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Correlation of defined lesions in the *N,N'*-dicyclohexylcarbodiimide-binding proteolipid with defects in the function and assembly of yeast mitochondrial H^+-ATPase and other respiratory enzyme complexes *

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Functional and assembly defects which occur in the H^+-ATPase complex of *mit*$^-$ mutants of *Saccharomyces cerevisiae* with mutations in the structural gene for subunit 9 of the enzyme complex have been investigated in order to define functionally important structural features of this subunit. The mutations in these strains have previously been characterised by DNA sequence analysis. A correlation between the alterations in the subunit 9 structure and the functional and/or assembly defects assign critical roles to the C-terminal arm of subunit 9 and to a glycine residue at position 18 in the assembly of the polypeptide. In addition, the assembly of subunit 9 to the H^+-ATPase modulates the synthesis of subunit 6 of the enzyme complex, and markedly affects the assembly of the respiratory enzyme complex, cytochrome oxidase. The level at which these secondary effects take place was investigated and shown to occur post-translationally in the case of the cytochrome *c* oxidase complex. The implications of this observation in the structural organisation of the inner mitochondrial membrane are discussed.

Introduction

The structure and function of the DCCD-binding proteolipid of mitochondrial H^+-ATPase has been the focus of intensive investigations in a number of laboratories because of the key role proposed for this subunit in the proton channel activity of the F_0 (membrane) sector of the enzyme complex [1–3]. The translocation of protons through the proton channel of the F_0 sector is coupled to ATP synthesis and hydrolysis on the F_1 (matrix) sector. In the yeast *Saccharomyces cerevisiae*, the proteolipid (subunit 9) is well characterized; it is a polypeptide of 76 amino-acid residues with a molecular weight of 7.6 kDa. The primary structure of the polypeptide has been determined [4] and shown to correspond exactly to the nucleotide sequence of the *oli*1 gene [5,6]. This proteolipid, together with two other components, subunit 6 ($M_r = 20000$) and subunit 8 ($M_r = 10000$) make up the F_0 sector in yeast; subunit 6 is believed to play a role in the coupling of oxidation to phosphorylation [7], whereas subunit 8

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Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; Cyt, cytochrome.

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may be involved in phosphate transport [8].

Little information, however, is available about the organisation of these three subunits in the F_0 sector and the manner in which they are integrated into the hydrophobic membrane environment. The DCCD-binding proteolipid, subunit 9, is envisaged to have a hair-pin structure with two proximal membrane spanning arms of hydrophobic residues and a hydrophilic loop, protruding into the mitochondrial matrix [9–11]. The N-terminal end also consists of hydrophilic residues and possibly protrudes into the intermembrane space. The identity of amino-acids which contribute to the maintenance of this proposed structure and of amino-acids important in proton channel activity is not, at present, well resolved. By analysing the amino-acid sequence of subunit 9 in a wide variety of organisms, Sebald [11] has assigned important roles in the structure and function of this subunit to three amino-acid residues (namely glutamic acid, proline and arginine) conserved at various places along the proteolipid molecule among at least seven different organisms analysed. Thus, DCCD binds covalently to a glutamic acid (or an aspartic acid) residue in the distal hydrophobic arm of the polypeptide at position 59 in yeast [12]; as a result, proton translocation across the membrane is inhibited [13,14]. A structural role has been assigned to the only proline residue which is at position 41 in yeast, since in the seven organisms analysed, its position is invariant. Several arginine residues are also highly conserved, and one of them at position 39 in yeast, is invariant. It has been suggested that this residue could have a catalytic function [11].

The use of genetically defined mutants of subunit 9 with specific lesions at various sites in the *oli*1 gene is a more direct way for defining functionally important structural features of the proteolipid. This approach has been used to define drug-binding domains within the proteolipid molecule [15,16]. Thus, results of DNA sequence analysis of the *oli*1 gene in a number of oligomycin and/or venturicidin resistant mutants suggest that the binding domains for these inhibitors span the C-terminal portion of the molecule (amino acids 53–65) together with a conserved Gly_{23} on the N-terminal arm [15,16]. Seven *oli*1 *mit*$^-$ mutants, which are defective in oxidative metabolism, and one temperature-conditional mutant (cold sensitive at 18°C), have recently been characterised by DNA sequence analysis in our laboratory [16,17]. The mutations occur at various places along the gene. We have now characterized these mutants for functional and assembly defects in the H^+-ATPase. By correlating the predicted alteration(s) in the subunit 9 polypeptide with the functional and assembly defects, we define domains on the molecule and particular amino-acid residues which are important in the function and assembly of the H^+-ATPase. In addition, we show that subunit 9 assembled to H^+-ATPase affects the assembly of other respiratory enzyme complexes, in particular cytochrome oxidase. This example of pleiotropy indicates that at least some of the inner mitochondrial membrane complexes are very closely associated, either physically or functionally.

