Biogenesis of mitochondria: DNA sequence analysis of *mit*⁻ mutations in the mitochondrial *oli1* gene coding for mitochondrial ATPase subunit 9 in *Saccharomyces cerevisiae*

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ABSTRACT

The nucleotide sequence of the yeast mitochondrial *oli1* gene has been obtained in a series of *mit*⁻ mutants with mutations in this gene, which codes for subunit 9 of the mitochondrial ATPase complex. Subunit 9 is the proteolipid, 76 amino acids in length, necessary for the proton translocation function of the membrane F₀-sector. These mutants were classified on the basis of their rescue by a petite strain shown here to retain the entire wild-type *oli1* gene. The mutation in one *mit*⁻ strain removes a positively charged residue (Arg₃₉ → Met) which is likely to be located in a segment of subunit 9 that protrudes from the inner mitochondrial membrane. In a second *mit*⁻ mutant, a negatively charged residue replaces a conserved glycine residue (Gly₁₈ → Asp) in a glycine-rich segment of the protein that is most likely embedded within the membrane. Other *mit*⁻ mutations result in frameshifts with predicted products 7, 65 and 68 amino acid residues long. In each *mit*⁻ mutant, there is the loss of one or more of the amino acid residues that are highly conserved among diverse species. The location and nature of specific changes pinpoint amino acid residues in subunit 9 essential to the activity of the mitochondrial ATPase complex.

INTRODUCTION

As a result of electron transport along the respiratory chain in the mitochondrion, a proton gradient across the inner mitochondrial membrane is generated. There is considerable evidence that the potential energy of this gradient is subsequently utilized to drive the conversion of ADP to ATP (1,2) by an oligomycin-sensitive mitochondrial ATPase (mtATPase) complex located on the inner mitochondrial membrane. On the basis of recent immunoprecipitation data it has been concluded that the mtATPase complex of *Saccharomyces cerevisiae* contains ten different polypeptides (3,4). Five of the seven subunits encoded by nuclear genes constitute the F₁-sector of the complex (5), which retains oligomycin-insensitive ATPase activity when isolated from the mtATPase complex and therefore harbours the ADP/ATP binding sites of the complex (6). The three mitochondrially encoded subunits are present in the F₀-sector of the complex (3,4). The F₀-sector

provides a proton channel embedded in the inner mitochondrial membrane in such a way that proton translocation is coupled to ATP synthesis in the intact mtATPase complex (6). The three yeast F_0 -sector subunits, exhibiting the properties of membrane proteins and denoted mtATPase subunits 9, 8 and 6, are products of mitochondrial genes *oli1* (7,8), *aap1* (9) and *oli2* (10-12), respectively.

Subunit 9 is thought to play a key role in providing the proton channel for the ATPase complex, but little is known about the way in which individual amino acid residues of subunit 9 contribute to that proton channel and its coupling to ATP synthesis. Dicyclohexylcarbodiimide (DCCD) inhibits ATPase complex activity (13) by inhibiting proton translocation across the membrane (14,15). The covalent binding of DCCD to yeast subunit 9 residue Glu₅₉ (16) seems to implicate that residue in proton translocation.

In this paper we identify additional specific amino acid residues of subunit 9 that play an essential role in the proper function of the mtATPase complex. These results are obtained from a nucleotide sequence analysis of the *oli1* gene in *mit*⁻ mutants which have lost the capacity to grow on a non-fermentable substrate.

MATERIALS AND METHODS

Yeast Strains

Saccharomyces cerevisiae strain J69-1B α *ade1 his5 [rho⁺]* (17) is the wild-type parent of the *mit*⁻ mutants 2422, 5102, 3861, 5726, 51223 and 5208, all of which were derived by mutagenesis with manganese chloride (18). Petite strain 23-3, which retains the mitochondrial *oli1* locus in a 1.35 kb segment of mtDNA, arose spontaneously from 70M *ade1 lye2 trp1 [rho⁺ oli2-23r]* (17). Growth of strains and genetic analyses were carried out as previously described (19). In petite marker rescue experiments between the *rho*⁻ mutant 23-3 and *mit*⁻ mutants, the resulting diploids were tested on media containing ethanol as the carbon source for their ability to grow as wild-type diploids.

