Amino acid substitutions in mitochondrial ATPase subunit 9 of *Saccharomyces cerevisiae* leading to oligomycin or venturicidin resistance

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Received 28 October 1985

A series of isonuclear oligomycin-resistant mutants of *Saccharomyces cerevisiae* carrying mutations in the mitochondrial *oli1* gene has been studied. DNA sequence analysis of this gene has been used to define the amino acid substitutions in subunit 9 of the mitochondrial ATPase complex. A domain of amino acids involved in oligomycin resistance can be recognized which encompasses residues in each of the two hydrophobic portions of the subunit 9 polypeptide that are thought to span the inner mitochondrial membrane. Certain amino acid substitutions also confer cross-resistance to venturicidin: these residues define an inner domain for venturicidin resistance. The expression of venturicidin resistance resulting from one particular substitution is modulated by nuclear genetic factors.

Proteolipid *Mitochondrial ATPase complex (Yeast)* *Amino acid substitution* *Cytoduction* *Drug-resistance domain* *Oligomycin* *Venturicidin*

1. INTRODUCTION

The *oli1* gene in mitochondrial DNA (mtDNA) of *Saccharomyces cerevisiae* codes for subunit 9 of the mitochondrial ATPase complex (mtATPase) [1,2]. Subunit 9 is a 76 amino acid proteolipid which plays a key role in the proton-translocating activity of the mtATPase; this subunit can be covalently bound by DCCD. The *oli1* gene was initially identified through the analysis of oli^R mutants. In previous work, direct amino acid sequencing of subunit 9 from spontaneously arising oli^R mutants revealed 3 possible amino acid substitutions [3]. These lie in the C-terminal portion of the protein, in close proximity to Glu_{59} where DCCD binds [4].

We recently studied [5] a set of oli^R mutants, from diverse parent strains, that had previously been subjected to extensive genetic analysis (including tests on cross-resistance to venturicidin). A key finding of our DNA sequencing analysis was the identification of amino acid substitutions at Gly_{23} (to Ala or Cys) in the N-terminal portion of subunit 9 that lead to resistance to both oligomycin and venturicidin. A domain of resistance to these drugs could thus be envisaged to encompass amino acids in both the N-terminal and C-terminal portions of subunit 9 [5]. In this view, the *S. cerevisiae* proteolipid folds into a hairpin structure that spans the membrane, as suggested for the homologous proteolipid subunits of other mitochondrial and bacterial ATPase complexes [6]. Differentiation of the resistance domains in yeast for oligomycin and

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Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; mtATPase, mitochondrial proton-translocating ATPase; oli^R, oligomycin-resistant (in reference to phenotype); TID, 3-(trifluoromethyl)-3-(*m*-[^{125}I]-phenyl)diazirin

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

venturicidin was made possible by the identification of those amino acid substitutions leading to oligomycin resistance without cross-resistance to venturicidin [5].

In this work, a further series of 7 oli^R mutants, all isolated from a common parent strain, have been studied by DNA sequence analysis of the *oli1* gene. We describe additional amino acid substitutions in subunit 9 leading to the oli^R phenotype. Other consequences of the mutations, including the degree of cross-resistance to venturicidin, are appraised.

2. MATERIALS AND METHODS

2.1. *Strains*

S. cerevisiae J69-1B *MATα ade1 his5 [rho$^+$]*, L410 *MATα ura1 his5 [rho$^+$]* (both from the Monash collection) and 8960-14B *MATa ura3 leu2 kar1-1 [rho$^+$]* (from G.R. Fink) are oligomycin-sensitive strains. The *rho°* derivatives (devoid of mtDNA) of each of these *rho$^+$* parent strains were derived by ethidium bromide treatment [7].

2.2. *Methods*

Growth of strains and genetic analyses [8], mutagenesis with $MnCl_2$ [9], cytoduction of mtDNA involving *kar1* mutants with *rho$^+$* donors and *rho°* recipients [10], and nucleotide sequence determination of the *oli1* gene [5] were carried out as previously described.

