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Mitochondrial adenosine triphosphatase in *mit*$^-$ mutants of *Saccharomyces cerevisiae* with defective subunit 6 of the enzyme complex

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***mit*$^-$ mutants carrying genetically defined mutations in the *oli2* region of the mitochondrial DNA were analysed. Most of these mutants demonstrated either the absence of subunit 6 or its replacement by shorter mitochondrial translation products which could be shown to be structurally related to subunit 6 by using a rabbit anti F_1F_0-antiserum, and by limited proteolytic mapping of the new mitochondrial translation products. Three representative *oli2 mit*$^-$ strains were analysed for the effects of a grossly altered subunit 6 or of a complete absence of this subunit on the activity and assembly of the H^+-ATPase. Our results suggest that this subunit is not required for the assembly of the proton channel of the enzyme complex. Thus, in the absence of subunit 6, the mitochondrial respiratory activities in the *oli2* mutants were found to be still sensitive to oligomycin, a specific inhibitor of the H^+-ATPase proton channel. Immunoprecipitation of the assembled H^+-ATPase subunits from these mutant strains using a monoclonal anti-β-subunit antibody indicates that subunit 6 is also not essential for the assembly of most F_1 subunits to components of the F_0 sector.**

Introduction

The H^+-ATPase, (ATP synthetase, ATP phosphohydrolase, EC 3.6.1.3) complex of the inner mitochondrial membrane is a mosaic consisting of mitochondrially made protein subunits as well as subunits imported from the extramitochondrial cytoplasm (see Ref. 1 and 2 for review). Because of this unique property, shared by only the mitochondrial ribosome and two respiratory enzyme complexes of the mitochondrial inner membrane, the biogenesis of the H^+-ATPase has been the subject of intensive studies in many laboratories.

Functionally, the enzyme complex can be divided into two sectors; the F_1 sector, which contains the catalytic site for the synthesis and hydrolysis of ATP, and the F_0 sector, which is an integral part of the mitochondrial inner membrane and has been suggested to act as a proton channel linking a transmembrane proton gradient, generated by the electron transport chain, to the synthesis of ATP on the F_1 sector [3]. The F_1 sector is now relatively well defined and has been shown to consist of five different protein subunits, all of which ware imported from the extramitochondrial cytoplasm. Despite many intensive investigations, on the other hand, the subunit composition of the F_0 sector is still uncertain. In the yeast *Saccharomyces*

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Abbreviation; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

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cerevisiae, the F_0 sector has been shown to contain three mitochondrially synthesised subunits: subunit 6 (in Tzagoloff nomenclature [4], with an apparent M_r of 20 kDa), subunit 8 (apparent M_r 10 kDa) and subunit 9 (a proteolipid, M_r 7.6 kDA). In addition, the yeast H^+-ATPase also contains two other cytoplasmically synthesised subunits with apparent M_r of 18 and 25 kDa [5] and at present it is not clear whether these polypeptides are subunits of the F_0 sector.

The study of the assembly of the F_0 sector has been hampered by the extremely hydrophobic nature of the components that make up this sector, and the reconstitution of the F_0 sector from its individual subunits has not been possible. Information regarding the involvement of F_0 subunits in the assembly of the H^+-ATPase complex, however, can also be obtained from the identification of functional and assembly defects in mutants in which one or more of these subunits are not synthesised or synthesised in a grossly altered form. As part of our general strategy to elucidate the processes involved in the assembly of the mitochondrial H^+-ATPase, we have recently isolated a series of monoclonal antibodies to various subunits of the enzyme complex [5]. Two of these monoclonal antibodies, which are specific for the β subunit of the F_1 sector, were found to be particularly effective in immunoprecipitating intact H^+-ATPase. These antibodies can therefore be used to define the extent to which the assembly of the H^+-ATPase can proceed in specific mutants.

We have previously reported [6] several *mit*$^-$ mutants of yeast in which subunit 6 is either not synthesised or replaced by immunologically related shorter polypeptides. The genetic lesions in these mutants, which presumably are in the structural gene of subunit 6, were mapped to the *oli2* region of the mitochondrial genome, the complete nucleotide sequence of which has been determined [7,8]. Workers in our laboratory have since isolated several more of these *oli2 mit*$^-$ mutants and the genetic lesions in these as well as in our original mutants have now been characterised in detail [8,9]. In the present study, we have investigated the participation of subunit 6 in the formation of the proton channel of the H^+-ATPase complex and the assembly of this sector to the F_1 sector, by defining the assembly and functional defects in the H^+-ATPase as the result of the alteration to subunit 6 in these *oli2 mit*$^-$ mutants. The *oli2 mit*$^-$ mutants analysed were found to have reduced ATPase activity which is insensitive to oligomycin. Our results suggest that the assembly of the proton channel of the membrane sector is not affected by the absence of subunit 6 or the presence of this subunit in a grossly altered form. Furthermore, the F_1-ATPase in the mutant strains appears to be membrane bound, and associated with a partly assembled membrane sector of the enzyme complex, although the binding is less tight than that of the wild-type strain. Mitochondria isolated from the *oli2 mit*$^-$ strains, however, have no detectable ATP-[^{32}P]P_i exchange activity.

Materials and Methods

Yeast strains

The *oli2 mit*$^-$ strains used in the present study were derived from the wild type strain J69-1B α *ade his* [rho$^+$] by mutagenesis with $MnCl_2$ [10]. The genetic properties of these mutants have been described elsewhere [8,9]. All yeast strains were stored on agar slopes containing glucose (20 g/l), yeast extract (10 g/l, Difco Laboratories, Detroit, MI), peptone (20 g/l, Oxoid Ltd., London) and agar (15 g/l) and subcultured at least every 3 months.

Growth conditions

For labelling of mitochondrial translation products, cells were grown aerobically at 28°C in 75 ml liquid batch cultures. The growth medium contained yeast extract (10 g/l), a salts mixture [11], glucose (10 g/l for the wild-type strain, 20 g/l for the *mit*$^-$ strains) and the auxotropic requirements of the individual strains (adenine, 100 mg/l; histidine, 50 mg/l; uracil, 50 mg/l; lysine, 50 mg/l). Cultures were harvested early in the stationary phase. To minimise the effects of catabolite repression, cells used for functional studies were grown in glucose-limited chemostat cultures at 28°C as previously described [12]. The dilution rate was 0.1 h^{-1}. Under these growth conditions, the steady-state glucose level of the cultures was maintained below that which induces catabolite repression [12].

Preparation of mitochondria and submitochondrial particles

For the analysis of mitochondrial translation products, mitochondria were isolated from cells which have been broken by manual shaking in the presence of glass beads essentially as described by Lang et al. [13]. Intact mitochondria used for the analysis of mitochondrial functions were isolated from spheroplasts which were prepared by zymolyase digestion of the cell wall as previously described [14]. Delipidated bovine serum albumin (2 mg/ml) was added to the buffer prior to breakage of the spheroplasts and in all the subsequent steps.