Materials and Methods

Yeast strains

The seven *mit*$^-$ strains used in this study, strains 38.6.1, 5726, 5102, 15B2, 5208, 51223 and 2422, were derived in this laboratory from the wild-type *Saccharomyces cerevisiae* strain J69-1B (*α adel his*5 [*rho*$^+$]) following mutagenesis with $MnCL_2$ [19]. They were selected for their inability to grow on a non-fermentable energy source. The conditional mutant 811 was isolated originally as an oligomycin resistant (4 μg/ml) strain growing on a nonfermentable carbon source at 28°C, but was later shown to be cold sensitive at 18°C for growth on a non-fermentable substrate. The mutations in these strains have been characterized by DNA sequencing of the *oli*1 gene [17]; the predicted amino-acid changes in each mutant are given in Table I.

All yeast strains were stored on solid agar media containing glucose (20 g/l; Ajax), yeast extract (10 g/l; Difco laboratories, Detroit, MI), peptone (20 g/l; Oxoid Ltd., London) and agar (15 g/l). The strains were subcultured at least every 2–3 months.

Cell growth

To minimize the effects of catabolite repression, cells used to study the synthesis and assem-

bly of mitochondrial translation products and for enzyme activities, were grown at 28°C to a steady-state in glucose-limited chemostat cultures in modified LKB ultraferm fermenters [20]. The growth medium contained glucose (20 g/l), yeast extract (10 g/l), a salts mixture [21] and the auxotrophic requirements, adenine (100 μg/ml; Sigma) and histidine (50 μg/ml; Sigma); the working volume was 500 ml. Each culture fermenter was inoculated with 20–30 colonies taken from a Petri dish culture and grown under batch culture conditions for 6–8 h. Fresh growth medium was then pumped into the cultures at a dilution rate of 0.1 h^{-1} for 40–45 h. Under these conditions, the steady state glucose level of the cultures was maintained below that which induces catabolite repression. All cultures were routinely checked for petite mutants and revertants.

Radioactive labelling of mitochondrial translation products and their analysis by SDS-polyacrylamide gel electrophoresis

For the analysis of mitochondrial translation products, cells were grown in glucose-limited chemostat cultures to ensure the maximum synthesis of mitochondrial translation products. These proteins were labelled in vivo for 60 minutes at 28°C in a low sulphate medium containing [^{35}S]sulphate and cycloheximide which specifically inhibits cytoplasmic protein synthesis [22]. After labelling, the cells were washed and resuspended in a buffer containing 0.3 M mannitol/0.26 M sorbitol/0.6 mM EDTA/12 mM Tris (pH 7.2). Mitochondria were isolated from mechanically ruptured cells [23]. The products of mitochondrial protein synthesis were separated electrophoretically on SDS-polyacrylamide gels [42] and visualized by autoradiography [43].

Immunoprecipitation of H^+-ATPase

For immunoprecipitation of H^+-ATPase, mitochondria were prepared as described above from cells labelled in the presence of cycloheximide, except that the buffer used to resuspend the cells before rupture contained the protease inhibitors, 10 mM *para*aminobenzamidine-HCl, 10 mM ε-amino-*N*-caproic acid and 2 mM phenylmethylsulphonyl fluoride [24]. F_0 subunits which are assembled to the F_1 sector were immunoprecipitated from Triton extracts of mitochondria essentially as described previously [24] using the Sepharose-conjugated monoclonal antibody RH 48.6 which recognizes an epitope on the β subunit. After an incubation of 1.0 h at 4°C with the monoclonal antibody, the antigen-antibody complex attached to the beads was washed three times in a buffer consisting of 0.1% Triton X-100, 0.1% delipidated bovine serum albumin and 5 mM Tris-acetate (pH 7.4). Mitochondrial translation products in the immunoprecipitates were visualised by fluorography [43] after electrophoresis [42].

Immunoprecipitation of cytochrome c oxidase complex

The cytochrome oxidase complex was immunoprecipitated from Triton extracts of ^{35}S-labelled mitochondria using a rabbit polyclonal antiserum raised specifically against the cytochrome *c* oxidase complex [25]. *Staphylococcus aureus* carrying protein A was used as an antibody absorbant.

Assays of mitochondrial enzyme activities

Mitochondria used in the study of mitochondrial function were isolated from spheroplasts prepared by zymolase digestion of the cell wall [26]. Delipidated bovine serum albumin was added (2 mg/ml) to the isolation buffer prior to rupture of the spheroplasts and in all subsequent steps, ensuring isolation of intact, coupled mitochondria.

Mitochondrial respiration was assayed polarographically at 28°C in 3 ml of 10 mm Tris/10 mM phosphate buffer (pH 7.4) containing 0.6 M sucrose/1.2 mM EDTA and delipidated bovine serum albumin (1.7 mg/ml). The concentration of mitochondria was 0.2–0.3 mg/ml and the reaction was started by the addition of 10 μl absolute ethanol.

Difference spectra of mitochondrial cytochromes were recorded at the temperature of liquid nitrogen at a concentration of 6 mg/ml in a 13 mM Tris buffer (pH 7.4) containing 0.6 M sorbitol/0.7 mM EDTA [27]. The spectra of sodium dithionite reduced mitochondria were recorded against potassium ferricyanide oxidized mitochondria.

Published procedures were employed to de-

termine the activities of NADH-cytochrome *c* reductase [28] and cytochrome *c* oxidase [29] and oligomycin-sensitive mitochondrial ATPase activity [30].

ATP-$^{32}P_i$ exchange activity was determined essentially as in Ref. 31 with minor modifications as described previously [7].

