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Molecular characterization of two spontaneous antimycin A resistant mutants of *Rhodospirillum rubrum*

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Abstract

Antimycin A is an inhibitor of cytochrome bc_1 complexes acting at the quinone reducing site (Q_i) of the cytochrome *b* subunit. We report here the isolation and molecular characterization of two spontaneous mutants of the purple non-sulfur bacterium *Rhodospirillum rubrum* resistant to this inhibitor. In the two mutants antimycin A resistance was found to be conferred by replacement of an aspartate residue at position 243 of the cytochrome *b* polypeptide chain, in one case by histidine and in the other by glutamate. The mutants exhibit cross-resistance to aurachin C but not to aurachin D. The exchange of Asp-243 does not only diminish the antimycin sensitivity of the isolated cytochrome bc_1 complexes but also has effects on the function of the quinone reducing site (Q_i). Oxidant-induced reduction of cytochrome *b*, requiring addition of antimycin A in the wild type, is already at a maximum in the absence of antimycin A. This indicates a diminished electron flow between heme *b*-566 and ubiquinone at the quinone reducing site (Q_i) of cytochrome *b*.

Keywords: Cytochrome bc_1 complex; Antimycin A resistance; Mutational analysis; (*Rsp. rubrum*)

1. Introduction

The cytochrome bc_1 complexes (EC 1.10.2.2) are membrane bound components of the mitochondrial and bacterial electron transport chains. In plants, algae and cyanobacteria the functionally and structurally similar cytochrome b_6f complexes are constituents of the photosynthetic electron transfer chains. In photosynthetic eubacteria cytochrome bc_1 complexes play a dual role: they serve as electron carriers in the respiratory chain as well as in the light driven cyclic electron flow under anaerobic conditions [1–4]. These complexes consist of at least three subunits, a *b*-type cytochrome with two heme groups, a cytochrome c_1 and an iron-sulfur protein known as Rieske iron-sulfur protein. In *Paracoccus denitrificans* [5], *Rhodobacter capsulatus* [6] and *Rhodospirillum rubrum* [7] the functional cytochrome bc_1 complex is composed of only these three subunits.

Cytochrome bc_1 complexes catalyze the two-electron oxidation of a quinol and the one-electron reduction of a soluble cytochrome *c* (or plastocyanin in cytochrome b_6f complexes). This electron transfer is coupled to a proton translocation across the membrane which is described in terms of a protonmotive Q-cycle mechanism that implies cytochrome *b* to be responsible for an electrogenic transmembrane electron transfer between the two *b*-heme groups and two ubiquinone oxidoreduction centers [8]. The oxidoreduction centers (Q_i- and Q_o-site), each in the vicinity of one of the two heme groups, have been proposed to be located near the electronegative and electropositive side of the membrane, respectively. These functional predictions are supported by models of the three-dimensional folding of the amino acid sequence of cytochrome *b* [9–11].

The experimental approach to confirm the Q-cycle mechanism was mainly based upon the use of specific inhibitors, which can be classified into Q_i- and Q_o-site inhibitors [12,13]. The molecular analysis of inhibitor resistant mutants leads to the identification of amino acids responsible for resistance thus providing data concerning the structural requirements of the Q-cycle mechanism and the environment of quinone and inhibitor binding sites [14–19]. Since most of the non-sulfur purple bacteria are not sensitive towards an-

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timycin A in vivo, whereas Q_i-site inhibitors are effective on chromatophores in vitro [18], no spontaneous mutants resistant towards antimycin A have been isolated up to now. The reason for this insensitivity in vivo is not known, but in Ref. [18] some possibilities are discussed. Photoheterotrophic growth of *Rs. rubrum* is inhibited almost completely by 10 μM antimycin A [20]. We report here the isolation and molecular characterization of antimycin A resistant mutants of *Rs. rubrum*.