Submitochondrial particles were prepared by sonication of a mitochondrial suspension (10–15 mg protein per ml) using a Branson Model B-30 Sonifier at setting 2 for 1 min on 60% pulse. Mitochondria which had not been broken by the sonication were removed by centrifugation at 12 500 rpm in a Sorvall SS34 rotor for 10 min. Submitochondrial particles were then collected from the supernatant by centrifugation in a Beckman Type 50 Ti rotor at 39 000 rpm for 30 min at 4°C.

Immunoprecipitation of H^+-ATPase complex

Cells were resuspended at 6 mg/ml in low sulphate medium [15] and total cell proteins were labelled with [^{35}S]sulphate (200 μCi/ml) at 28°C for 4 h in the absence of antibiotics. Mitochondrial translation products were labelled for 1 h in the presence of cycloheximide, which allows the incorporation of [^{35}S]sulphate into products of mitochondrial protein synthesis only [15]. Immunoprecipitation of mitochondrial translation products associated with the H^+-ATPase complex was carried out with a rabbit antiserum raised specifically against F_1F_0-ATPase. *Staphylococcus aureus* carrying protein A was used as an antibody absorbent, as previously described [16]. Immunoprecipitation of assembled subunits of the H^+-ATPase was carried out by using a monoclonal anti-β- antibody coupled to CNBr-activated Sepharose 4B beads as described in [5]. Immunoprecipitates were analysed by polyacrylamide slab gel electrophoresis in the presence of SDS as described by [15] and visualised by fluorography [17].

Peptide mapping by limited proteolysis

Peptide mapping of proteins by limited proteolysis was carried out according to the method of Cleveland et al. [18]. ^{35}S-labelled mitochondrial proteins (about $5 \cdot 10^5$ dpm) were first separated on 12.5% polyacrylamide gels as described above. Gels were either dried directly without fixing or mildly treated in a fixer solution containing 50% (v/v) methanol and 10% (v/v) acetic acid and then dried. The gels were directly autoradiographed without further treatment. Bands of interest were precisely located by using the autoradiogram and sliced out from the dried gels. Gel slices were then placed on the sample wells of a 17.5% polyacrylamide gel with a long stacker (5 cm). Spaces around the gel slices were filled with 0.125 M Tris-HCl (pH 6.8)/0.1% SDS/1 mM EDTA/20% (w/v) glycerol. The slices were then overlaid with 50 μg chymotrypsin (Sigma, St. Louis, MO) in 0.125 M Tris-HCl (pH 6.8)/0.1% SDS/1 mM EDTA/10% (w/v) glycerol. Electrophoresis was performed at 30 mA until the dye front was three-quarters of the way through the stacker. The current was then turned off and digestion was allowed to proceed for 1 h at room temperature. After this period of time, electrophoresis was resumed until the dye front reached the bottom of the gel. The gel was treated for fluorography as described above.

Preparation of F_1-ATPase

F_1 was prepared essentially as according to the method of Beechey et al. [19]. Submitochondrial particles were suspended to a concentration of 5 mg/ml in a buffer comprising 0.25 M sucrose/10 mM Tris-sulphate/1 mM EDTA (pH 7.5). Chloroform (analytical grade; 0.5 vol.) was added and the two phases were vigorously mixed for 30 s on a vortex mixer. The emulsion was broken and the two phases were separated by centrifugation in an MSE bench centrifuge at room temperature for 5 min. The aqueous layer so obtained was then centrifuged at 36 000 rpm in a Type 50Ti rotor for 30 min at 20°C. The supernatant contained oligomycin-insensitive ATPase activity of the F_1-ATPase.

Other analytical procedures

Published procedures were employed to de-

termine the activities of mitochondrial respiration and cytochrome oxidase [20], ATPase [21] and NADH-cytochrome *c* reductase [22]. ATP-[^{32}P]P_i exchange activity was assayed by a modification of the method of Ryrie [23]. The assay medium is made up of 0.25 M sucrose, 10 mM ATP, 20 mM $MgSO_4$, delipidated bovine serum albumin (4 mg/ml), 50 mM Tricine and 10 mM KH_2PO_4 (containing $5 \cdot 10^6$ cpm $^{32}P_i$: carried free from the Australian Atomic Energy Commission, Lucas Heights, N.S.W.). Mitochondria was added at a concentration of 100–200 μg in 0.5 ml of the assay medium and incubation at 20°C with continuous agitation for 10 min. The reaction was stopped with 50 μl of 50% (w/v) trichloroacetic acid solution and the reaction mixture was centrifuged at $1500 \times g$ for 5 min in an MSE bench centrifuge. The unreacted inorganic phosphate was extracted from the supernatant according to the method of Avron [24].

Protein concentration was estimated essentially as described by Lowry et al. [25] except that the reaction mixture was incubated in a bath of boiling water for 45 s instead of at room temperature for 30 min.

Results

Structural alteration to H$^+$-ATPase subunit 6 in the oli2 mit$^-$ mutants studied

the structural alteration to the H$^+$-ATPase subunit 6 in the *oli2 mit*$^-$ mutants used in the present study was determined by isolating the enzyme complex from cells of the wild-type and *oli2 mit*$^-$ strains which have been labelled in vivo with [^{35}S]sulphate in the presence of cycloheximide, which allows the incorporation of [^{35}S]sulphate into mitochondrial translation products only. The H$^+$-ATPase complex was then isolated from the triton extracts of mitochondria, isolated from the