Results

Description of mutants

The mutations in the seven *mit*$^-$ mutants and one temperature-sensitive mutant are described in Table I. The mutant strains can be divided into three groups on the basis of the nature of their mutations. (a) Base substitution mutations which give rise to full-length subunit 9 polypeptides; the mutations occur at position 39 where a conserved positively charged Arg is substituted by an uncharged Met, at position 18 where a conserved Gly is replaced by Asp and at position 23, where again a conserved Gly is replaced by either Asp or Cys. (b) Frameshift mutations which lead to premature termination such that truncated polypeptides are synthesized; two such mutations result in polypeptides of 7 and 65 amino-acids residues. (c) Mutations to a stop codon which also give rise to truncated polypeptides, here, two identical mutations predict products of 68 amino acids.

Irrespective of the type and nature of their mutations, none of the *mit*$^-$ mutants are able to grow by oxidative phosphorylation. These mutants only grow to a cell density of around 2 mg/ml in a growth medium containing 2% glucose as an energy source, compared to a cell density of 6 mg/ml for the wild-type strain. The growth yield of the *mit*$^-$ mutants is indeed comparable to a *rho*^{0} petite strain completely devoid of mtDNA and which can, therefore only grow by fermentative metabolism. In contrast, the temperature-conditional mutant strain, was found to grow to some extent by oxidative metabolism. However, its generation time at 18°C is about three times that of the wild-type strain at that temperature.

TABLE I

NATURE OF ALTERATIONS OF THE SUBUNIT 9 MOLECULE IN THE *oli*1 *mit*$^-$ MUTANT STRAINS

The mutations in the mutant strains were defined by DNA sequencing [17]. Amino-acid residue change and length of subunit 9 were deduced from the DNA sequence.

Strain	Amino-acid residue change	Predicted length of polypeptide (amino-acid residues)
J69-1B	none	76
2422	$Arg_{39} \rightarrow$ Met	76
5102	$Gly_{18} \rightarrow$ Asp	76
15B2	$Gly_{23} \rightarrow$ Asp	76
38.6.1	frameshift [a]	7 [a]
5726	frameshift [b]	65 [b]
51223	$Ser_{69} \rightarrow$ stop	68
5208	$Ser_{69} \rightarrow$ stop	68
811	$Gly_{23} \rightarrow$ Cys	76

[a] In strain 38.6.1, the insertion at nucleotide 13 causes a frameshift resulting in a polypeptide with predicted carboxy-terminal residues 5–7: FSS.

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

Impairment in function of the H$^+$*-ATPase complex*

The effect of the mutations on the function of the H$^+$-ATPase was analysed (a) by determining the oligomycin sensitivity of the ATPase activity and (b) by directly measuring ATP-$^{32}P_i$ exchange activity of mitochondria isolated from the mutants.

All the mutant strains studied were found to have significant although reduced levels of mitochondrial ATPase activity (Table II). Thus, while the mitochondrial ATPase activity in the wild-type strain is around 0.97, the activity in the *mit*$^-$ mutant strains varies between 0.21 and 0.59. However, the ATPase activity in the mutant strains was found to be significantly less sensitive to inhibition by oligomycin, varying between 11–32% as compared to 80% inhibition in the wild-type strain. In fact, the level of inhibition by oligomycin in the *oli*1 mutants is comparable to that in a mtDNA-less petite strain, which lacks the F_0 sector of the H$^+$-ATPase. Lack of sensitivity to oligomycin was observed not only for major alterations in the structure of subunit 9, as in cases where the polypeptide is truncated (strains 38.6.1, 5726, 51223, 5208), but also in mutants where the structural alteration consists of a single base substitution in a full-length product (strains 2422, 5102, 15B2, 811). This observation suggests that in

TABLE II

ATPase ACTIVITIES AND ATP-$^{32}P_i$ EXCHANGE ACTIVITIES OF MITOCHONDRIA ISOLATED FROM THE *oli*1 MUTANT STRAINS

The wild-type and *mit*$^-$-mutant strains were grown in glucose-limited chemostat cultures at 28°C at a dilution rate of 0.1 h^{-1}. The temperature-sensitive strain 811 was grown at the non-permissive growth temperature of 18°C under similar conditions. Intact mitochondria were prepared from spheroplasts; ATPase activity was measured at 28°C as described previously [30] and oligomycin was added at about 100 μg/mg mitochondrial protein. The values shown correspond to the mean and standard deviation obtained from at least four independent experiments per strain. ATP-[^{32}P]P_i exchange activity was determined at 28°C as described in Ref. 7; the values shown correspond to the mean and standard deviation obtained from at least three independent experiments per strain. Oligomycin was at 100 μg/mg mitochondrial protein; in the wild-type strain, the activity was about 55% sensitive to SF6847 (used at 20 μg/mg protein) and about 25% sensitive to KCN (used at 2 mg/mg protein).