Nucleotide sequencing strategy

The mtDNA of each strain was isolated and purified on KI density gradients containing the dye Hoechst compound 33 258 (20). The purified mtDNA was digested with HpaII and cloned into the AccI site of M13mp10 (21). Plaques of recombinants containing DNA fragments from the *oli1* gene region were selected by hybridization (22) to radioactively labelled DNA

probes. These probes were either (a) 23-3 mtDNA, labelled by nick-translation or by a 3'-terminal fill-in reaction of HpaII digested DNA using [α - 32 P]dGTP and DNA polymerase I (Klenow fragment) or (b) synthetic oligonucleotides labelled at the 5'-end using [γ - 32 P]ATP and polynucleotide kinase. Orientation of the cloned fragments in the M13 vector was determined by hybridization to the 5'-end labelled synthetic oligonucleotides. The nucleotide sequence of the selected recombinants was determined by the chain-termination method (23) using synthetic oligonucleotides as primers (Table 1). Oligonucleotides were synthesized using an Applied Biosystems 380A DNA synthesizer.

RESULTS

Initial characterization of the mutants

The *mit*⁻ mutant strains 2422, 5102, 3861, 5726, 51223 and 5208 were initially selected for their inability to grow on a non-fermentable carbon source. These respiratory deficient strains, when mated to the respiratory deficient petite 23-3, give rise to respiratory competent diploid progeny. This result localizes these mutations to the 1.35 kbp segment (17,24) of the mitochondrial genome retained in petite 23-3. In addition, the altered electrophoretic mobility of mtATPase subunit 9 in strain 2422 and the failure to detect subunit 9 in strains 3861 and 5726 (3,25) suggest that the mutations of some or all of these mutants occur in the *oli1* gene. A study was initiated to determine whether the sites of mutation in these *oli1 mit*⁻

Table 1. Synthetic oligonucleotides used as primers for sequencing the *oli1* gene.

Primer	Length in Nucleotides	Nucleotide Sequence	Location ^a	Strand Hybridized
1	25	5'-ATAATATAAAATAAGAATAGATTA-3'	-22 to -46	non-mRNA-like
2	13	5'-AGTATTAGCAGCT-3'	9 to 21	non-mRNA-like
3	25	5'-TATTAATTTTCATTATTTTTTATTTA-3'	252 to 276	mRNA-like
4	15	5'-ACACCGAATAATAAT-3'	213 to 227	mRNA-like
5	30	5'-CCTGCTCCAATATATTTAGCTGCTAATACT-3'	9 to 38	mRNA-like

^a The location of the nucleotide sequence to which the primer hybridizes is specified with reference to numbering in Figure 2.

Figure 1. The strategy employed to obtain the nucleotide sequence of mtDNA from wild-type J69-1B, *olil* *mit*⁻, and petite strains in the vicinity of the *olil* gene. The nucleotide sequence was obtained by the chain termination method using one of the primers, 1-5 (see Table 1), with the appropriate single-stranded M13 recombinant template. The location of each primer is indicated with respect to the *olil* gene (hatched); numbers refer to the sequence shown in Figure 2. The flanking HpaIII sites are 1.1 kb apart on J69-1B mtDNA. Arrows indicate the extent, and direction, of the particular mtDNA sequences determined.

mutants are located within the coding region of the *olil* gene and, if so, the effect of such mutations on the amino acid sequence of the mtATPase subunit 9 that would be synthesized in the mutants.

The approach for determining the nucleotide sequence of mutations in the *olil* gene

The objectives of this study required that the nucleotide sequence in the vicinity of a particular gene be determined in a series of mutant strains. In order to accomplish this most conveniently, a strategy was chosen that involved the M13 chain-termination nucleotide sequencing method utilizing synthetic oligonucleotides unique to the *olil* gene region (Table 1 and Figure 1). The mtDNA from wild-type strain J69-1B and from the *mit*⁻

Figure 2. The nucleotide sequence of the yeast mitochondrial *oli1* gene in strain J69-1B. The coding region has been translated using the yeast mitochondrial genetic code (26). For the region of the J69-1B mitochondrial genome shown here, the sequence of petite strain 23-3 is identical. Numbering of nucleotides and amino acids is shown below and above each line, respectively. Asterisk indicates termination codon.

mutants was digested with HpaII and cloned into the AccI site of M13mp10. In most cases, both orientations of the relevant fragment inserted into the phage DNA were obtained, using the synthetic oligonucleotides as strand-specific hybridization probes to identify them. It should be noted that the HpaII sites bounding the 1.1 kb fragments of mtDNA cloned in this manner lie at least 300 bp from the coding region of the *oli1* gene.