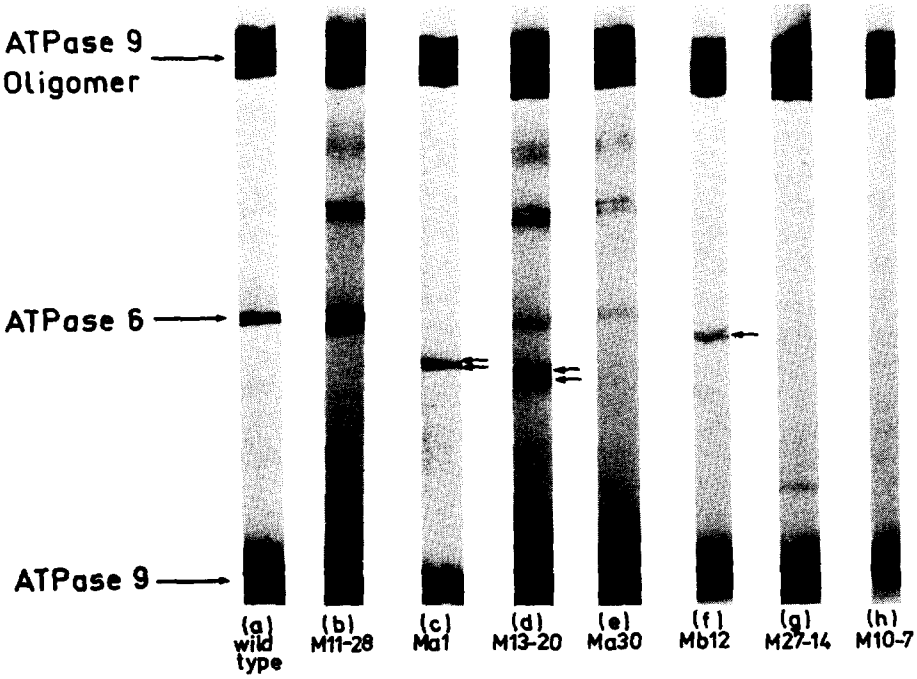

Fig. 1. Mitochondrially synthesized H$^+$-ATPase subunits in *oli2 mit*$^-$ mutants of yeast. Mitochondrial translation products were labelled in vivo with [^{35}S]sulphate in the presence of cycloheximide, and mitochondria were isolated. The ATPase complex was extracted from the mitochondria with Triton X-100 (0.5%) in a 4 mM Tris-acetate buffer (pH 7.5).25 M sucrose at a protein concentration of 6.25 mg/ml and precipitated in the presence of *Staphylococcus aureus* cells with rabbit antiserum raised against purified wild-type F_1F_0-ATPase. Samples of the immunoprecipitates were solubilised with SDS and electrophoresed in 12.5% polyacrylamide slab gels in the presence of SDS [15]. Mitochondrially synthesised proteins were detected by fluorography [17]. Arrows indicate new mitochondrial translation products associated with the mitochondrial ATPase of the mutant strains.

^{35}S-labelled cells, by immunoprecipitation using a specific antiserum against F_1F_0-ATPase. This antiserum was characterised by western immunoblotting and was found to contain antibodies to the α, β and γ subunits of the F_1 sector as well as subunit 6 (data not shown). Mitochondrially synthesised subunits of the immunoprecipitated enzyme complex were analysed by electrophoresis on SDS-polyacrylamide gels. As shown in Fig. 1, the three prominent mitochondrially synthesised polypeptides, observed in the immunoprecipitate from the wild-type mitochondria are the oligomeric and monomeric forms of the proteolipid subunit 9 (apparent M_r 50 and 7.6 kDa, respectively), and subunit 6 (apparent M_r 20 kDa). The resolution of the polyacrylamide gel used in this particular experiment did not allow the separation of subunit 8 (apparent M_r in SDS-polyacrylamide gel 10 kDa) from subunit 9. Analysis of the immunoprecipitates of the *oli2 mit*$^-$ strains show that in all but one of these mutants, the H^+-ATPase subunit 6 is not present (Fig. 1). In four of the *mit*$^-$ strains, the absence of subunit 6 is accompanied by the appearance of new translation products with apparent molecular weights lower than 20000. The apparent molecular weights of the new mitochondrial translation products in these strains range from approx. 12000 to 19000. The presence of these new mitochondrial translation products in the immunoprecipitates indicates that they either cross-reaction with the anti-ATPase antibodies or are associated with other ATPase subunits which are antigenic.

One mutant, strain M11-28, contains an apparently normal subunit 6. However, any small alteration, such as a single amino acid substitution as a result of point mutations, would not have been detected in a one-dimensional gel system as used in the present study. In fact, we have previously shown that the subunit 6 in this mutant strain has an altered p*I* when analysed by a two-dimensional gel system [16]. The new translation

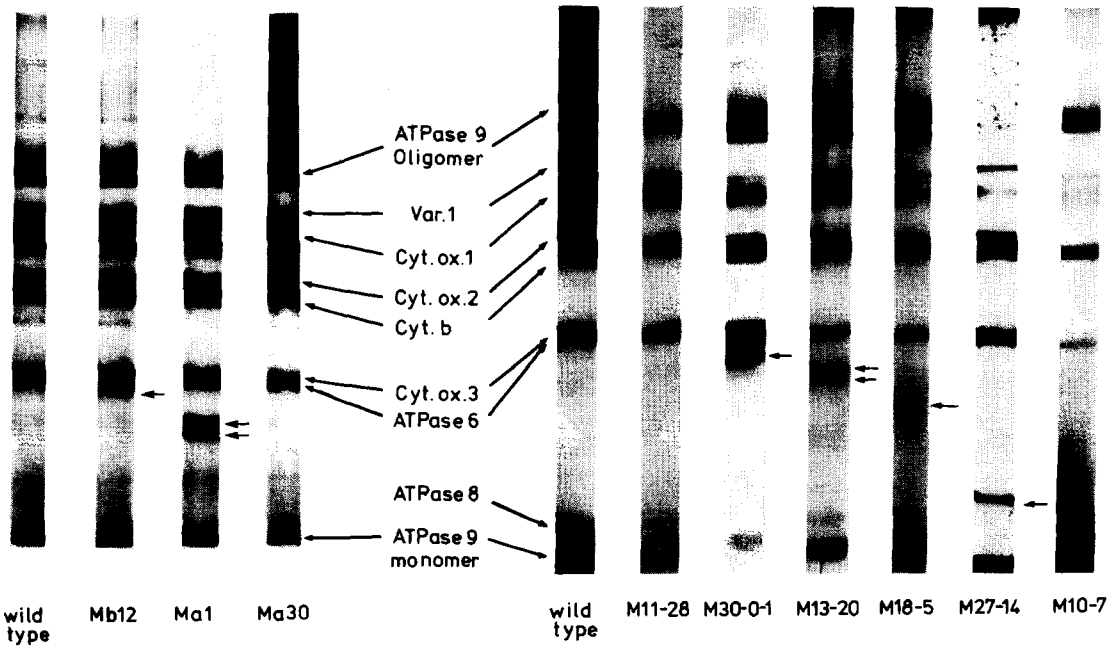

Fig. 2. Mitochondrial translation products in the *oli2 mit*$^-$ mutant strains. Strains were labelled in vivo with [^{35}S]sulphate in the presence of cycloheximide and mitochondria isolated as described in Materials and Methods. Mitochondrially synthesised proteins were solubilised with SDS, electrophoresed in 12.5% SDS-polyacrylamide gels in the presence of SDS [15] and visualised by fluorography [17]. Positions of mitochondrially synthesised protein components of H^+-ATPase (oligomeric and monomeric forms of subunit 9, subunit 6 and subunit 8), cytochrome oxidase (cyt. ox. I, II and III) and cytochrome *b* apoprotein (cyt. *b*) are indicated. Small arrows indicate new translation products found in the *mit*$^-$ strains.

products observed in the immunoprecipitates of the *oli2* mutant strains could also be seen when mitochondrial translation products from whole mitochondria of the mutant strains were analysed on SDS-polyacrylamide gels (Fig. 2). However, since in this gel H^+-ATPase subunit 6 co-migrates with subunit III of cytochrome oxidase, the absence of either one of these subunits would not be immediately evident from the display of the total mitochondrial translation products.

The observation that ATPase subunit 6 is replaced in some of the mutants studied by shorter polypeptides suggests that the subunit 6 lesions in these *mit*$^-$ strains are probably due to nonsense mutations resulting in early termination during the synthesis of this subunit. If this were the case, the new translation products would be structurally related to subunit 6.