Strain	ATPase activity (μmol/min per mg)		ATP-$^{32}P_i$ exchange activity (nmol/min per mg)	
	no oligomycin	% inhibition by oligomycin	no addition	+ oligomycin
J69-1B (wild-type)	0.97 ± 0.46	80.0 ± 8.3	49.80 ± 19.80	1.78 ± 1.05
2422	0.32 ± 0.07	27.1 ± 16.3	0.70 ± 0.50	0.57 ± 0.54
5102	0.57 ± 0.23	20.5 ± 15.1	0.90 ± 0.45	1.93 ± 1.62
15B2	0.21 ± 0.15	31.6 ± 10.2	0.05 ± 0.3	0.50 ± 0.30
38.6.1	0.59 ± 0.19	21.3 ± 15.1	0.50 ± 0.50	0.30 ± 0.30
5726	0.36 ± 0.16	14.1 ± 9.9	2.37 ± 0.28	1.43 ± 1.08
51223	0.36 ± 0.08	21.6 ± 18.6	1.23 ± 0.99	1.17 ± 0.98
5208	0.26 ± 0.12	11.6 ± 10.6	1.35 ± 0.25	0.50 ± 0.50
811	0.41 ± 0.10	22.4 ± 8.0	8.0 ± 3.0	2.0 ± 1.0
EJO (*rho*^{0})	0.80 ± 0.39	12.8 ± 8.4	0.85 ± 0.12	0.63 ± 0.21

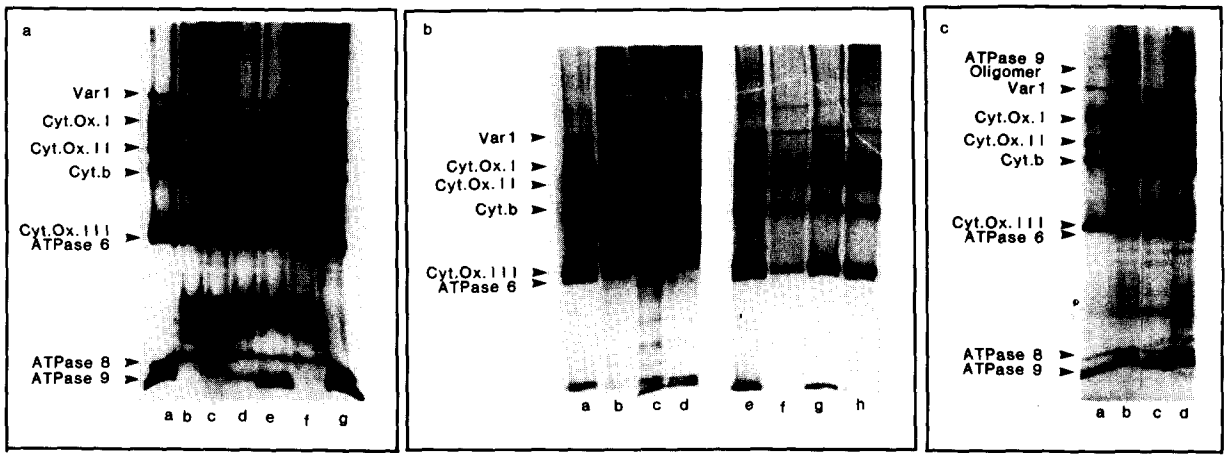

Fig. 1. Mitochondrial translatin products in *oli*1 *mit*$^-$ and temperature-conditional mutants. Cells were labelled in vivo with [^{35}S]sulphate in the presence of cycloheximide and mitochondria isolated as in Materials and Methods. Mitochondrial proteins were solubilised in SDS, electrophoresed on 12% polyacrylamide gels in the presence of SDS and mitochondrial translation products were visualized by autoradiography. (The gel size was 27×16×0.15 cm and electrophoresis was at 15 mA for 15–20 h; under these conditions, subunit 6 of H^+-ATPase ran slightly faster than subunit III of cytochrome oxidase.) Autoradiogram a illustrates total mitochondrial products from *mit*$^-$ mutants; (a) J69-1B, (b) 5726, (c) 5102, (d) 51223, (e) 2422, (f) 38.6.1, (g) 15B2. Autoradiogram b illustrates mitochondrial translation products with ATPase subunit 8 and subunit 9 having been electrophoresed to the bottom of the gel for better resolution of cytochrome oxidase subunit III and ATPase subunit 6; (a) J69-1B, (b) 5726, (c) 5102, (d) 51223, (e) J69-1B, (f) 38.6.1, (g) 2422, (h) 15B2. Autoradiogram c illustrates mitochondrial products from the wild-type strain and the temperature conditional mutant; (a) J69-1B, grown and labelled at 28°C, (b) 811, grown and labelled at 28°C, (c) J69-1B, grown and labelled at 18°C, (d) 811, grown and labelled at 18°C.

the mutant strains the ATPase activity of F_1 is no longer coupled to the H^+-translocating activity of F_0. The ATP-^{32}P exchange activity of the mutant strains shown in Table II is consistent with this explanation. Thus, a level of incorporation of 50 nmol $^{32}P_i$/min per mg of protein was observed for the wild-type strain, whereas none of the *oli*1 *mit*$^-$ strains have any significant ATP-$^{32}P_i$ exchange activity. In contrast, the temperature conditional mutant strain 811 retains exchange activity even when grown at the restrictive growth temperature of 18°C, although at a rate (at an assay temperature of 28°C) only 20% of the wild-type strain. This result is consistent with the fact that the mutant strain is able to grow to some extent by oxidative metabolism at the restrictive temperature. At the permissive temperature, ATPase and ATP-$^{32}P_i$ exchange activities appear normal (data not shown).

