultures were diluted and plated on atrazine containing agar plates (medium 27, 1.5% agar, 0.3 mM atrazine).

Genomic DNA from mutant *Rps. viridis* strains was isolated from 1.0 l cultures as described by Sinning and Michel [11]. Cloning of an 1.9 kb *Eco*RI/*Sal*I restriction fragment, containing the L subunit gene, major part of the M subunit gene and a small part of the LHC α subunit gene was performed as essentially described by Sinning and Michel [11] except that a non-radioactive DNA labeling and detection kit (Boehringer, Mannheim) was used to select *Escherichia coli* colonies bearing the desired recombinant plasmid. For that purpose a DNA library was generated by cloning *Eco*RI/*Sal*I restriction fragments of 1.8–2.0 kb size from *Rps. viridis* genomic DNA into the vector pBS– (Stratagene, San Diego) using *E. coli DH5α* as a host for recombinant plasmids. Colonies were grown on nitrocellulose filters placed on LB agar plates (ampicilline 100 μg/ml) [15], lysed and the denatured DNA baked to the filter [16]. A 464 bp *Bam*HI/*Sca*I restriction fragment containing part of the L subunit gene was labeled with digoxigenin by random primed DNA synthesis in the presence of digoxigenin-11-dUTP using heptamer oligonucleotides and Klenow polymerase and hybridized to the DNA on the NC filters. Subsequent incubation with a digoxigenin-specific antibody coupled to alkaline phosphatase and addition of NBT and BCIP allowed identification of positive clones by the formation of a blue dye precipitate (non-radioactive DNA labeling and detection kit, Boehringer Mannheim, User's manual).

DNA sequence analysis of cloned 1.9 Kbp *Eco*RI/*Sal*I restriction fragments from atrazine-resistant mutants was performed using the Sanger dideoxy method [17]. The strand synthesis was carried out at 50 °C with Klenow enzyme or T7 DNA polymerase (T7 Sequencing Kit, Pharmacia) using eight 17mer oligonucleotides obtained from I. Sinning as primers for the sequencing reaction.

Results and Discussion

The X-ray structure analysis of the reaction centers from *Rps. viridis* [5] shows the herbicide-binding pocket being formed by the connecting loop between the fourth and the fifth transmembrane helices of the L subunit (L119–L225). However, since one terbutryn-resistant mutant showed two mutations (in L216 and M263 [9]) the complete L subunit and most of the M subunit of the atrazine-resistant mutants were sequenced. The mutants except one (MAV 3) are different from those obtained as terbutryn-resistant strains [9]. A survey is given in Table I.

The mutant MAV 2 with the mutation L212-Glu → Lys is the most interesting mutant presented here. L212-Glu is conserved in all reaction centers from purple bacteria [18–20] and *Chloroflexus aurantiacus* [21]. Its side chain forms a large part of the bottom of the Q_B binding site and is assumed

Table I. Comparison of the different atrazine-resistant mutants.

Mutant	Mutation	Nucleotide change
MAV 2	L212-Glu → Lys	GAG → AAG
MAV 3	L216-Phe → Ser	TTC → TCC
MAV 4 = MAV 5	L217-Arg → His	CGT → CAT
	L220-Val → Leu	GTT → CTT

to be involved in donating protons to the reduced ubiquinone [5]. Evidence for its participation has been presented by Paddock *et al.* [22]. They have investigated the proton transfer to the fully reduced Q_B^{2-} in reaction centers from *Rhodobacter (Rb.) sphaeroides* by exchange of the protonable glutamic acid into the non-protonable glutamine *via* site-directed mutagenesis (L212-Glu → Gln). Cytochrome turnover kinetic measurements of the mutants showed a fast initial photooxidation (2.9 ± 0.2 Cyt *c* per reaction center, accompanying the reduction of DQ_AQ_B to $DQ_A^-Q_B^{2-}$), followed by a slower phase (rate constant = 7 ± 2 s^{-1}) indicating a block in the turnover of the quinone.

Both the electron transfer rate $DQ_A^-Q_B \rightarrow DQ_AQ_B^-$ and the recombination reaction rate $D^+Q_AQ_B^- \rightarrow DQ_AQ_B$ are pH-independent in the L212-Glu → Gln mutant and pH-dependent in native *Rb. sphaeroides* reaction centers [22]. This pH dependence of the wild type strain is proposed to be caused by L212-Glu which then has an anomalously high pK_a value of 9.5 due to its environment [22]. In our mutant a change of charge has taken place (L212-Glu → Lys). Reasons for the atrazine resistance may be structural rearrangements as well as possible electrostatic repulsions between the side chain of L212-Lys and the potentially positively charged nitrogen of the herbicide's aminoethyl group [23]. Further investigations will show its influence on the rate of proton transfer to Q_B^{2-} as well as the $Q_A \rightarrow Q_B$ electron transfer rates and the rate of charge recombination ($Q_B^- \rightarrow P960^+$). The positive charge of the side chain of L212-Lys may accelerate Q_B^{2-} protonation, and slow down the recombination rate $P^+Q_B^- \rightarrow PQ_B$, and therefore stabilize $P^+Q_B^-$.

The same mutant as MAV 3 (L216-Phe → Ser) has been obtained and characterized as terbutryn-resistant [11]. The double resistance of MAV 3, respectively T6 [11] as well as the strong cross resistance of weeds to all symmetrical triazines and asymmetrical triazinones [24] indicates the presence of just one herbicide-binding pocket. The L subunit of *Rb. sphaeroides* was found to contain the atrazine-binding site by photoaffinity-labeling experiments with azidoatrazine [25, 26]. Pretreatment with terbutryn resulted in reduction of ^{14}C incorporation due to competition between the different triazines for the same binding pocket [25]. On the other hand kinetic measurements of the back reactions $P^+Q_A^- \rightarrow PQ_A$ and $P^+Q_AQ_B^- \rightarrow PQ_AQ_B$ on membranes (*Rb. sphaeroides* [25]; *Rps. viridis* [27]) as well as on isolated reaction centers [28] show essential differences in the sensitivity of triazine type herbicides and others. Bylina *et al.* have produced 28 *Rb. capsulatus* mutants by site-directed mutagenesis [12, 13]. Analysis of resistance and cross resistance shows different sensitivities to closely related triazine type herbicides suggesting different but partially overlapping binding sites for atrazine and terbutryn in purple bacteria.

The double mutation L217-Arg → His, L220-Val → Leu was found twice (MAV 4, MAV 5). As described previously [5] L220-Val (present in *Rb. sphaeroides* [18], Met in *Rb. capsulatus* [19]) interacts strongly with terbutryn in *Rps. viridis* wild type reaction centers. As a consequence of the likely partial overlap of the atrazine and terbutryn binding sites the mutation L220-Val → Leu may change the binding affinity for atrazine; additionally leucine could cause steric hindrance due to an extra methylene group. The substitution of L217-Arg (conserved in both *Rb. sphaeroides* [18] and *Rb. capsulatus* [19]) for His may compensate for detrimental effects of the exchange L220-Val → Leu [9]. Since Met in *Rb. capsulatus* is even slightly larger than Leu in our atrazine-resistant mutant, it will be of interest to see if *Rb. capsulatus* is naturally resistant to atrazine. L223-Ser which is supposed to form a hydrogen bond to Q_B [7] has not mutated.

The presumption of Bylina *et al.* that atrazine causes a spectrum of resistant mutants mostly being different to that of other triazines [12] seems to prove true. Crystal structure analysis of atrazine binding to the wild type *Rps. viridis* reaction center as well as of the mutants will give more information about the action of these herbicides.

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