Limited proteolytic digestion of the new translation products of oli2 mit$^-$ *strains Ma1 and M13-20, and subunit 6 of the wild-type strain*

Due to the almost similar electrophoretic mobility of cytochrome oxidase subunit III and H^+-ATPase subunit 6 on SDS-polyacrylamide gel, the isolation of subunit 6 for proteolytic mapping from a gel display of total mitochondrial translation products is difficult. To circumvent this problem, we have used an *oxi2 mit*$^-$ mutant (strain 2008), which does not synthesise cytochrome oxidase subunit III, as the source of ATPase subunit 6. The absence of cytochrome oxidase subunit III in this *mit*$^-$ mutant has previously been shown on a two-dimensional gel display of the total mitochondrial translation products [26]. Hence, the 20 kDa band observed in polyacrylamide gel electrophoretic display of total mitochondrial translation products of this strain consists of only subunit 6 of H^+-ATPase.

The 15 and 16 kDa new translation products from one of the *oli2 mit*$^-$ mutants (strain Ma1) were separated on SDS-polyacrylamide gels, sliced and digested with chymotrypsin. Their digestion patterns were then compared with that obtained from wild-type subunit 6. As shown in Fig. 3a, the two translated products individually or as a mixture yielded very similar patterns to that of the wild type subunit 6. Similar analysis of the two new mitochondrial translation products from strain

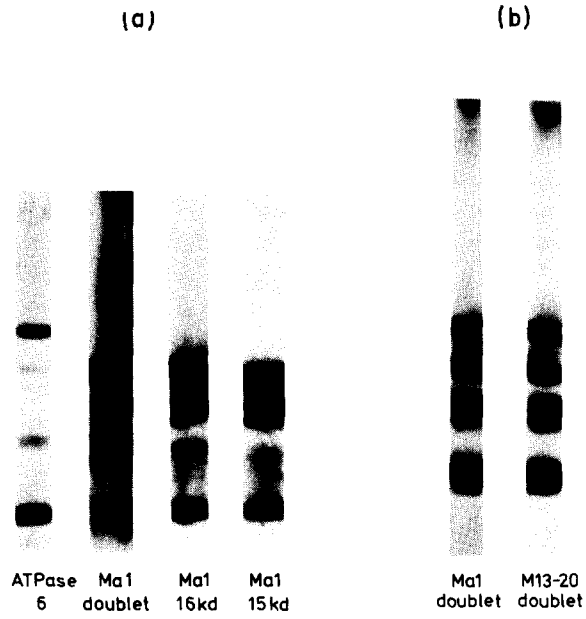

Fig. 3. Proteolytic digestion patterns of subunit 6 and the new translation products of *oli2 mit*$^-$ strains Ma1 and M13-20. Subunit 6 from the *oxi2 mit*$^-$ strains 2008 and the new translation products from the *mit*$^-$ strains Ma1 and M13-20 were obtained from a display of ^{35}S-labelled mitochondrially synthesised proteins on SDS-polyacrylamide gels. Proteolytic digestion was carried out in a 17.5% polyacrylamide gel for 1 h with 50 μg of chymotrypsin as described in Materials and Methods. Fragmentation patterns were visualized by fluorography. (a) Chymotryptic digest patterns of subunit 6 and the new translation products of strain Ma1; and (b) Chymotryptic digest patterns of the new translation products of strains Ma1 and M13-20.

M13-20 also yielded chymotrypsin digest patterns similar to that of subunit 6 (Fig. 3b). Hence, the new translation products from strains Ma1 and M13-20 appear to represent shorter forms of subunit 6 which presumably arose as the result of an early termination during the systhesis of this subunit.

oli2 mit$^-$ *mutants contain mitochondrial ATPase activities which are not sensitive to oligomycin*

Three representative *oli2 mit*$^-$ mutants described above were analysed for their respiratory enzyme and ATPase activities. These mutants are strains Mb12 and Ma1, which have altered subunit 6 (M_r approx. 19 and 15 + 16 kDa, respectively), and strain M10-7, which completely lacks this

subunit. Unlike the previously reported *pho1* mutants (also carrying lesions in the *oli2* region of the mtDNA) [27,28], which can grow by oxidative metabolism, albeit at a much reduced rate, all the *oli2 mit*$^-$ strains analysed were unable to grow in batch cultures with ethanol as the energy source (data not shown). To minimise catabolite repression, cells used in the present studies were grown in the presence of glucose in glucose-limited chemostat cultures. At a dilution rate of 0. $1h^{-1}$, it has been shown that the glucose concentration in the steady state is lower than that which induces catabolite repression [12]. As shown in Table I, the steady-state growth yields of all the mutant strains are comparable to that of a mtDNA-less petite (*rho*°) strain which completely lacks oxidative phosphorylation, confirming the conclusion from the batch culture growth experiments that the *oli2 mit*$^-$ strains are not capable of growth by oxidative metabolism.

The mitochondrial ATPase activities of the *oli2* mutants were determined in order to investigate the effect of the alteration in subunit 6 structure (or loss of subunit 6) on the function of the complex. As shown in Table II, all the mutants analysed contained significant although reduced rates of ATPase activity. The extent of the alteration to subunit 6 structure appears to bear no relation to the loss of the activity of this enzyme complex. The mutants ATPase activities were found to be insensitive to inhibition by oligomycin. Thus, while the maximal inhibition by oligomycin for the wild-type strain was 83%, that for the mutant strains did not exceed 16%. This degree of inhibition is only slightly higher than that of the H^+-ATPase activity from a mtDNA-less *rho*° strain (see Table II) which, because of the absence of mitochondrial protein synthesis lacks all the mitochondrially synthesised subunits.

The mitochondrial respiratory activities of the *oli2 mit*$^-$ mutants were also analysed and found to be lower than that of the wild-type strain. Strains Ma1 and M10-7 exhibited 59% and 41% of the wild-type respiratory rates while strain Mb12 exhibited a rate which is only 30% of that of the wild-type strain (Table III). The addition of ADP to the respiring mitochondria of the mutant strains did not produce any effect, indicating the lack of respiratory control in these strains. In contrast, the wild-type respiration was stimulated by about 90% on the addition of ADP. The lower respiration rates in the *oli2 mit*$^-$ strains analysed could be partly due to the large number of respiratory deficient petite mutants formed during growth (vary between 40–45% of the total cell population). However, the high petite frequency alone cannot completely account for the reduction in the respiratory activity. For example, in mutant strain Mb12, the proportion of respiratory deficient petite cells in the culture was about 45%. The respiration rate (in the presence of ADP) was, however, not 45% of that of the wild type but only 16%. Thus it appears that the mutations in the *oli2 mit*$^-$ strains analysed have an indirect effect on the assembly and/or the activity of one or more respiratory enzyme complexes.

To determine the complex(es) affected, the NADH-cytochrome *c* reductase and cytochrome *c* oxidase activities of the mutant strains were analysed. As shown in Table III, the NADH-cytochrome *c* reductase activity in strains Ma1 and Mb12 was reduced by about 60% and 70%, respectively. If petite frequency is taken into consideration, these rates represent a reduction of only about 40% to that of the wild-type strain. The cytochrome *c* oxidase activity, however, appears to be more significantly affected by the mutations in these strains. Thus, strains Ma1 and M10-7 exhibited about 18% of the wild-type cytochrome *c* oxidase activity, while strain Mb12 contained only

TABLE I

STEADY-STATE GROWTH YIELDS OF *oli2 mit*$^-$ STRAINS IN GLUCOSE-LIMITED CHEMOSTAT CULTURES

Cells were grown in glucose-limited chemostat cultures at a dilution rate of 0.1 h^{-1}. The petite frequencies of the *mit*$^-$ strains varied from 26 to 40% in these cultures. Figures are the mean ± S.D. of at least three independent experiments.