Synthesis of mitochondrial translation products

The mitochondrial translation products of the mutant strains were analysed by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 1, in two of the mutant strains, namely strains 38.6.1 and 5726, the subunit 9 polypeptide (M_r 7600) is apparently absent. In all the other strains, subunit 9 is synthesised, although in varying amounts. In strain 2422 the electrophoretic mobility of subunit 9 seems to be slightly faster than that of the other strains, and in strain 5102 it appears to be slightly slower. In strain 811, the mobility of the monomeric form of subunit 9 seems to be slower than that of the other strains irrespective of the temperature at which the strain was grown and labelled. In addition, the oligomeric form of subunit 9 is very prominent in this strain.

The amount of H^+-ATPase subunit 9 synthesised in the mutant strains was quantitatively estimated in a laser densitometer by scanning autoradiograms from a number of similar, but independent experiments. For each scan, the amount synthesized was calculated from the area of the peak and expressed relative to the amount of cytochrome *b*, which appeared fairly constant in all the strains. Thus, it was found that the amount of subunit 9 synthesised in strains with a full-length subunit 9 product (strains 2422 and 5102) was about 50% of the amount in the wild

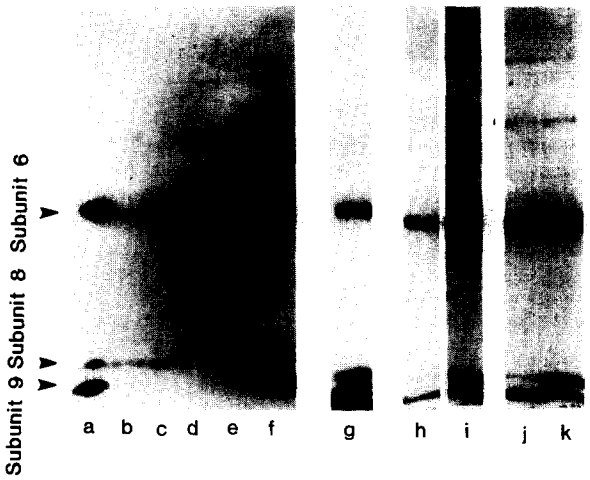

Fig. 2. Assembly of mitochondrially synthesized components of H^+-ATPase in *oli*1 *mit*$^-$ and temperature-conditional mutants. Mitochondria isolated from cells labelled in vivo (Fig. 1) were solubilised with Triton X-100 (0.25%) at a protein concentration of 6.25 mg/ml. H^+-ATPase was immunoprecipitated from Triton extracts with an anti-β monoclonal antibody [24]. Mitochondrially synthesized proteins associated with H^+-ATPase were visualized by fluorography after electrophoresis on 12% SDS-polyacryalamide gels (a) J69-1B, (b) 5726, (c) 5102, (d) 51223, (e) 38.6.1, (f) 2422, (g) 15B2 (h) J69-1B, grown and labelled at 28°C, (i) 811, grown and labelled at 28°C, (j) J69-B1, grown and labelled at 18°C, (k) 811, grown and labelled at 18°C.

type, while in stains with a truncated subunit 9 product (strains 51223 and 5208), it was about 25%. In strains carrying a frameshift mutation (strains 5726 and 38.6.1), no peak corresponding to subunit 9 could be detected on the scan.

A feature of particular note in Fig. 1b is the obvious reduction in the net level of synthesis of subunit 6 in most of the mutant strains. In mutant strains 5726 and 38.6.1 subunit 6 cannot be detected on the gel, while in strains 51223 and 5102 the amount present is considerably less than in the wild-type strain. Strains 2422 and 15B2, however, appear to synthesize the protein normally. Genetic tests have shown that the reduction in the synthesis of subunit 6 is not due to secondary mutations in the *oli*2 gene.

Assembly of the H^+-ATPase complex

To investigate the assembly of the F_0 sector in the *oli*1 mutants, a monoclonal antibody specific for the β subunit of the F_1 sector, RH 48.6 [24],

