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Two changes of the same nucleotide confer resistance to diuron and antimycin in the mitochondrial cytochrome *b* gene of *Schizosaccharomyces pombe*

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Diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea) and antimycin, both inhibitors of mitochondrial respiration, block electron flow between cytochromes *b* and c_1. Mutants resistant to either drug have been selected using *Schizosaccharomyces pombe* strains with an extrachromosomally inherited mutator. In analogy to *Saccharomyces cerevisiae* these mutational sites were assumed to map in the cytochrome *b* gene. DNA sequence analysis showed that two changes in the same nucleotide are responsible for resistance to antimycin and diuron. Analysis of resistant and sensitive progeny of crosses between the mutants and the wild type confirmed the correlation between mutational alteration and resistant phenotype.

Diuron resistance; Antimycin resistance; Mitochondrial cytochrome *b* gene; DNA sequence analysis; Mutational alteration; Mutator strain; (*Schizosaccharomyces pombe*)

1. INTRODUCTION

After treatment with *N'*-nitro-*N*-nitrosoguanidine several mitochondrial mutants have been isolated in the fission yeast *Schizosaccharomyces pombe* strain *ade7-50h*$^-$ (strain *50* for short), which are resistant to antimycin both in vivo and in vitro [1,2]. Concomitantly, a mutator mutation was induced in all of them. This mutator causes an increased susceptibility of the mitochondrial genome towards point mutations and deletions of the mitochondrial DNA [3-5]. In analogy to *Saccharomyces cerevisiae*, the antimycin resistant mutants have been allocated to the cytochrome *b* gene [6]. In *Sc. pombe*, the cytochrome *b* gene (*cob*) is split by a group II intron [7]. In order to facilitate sequencing of drug-resistant mutants in the *cob* gene, we have constructed an intronless *cob* gene by intron DNA splicing [8]. In this strain we have selected mutants resistant to diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea), which is also an inhibitor of the respiratory chain between cytochromes *b* and c_1 [9]. In this paper we describe the base changes leading to diuron- and antimycin-resistance and discuss the results in relation to previous genetic data from mutant selection and from crosses between resistant mutants [10]. The mutational alterations will also be discussed concerning a modification of the current model for protein folding of the cytochrome *b* apoprotein [11].

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2. MATERIALS AND METHODS

2.1. *Strains and plasmid*

The *Sc. pombe* mutant *ade7-50h*$^-$ was from the collection of U. Leupold, Berne. The meiosis deficient strain *mei1 ade6* was kindly provided by R. Egel, Copenhagen. The diuron resistant strain *diu*^{r}*-301* is described in [12]. The mutant strains *ana*^{r}*-X39* and *ana*^{s}*-X39* are described in this paper. The *E. coli* strain *JM 101 lac*$^-$, *pro*$^-$, *amp*^{s}, *F*$^+$, *pro*$^+$ was used for cloning. Plasmid *pFM71* is described in [13].

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2.2. *Genetic techniques*

Media, selection of mutants, construction of stable diploids, mitotic segregation analysis, random spore analysis and meiotic crosses are described in [2].

2.3. *Molecular techniques*

For preparation of mitochondrial DNA, restriction enzyme digestion, agarose gel electrophoresis, electroelution, nick-translation, Southern hybridization, M13 cloning and DNA sequencing according to Sanger, standard procedures were used; relevant modifications of these techniques are given in [13]. Hydrophobicity patterns were evaluated according to [14] and protein secondary structures determined according to [15] using an IBM-AT computer.

3. RESULTS AND DISCUSSION

3.1. *The diuron-resistant mutant* diu^r-301

The nucleotide sequence of the entire *cob* gene has been determined for strain *ade7-50h*$^-$ (referred to as the mitochondrial wild-type), from which the antimycin-resistant mutant *ana*^{r}*-14* has been derived by nitrosoguanidine mutagenesis [12]. *Diu*^{r}*-301* is a spontaneous mutant of *ana*^{r}*-14* which has lost antimycin resistance - a phenomenon which will be discussed later. The two exonic regions of the *cob* gene have been sequenced and compared with the homologous sequences in the wild type. In mutant *diu*^{r}*-301* the C in position 110 (position 1 is the A of the ATG initiation codon) is changed into G. This base change leads to the replacement of an alanine (Ala) by glycine (Gly) in position 37.