Strain	Y_{glu} (g cell dry wt./ml glucose)
J69-1B (wild type)	120.7 ± 5.9
Ma1	25.2 ± 2.7
Mb12	24.3 ± 3.0
M10-7	26.1 ± 1.4
EJO (*rho*°)	23.4 ± 2.7

TABLE II

ATPase AND ATP-[^{32}P]P_i EXCHANGE ACTIVITIES OF MITOCHONDRIA ISOLATED FROM THE *oli2 mit*$^-$ MUTANT STRAINS

The wild-type and mutant strains were grown in glucose-limited chemostat cultures at 28°C at a dilution rate of 0.1 h^{-1}. Intact mitochondria were isolated from spheroplasts, and ATPase and ATP-[^{32}P]P_i exchange assays carried out as described in Materials and Methods. The concentration of oligomycin used in ATPase assays was about 200 μg/mg mitochondrial protein. n.d., not determined.

Strain	Apparent M_r of subunit 6 (kDa)	ATPAse		ATP-[^{32}P]P_i exchange (nmol/min per mg)
		Specific activity (μmol/min per mg)	Inhibition by oligomycin (%)	
J69-1B (wild type)	20	0.79	83	156
Ma1	15+16	0.28	15	0
Mb12	19	0.40	16	1
M10-7	absent	0.42	10	n.d.
EJO (*rho*°)	absent	0.43	5	0

about 5% of the wild-type activity.

Since the *oli2* mutations in these *mit*$^-$ strains have been shown to affect specifically subunit 6 of the H^+-ATPase, the reduction in the activities of the respiratory enzyme complexes reflects secondary effects of the mutations. This phenomenon is quite common in yeast mitochondria whereby a mutation in one gene (e.g., the structural gene for cytochrome *b*) can affect the production of another protein (e.g., cytochrome oxidase subunit I) encoded in a separate region of the mtDNA [29–32]. The mechanism(s) for such interactions which are also observed between other mitochondrial genes [33] is still not fully understood.

Since the *mit*$^-$ strains retain significant levels (although reduced) of respiratory activity, the inability of the *mit*$^-$ strains to grow by oxidative metabolism is probably also partly due to the absence of coupling between the electron-transport chain and the synthesis of ATP by the ATPase complex (since ADP cannot stimulate mitochondrial respiration). Indeed, when the ATP-[^{32}P]P_i exchange activities of the ATPase complex in the *oli2 mit*$^-$ mutants were analysed, the *mit*$^-$ strains were found to be incapable of catalysing ATP-[^{32}P]P_i exchange (Table II). Thus, although

TABLE III

MITOCHONDRIAL RESPIRATORY ACTIVITIES OF *oli2 mit*$^-$ strains

Intact mitochondria were isolated from cells grown in chemostat cultures at 28°C and respiratory enzyme activities determined as described in Materials and Methods. The mitochondrial respiration experiment was independent of the experiment where NADH-cytochrome *c* reductase and cytochrome *c* oxidase assays were carried out. Results shown are the representative of at least three independent experiments except for the petite frequencies, which are expressed as the mean ± S.D. n.d., not determined.

Strain	Mitochondrial respiration (nmol O_2/min per mg)		Petite frequency (% of total population)	NADH-cytochrome *c* reductase (μmol/min per mg)	Cytochrome *c* oxidase (μmol/min per mg)
	+ EtOH	+ EtOH + ADP			
J69-1B	79.2	148.9	6 ± 3	1.06	1.2
Mb12	23.4	23.7	45 ± 15	0.32	0.06
Ma1	46.6	49.0	42 ± 8	0.43	0.21
M10-7	32.8	32.8	40 ± 14	n.d.	0.22

the mutant strains have significant levels of ATPase and electron transport chain activities, the ATPase complex apparently is unable to utilise the energy obtained from the proton gradient generated by respiration, for the synthesis of ATP. One possible explanation is that in the absence of subunit 6 or its presence in a grossly altered state, there is a gross defect in the assembly of membrane sector components of this enzyme complex. Alternatively, it is possible that the components of the F_1 and the F_0 sectors are assembled normally, but subunit 6 is required for the coupling of the proton channel activity of the F_0 sector to the ATPase activity of the F_1 sector. The suggested possibilities were investigated.

Subunit 6 does not appear to be required for the assembly of the H^+-ATPase proton channel

The proteolipid subunit 9 of the F_0 sector of the H^+-ATPase complex has been reported [34–36] to be involved in the proton channel of the enzyme complex, but whether this subunit in itself is sufficient to form the channel or whether other F_0 subunits are also required to form this channel is not firmly established. It would therefore be of great interest to determine whether subunit 6 plays a role in the activity or the assembly of the H^+-ATPase protein channel. As shown in the previous section, the addition of ADP to the respiring mitochondria of the mutant strains did not produce any effect, indicating the lack of respiratory control (also called acceptor control, to distinguish it from respiratory control by oligomycin) in these strains. However, when oligomycin was subsequently added to the reaction mixture, the antibiotic was found to inhibit respiration in the *mit*$^-$ strains to the same extent as it did on the ADP stimulated respiration of the wild-type strain (Table IV). Oligomycin exerts its action on respiration through the proteolipid subunit 9 by blocking the proton channel of the H^+-ATPase complex [37,3], resulting in a build-up of protons outside the mitochondria which in turn inhibits respiration. The observation that oligomycin inhibited respiration in the *mit*$^-$ strains therefore indicates that the oligomycin-binding subunit 9 is assembled in these mutant strains into the proton channel of the F_0 sector. The uncoupler CCCP (which acts as a proton carrier) reverses the inhibition of respiration by oligomycin in the mutant strains as well as that in the wild type, presumably by allowing free translocation of protons across the membrane. From these indirect assays, it therefore appears that the assembly of the proton channel is not affected by the absence of subunit 6 or the presence of a grossly altered subunit 6.

TABLE IV

EFFECT OF OLIGOMYCIN ON STATE III (ADP STIMULATED RATE) RESPIRATION IN MITOCHONDRIA ISOLATED FROM THE *oli2 mit*$^-$ STRAINS

Cells were grown in chemostat cultures and intact mitochondria isolated. Mitochondrial respiration was measured polarographically at 28°C in 2.5 ml of 10 mM Tris-phosphate buffer (pH 7.4) containing 0.6 M sucrose, 2 mM EDTA and 5 mg delipidated bovine serum albumin. Addition of 5 μl absolute ethanol initiated the reaction. Subsequent additions were 0.5 μmol ADP, 30 μg oligomycin and 30 nmol CCCP. Data presented are the mean ± S.E. of the means of more than five independent experiments.