2. Materials and methods

2.1. Enzymes and chemicals

Restriction endonucleases, calf intestinal alkaline phosphatase, T4 DNA ligase, antimycin A, myxothiazol and dodecyl maltoside were obtained from Boehringer Mannheim (Germany). T7 DNA polymerase was purchased from Pharmacia. Taq polymerase and the appropriate buffer were obtained from Promega (Munich, Germany). Radiochemicals were obtained from Amersham Buchler (Braunschweig, Germany). Ubiquinol 2 was a gift from W. Oettmeier (Ruhr-Universität Bochum). Aurachin C and aurachin D, isolated from *Stigmatella aurantiaca* [21] were a gift from G. Höfle (GBF, Braunschweig).

2.2. Bacterial culture and mutant selection

Cultures of *Rs. rubrum* FR1 (DSM 1068) were grown under anaerobic photoheterotrophic conditions according to Ormerod et al. [22]. In the end-logarithmic phase they were harvested by centrifugation at 10000 $\times g$ for 20 min and washed twice in 10 mM Tris-HCl (pH 7.6). Antimycin-resistant mutants were selected on agar plates containing 10 μM antimycin A.

2.3. Purification of the cytochrome bc_1 complex

Chromatophores were prepared by the method of Ljungdahl et al. [23], modified according to Majewski [7]. French-pressure cell extracts were centrifuged at 10000 $\times g$ for 20 min, followed by centrifugation of the supernatant at 120000 $\times g$ for 75 min. Membranes were washed in 50 mM Tris-HCl (pH 7.6), 10 mM $MgSO_4$, 1 mM EDTA, 100 mM NaF and pelleted a second time. Chromatophores were resuspended in 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM PMSF, 20% glycerol and diluted to 10 mg/ml protein. After addition of dodecyl maltoside to a final concentration of 7 mg/ml the mixture was stirred on ice for 30 min and then centrifuged at 120000 $\times g$ for 75 min. The supernatant was decanted and reserved, and the pellet was reextracted under the same conditions. The combined supernatants were loaded onto a DEAE-Bio-Gel A column (Pharmacia) equilibrated with 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 20% glycerol, and 0.1 mg/ml dodecyl maltoside. After washing the column with 3 column volumes of the equilibration buffer, elution was carried out using a 100–300 mM NaCl linear gradient. Fractions containing maximal ubiquinol : cytochrome *c* reductase activity were pooled, concentrated by ultrafiltration using Amicon Diaflo PM10 membranes, diluted 1 : 1 with glycerol and stored at −70°C.

2.4. Enzyme activity

Enzyme activity was measured as ubiquinol 2:cytochrome *c* reductase activity at 550–540 nm in 40 mM Na_2HPO_4/NaH_2PO_4 (pH 7.6), 0.5 mM EDTA, 20 mM malonat, 50 mM cytochrome *c* [24] using Sigma ZWS II spectrophotometer (Biochem, Munich, Germany).

2.5. Redox difference spectra

Redox difference spectra were recorded using a Hitachi U-3210 spectrophotometer. The sample was diluted in 50 mM Mes/KOH (pH 6.5), 1 mM $MgSO_4$, 0.1 mg/ml dodecyl maltoside and oxidized by ferricyanide to a final concentration of 75 mM. Solid ascorbate and dithionite were added in the smallest possible amounts.

2.6. Oxidant induced reduction of cytochrome b

Purified cytochrome bc_1 complex was diluted to a final concentration of 1.75 mM cytochrome *b* with 50 mM Mes/KOH (pH 6.5), 1 mM $MgSO_4$, 0.1 mg/ml dodecyl maltoside. Reduction of cytochrome *b* was monitored at 560–577 nm in a Sigma ZWS II spectrophotometer with a 3 nm band pass. Substrate and inhibitors were added as specified in the figure legends.