3. RESULTS AND DISCUSSION

3.1. *Characterization of oligomycin-resistant mutants*

A series of oli^R mutants was isolated from strain J69-1B following mutagenesis with $MnCl_2$. The mutant isolates were classified initially into 2 groups: those showing mitochondrial or nuclear inheritance [8] of the oli^R phenotype (more than 95% of these isolates were mitochondrial by the applied criteria of mitotic segregation and ethidium bromide elimination of mtDNA). The mitochondrial mutants were further classified as *oli1* or *oli2* mutants by mating to reference oli^R strains carrying mutations at the *oli1* or *oli2* loci. Some mutants showed tight linkage to *oli2* markers (less than 5% recombination) and were classified as *oli2*; the majority showed linkage to *oli1* alleles in recombination tests, but little linkage to *oli2*. The latter mutants were further classified by tests of their cross-resistance to venturicidin and their temperature sensitivity; 7 putative *oli1* mutants with various phenotypes (table 1) were selected for further study. Each such mutant was mated to petite 23-3 carrying the *oli1* gene (that would confer the oli^S phenotype) [11]; in all cases significant segre-

Table 1

Nucleotide changes in *oli1* gene and resulting amino acid substitutions in oligomycin-resistant strains

Isolate number	Phenotype	DNA sequence number	Nucleotide change	Codon number	Codon change	Amino acid change	Allele
0811	O^R V^{R^a}	+ 67	G→T	23	GGU→UGU	Gly→Cys	*oli1-704*
0812	O^R V^R	+ 67	G→A	23	GGU→AGU	Gly→Ser	*oli1-705*
0814	O^R V^S	+159	A→T	53	UUA→UUU	Leu→Phe	*oli1-706*
0821	O^R V^S	+159	A→T	53	UUA→UUU	Leu→Phe	*oli1-706*
0850	O^R V^{R^b}	+166	G→A	56	GCC→ACC	Ala→Thr	*oli1-707*
0807	O^R V^{R^c}	+192	C→A	64	UUC→UUA	Phe→Leu	*oli1-708*
0851	O^R V^{R^c}	+192	C→A	64	UUC→UUA	Phe→Leu	*oli1-708*

Phenotypes: O^R, oligomycin-resistant; V^R or V^S, venturicidin-resistant or -sensitive, respectively (with the following qualifications: [a]strongly cold-sensitive, i.e. very little growth on non-fermentable substrates at 18°C; [b]weakly cold-sensitive; [c]weakly cold-sensitive with an ambiguous cross-resistance to venturicidin). Allele numbers are assigned as in [5], except that identical mutations in the isogenic strains analyzed here are given the same allele number. Note that as mutants were selected for analysis because of their different phenotypes, the distribution of mutations in this table does not reflect their frequency of occurrence

gation of *rho*$^+$ *oli*^{S} diploids was observed, thus confirming the assignment of the oli^R mutation to the *oli1* region.

3.2. *Sequence changes in oligomycin-resistant mutants*

The sequence of the *oli1* gene in each of the 7 mutants was determined. The single nucleotide changes observed relative to the wild-type sequence, together with the consequent amino acid substitutions, are shown in table 1. Two isolates (0811 and 0812) carry substitutions at amino acid residue 23. In the case of mutant 0811 (Gly_{23}→Cys) the mutation is identical to one previously identified in strain L748 [5]; the cold-sensitive phenotype and other properties of these 2 mutants, including the reduced mobility of subunit 9 on polyacrylamide gels [12], are very similar. The other mutant (0812) in which amino acid 23 is altered (Gly_{23}→Ser), represents a second case of a substitution at this position leading to oligomycin and venturicidin resistance with no concomitant temperature sensitivity. We previously identified [5] a Gly_{23}→Ala substitution conferring similar properties. It is of interest that the radical substitution Gly_{23}→Asp identified in *mit*$^-$ strain 15B-2 leads to non-functional subunit 9 [13].