Sequential additions to mitochondria	Respiration rate (% CCCP stimulated rate)		
	J69-1B	Ma1	M10-7
Ethanol	45.8 ± 2.9	89.7 ± 8.2	94.0 ± 2.9
ADP	93.3 ± 10.9	89.7 ± 8.2	95.7 ± 5.2
Oligomycin	59.5 ± 6.9	61.0 ± 5.4	67.7 ± 2.9
CCCP	100	100	100

Assembly of the proton channel to F_1-ATPase

One way to determine whether the ATPase activities in the *oli2 mit*$^-$ strains are those of free F_1 or those of F_1F_0-complex is by studying the cold stability of the enzyme complex in these strains. Solubilised F_1-ATPase is oligomycin-insensitive and cold-labile, and differs in these respects from the membrane-bound enzyme, which is oligomycin-sensitive and cold-stable [38,39]. Thus, as shown in Fig. 4a, the completely oligomycin-insensitive F_1-ATPase solubilised from submitochondrial particles of the wild-type strain by using a chloroform/methanol extraction procedure lost more than 50% of its original activity at 4°C in just 1 h after the solvent extraction, while the mitochondria of the wild-type strain still retained about 70% of their original activity, even after 40 h incubation in the cold. Analysis of the mutants which have lost subunit 6 (strain M10-7)

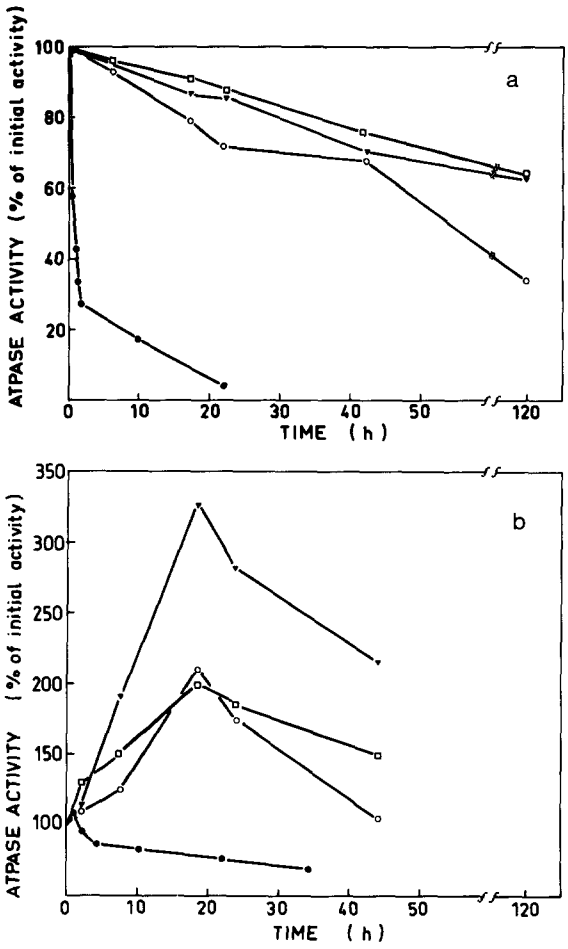

Fig. 4. Cold stability of the mitochondrial ATPase in *oli2 mit*$^-$ mutant strains. Strains were grown in glucose-limited chemostat cultures at 28°C at a dilution rate of 0.1 h^{-1} and intact mitochondria were isolated as described in Materials and Methods. F_1-ATPase was extracted from the wild-type strain with chloroform according to the method of Beechey et al. [19]. The specific activity of the F_1-ATPase preparation was 7.05 μmol ATP/min per mg protein and its ATPase activity was insensitive to inhibition by oligomycin. Mitochondria and solubilised F_1-ATPase were incubated at 4°C (a) and room temperature (b). Aliquots were taken at intervals and assayed for the ATPase activity (○) Strain J69-1B; wild type. (□) Strain Ma1; *oli2 mit*$^-$, grossly altered subunit 6. (▲) Strain M10-7; *oli2 mit*$^-$, subunit 6-less. (●) Solubilised wild-type F_1-ATPase activities.

or have an altered subunit 6 (strain Ma1) revealed mitochondrial ATPase activity which exhibits the same cold stability as that of the wild-type strain (Fig. 4a).

The mechanism responsible for the cold-lability of the F_1-ATPase is not well understood. Two yeast proteins (apparent M_r 9 and 15 kDa), which were recently sequenced [40], have been shown to promote the binding of the intrinsic H^+-ATPase inhibitor protein to the F_1F_0-ATPase and stabilise the ATPase-inhibitor complex [41]. The 9 and 15 kDa stabilising factors had no affect on the stabilisation of the complex between purified F_1-ATPase and the inhibitor, and were therefore suggested to be involved in the binding of the F_1-ATPase-inhibitor complex to the F_0 membrane sector. Thus these two factors might be involved in the conferral of the cold-stability to the F_1F_0-ATPAse.

Our results indicate that the ATPase activity observed in the absence of subunit 6 or in the presence of a grossly altered subunit 6 is not of a free F_1-ATPase but of a complex consisting of the F_1-ATPase (as well as other associated factors such as the recently reported 9 and 15 kDa stabilising factors [41]) and one or more of the other F_0 subunits which confer cold stability to the enzyme complex. The stability of solubilised F_1-ATPase at room temperature is demonstrated in Fig. 4b: its specific activity at this temperature was found to be elevated before decreasing to about 80% of its original activity after 20 h. The increase in specific activity followed by a decrease with incubation at room temperature was also observed in the membrane-bound ATPase of the wild-type strain as well as in the *oli2 mit*$^-$ strains. The observed phenomenon probably reflects the dissociation of the F_1 inhibitor protein [42,43] and/or the 9 and 15 kDa stabilising factors [41] and the subsequent dissociation of the F_1 subunits and their degradation by proteinases.

The above results suggest that subunit 6 of the H^+-ATPase is not essential for the attachment of the F_1 sector of the enzyme complex to the mitochondrial membrane. It is therefore of interest to determine the extent to which the ATPase assembly can still proceed in the absence of subunit 6 or its presence in a grossly altered state. Workers in our laboratory have recently produced and characterised a number of monoclonal antibodies against various subunits of the mitochondrial H^+-ATPase [5]. A monoclonal antibody, RH48-6, which is specific for the β-subunit of the F_1-sector was used in the present study to isolate the enzyme

complex from the *oli2 mit*$^-$ strains in order to establish the H^+-ATPase assembly defect in these mutants. This monoclonal antibody was used because it was found that, unlike many polyclonal antibodies, it can efficiently precipitate the ATPase complex from detergent-solubilized mitochondria without causing the dissociation of the subunits of the enzyme complex [5].