2.7. Cloning and sequencing of the mutant petB gene

Genomic DNA from *Rs. rubrum* was isolated according to Silhavy et al. [25], and further purified by CsCl gradient centrifugation. After restriction with suitable restriction enzymes the fragments were "shot gun" ligated into pBluescript KS^+ (Stratagene, Heidelberg) and cloned into *Escherichia coli* XL1-Blue or *E. coli* TG1. Positive clones were identified by colony filter hybridization according to Sambrook et al. [26] with the wild-type *petB* gene as homologous hybridization probe. Labeling of the probe was done by polymerase chain reaction (PCR) amplification with digoxygenin (Dig)-dUTP (Boehringer, Mannheim). The PCR

was performed according to Saiki et al. [27]. The reaction mixture consisted of 200 mM Dig-dNTP labeling mixture (Boehringer, Mannheim), 2 mM $MgCl_2$, 20–50 pmol of each primer, 1 mg genomic wild-type DNA, 2.5 U Taq polymerase in 1 × Taq polymerase buffer. Denaturation: 94°C, 30 s; annealing: 48°C, 30 s; elongation: 72°C, 2 min.

The labeled DNA was detected using the DIG Nucleic Acid Detection Kit from Boehringer (Mannheim, Germany). DNA sequencing was performed by the dideoxy chain-termination procedure of Sanger et al. [28] using [α-^{35}S]dATPαS (600 Ci/mmol) with the T7 DNA polymerase and Deaza ^{T7}Sequencing Mixes (Pharmacia, Freiburg).

2.8. Primers for PCR and DNA sequencing

Oligonucleotides were synthesized by Peter Cichocki, Ruhr-Universität Bochum, with the Gene Assembler (Pharmacia, Freiburg) using the phosphoramidite method: I: 5′-CCGTATGCATTCACGGACGACACCAC-3′;
II: 5′-GATTCCGTCGAGCGCATCATG-3′;
III: 5′-TCAGCGTTGATAACCCGACG-3′;
IV: 5′-ACCCACATCGTTCCGGAATG-3′;
V: 5′-GCTAGCAGTTTGCCCGGGATCAC-3′;
VI: 5′-CAGCACGAACAAGATCAAG-3′;
VII: 5′-GATGGTATCGAACGGACCCTT-3′;
VIII: 5′-AAGACATAGCCCATGAAGG-3′.

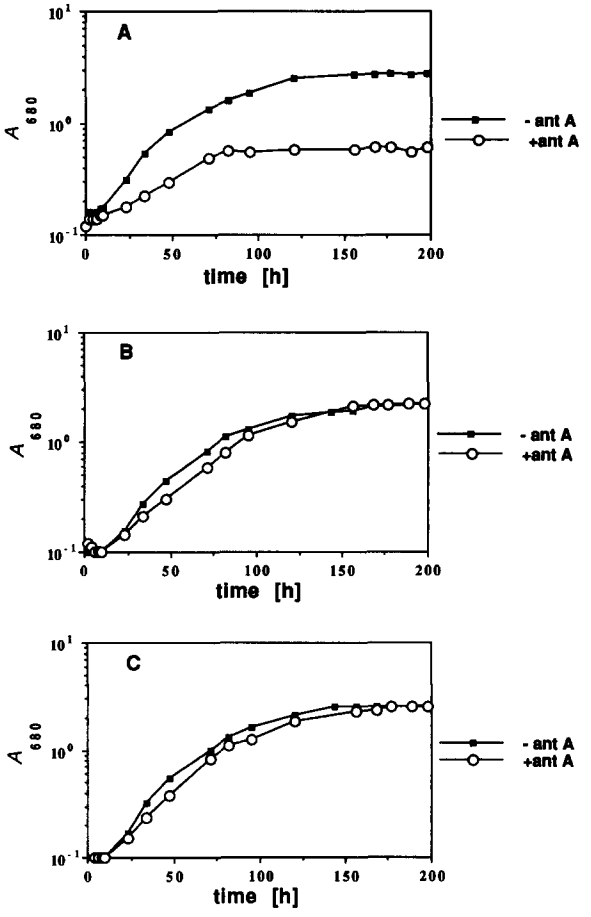

Fig. 1. Photoheterotrophic growth of *Rhodospirillum rubrum* FR1 without inhibitor (square) and in the presence of 10 μM antimycin A (circle). A: wild type; B: mutant D243H; C: mutant D243 E.

3. Results

Antimycin A that affects the quinone reduction site (Q_i) of the cytochrome bc_1 complex inhibited photoheterotrophic growth of *Rs. rubrum* strain FR1 almost completely at a final concentration of 10 μM.