The other substitutions in oli^R mutants lie in the C-terminal arm of subunit 9. Two mutants 0814 and 0821 carry one of the most commonly encountered substitutions in subunit 9, namely Leu_{53}→Phe [1,2,5,14]. The substitution Ala_{56}→Thr in mutant 0850 has not been previously recognized. This mutation confers oligomycin resistance with a strong cross-resistance to venturicidin. The final 2 mutants 0807 and 0851 carry the same substitution

Table 2

Oligomycin and venturicidin phenotype in mutant strains and derived cytoductants

Group		Strain constitution		Phenotype		
		Nucleus	Mitochondrion	OLI(4)	VEN(1)	VEN(5)
(A)	Mutants	L4100	L4100	R	R	S/R
		J69-1B	0851	R	S/R	S/R
		J69-1B	0807	R	S/R	S/R
		J69-1B	0814	R	S	S
		J69-1B	0850	R	R	R
(B)	Cytoductants	L410	L4100	R	R	R
		L410	0851[a]	R	R	R
		J69-1B	L4100	R	S/R	S/R
		J69-1B	0851[a]	R	S/R	S/R
		8960-14B	L4100[b]	R	R	S/R
		8960-14B	0851[a,b]	R	R	S/R

[a] Cytoductants derived from mutant 0807 show same phenotype as those from 0851

[b] Cytoductants with the 8960-14B nuclear background show reduced growth on control YEPE plates (no drugs) relative to *rho*$^+$ strains with the other nuclear backgrounds tested here

Cytoductants were made by crossing mutants to the *rho*° derivative of strain 8960-14B *(kar1)* then selecting Leu$^+$ Ura$^+$ *rho*$^+$ haploid clones. These primary cytoductants were then crossed back to *rho*° derivatives of strains L410 or J69-1B, selecting haploid *rho*$^+$ clones with the appropriate auxotropic requirements. Drug resistance was tested by dropping cell suspensions onto YEPE plates [8] with the following concentrations of drugs: OLI(4), oligomycin 4 μg/ml; VEN(1), venturicidin 1 μg/ml; VEN(5), venturicidin 5 μg/ml. Phenotypes were scored after 7 days at 28°C as follows: R, unambiguous resistance (similar growth to control spots plated in absence of drugs); S, unambiguous sensitivity (no growth on drug plate); S/R, ambiguous phenotype (detectable growth, but inhibited relative to control)

(Phe$_{64}$→Leu). In this case the venturicidin resistance is barely detectable. Precisely the same mutation has been identified in strain L4100 [5]; in that case an appreciable resistance to venturicidin was observed. It was important to resolve this anomaly as residue 64 lies near the boundary of the domain of venturicidin resistance that we have proposed [5]. The genetic analysis described in section 3.3 reveals the influence of nuclear genes on the expression of venturicidin resistance at residue 64.

3.3. *Expression of venturicidin resistance can be subject to nuclear influence*

Table 2A indicates phenotypes of the 3 strains (L4100, 0851, 0807) carrying the mutation Phe$_{64}$→Leu. As noted in [5], strain L4100 is resistant to venturicidin at 1 μg/ml, but shows little resistance to this drug at 5 μg/ml. By contrast mutants 0851 and 0807 show little or no resistance to venturicidin even at 1 μg/ml. For comparative purposes, the behaviour of 2 other oli^R mutants derived from strain J69-1B is shown in table 2A, namely 0814 and 0850 which show, respectively, complete sensitivity or strong resistance to venturicidin.

The technique of cytoduction, utilizing crosses involving haploids carrying the *kar1-1* mutation that prevents efficient nuclear fusion [15], enables mitochondrial genomes to be transferred to specified nuclear backgrounds in a systematic way [10]. Table 2B indicates the phenotypes of cytoductants constructed so as to place the mtDNA genomes of mutants L4100 and 0851 in common nuclear backgrounds, namely those of strains J69-1B, L410 and 8960-14B (the last-named is the *kar1* mutant strain used in the cytoduction manipulations). It is clear from the results that the critical determinant of venturicidin resistance of the Phe$_{64}$→Leu mutants is the nuclear background in which the mtDNA is resident. For example, mtDNA from mutant 0851 in the L410 background now elicits marked resistance to venturicidin even at 5 μg/ml, indistinguishable from that of L4100 mtDNA cytoduced into the same L410 nuclear background. Furthermore, when L4100 mtDNA is placed in the J69-1B nuclear background the venturicidin resistance pattern (now very weak) corresponds exactly to that of a cytoductant carrying 0851 mtDNA introduced into the J69-1B background (note that this pattern mimics exactly that of the original mutant 0851). Although the growth rate on non-fermentable substrates of cytoductants in the 8960-14B nuclear background is reduced relative to that of other strains (donors, or cytoductants in other backgrounds), a similar pattern of resistance to venturicidin conferred by mtDNA genomes of both L4100 and 0851 is observed (table 2B).

