Sequence Analysis of Mutants from *Rhodopseudomonas viridis* Resistant to the Herbicide Terbutryn

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Several mutants resistant to terbutryn (2-thiomethyl-4-ethylamino-6-*t*-butylamino-*s*-triazine) from the purple bacterium *Rhodopseudomonas viridis* have been isolated. The sequence analysis of that part of the DNA coding for the herbicide binding site in the photosynthetic reaction centre showed that only two genetically different mutant strains were obtained. The sequence data are discussed with respect to recent crystallographic results.

Introduction

The photosynthetic reaction centres (RCs) from the purple bacteria are well characterized (for review see [1]). The RC from *Rps. viridis* consists of four subunits: H (high), L (low) and M (medium), so called after their apparent molecular weights as determined by sodium dodecyl sulphate polyacrylamide-gel electrophoresis, and of a tightly bound cytochrome molecule [2]. The RC from *Rps. viridis* contains four bacteriochlorophyll *b* molecules, two of them forming the primary electron donor ("special pair"), two bacteriopheophytin *b*, one menaquinone-9 ("primary quinone" or "Q_A"), one non-heme iron and one ubiquinone-9 ("secondary quinone" or "Q_B") [3]. The RC from *Rps. viridis* could be crystallized [4]. The crystallographic analysis of the RC crystals provided a complete picture of pigment arrangement, protein structure and the pigment binding-sites [5–7]. The crystal structure of the *Rps. viridis* RC and sequence homologies suggest that the core of the RC of photosystem II (PS II) resembles very much the RC from purple bacteria [6, 8, 9]. In particular the D1 protein (also called 32 kDa protein, Q_B protein or herbicide binding protein) seems to correspond to the L subunit and the D2 protein to the M subunit. This proposal is in agreement with the observation that the triazine herbicides atrazine (2-chloro-4-ethylamino-6-isopropylamine-*s*-triazine) and terbutryn inhibit the photosynthetic electron transport in both, the RCs of purple bacteria and PS II of chloroplasts.

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These commercially used herbicides appear to block the electron flow between Q_A and Q_B in both systems [10]. Furthermore, photoaffinity labelling using azido-atrazine leads to covalent attachment of the label to the D1 protein in PS II and to the L subunit in the bacterial RC [11, 12].

Since in *Rps. viridis* the binding site for the herbicide terbutryn has been established by X-ray crystallography [7], *Rps. viridis* is certainly the best suited organism to understand the mechanism of herbicide resistance.

Material and Methods

Herbicide resistant mutants were obtained by photosynthetic growth of *Rps. viridis* DSM 133 on succinate-based medium containing 100 μM terbutryn. The herbicide was added in a 0.1 M ethanolic stock solution after autoclaving. Starting with 50 ml inoculum photosynthetic growth was observed in individual 0.7 l culture bottles in the presence of the herbicide after a few weeks at room temperature. One single mutant clone was picked from each bottle after streaking on agar plates containing the herbicide, which were incubated in the light at 30 °C. These single colonies were added to 10 ml media and after a few months of growth in the presence of the herbicide the streaking procedure was repeated once.

Genomic DNA was isolated by standard procedures from 0.7 l cultures [9]. DNA was digested using the restriction endonucleases *Eco*RI and *Sal*I and sitze fractionated by agarose gel electrophoresis. Fragments of 1.9 Kb size were ligated into Bluescribe M13$^-$ vector (from: vector cloning systems,

San Diego, CA). Screening was done by colony hybridization. The 1.9 Kb *Eco*RI/*Sal*I fragment from *Rps. viridis* "wild type" coding for the L and most of the M subunit of the RC was labelled with [^{32}P]dCTP by nick translation and used for hybridization at 48 °C [13]. After plasmid isolation, the 1.9 Kb fragment was cleaved using the two *Pst*I and *Sma*I sites on this fragment. Size fractionation was done as above. The M13mp9 system was used for sequence analysis by the dideoxy method of Sanger as described [9].

Results and Discussion

So far, seven independent terbutryn-resistant mutant clones have been isolated. When the DNA sequence work was started, no data about herbicide or quinone binding to the isolated RC were available. However, the terbutryn binding site in the wild type *Rps. viridis* RC was known by X-ray crystallographic analysis in detail [7]: Only amino acid residues of the connecting loop between the fourth and fifth transmembrane helices of the L subunit form the terbutryn binding site. Mutations leading to terbutryn resistance were expected to be located in this region. Therefore morethan 50% of the L subunit genes of five mutants, coding for the entire terbutryn-binding site, were sequenced. This could conveniently be done by sequencing a 86 bp *Sma*I fragment and two mixed *Pst*I/*Sma*I-fragments (114 bp and 487 bp). All observed base pair changes were located at the beginning of the mixed 487 bp *Sma*I/*Pst*I-fragment.