Nucleotide sequence in wild-type *oli1* gene

The nucleotide sequence of the entire *oli1* gene coding region in *Saccharomyces cerevisiae* strain J69-1B was determined (Figure 2). This coding region, translated using the yeast mitochondrial genetic code (26), specifies a polypeptide of 76 amino acids identical to subunit 9 of the mtATPase complex, the DCCD-binding proteolipid (27). We report here the first complete wild-type (oligomycin sensitive) sequence of the *oli1* gene (Figure 2). The *oli1* gene sequence previously reported was obtained from oligomycin resistant (*oli*^R) strains KL14-4A (7) and D273-10B/A21 (8). In these *oli*^R strains, codon 53 (UUU) specifies phenylalanine as confirmed by amino acid composition data of one of the corresponding mutant strains

Table 2. Nucleotide sequence changes in *oli1 mit⁻* mutant strains.

Strain	Nucleotide Number ^a	DNA Sequence Change	Codon Number ^a	Codon Change	Amino Acid Residue Change	Predicted Length ^b
J69-1B	none	none	none	none	none	76
2422	116	G → T	39	AGA → AUA	Arg → Met	76
5102	53	G → A	18	GGU → GAU	Gly → Asp	76
3861	13	T inserted	5	UUA → UUU	frameshift ^c	7 ^c
5726	169	T deleted	57	UUA → UAU	frameshift ^d	65 ^d
51223	206	C → A	69	UCA → UAA	Ser → stop	68
5208	206	C → A	69	UCA → UAA	Ser → stop	68

^a The nucleotide and codon numbering refer to Figure 2.

^b The predicted gene product length is stated in terms of amino acid residues.

^c In strain 3861, the insertion at nucleotide 13 causes a frameshift resulting in a polypeptide with predicted carboxy-terminal residues 5-7: FSS.

^d In strain 5726, the deletion at nucleotide 169 causes a frameshift resulting in a polypeptide with predicted carboxy-terminal residues 57-65: YQKTQVYSV.

(28). In addition, when these *oli1* gene sequences of the *oli^R* mutants were published, it was not yet known that the yeast mitochondrial genetic code differed from the universal code and that codon 46 (CUA), codes for threonine instead of leucine in yeast mitochondria (29).

Nucleotide sequence changes in *oli1 mit⁻* mutants

Each of the *oli1 mit⁻* mutants investigated here was shown to contain a single mutation in the *oli1* gene coding region (Table 2). The mutations observed include base transition, base transversion, and the insertion or deletion of a base.

A base transversion G → T occurs at nucleotide +116 in strain 2422. This results in a missense mutation affecting codon 39, which in wild-type is AGA coding for arginine. In strain 2422 codon 39 becomes AUA which has been suggested (26) to specify methionine in *S. cerevisiae* mitochondria. Amino acid sequencing data on the subunit 9 polypeptide isolated from strain 2422 (D. Tweeddale, unpublished data) indicate that a methionine is indeed found at position 39, thus confirming this codon assignment for AUA. Thus, residue 39 of subunit 9 in this mutant undergoes a change from an amino acid that is positively charged at neutral pH to one that is hydrophobic and uncharged. Strain 5102 has undergone a base transition G → A at nucleotide +53. This engenders a missense mutation resulting in the change of glycine (GGU) to a negatively charged aspartic acid (GAU) at position 18 in the

Strain	Predicted Amino Acid Sequence	Predicted Length
J69-1B wild type	MQLVLAAKYI GAGISTIGLL GAGIGIAIVF AALINGVSRN PSIKDTVFPN AILGFALSEA TGLFCLMVSF LLLFGV	76
2422 mutant	_____ M _____	76
5102 mutant	_____ D _____ 39	76
3861 mutant	_____ 18 _____	7
5726 mutant	FSS _____ YQKT QVYSV	65
51223 mutant	_____ 57 _____	68
5208 mutant	_____	68

Figure 3. Predicted amino acid sequences of the *oli1* gene products in wild-type and *mit⁻* mutant strains. The lines indicate regions identical to the wild-type sequence and only residues that differ from wild-type are indicated, together with the position (number) of the altered amino acid or the first altered amino acid in a run. Predicted length is stated in terms of amino acid residues.