3.4. *Domains in subunit 9 for oligomycin and venturicidin resistance*

A compilation is made in fig.1 of all reported

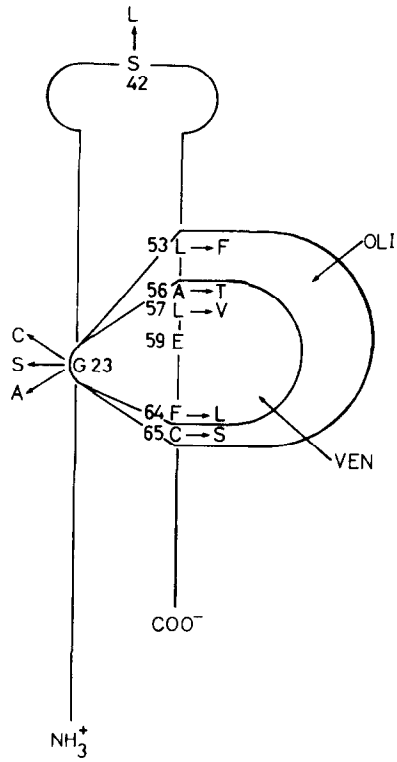

Fig.1. Domains of oligomycin and venturicidin resistance in yeast mtATPase subunit 9. The subunit 9 polypeptide is represented in a hairpin configuration [5,11], with the 2 membrane-spanning domains indicated as vertical straight lines. Amino acids relevant to drug resistance domains are indicated here by residue number; using single-letter code, the wild-type residues and substitutions in sequenced mutants ([3,5]; this paper) are shown. The venturicidin-resistance domain (VEN) overlaps with and lies entirely within the oligomycin-resistance domain (OLI). Also indicated are the positions of Glu$_{59}$ (the DCCD-binding site) and Ser$_{42}$ which lies in the hydrophilic loop, but which is not thought to lie directly in the oligomycin-binding domain [5].

amino acid substitutions in mtATPase subunit 9 of *S. cerevisiae* leading to oligomycin or venturicidin resistance. Domains of amino acid considered to be involved in the binding sites for these 2 drugs [5] are indicated. The newly sequenced mutants fall largely within these domains as previously identified, with the extension that the venturicidin domain now includes residue 56. A further substitution at residue 23 (Gly→Ser) has also been identified.

Recently, Sebald et al. [16] have carried out chemical modification studies on *Neurospora crassa* subunit 9 using a lipid-soluble carbene (TID) as a photoaffinity probe [17]. About 10 residues scattered through the 2 hydrophobic stems of the polypeptide were found to be labelled. The chemical modification of those residues around Glu_{65} (DCCD-binding site in the *Neurospora* peptide) was blocked by oligomycin, leading to the conclusion that certain residues in the region Ser_{55}–Phe_{70} (corresponding to yeast residues Pro_{49}–Phe_{64}) are involved in binding oligomycin. This chemically defined binding domain corresponds broadly to the resistance domain on the C-terminal stem shown in fig.1. Surprisingly, oligomycin was not found to protect any residues in the N-terminal stem, particularly those in the vicinity of residues equivalent to yeast Gly_{23}. This may reflect the failure of TID to bind covalently to amino acids in the putative oligomycin-binding domain on the N-terminal stem. Nevertheless, it cannot yet be excluded that Gly_{23} does not participate directly in binding oligomycin and that substitutions at this position lead to the oli^R phenotype by interactions that distort the conformation of the binding domain on the C-terminal stem.

ACKNOWLEDGEMENTS

This work was supported by the Australian Research Grants Scheme. We thank Professor A.W. Linnane for useful discussions.

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