Spontaneous mutants resistant to antimycin A were selected under photoheterotrophic growth conditions on agar plates containing 10 μM antimycin A. Two of the mutants (D243H and D243E) have been characterized by molecular biological and biochemical means.

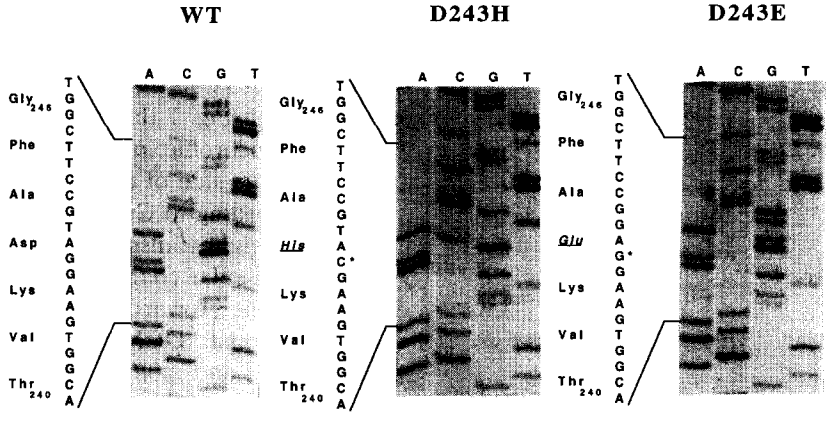

Fig. 2. Sequencing gels from wild-type and antimycin A resistant *petB* genes. A: adenine; C: cytosine; G: guanine; T: thymine; *mutated base. Antimycin A resistance results in a change from G to C in D243H and from T to G in D243E. In both mutants the single point mutation leads to an exchange of Asp-243.

Their photoheterotrophic growth was not affected by antimycin A at a concentration of 10 μM (Fig. 1).

To determine whether inhibitor resistance is due to mutations in the gene coding for the cytochrome *b* subunit of the cytochrome bc_1 complex the *petB* genes of the two mutants have been cloned and sequenced. Comparison with the wild-type sequence [29] revealed that both mutant *petB* genes contain a single point mutation that leads to the exchange of the Asp-243 in the deduced polypeptide chain of cytochrome *b* (Fig. 2). Asp-243 is replaced by His and Glu, respectively. These results have been confirmed by the analysis of restriction fragments of genomic DNA. The mutations create new restriction sites for the restriction endonucleases *Sph*I or *Stu*I, respectively (Fig. 3).

The biochemical characterization of the mutant cytochrome bc_1 complexes was carried out with enzyme preparations purified according to Majewski [7]. The catalytic activity of the isolated cytochrome bc_1 complexes was measured as ubiquinol 2:cytochrome *c* oxidoreductase activity. The turnover numbers calculated for the mutant enzymes were 93.6 and 106.7 μmol cytochrome *c* reduced per μmol heme *b* × min, i.e. 60% and 68%, respectively, of the turnover of the wild-type enzyme (155.5 μmol cytochrome *c* reduced per μmol heme *b* × min, see Table 1). Fig. 4 shows the dependence of the catalytic activity of the isolated cytochrome bc_1 complexes on the concentration of antimycin A.

The inhibitory effects of antimycin A and three other cytochrome bc_1 complex inhibitors are summarized in Table 2. Fig. 5 shows the structures of the