In order to correlate the mutational change with the resistance phenotype, mutant *diu*^{r}*-301* was crossed with the meiosis deficient strain *mei1 leu2*. The diploid progeny showed mitotic segregation of resistance versus sensitivity to diuron (not shown) thus indicating extrachromosomal inheritance. Resistant diploids were purified three times on diuron medium and sensitive diploids were checked by replica plating on drug medium. Cloning and sequencing of the relevant part of the *cob* gene revealed that the C to G transversion is only found in the resistant clones.

3.2. *The antimycin-resistant mutant* ana^r-X39

In order to facilitate cloning and sequencing of the *cob* gene, a derivative of mutant *ana*^{r}*-14*, mutant *ana*^{r}*-X39*, was used in which the *cob* intron has been removed by intron DNA splicing [8]. After sequencing the *cob* gene only one base change was discovered, concerning the same nucleotide as in the diuron-resistant mutant: in this case the C was replaced by a T. This transition leads to the replacement of alanine (Ala) by valine (Val). Analysis of diploids issued from mitotic segregation (see above) confirmed that the C to T transition is correlated with the antimycin resistance (not shown).

3.3. *Origin and allelism of drug-resistant mutants in the* cob *gene*

A series of diuron-resistant mutants has been isolated in *ana*^{r}*-14*, which could be classified in 3 types. Type I isolates (60%) could grow on both drug media (antimycin and diuron), type II isolates (35%) were initially able to grow on both drug media, but not on the double medium. These mutants very likely contained mixtures of two different mitochondrial genomes. Type III isolates (5%), like mutant *diu*^{r}*-301*, have lost the capacity to grow on antimycin medium [12]. Analysis of 6040 spores from a cross between *diu*^{r}*-301* and *ana*^{r}*-14* did not yield any double resistant recombinant. Both observations can now be explained by the fact, that the same nucleotide has mutated in the two resistant strains.

3.4. *Consequences of the amino acid changes in the cytochrome* b *protein*

The changes from Ala (position 37) to Gly in *diu*^{r}*-301* and from Ala to Val in *ana*^{r}*-X29* slightly changes the hydrophobicity of the transmembrane segment I (fig.1). Alanine carries an uncharged R group (CH_3), and the additional methyl group of V slightly increases hydrophobicity. Gly (in mutant

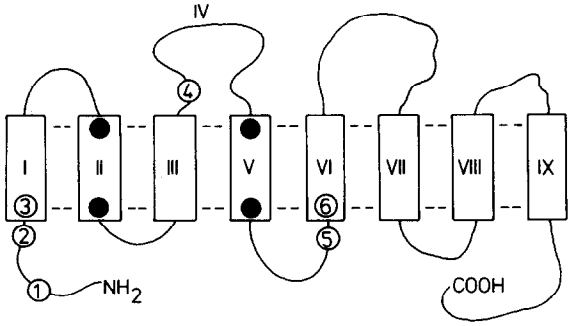

Fig.1. Revised model for folding of apocytochrome *b* according to [11]. The filled circles are the four invariant histidine residues which bind the two heme groups. The numbers refer to the amino acid changes listed in table 2.

Table 1

Protein secondary structure in the antimycin domain

Strain	Amino acid sequence	Conformation
	37	37
	*	*
ade7-50h⁻	FWNFGSLLACVLVIQ	EEETTTCHHHEEEEE
diuʳ-301	********G******	EEETTTCTTEEEEEE
anaʳ-X39	********V******	EETTTTCEEEEEEEE

The protein secondary structure was determined according to [14]. H = α-helical, E = β-sheet, T = α-turn, C = no ordered configuration

anaʳ-X39) leads to a decrease in hydrophobicity due to its polar and neutral properties. It is unlikely that local changes in the hydrophobicity profile are responsible for differential drug binding. It is more likely that alterations in the secondary structure of the first transmembrane segment play a role in inhibitor binding. The local protein secondary structure was determined according to [14] and the results are summarized in table 1. In the wild type (strain *50*) the Ala in position 37, together with the surrounding amino acids, form a short α-helical segment. The amino acid changes in both resistant mutants lead to destruction of this helical segment, as indicated in table 1. These conformational changes very likely influence the differential binding of the drugs in wild type and mutants.