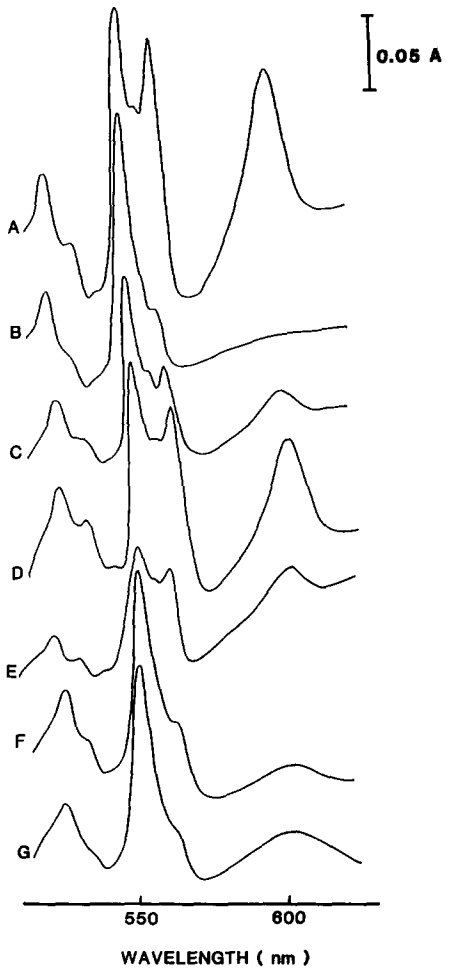

Fig. 3. Absorption cytochrome spectra of mitochondria isolated from wild-type and mutants. Cells were grown in glucose chemostat cultures and mitochondria isolated from spheroplasts. Difference cytochrome spectra (sodium dithionite reduced vs. potassium ferricyanide oxidized) were recorded at the temperature of liquid nitrogen in all mutants strains. A J69-1B, B 5726, C 2422, D 811 grown at 28°C, E 811 grown at 18°C, F M10-7, G M2610. The cytochrome spectra from the other *mit*$^-$ mutants were also recorded; cytochrome spectra from strain 15B2 were identical to those from strain 2422 and spectra from strains 51223, 5208 and 38.6.1 were identical to those from strain 5726.

was used to immunoprecipitate assembled H^+-ATPase subunits from Triton extracts of mitochondria. These were prepared from cells which have been labelled with [^{35}S]sulphate in the presence of cycloheximide. From the results shown in Fig. 2 the mutants can be divided into two categories: those able to assemble an apparently normal, although presumably nonfunctional, F_0 sector, and those in which the assembly of the F_0 sector is defective. In two *mit*$^-$ strains where the mutations give rise to a full-length subunit 9 product (strains 15B2 and 2422) all three mitochondrial synthesized components of the F_0 sector, namely subunits 9, 8 and 6 are clearly associated with the enzyme complex as is the case in the wild-type strain J69-1B. The same situation exists in the temperature-conditional mutant 811 grown and labelled at the restrictive temperature; in this strain, the mutation also leads to a full-length product with a single amino-acid substitution.

In the other four strains subunit 9 does not appear to be assembled, either in strains 5726 and 38.6.1, where the polypeptide is not synthesised, or in strains 51223 and 5102 where the proteolipid is synthesised, but in an altered form. It is of interest that in strain 5102 ($Gly_{18} \rightarrow$ Asp) subunit 9 is not assembled, whereas in strains 15B2 and 811 ($Gly_{23} \rightarrow$ Asp and Cys) the polypeptide is assembled in the complex.

In addition, mutants which fail to assemble subunit 9 also show a marked reduction in the amount of subunit 8 assembled to the complex.

The activity of the electron-transport chain

A surprising and interesting finding of our current investigation is the observation that the respiratory enzyme activities, in particular those of the cytochrome *c* oxidase complex, are much reduced in the *oli*1 mutants. Table III shows that the rate of mitochondrial respiration is only about 10% of the wild-type in all the mutants studied, except in strains where a full-length subunit 9 is synthesized and assembled (strains 2422, 15B2 and 811); here the activity is between 20% and 30%. Consistent with the respiration data are the low activities of the NADH-cyt *c* reductase and the cytochrome *c* oxidase. The NADH-cyt *c* reductase activity ranges from 10% of the activity of the wild-type for strains with a truncated subunit 9 (strains 5726, 5208, 51223 and 38.6.1) to about 30% in strains with a full-length assembled product. However, the cytochrome *c* oxidase activity is virtually negligible in the *mit*$^-$ mutant strains except in strains 2422, 15B2 and 811 which have specific activities of 0.07–0.30 μmol/min per mg,

TABLE III

MITOCHONDRIAL RESPIRATORY ACTIVITIES OF THE MUTANT STRAINS

Cells were grownin glucose-limited chemostat cultures at 28°C and mitochondria were prepared from spheroplasts. Electron transfer was assayed as described in Materials and Methods; wild-type mitochondrial respiration was 100% KCN sensitive (10 μmol); wild-type NADH-Cyt *c* reductase activity was 90% antimycin A sensitive (0.02 μmol); wild-type cytochrome *c* oxidase activity was 100% KCN sensitive (3 μmol).

Strain	Mitochondrial respiratory activity (ng atoms O/ min per mg)	NADH-Cyt *c* reductase activity (μmol/ min per mg)	Cytochrome oxidase activity (μmol/ min per mg)
J69-1B (wild-type)	128	0.35	1.1
2422	33	0.10	0.09
5102	18	0.05	0.006
38.6.1	13	0.04	0.005
5726	15	0.04	0.005
51223	16	0.03	0.006
5208	16	0.03	0.006
15B2	24	0.11	0.08
811	32	0.30	0.30
M10-7 (*oli*2)	42	0.13	0.20
M26-10 (*aap*1)	30	0.10	0.12
EJO (*rho*^{0})	8	0.04	0

compared to 1.1 μmol/min per mg in the wild-type strain. This observation is further supported by cytochrome spectra recorded from the mutant mitochondria. As shown in Fig. 3, no spectral cytochrome aa_3 could be detected in strains with truncated products (5726 and 51223), while in strains 2422 and 15B2, the cytochrome aa_3 peak could be detected, although smaller in size than in the wild-type strain. In all cases, spectral cytochrome *b* and c_1 could be clearly detected, although significantly lower in amount. Spectral cytochrome *c*, on the other hand, appears to be normal. It seems, therefore, that the assembly of subunit 9 into the H^+-ATPase is important for the assembly of a functional cytochrome oxidase complex.