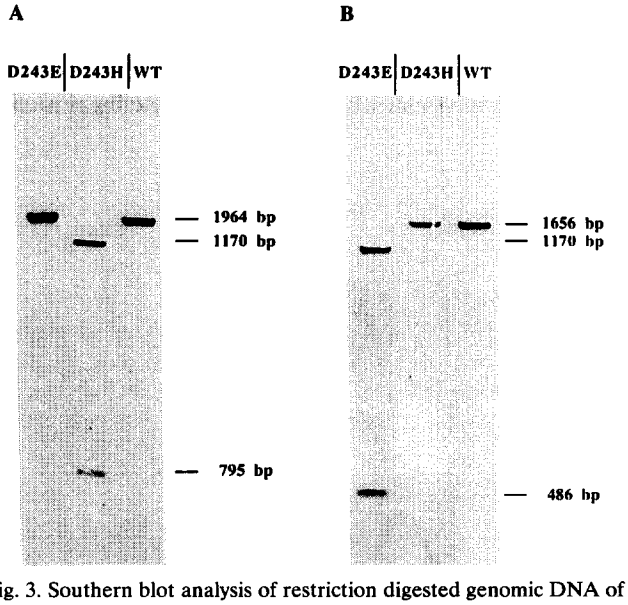

Fig. 3. Southern blot analysis of restriction digested genomic DNA of the wild type and the two mutants D243H and D243E. The blot was probed with a non-radioactively labeled PCR product of the wild-type *petB* gene. A: restriction with *Eco*RI, *Cla*I and *Sph*I; B: restriction with *Eco*RI, *Cla*I and *Stu*I.

Table 1
Turnover numbers of the isolated cytochrome bc_1 complexes of wild-type and antimycin A resistant mutants (s^{-1})

Wild-type FR 1	D243H	D243E
155.5	93.6	106.7

inhibitors. The sensitivity of the mutant cytochrome bc_1 complexes towards antimycin A is decreased to two orders of magnitude compared with the wild-type enzyme. While the inhibitory effects of myxothiazol, an inhibitor acting at the Q_o-site of the cytochrome bc_1 complex, is unchanged as well as that of aurachin D, the mutant cytochrome bc_1 complexes exhibit a significantly increased tolerance towards aurachin C.

Redox difference spectra and subunit composition on SDS-PAGE of the purified cytochrome bc_1 complexes revealed no differences between the mutant and the wild-type enzymes (data not shown).

A characteristic feature of the Q-cycle pathway in the cytochrome bc_1 complex is the oxidant induced reduction of cytochrome *b*. In the wild-type enzyme an "extra reduction" of cytochrome *b* is induced by the addition of a high potential oxidant such as ferricyanide when electron flow is blocked at the Q_i-site by antimycin A. Both mutant cytochrome bc_1 complexes show this "extra reduction" even in the absence of antimycin A (Fig. 6).

4. Discussion

Two mutants of *Rs. rubrum* resistant to antimycin A have been isolated and analyzed. These are the first spontaneous mutants of non-sulfur purple bacteria resistant to a Q_i-site inhibitor. In both cases antimycin A resistance is conferred by a single point mutation in the *petB* gene that leads to the exchange of amino acid Asp-243 to His and Glu, respectively, in the deduced polypeptide chain of the cytochrome *b* subunit. Asp-243 is conserved in all known sequences of cytochrome *b* (and subunit IV in the cytochrome b_6f complexes) [30]. As shown in Fig. 7 this residue is localized in a region of the cytochrome *b* subunit that is located at the cytoplasmatic side of the membrane in a cytochrome *b* model according to Crofts et al. [10] and Brasseur [11], displaying eight transmembrane helices.

The molecular characterization of mutations conferring resistance towards antimycin A and other Q_i-site inhibitors like DCMU and funiculosin in yeast [14,15,17,31] and mouse [32,33] has led to the identification of two regions of the protein which are proposed to be part of the inhibitor binding niche. One of these regions comprises the amino acids Val-30, Asn-43 and Gly-49, the second region is determined by Met-