For this purpose, the wild-type strain J69-1B and the *oli2 mit*$^-$ mutant strains Ma1 and M10-7 were labelled with [^{35}S]sulphate for 4 h in low sulphate medium in the absence of any antibiotics, as described in Materials and Methods. Assembled F_1 and F_0 subunits were immunoprecipitated with the monoclonal antibody from the Triton extracts of mitochondria isolated from the ^{35}S-labelled cells. As shown in Fig. 5A, in the wild-type strain, this antibody precipitated all the F_1 subunits (α, β, γ, δ and ϵ: apparent M_r 56, 54, 31, 14 and 12 kDa, respectively) and the mitochondrially synthesised subunits of the F_0 sector (subunits 6, 8 and 9: apparent M_r 20, 10 and 7.6 kDa, respectively). In addition, two other protein bands (apparent M_r 18 and 25 kDa) could be observed in the immunoprecipitate, and since these additional polypeptides are always associated with the immunoprecipitates obtained with monoclonal anti-β-antibodies, it has been suggested that they are probably genuine subunits of the enzyme complex [5]. The additional protein bands observed between the β and the γ subunits have previously been shown to be breakdown products of the β subunit.

Analysis of the immunoprecipitates from the two *oli2 mit*$^-$ mutant strains shown in Fig. 5A reveals the presence of the four largest subunits of F_1-ATPase (α, β, γ and δ) and subunits 8 and 9 of the F_0 sector. Hence, it appears that the F_1 sectors

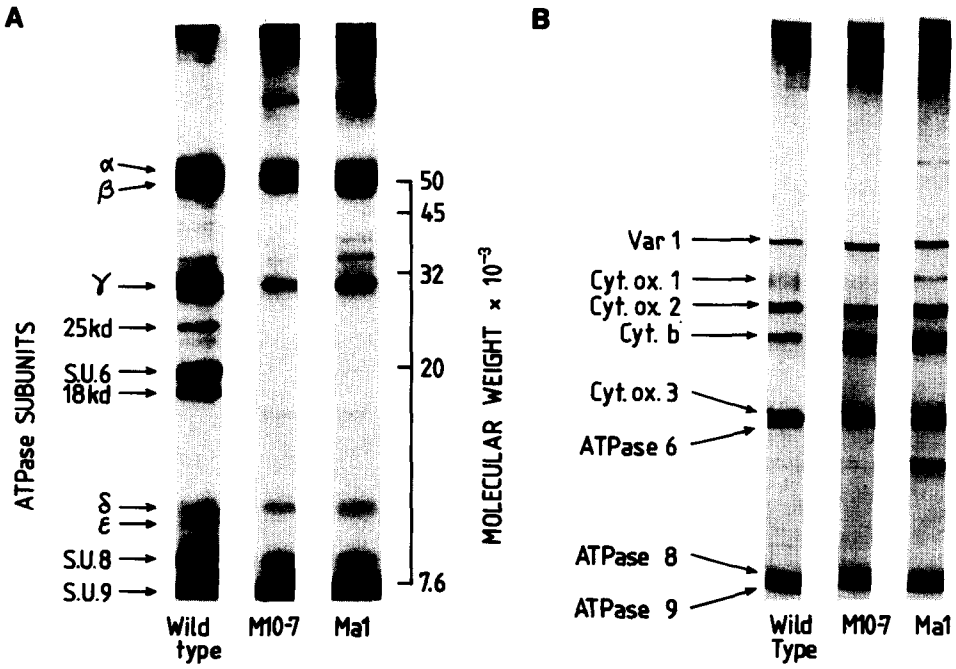

Fig. 5. Assembled subunits of the H^+-ATPase in the *oli2 mit*$^-$ mutants. Wild type and *oli2 mit*$^-$ strains (strain M10-7 which lacks subunit 6 and strain Ma1 which has a grossly altered subunit (6) were grown in glucose-limited chemostat cultures at 28°C. Cells were harvested in the steady state and labelled in vivo with [^{35}S]sulphate for 4 h in the absence of antibiotics. Mitochondria were isolated and Triton extracts prepared from the mitochondria. Assembled subunits of the H^+-ATPase complex were then immunoprecipitated from the Triton extracts by using a monoclonal anti-β antibody RH48.6, which has been coupled to CNBr-activated Sepharose 4B beads as in Materials and Methods. The immunoprecipitated samples were displayed on a 12.5% polyacrylamide gel [15], and radioactively labelled bands were detected by fluorography [17]. (A) H^+-ATPase subunits associated with the immunoprecipitate. (B) Total mitochondrial translation products (labelled with [^{35}S]sulphate in the presence of cycloheximide as in Fig. 2) in strains used for the immunoprecipitation.

Analysis of the immunoprecipitates from the two *oli2 mit*$^-$ mutant strains shown in Fig. 5A reveals the presence of the four largest subunits of F_1-ATPase (α, β, γ and δ) and subunits 8 and 9 of the F_0 sector. Hence, it appears that the F_1 sectors of the *oli2 mit*$^-$ mutants are indeed associated with components of the F_0 sector of the H^+-ATPase enzyme complex. A number of H^+-ATPase subunits, however, could not be detected in these immunoprecipitates. these include the ϵ subunit of the F_1-ATPase, and the 18 and 25 kDa cytoplasmically synthesised subunits. In addition, trace amounts of a polypeptide with the mobility of a 16 kDa protein was found to be associated with the *oli2 mit*$^-$ immunoprecipitates. The significance of this observation is not clear at present. In strain Ma1, the 16 kDa polypeptide might represent a small amount of assembled altered subunit 6 (apparent M_r 15–16 kDA) which is a major component in the display of the total mitochondrial translation products of the mutant strain (Fig. 5B). However, a polypeptide with a similar mobility was also detected in the immunoprecipitate of strain M10-7 (Fig. 5A), which does not synthesise an altered subunit 6 (Fig. 5B).

Discussion

The oli2 gene codes for H$^+$-ATPase subunit 6

Although it is widely accepted that the *oli2* gene codes for the mitochondrial H^+-ATPase subunit 6, the evidence for this is actually very limited. Thus, while the nucleotide sequence of the *oli2* gene has been completely determined and it has been possible to predict from the nucleotide sequence that the *oli2* gene product is a hydrophobic protein of 259 amino acids long (approx. 28 kDa), no information is as yet available on the amino acid composition of subunit 6, or on its amino acid sequence, to allow any direct comparison to be made between the *oli*2 gene sequence and its putative gene product. The evidence for the gene-gene product relationship between the *oli2* gene and H^+-ATPase subunit 6 is in fact very indirect and based on a preliminary report from our laboratory that some *mit*$^-$ mutants of yeast, with lesions mapping in the *oli2* region of the mitochondrial genome, do not synthesize subunit 6. The results presented in the present report, therefore, are of a particular significance, as they confirm and extend the previous observation.

A total of nine *oli2 mit*$^-$ strains which are well characterized genetically have been analysed. the mutations in these strains have been more precisely mapped and shown to be located within the *oli2* coding region. In particular, the mutation in one of these strains (strain Mb12) has been shown to be at an *Eco*RI restriction site which is located within the *oli2* reading frame.

In several of these mutants, subunit 6 was found to be replaced by new mitochondrial translation products with apparent molecular weights of lower than 20 kDa. The new polypeptides co-precipitated with mitochondrial ATPase when the enzyme complex was isolated with rabbit anti-holo ATPase, indicating that the new polypeptides retain antigenic determinants of an ATPase subunit or are physically associated with a subunit of the enzyme complex which is antigenic.