Such pleiotropic effect, whereby a mutation in one mitochondrial gene affects multiple enzymatic complexes is not uncommon in yeast. For example, mutations in the cytochrome *b* gene are known to affect the synthesis of subunit I of cytochrome oxidase and vice versa [33–36]. Moreover, mutations in the yeast mitochondrial H^+-ATPase subunit 6 gene have also been shown to affect the activity of the respiratory chain [7]. In this study, however, the effect of the *oli*1 *mit*$^-$ mutations on the respiratory enzyme activities is more severe than previously reported. This is evidenced in Fig. 3 and Table III, where mutations affecting subunits 6, 8 and 9 of the yeast mitochondrial H^+-ATPase are compared simultaneously. For example, whereas spectral cytochrome aa_3 can be recorded in *oli*2 and *aap*1 *mit*$^-$ mutant strains (although in reduced amounts), it is not in strain 5726 as described above. The extreme pleiotropic effects of the *oli*1 mutants is not related to the number of petite mutants in the cultures because the petite frequency of all the *mit*$^-$ strains was comparable ranging from 60–70% for strains 5726

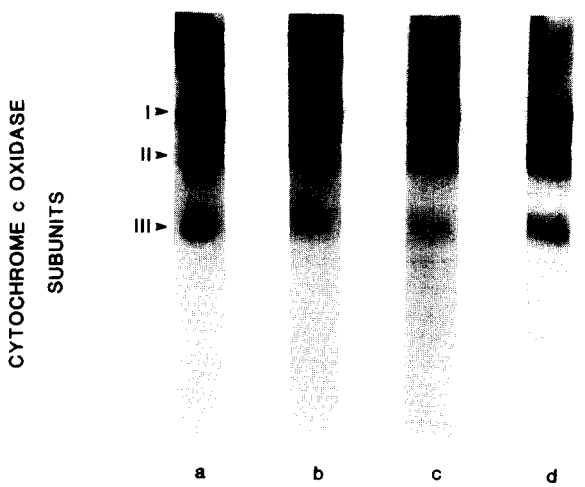

Fig. 4. Synthesis of mitochondrially synthesised components of cytochrome *c* oxidase in *oli*1 *mit*$^-$ mutants. Mitochondria isolated from cells labelled in vivo were solubilised with Triton X-100 (0.25%) at a protein concentration of 6.25 mg/ml. Cytochrome *c* oxidase was immunoprecipitated from Triton extracts with a rabbit antiserum against the holocytochrome *c* oxidase [25]. Mitochondrially synthesised subunits of cytochrome *c* oxidase were visualised by fluorography after electrophoresis on 12% SDS-polyacrylamide gels. (a) J69-1B; (b) 38.6.1; (c) 5726; (d) 5102. Subunits I, II and III of cytochrome *c* oxidase were present in strains 51223, 2422 and 15B2 also.

and 26–210 to 60–75% for strain 10-7.

We have further confirmed that the absence of spectral aa_3 in all the strains that do not assemble subunit 9 is not due to a defect in the synthesis of cytochrome oxidase subunits. For this purpose, cytochrome oxidase subunits were immunoprecipitated from Triton extracts of mitochondria with a rabbit antiserum against the holocytochrome *c* oxidase. Fig. 4 shows that the mitochondrially synthesized subunits I, II and III are present in all the mutant strains. Further, immunoprecipitation of the subunits from cells labelled with [^{35}S]sulphate in the absence of inhibitors of protein synthesis revealed the presence of subunits IV, V, VI and VII, which are nuclearly coded, in addition to subunits I, II and III (results not shown).

Discussion

Functionally-important structural features of subunit 9

Results of our analysis of the *oli*1 mutants in which subunit 9 is produced in a truncated form allows the definition of functionally important domains on the molecule. These results suggest that the C-terminal arm of subunit 9 is essential for the assembly of the protein into the H^+-ATPase complex. Thus, in two premature termination mutants where 85% of the length of the molecule is still synthesized, the polypeptide can no longer assemble to the complex.

An interesting observation is that in one mutant (strain 5726) where a frameshift mutation is predicted to give rise to a 65 amino-acid long product, no truncated protein can be detected on our SDS-polyacrylamide gels. Yet, a mutant protein predicted to be 68 amino acids long is detected reproducibly (strain 51223). In the former mutant 5726, a frameshift mutation at amino acid 56 introduces a positive lysine residue at position 59 in place of the negative glutamic acid residue. Thus, a possible explanation for the apparent lack of ATPase subunit 9 synthesis in this strain, is that the new positive charge may have prevented the insertion of the mutant protein into the membrane so that it is rapidly degraded after synthesis.

Mutations leading to single amino-acid substitutions are valuable in defining functionally and/or structurally important residues in the polypeptide. In this communication, we have described four amino-acid substitutions, three of which lie in the membrane-spanning portion of the N-terminal arm of the proposed structure for subunit 9 and the other is in the hydrophilic loop proposed to protrude into the mitochondrial matrix [11].