Only two different mutants were observed: The first one showed a double mutation with changes of two base pairs (CGT to CAT and TCG to GCG) producing the substitution of two amino acids: arginine L217 is replaced by histidine and serine L223 by alanine. Only one base pair was altered in the second mutant (TTC to TCC), which results in a change of phenylalanine L216 to serine.

The three amino acids phenylalanine L216, arginine L217 and serine L223 are part of the connecting loop between the fourth and fifth transmembrane helices of the L subunit in the *Rps. viridis* wild type RC.

Fig. 1 reproduces the binding site of terbutryn as determined by X-ray crystallography [7]: a hydrogen bond is likely between the peptide-nitrogen of isoleucine L224 and N3 of the *s*-triazine ring system of terbutryn. A second hydrogen bond with the ethyl-

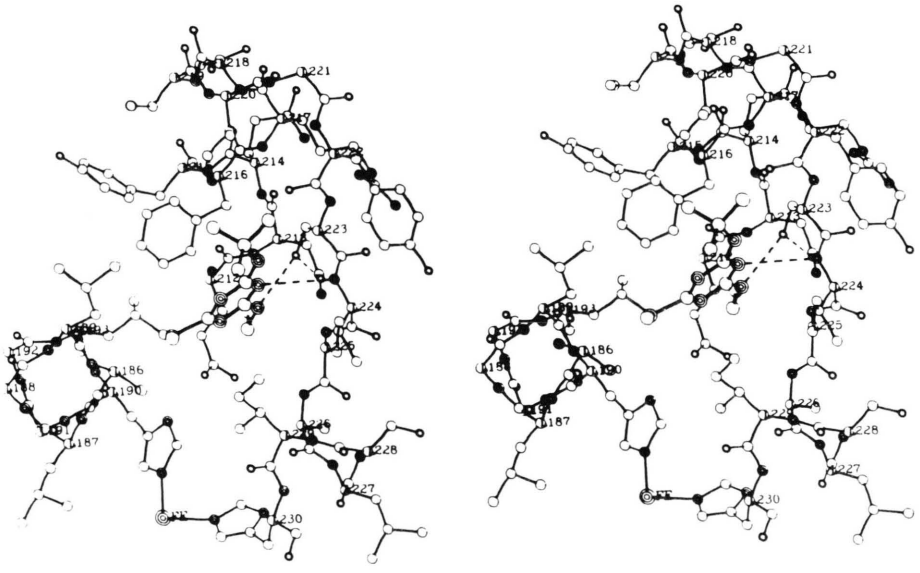

Fig. 1. (Stereo pair) Terbutryn and protein residues forming the binding pocket: Residue numbers indicated at C_α-positions. Empty circles: carbons; half filled small circles: oxygens, filled circles: nitrogens and other atoms; possible hydrogen bonds: dashed lines. The figure is part of figure 4 from ref. 7 and was prepared by J. Deisenhofer using a computer program written by Lesk and Hardman [16].

amino-nitrogen of the herbicide as hydrogen bond donor and the side chain oxygen of serine L223 as acceptor can be observed.

From these data the mutation of serine L223 to alanine could be expected, because it abolishes one of the two hydrogen bonds and, therefore, must decrease the binding of terbutryn. A decrease in binding of this herbicide in a mutant strain from *Rhodobacter (Rb.) sphaeroides* has indeed been observed, where resistance is caused by the change of serine L223 to proline [14].

The replacement of arginine L217 by histidine was unexpected because this residue does not participate in the terbutryn binding directly. We think that this mutation is somehow able to compensate for a detrimental effect of the first mutation.