polypeptide, which remains the same length as in wild-type. Identical nonsense mutations occur in strains 51223 and 5208 in which the C → A change at nucleotide +206 creates a termination codon from serine codon 69; the predicted polypeptide has a shortened length of 68 amino acids. The mutation in strain 5726 is caused by the deletion of a single T at nucleotide +169. The resulting frameshift results in a predicted polypeptide 65 amino acids long, consisting of the 56 N-terminal amino acid residues of mtATPase subunit 9 followed by nine further residues from out-of-frame translation (Table 2, Figure 3). Not only is the altered gene product in strain 5726 shorter, with multiple incorrect residues at its C-terminal end, but in addition there is a positively charged lysine in the position of the normal negatively charged glutamic acid at amino acid residue 59. Strain 3861 has the insertion of a T at nucleotide 13; this produces a highly truncated polypeptide 7 amino acids in length, of which only the first 4 correspond to those at the N-terminus of wild-type subunit 9.

DISCUSSION

General aspects of mtATPase subunit 9 structure

Yeast mtATPase subunit 9 is a membrane protein which has a major role in providing the channel for proton translocation in the mitochondrial mtATPase complex. One residue identified as important in this function is glutamic acid residue 59 to which DCCD covalently binds in inactivating the mtATPase complex (16). Other residues in subunit 9 are undoubtedly important in its function but to date there is little direct evidence that

Figure 4. Amino acid sequence of the *olil* gene product indicating the consequences of mutation in *olil mit-* strains. The amino acid sequence (using single letter code) is drawn to reflect the membrane spanning potential of two segments of the polypeptide. It has been suggested that the two vertical arms lie within the membrane while the loop at the top of the figure and the N-terminus at the bottom protrude from either side of the membrane (30,32). Current models indicate the loop of the hairpin to face the F_1 side of the membrane. Evolutionarily conserved residues are based on the amino acid sequences of proteolipid from *Neurospora crassa*, bovine and *Saccharomyces cerevisiae* mitochondria, spinach chloroplasts, and cell membranes of *Mastigocladus laminosus*, *Escherichia coli* and the thermophilic bacterium PS-3 (30); (X), absolutely conserved amino acid residue X; (X), highly conserved amino acid residue X (two alternatives only, alternate specified in small character); (+), (-), charge is absolutely conserved; (*), conservation of uncharged polar residue.

indicates the significance of particular amino acid residues. The amino acid sequence of yeast mtATPase subunit 9 has been compared with the sequence of the analogous protein from diverse species (30). This

comparison reveals a number of conserved amino acid residues at which there are either only one or two alternatives observed (Figure 4). Two clusters of conserved residues are present in two hydrophobic, potentially membrane spanning segments of subunit 9 (30-32). In one case, alternating glycines are conserved in a highly glycine-rich segment; in the other case, the conserved residues are clustered around a negatively charged residue. In the case of *S. cerevisiae*, this is the glutamic acid residue at position 59, to which DCCD can covalently bind (16). As mentioned above, DCCD inhibits the ATPase complex activity by inhibiting proton translocation across the membrane. A third cluster of conserved residues exists in the vicinity of the absolutely conserved arginine residue 39, that is likely to be outside of the membrane on the basis of its hydrophilicity (cf. Figure 4).

Implicit in the hairpin structure envisaged for this protein (30,32), as represented in Figure 4, is the proximity of the two membrane-spanning arms. In the case of *E. coli*, this physical proximity has been inferred from the identification of amino acid substitutions in the relevant *E. coli* ATPase subunit (the proteolipid subunit c) from DCCD-resistant mutants (33). Several mutants of this type carry substitutions of the isoleucine at position 28, which lies in the N-terminal arm of the proteolipid opposite to the aspartic acid residue at position 61 (in the other arm) to which DCCD normally binds. In the yeast mtATPase complex, the proximity of the two putative membrane-spanning arms of subunit 9 is suggested by observations that resistance to oligomycin can be elicited by amino acid substitutions within the conserved regions in each of the two arms of this protein (34).