The conserved Gly at position 23 is substituted in one mutant by Cys and by the negatively charged Asp in a second mutant. The Gly → Cys substitution gives rise to a temperature-conditional mutant which, to a limited extent, can still make use of the proton gradient generated from electron transport to synthesize ATP at the restrictive temperature. The Gly → Asp substitution leads to a *mit*$^-$ phenotype such that the mutant strain is unable to metabolise a non-fermentable substrate. In a previous communication [15], it has been reported that an Ala substitution at Gly_{23} leads to oligomycin resistance. We observe, therefore, a gradation of functional defects between the Ala, the Cys and the Asp replacements. The insertion of the negatively charged Asp is more likely to interfere with the structural stability of the Gly rich membrane spanning segment than the insertion of Cys or Ala; moreover, Asp might also interfere with adjacent charged moieties in its vicinity including Glu_{59} in the other hydrophobic arm of the proteolipid. This latter residue has been implicated in the proton translocating activity of the H^+-channel [12–14]. However, neither the Cys nor the Asp substitution affects the assembly of subunit 9 into the H^+-ATPase implying that the substitutions have not resulted in a major conformational change as to prevent the integration of subunit 9 with subunit 8 and and subunit 6 into the F_0 sector.

Another conserved Gly, this time at position 18, is also substituted by the negative Asp in another mutant. Like the mutation at Gly_{23}, this leads to a *mit*$^-$ phenotype. Here, however, the strain is unable to assemble subunit 9; suggesting that Gly at position 18 has a very critical role in the assembly of subunit 9 into the H^+-ATPase, either by directly interacting with other subunits of the complex or by maintaining the correct conformation in the interacting domain(s) of the molecule.

The last amino-acid substitution investigated takes place at position 39 of the hydrophilic loop where the positive charged Arg is replaced by an uncharged Met. The mutation gives rise to a *mit*$^-$ condition and, since in this strain the H^+-ATPase complex appears to be normally assembled, it seems that either the Arg residue itself or the positive charge, is vital for H^+-ATPase function. A revertant of strain 2422 ($Arg_{39} \rightarrow Met$) was found to carry an Arg → Lys substitution (Willson, T., personal communication), thus emphasising the importance of the positive charge in H^+-ATPase function.

Secondary effects of mutations in the oli1 gene

A surprising conclusion reached in this study is that when subunit 9 is not assembled into the H^+-ATPase complex, major secondary pleiotroic effects on the synthesis of subunit 6 of the H^+-ATPase complex and on the functional assembly of the cytochrome oxidase complex take place.

In mutant strains where subunit 9 is not assembled, levels of synthesis of subunit 6 are very low, whereas in those mutants where subunit 9 is assembled, albeit in a defective form, subunit 6 is synthesised in amounts comparable to that in the parental wild-type strain. This observation points to a regulatory interaction operating between subunit 9 and subunit 6, so that when subunit 9 is not assembled, either the synthesis of subunit 6 is prevented or its degradation takes place rapidly under conditions in which it cannot be immediately integrated into the membrane.

To gain a better understanding of the level at which the regulatory interaction is exerted, we are at present investigating the transcriptional patterns of the *oli*2 gene (coding for subunit 6) in these *oli*1 *mit*$^-$ mutants. Results suggest that the mechanism is operating posttranscriptionally, since the two mature *oli*2 transcripts of length 5.1 and 4.5 kb are present in all the mutant strains [44].

In the case of the formation of cytochrome oxidase, we have shown that the effect arising from the lack of assembled subunit 9 must be post-translational, since all the apoprotein components of the enzyme complex are synthesized. The absence of spectral cytochrome aa_3 is therefore not a consequence of a lack of the apoproteins required for the binding of heme *a* to form holocytochrome aa_3 but must reside in a defect in heme *a* synthesis or attachment to the apoprotein. Protoheme is the precursor of heme *a* and its synthesis involves several steps, of which the first and last take place in the inner mitochondrial membrane in yeast [37]. It is possible, therefore, that the absence of assembled subunit 9 of H^+-ATPase deranges the inner mitochondrial membrane in such a way that protoheme synthesis and/or heme *a* binding to the apoproteins of cytochrome oxidase are affected.

The absence of a functionally assembled cytochrome oxidase complex in some *oli*1 *mit*$^-$ mutants suggests that a tight assoiation exists between the H^+-ATPase and the cytochrome *c* oxidae complexes. There are at present several lines of evidence suggesting a functional association between these complexes. Thus, Deléage et al. [38] showed that the protons effective in ATP synthesis appear to be localized in a 'kinetic compartment' and are the result of a rapid production of protons either by substrate oxidation or ATP hydrolysis. It has also been shown that the respiratory chain and ATP synthase function as a single directly coupled unit in bovine heart submitochondrial particles [39]. Localized coupling of photophosphorylation has been demonstrated in the bacteria *Rhodopseudomonas sphaeroides* [40].

Boutry and Goffeau [41] have reported nuclear ATPase mutants of *Schizosaccharomyces pombe* which have an altered α on β subunit of the F_1 sector and which also exhibit pleiotropy on the cytochrome oxidase activity. In those mutants, the decreased cytochrome oxidase activity is associated with the presence of abnormally translated subunits of the complex.

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