An explanation why the mutation of phenylalanine L216 to serine also causes herbicide resistance is less obvious. This mutation might lead to an increase in the polarity of one part of the terbutryn binding site. As can be seen in Fig. 1 the hydrophobic *t*-butyl and the thio-methyl side chains of the herbicide are close to phenylalanine L216. The correct binding to the protein at this side might be achieved by hydrophobic interactions. A serine in this region might increase the polarity and result in a decreased terbu-

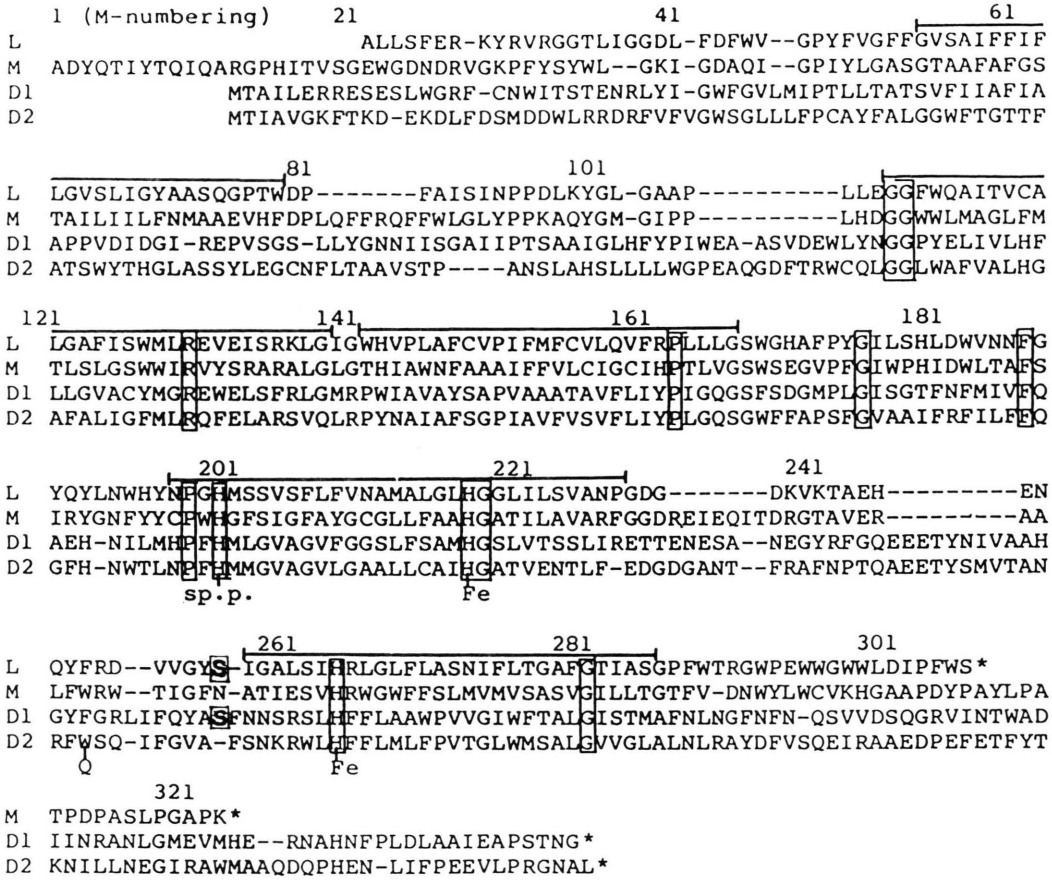

Fig. 2. Alignment of amino acid sequences of the L and M subunits from *Rps. viridis* and the D1 and D2 proteins from spinach chloroplasts taken from ref. [9], slightly modified. Numbers refer to the M subunit from *Rps. viridis*. The transmembrane helices in *Rps. viridis* are indicated by bars above the L subunit sequence. Residues binding to the special pair (sp.p.) and to the non heme iron (Fe), and residues forming the major part of the quinone binding sites (Q) are indicated. The serine L223 and serine D1-264 are marked.

tryn binding. If this explanation is correct this mutant might be less resistant to atrazine, which does not possess the *t*-butyl and thio-methyl side chains. Alternatively, replacing the large phenylalanine side chain by a small serine side chain, and the tendency of the serine side chain to form a hydrogen bond, may lead to a change in the protein conformation, which also could cause terbutryn resistance. A discrimination between these possibilities should be possibly by X-ray crystallography.

Interestingly, changes of serine D1-264 to alanine and phenylalanine D1-255 to tyrosine were reported in triazine resistant mutants from the green algae *Chlamydomonas* [15]. The amino acids sequences of the L and M subunits from *Rps. viridis* and the D1 and D2 RC subunits from spinach chloroplasts are aligned in Fig. 2. Serine L223 and phenylalanine L216 are homologous to serine D1-264 and phenylalanine D1-255. We therefore suggest that the mode of terbutryn binding in the RC of purple bacteria is the same as in the PS II RC.

Acknowledgements

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