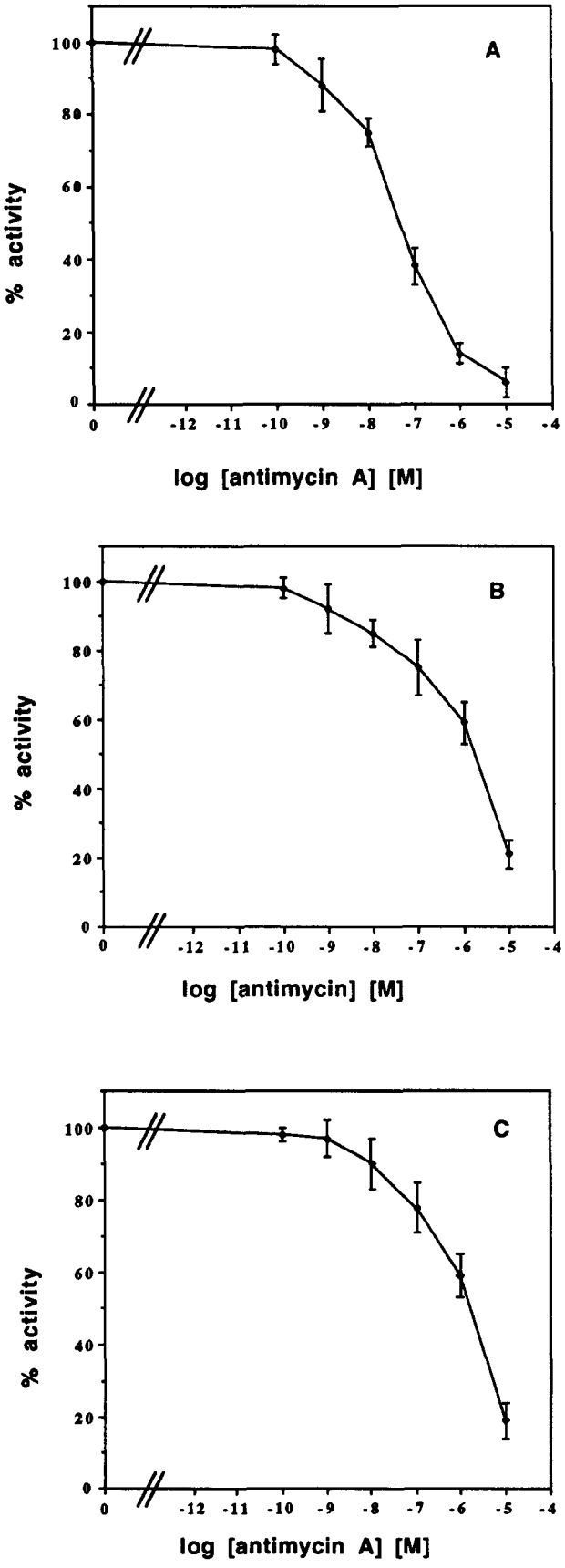

Table 2
Inhibitory effects of antimycin A and three other cytochrome bc_1 complex inhibitors on the ubiquinol 2:cytochrome *c* oxidoreductase activity of the isolated wild-type and mutant enzymes displayed as pI_{50} values

	Wild-type FR1	D243H	D243E
Antimycin A	7.8	5.8	5.7
Myxothiazol	8.0	7.99	7.75
Aurachin C	7.8	6.67	6.65
Aurachin D	7.3	7.15	7.22

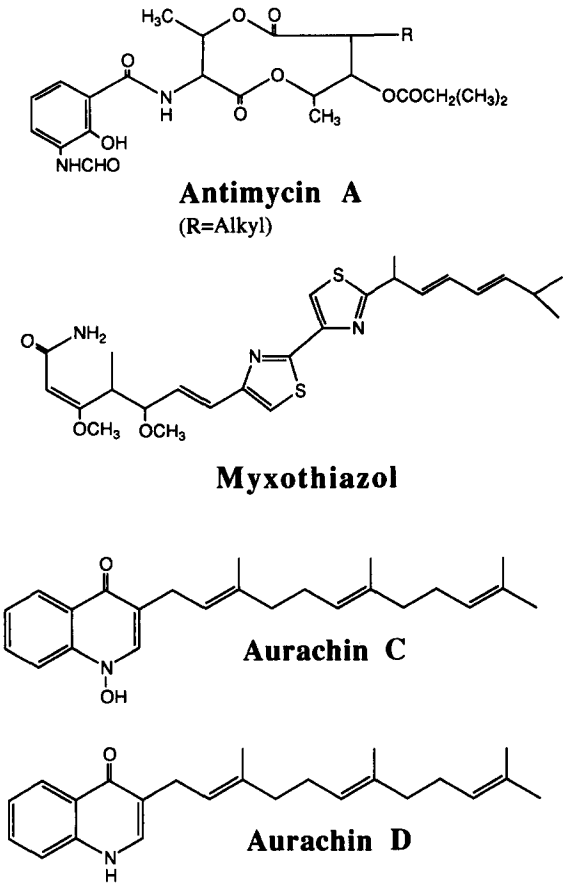

Fig. 5. Structures of cytochrome bc_1 complex inhibitors used in the present work.