The new mitochondrial translation products are probably the result of an early termination during the translation of ATPase subunit 6 in the mutant strains. Comparison of peptide maps of the new translation products with that of subunit 6 showed good structural homology between two of the new products examined (from strains Ma1 and M13-20) and subunit 6. In addition, there is a good overall relationship between the reported mapping positions on the *oli2* gene of the mutations in the strains analysed [8] and the size of the new products observed.

Together with our genetic mapping and DNA sequencing data [8,9], results presented in this communication provide the experimental evidence for the widely accepted assumption that H^+-ATPase subunit 6 is the product of the *oli2* gene. Many questions, however, remain unanswered. For example, the apparent molecular weight of subunit 6 as determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate is significantly smaller than the molecular weight predicted from the DNA sequence of the *oli2* gene. This apparent discrepancy might be simply due to the hydrophobic nature of subunit 6. Many hydrophobic proteins have been reported to behave abnormally in SDS-polyacrylamide gel electrophoresis, and similar discrepancies have been reported between the apparent molecular

weight of several other yeast mitochondrial translation products and their molecular weight as predicted from the nucleotide sequence of their structural genes, for example, cytochrome oxidase subunit III, apparent molecular weight 20 000, predicted molecular weight 30 000 [44,45].

It is also possible, however, that subunit 6 is synthesized as a 28 kDa precursor molecule which is then processed by partial proteolysis to produce a smaller mature subunit. Determination of at least the N-terminal and the C-terminal amino acid sequences of subunit 6 is essential to resolve some of these questions. Direct amino acid sequencing might also explain the observation in our laboratory that the H^+-ATPase subunit 6 can be separated by isoelectrofocusing into two components of different p*I*, but almost similar apparent molecular weight [16,46]. Both components are simultaneously altered by single *mit*$^-$ mutations in the *oli2* gene and one-dimensional papain-generated peptide maps of these components showed that they are identical except for the mobility of a single fragment. These observations suggest that the two components of subunit 6 are different forms of a single protein.

Assembly of the H^+-ATPase

The present study represents the first detailed biochemical characterization of *oli2 mit*$^-$ mutants in which the alterations that occur to the H^+-ATPase subunit 6, as the consequence of the mutations in its structural gene, are well defined. A *pho1* mutant, which also carries mutations in the *oli2* region of the mtDNA, has previously been biochemically analysed [28]. However, this *pho1* mutant was shown to retain some capacity to grow by oxidative metabolism, and most likely, therefore, to contain assembled, albeit only partially semi-functional, subunit 6.

Results presented in this communication provide some information on the involvement of H^+-ATPase subunit 6 in the assembly of the enzyme complex. Firstly, this subunit which is a hydrophobic protein component of the membrane sector of the enzyme complex, does not appear to be required for the assembly of the ATPase proton channel. Thus, in the absence of a functional subunit 6, the mitochondrial respiratory activities in the *oli2 mit*$^-$ mutants were found to be still sensitive to inhibition by oligomycin, a specific inhibitor of the H^+-ATPase proton channel. This observation is of particular significance because, although the involvement of the ATPase subunit 9 in the proton translocation step of oxidative phosphorylation is now well documented, it is by no means certain that this proteolipid is the only component of the H^+-ATPase proton channel. It is interesting to note that, in contrast to our finding, a mutant of *Escherichia coli* (*unc* B402) which synthesizes a shorter form of the *a* subunit of the H^+-ATPase (homologous to the yeast H^+-ATPase subunit 6 (see Ref. 47) as the result of a chain terminating mutation in the *unc* B gene, has been shown to have low F_0-mediated H^+-translocation activity [48]. More *unc* B mutants, including those in which no synthesis of the *a* subunit or its altered form is detectable, have to be analysed to resolve the discrepancy. Direct measurement of the proton channel activity in the *oli2 mit*$^-$ mitochondria is also required to confirm our conclusion. The extension of such study to the investigation of the mitochondrial H^+-ATPase proton channel activity in *oli1* and *aap1 mit*$^-$ mutants which specifically lack the H^+-ATPase subunit 9 and subunit 8 respectively [49], will significantly contribute to our understanding of the role of these subunits in the assembly of the ATPase proton channel.

Secondly, our results also indicate that subunit 6 is not essential for the assembly of most of the F_1 subunits to the mitochondrially synthesized subunits 8 and 9 of the membrane sector of the enzyme complex. Immunoprecipitation of the assembled complex from the *oli2 mit*$^-$ mutants with a monoclonal anti-β-subunit antibody clearly demonstrates that the complex contains subunits 8 and 9, as well as four of the F_1 subunits (α, β, γ and δ). This finding is consistent with our observation that the partially assembled complex is not cold-labile as would be expected if the ATPase activity detected in the mutant mitochondria were that of a free F_1 sector. The binding of the F_1-subunits to the membrane sector in the mutant strains, however, is less tight than that of the wild-type. Thus, more than 90% of the ATPase activity was released from strain Ma1 mitochondria when these mitochondria were subjected to ultrasonic vibration in a Branson sonifier under conditions which

released only 30% of the activity from the wild-type mitochondria (data not shown).

It is not possible as yet to firmly establish the exact subunit composition of the partially assembled complex. While the monoclonal anti-β-subunit antibody used has been shown to precipitate reproducibly the assembled subunits of the H^+-ATPase complex in the wild-type strain, it is quite possible that some of these subunits bind less tightly to the complex in the absence of subunit 6, and therefore might have become dissociated during immunoprecipitation. The F_1 subunits and subunits 8 and 9 observed in the immunoprecipitates of the *oli2 mit*$^-$ mitochondrial ATPase, therefore, can only be considered at the moment to be the minimum composition of the defective complex. In a number of other *oli2 mit*$^-$ immunoprecipitates, for example, small amounts of the ϵ and the 18 kDa subunits could sometimes be detected. Furthermore, we have recently observed that all five subunits of the F_1 sector are assembled in a mtDNA-less *rho*° mutant of yeast which do not have an active mitochondrial protein synthesis and therefore cannot synthesise the three mitochondrially synthesised subunits of the enzyme complex. Further analysis involving the use of other monoclonal antibodies and the refinement of the immunological procedure is required to resolve this question.

Note added in proof (Received January 3rd, 1985)

The nature of the mutations in a series of *oli2 mit*$^-$ strains has recently been determined (John, U.P., Gabrielle, McMullen, L., Novitski, C.E., and Nagley, P., unpublished data). In each of three *mit*$^-$ mutants described above, Mb12, Ma1 and M10-7, the mutation is found to be either the insertion or the deletion of a single base that leads to a reading frameshift and a consequent premature termination of translation. The electrophoretic mobility of the altered subunit 6 polypeptide in strains Mb12 and Ma1 is commensurate with the predicted altered *oli2* gene product (246 and 211 amino acids in length, respectively), whilst in strain M10-7 (no subunit 6 detectable), the predicted *oli2* gene product is only 47 amino acids long.

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