3.5. *Segment I is part of the antimycin-binding domain*

Table 2 summarizes the published sequence data of various drug-resistant mutants in mouse, *S. cerevisiae* and *Sc. pombe* mitochondria. It is interesting to note that four different mutational sites concern the amino acid position 37. A comparison of this segment from various organisms (table 3) reveals that *Sc. pombe, Aspergillus nidulans, Neurospora crassa* and *Rhodobacter* possess an Ala at this position, whereas the majority of organisms (including *S. cerevisiae*) has a Gly. It is tempting to speculate that the Ala in position

Table 3

Comparison of the amino acid sequences of the antimycin domain from different organisms

Organisms	Antimycin domain
	37
	*
Schizosaccharomyces	FWNFGSLLACVLVIQ
Aspergillus	LWNFGSLLALCLGIQ
Neurospora	LWNFGSLLACCLIIQ
Rhodobacter	WWIWGSLLAFTLVLQ
Saccharomyces	WWNMGSLLGLCLVIQ
Drosophila	WWNFGSLLGLCLIIQ
Xenopus	LWNFGSLLGVCLIAQ
Mouse	WWNFGSLLGVCLMVQ
Bovine	WWNFGSLLGICLILQ
Human	WWNFGSLLGACLILQ
Maize	WWGFGCLAGICLVIQ
Wheat	WWGFGSLAGICLVIQ
Trypanosoma	IYGVGFSLGFFIALQ
Spinach chloroplast b_6	FYCLGGITLTCFLVQ
Tobacco chloroplast b_6	FYCLGGITLTCFLVQ
Liverwort chloroplast b_6	FYCLGGITLTCFLVQ

The sequences are aligned relative to the *Sc. pombe* sequence. Taken from [11], where all references can be found

Table 2

Compilation of amino acid changes in inhibitor-resistant mutants

Number	Amino acid position	Organism	Resistance towards	Amino acid change	Reference
1	17	*S. cerevisiae*	diuron	I → F	[18]
2	31	*S. cerevisiae*	diuron	N → K	[18]
3	37	mouse	antimycin	G → V	[11]
3	37	*S. cerevisiae*	antimycin	G → V	[11][a]
3	37	*Sc. pombe*	antimycin	A → V	this work
3	37	*Sc. pombe*	diuron	A → G	this work
4	141	mouse	myxothiazol	G → A	[18]
5	225	*S. cerevisiae*	diuron	F → S	[18]
6	230	mouse	HQNO	G → D	[18]

[a] Colson, A.-M., personal communication, quoted in [11]

Amino acid positions are according to *Sc. pombe*. The numbers refer to the locations of amino acid changes in fig.1

37 might correlate with the much higher resistance in vivo and in vitro of antimycin-resistant mutants of *Sc. pombe* compared to those of *S. cerevisiae*. In the chloroplast cytochrome b_6 proteins, there is a leucine residue at the homologous position which might be responsible for the 1000-fold higher resistance to antimycin compared with mitochondria. In this context it should be mentioned that it has been described in a previous paper [12] that the antimycin-resistant mutant *ana*^{r}*-14* is more sensitive to diuron than the corresponding wild type. Only half of the amount of diuron is needed in the mutant to reduce the growth rate to 50%. It could be assumed that the change from Ala to Val simultaneously increases resistance towards antimycin and decreases the tolerance to diuron.

3.6. *The revised model of the folding of apocytochrome* b

The basic features of the models of Widger et al. [16] and Saraste [17] are the nine hydrophobic domains spanning the inner mitochondrial membrane. Analysis of drug-resistance mutations has led to serious inconsistencies in regard to their location on both sides of the membrane and in relation to the heme group [11]. Howell and Gilbert [11] therefore suggested a modification of the Widger-Saraste model which is depicted in fig.1 and discussed in detail in [11]. The basic differences between the old and the revised model are (i) the inverse orientation of the amino-terminus and consequently of segments I–III in the membrane, and (ii) that domain IV is located outside the membrane. Fitting all available data on mutants, including those described in this paper, in the revised model, it appears that the amino acid changes of all mutants resistant to drugs with related properties (antimycin, diuron and HQNO) are located at the inside, whereas the target for myxothiazol is at the outside of the inner membrane.

3.7. *Concluding remarks*

Mitochondrial mutants conferring to drugs inhibiting the electron transport provide an excellent tool to elucidate the architecture of membrane bound enzyme complexes. The molecular analysis of all available drug-resistant mutants in the cytochrome *b* gene will certainly deepen our understanding of the structure and function of the respiratory chain.

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