210, Asn-219, Phe-235, Tyr-239, Lys-242, Asp-243 and Gly-246 of the *Rs. rubrum* polypeptide chain. These two regions are considered to be involved.

In addition to their diminished sensitivity towards antimycin A the mutant cytochrome bc_1 complexes described here exhibit alterations in their biochemical properties.

Fig. 4. Effect of antimycin A on ubiquinol:cytochrome *c* oxidoreductase activity of the isolated wild-type and antimycin A resistant mutant cytochrome bc_1 complexes. A: wild type; B: mutant D243H; C: mutant D243E. Catatytic activity is displayed as percentage of the control rate without inhibitor.

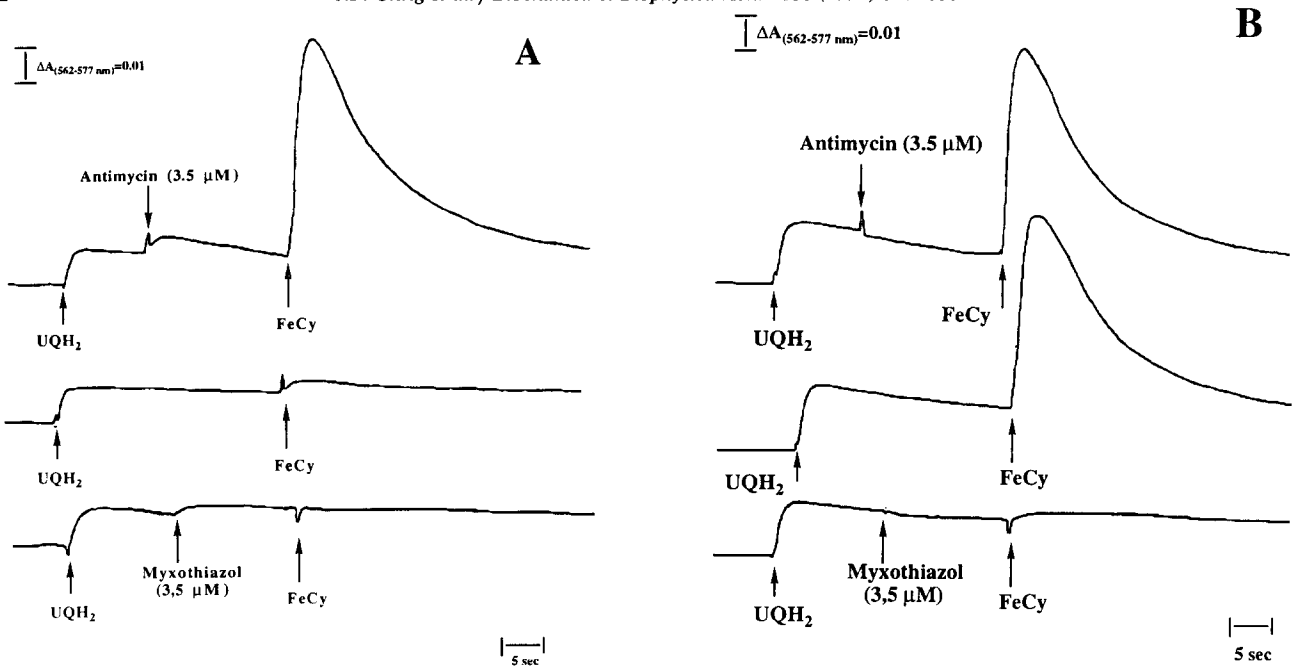

Fig. 6. Oxidant induced reduction experiments showing the redox status of heme *b*-562 for the wild-type control and the antimycin A resistant mutants. A: wild type; B: mutant D243H. The mutant D243E showed the same oxidant induced reduction behavior as the mutant D243H (data not shown). Concentrations of substrate and oxidant: 30 μM UQH_2:20 μM ferricyanide. Concentrations of inhibitors are indicated in the figures.