Arg₃₉ → Met

The replacement of arginine at residue 39 by methionine in subunit 9 of *mit⁻* mutant strain 2422 indicates that the arginine residue is essential for the function of the mtATPase complex. As pointed out above, residue 39 lies in a hydrophilic sector between the two hydrophobic sectors and thus it is likely that it protrudes out of the inner mitochondrial membrane. If the residue is exposed to the matrix side of the membrane, then it may serve a role in binding ligands near the F₁-sector of the complex. Alternatively this region might be involved in binding the mtATPase subunits together properly. In this case, however, the arginine cannot be essential for the association of the eight majority subunits (F₁ and F₀) of the complex of strain 2422 since immunoprecipitation of the mtATPase complex of that strain with monoclonal antibody to the β subunit of the F₁-sector precipitates the five F₁ and three F₀ polypeptides (25).

Gly₁₈ → Asp

In strain 5102 the highly conserved glycine at residue 18 is replaced by a negatively charged aspartic acid residue. This substitution occurs in the glycine-rich membrane-spanning segment which, as discussed above, is possibly adjacent to the glutamic acid membrane-spanning segment. It is well established that glycine residues contribute a unique compactness and flexibility to polypeptides containing them and it may be the loss of these characteristics which inactivates the mtATPase complex. Another critical factor is the insertion of a negatively charged residue which could interfere with the stability of that region of the protein within the membrane or could interfere with other charged moieties such as Glu₅₉.

In evaluating this mutant in more detail, it is profitable to consider here the possible secondary structure of this segment of subunit 9 in its hydrophobic, membrane-spanning environment in the mtATPase complex. It has been suggested that membrane-spanning regions of certain proteins must be α -helical in order to avoid the energy cost of locating hydrogen bond donors or acceptors in a nonpolar environment (35), as is reported for bacteriorhodopsin (36). Assuming that membrane-spanning segments are α -helical, and given that glycine is generally considered a strong helix breaker in globular proteins (37), it has been recommended that arbitrary changes in the criteria of secondary structure predictions regarding glycine be made in order to obtain a prediction of α -helical content of this glycine-rich stretch of subunit 9 (31). Such an approach might be justifiable but there may also be another possibility worthy of consideration. The conserved, alternating glycines in this region of subunit 9 (see Figure 4) bear some resemblance to the (gly-ser-gly-ala-gly-ala)_n sequence found in silk fibroin and known to exist in β -sheets with glycine facing one side (38). Furthermore, mtATPase subunit 9 has an estimated stoichiometry of more than 3 (39) and probably 6 in the yeast mtATPase complex on the basis of the observed multimeric form of subunit 9 (40) and inhibitor binding sites (28). It may therefore be feasible that yeast mtATPase subunit 9 is in a β -barrel configuration (41). Other polypeptide membrane-spanning segments, such as the subunit 9 region around Glu₅₉, could perhaps be involved as well. The glycine to aspartic acid change at residue 18 of mtATPase subunit 9, could then be the alteration of a residue on the barrel surface, inside or outside. Such an alteration on the interior could block the proton channel or inhibit certain conformations of the channel whereas alteration to the exterior might disrupt the

association of subunit 9 with another subunit; such possibilities are amenable to future tests.

Frameshift Mutations

It is possible to study not only the effects of single amino acid residue substitutions, but also the effects of the removal of substantial segments of the protein. Such alterations can arise from premature termination or frameshift mutations. The observation that strains 51223 and 5208 are *mit⁻* in character demonstrates that the C-terminal eight residues of mtATPase subunit 9 are essential for growth of cells on a non-fermentable substrate. These eight residues include two highly conserved phenylalanine residues (Figure 4). The number of wild-type residues of subunit 9 retained in the mutant *oli1* gene product ranges from 4 out of a total of 76 in strain 3861, to 65 in strain 5726, and 68 in strains 51223 and 5208.

Upon further analysis of the strains, these strategically located mutations promise to yield insight into the extent to which the truncated polypeptide segments play a role in the assembly and integrity of the multi-subunit mtATPase complex in yeast. It is already apparent (25) that mutations of this type can have a much more severe effect on the assembly of the mtATPase complex and on mitochondrial membrane function in general as compared to the situation in strain 2422, in which the mtATPase complex is assembled (but non-functional) and there is relatively little pleiotropic effect on other respiratory functions. By contrast, in those *oli1* mutants where the subunit 9 polypeptide is extensively altered or effectively absent, not only are there defects in the assembly of the F_0 -sector but there are also interesting pleiotropic effects on the assembly of functional cytochrome *b* c_1 and cytochrome *c* oxidase complexes (25).

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