In the wild-type cytochrome bc_1 complex antimycin A is known to cause the oxidant induced reduction which is due to the impaired reoxidation of heme *b*-562. This phenomenon was introduced by Bowyer and Trumpower [34].

An oxidant induced reduction of the mutant cytochrome *b* was observed even in the absence of antimycin A. The reason for this alteration of the reoxidation kinetic of cytochrome *b* may be a shift of the heme *b*-562 redox potential or a destabilization of the semiquinone anion at the Q_i-site. The destabilization of the semiquinone anion results in a decrease of the potential of the quinone/semiquinone redox couple [35]. This could lead to an impaired electron transfer from the *b*-562 to quinone and therefore to a more stable reduction of cytochrome *b*. In this case Asp-243 is involved not only in antimycin A binding but also in the stabilization of the semiquinone anion in the Q_i binding niche. The altered reoxidation kinetic of cytochrome *b* at the Q_i-site is likely to be the reason for

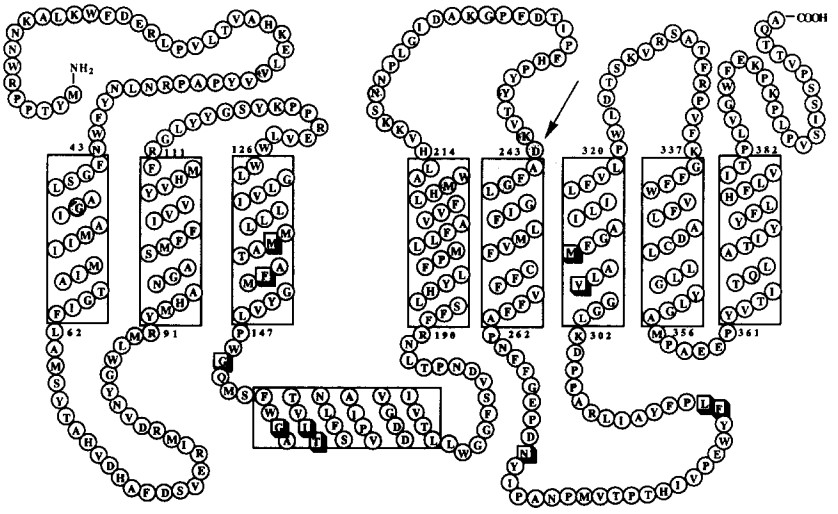

Fig. 7. Cytochrome *b* folding model of *Rhodospirillum rubrum*. The model displays eight transmembrane helices according to Crofts et al. [10] and Brasseur [11]. The loci of mutants exhibiting inhibitor resistance for the Q_o-site (outlined squares) or the Q_i-site (grey circles) are shown. These include mutations from yeast [14–17,31], mouse [32,33] and *Rb. capsulatus* [18]. The mutations in *Rs. rubrum* that specifically confer resistance to antimycin A are located at Asp-243 (arrow).

the diminished catalytic activity of the mutant cytochrome bc_1 complexes.

Single flash experiments with chromatophores and intact cells confirmed the result of the oxidant induced reduction experiments with the isolated enzyme complexes (data not shown).

The corresponding Asp-252 residue of the *Rb. capsulatus* cytochrome *b* has recently been exchanged to Asn by site-directed mutagenesis [36]. In flash kinetics with chromatophores comparable results concerning the altered function of the Q_i-site of the mutant cytochrome bc_1 complex have been obtained.

The cross-resistance of the two *Rs. rubrum* mutants towards aurachin C confirms the supposition that it belongs to the group of Q_i-site inhibitors [37]. Although there is only very little structural difference between aurachin C and aurachin D (aurachin C is the *N*-oxide of aurachin D) the sensitivity of the mutant cytochrome bc_1 complexes towards aurachin D is not affected. The site of action of aurachin D in cytochrome bc_1 complexes has not yet been fully identified [37]. Our results show clearly that it does not act at the Q_i-site but rather at the Q_o-site.

Acknowledgements

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