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BIOGENESIS OF MITOCHONDRIA

DEFECTIVE ASSEMBLY OF THE PROTEOLIPID INTO THE MITOCHONDRIAL ADENOSINE TRIPHOSPHATASE COMPLEX IN AN *oli2 mit*$^-$ MUTANT OF *SACCHAROMYCES CEREVISIAE* *

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A single mutation in the *oli2* region of the mitochondrial DNA causes a charge alteration in a mitochondrially translated subunit of the mitochondrial ATPase (subunit 6; apparent M_r 20 000; apparent p*I* 6.9 and 7.1). This alteration leads to the defective assembly of the proteolipid subunit into the enzyme complex. The mutant, which is able to grow only very slowly by oxidative metabolism at 28°C offers new possibilities for studying the assembly of the membrane sector (F_0) into the mitochondrial ATPase complex and the role of subunit 6 in this process.

Introduction

The assembly of multi-subunit enzyme complexes of the mitochondrial inner membranes is a complicated process, involving the synthesis and assembly of protein components made within mitochondria as well as those imported from the extra-mitochondrial cytoplasm [1]. The process is still poorly understood even though there have been extensive investigations using a variety of experimental approaches. These include: (a) the chemical dissection of the membrane into individual units followed by a careful investigation of the conditions required to reconstitute these units into functional membranes, and (b) in vivo studies in yeast of the formation of functional mitochondrial membranes when cells are released from anaerobiosis or catabolite repression.

Information on the various controls of the assembly of the mitochondrial membrane can also be obtained by extensive characterisation of mutants which are defective in mitochondrial assembly as the result of well-defined single mutational events (affecting either the nuclear or the mitochondrial DNA) [2]. Although potentially very useful, this last approach has been employed less frequently due mainly to the difficulty encountered in isolating assembly mutants.

In this paper we describe a *mit*$^-$ mutant of *Saccharomyces cerevisiae* which is of particular interest; the mutation in this strain, which maps in the *oli2* region of the mtDNA, causes a change in the apparent p*I* of a subunit of mitochondrial ATPase which has a molecular weight of approx. 20 000 (subunit 6 inTzagoloff's nomenclature [3]). The alteration does not affect the assembly of this subunit with the F_1-ATPase but leads to an extremely low rate of assembly of the proteolipid into the F_0F_1-ATPase complex. The mutant can, therefore, be used to study the assembly of the membrane sector (F_0) of the mitochondrial ATPase into a functional enzyme complex and to obtain further information on the role

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Abbreviations: SDS, sodium dodecyl sulphate; CCCP, carbonylcyanide *m*-chlorophenylhydrazone.

played by the two mitochondrially synthesized subunits of the F_0 sector of the mitochondrial ATPase in the biogenesis and the function of the enzyme complex.

Methods

Yeast strains and cell growth

S. cerevisiae strain J69-1B (*α adel his* [*rho*$^+$]) is the parental strain from which the *oli2 mit*$^-$ mutants, M13-20, M10-7 (described previously [4]) and M11-28 were generated by mutagenesis with Mn^{2+} [5].

In contrast to strains M13-20 and M10-7 which cannot grow on non-fermentable carbon source, strain M11-28 exhibits a 'leaky' *mit*$^-$ phenotype, growing very slowly on ethanol at 28°C.

The mutation in strain M11-28 is tightly linked to a group of mutations (including M13-20 and M10-7) which have been previously mapped in the *oli2* region of mtDNA [4]. It also generates diploid progeny with a wild-type phenotype in a cross with a *rho*$^-$ strain retaining a short segment of mtDNA which contains part of the *oli2* gene [4]. Cells used for the study of enzyme activities were grown to steady state in glucose-limited chemostat cultures [6] and those used for radioactive labelling of mitochondrial translation products were grown aerobically to early stationary phase in 75 ml batch cultures in a growth medium containing a salts mixture, yeast extract (10 g/l), 2% glucose as an energy source, and the strain auxotrophic requirements [7].

Electrophoretic analysis of mitochondrial translation products

Mitochondrial translation products from wild-type and mutant strains were pulse labelled in vivo for either 60 or 180 min as indicated in a low sulphate medium containing cycloheximide (500 μg/ml) and $H_2{}^{35}SO_4$ (500 μCi/ml) as previously described [8]. Yeast cells were broken using the manual shaking method [9] and mitochodria were isolated in the presence of protease inhibitors according to the method of Roberts et al. [10].

SDS-polyacrylamide gel electrophoresis of the labelled samples was carried out on 12.5% gels as previously described [8]. Two-dimensional electrophoretic analysis of the mitochodrial translation products was performed following the method recently described by Stephenson et al. [11], except that the time of equilibration of the isoelectric focused gel in the SDS/Tris-HCl buffer, prior to electrophoresis on the second dimension, was reduced from 10 min to 2 min. Although this modification results in occasional streaking of the major spots, it has the advantage of conserving some components focused at the basic end of the first-dimension gel which can otherwise be eluted out during equilibration. The ^{35}S-labelled mitochondrial translation products electrophoresed on one- and two-dimensional gels were visualised by fluorography [12] on Kodak X-O-MAT film.

Immunoprecipitation procedure

Rabbit specific antiserum against yeast F_1-ATPase was a gift from Dr. G. Schatz. Rabbit and F_0F_1-ATPase was prepared in this laboratory as previously described [13]. ATPase subunits were immunoprecipitated from Triton extracts of the ^{35}S-labelled mitochondria obtained by incubating the mitochondrial sample on ice for 15 min in 5.4 mM Tris-acetate buffer, pH 7.5, containing 62 mM sucrose and 0.5% (w/v) Triton X-100, at a concentration of 6.25 mg mitochondrial protein/ml.

Immunoprecipitation was performed in the presence of *Staphylococcus aureus* carrying protein A as an antibody adsorbant. Cells of *S. aureus* Cowan strain 1 were grown, heat killed, formaldehyde-fixed and stored as described by Kessler [14]. Immediately before immunoprecipitation, cells (10 ml packed cell volume/100 ml suspension) were collected by centrifugation, resuspended to the same cell concentration in 5 mM Tris-acetate buffer, pH 7.5, containing 0.5% (w/v) Triton X-100 (immunoprecipitation buffer) and incubated at 23°C for 15 min in a shaking incubator. The cells were washed once with the immunoprecipitation buffer and resuspended again in the same buffer.

To minimize nonspecific precipitation by the *S. aureus* cells, 50 μl Triton extract, diluted to 200 μl with the immunoprecipitation buffer, was mixed vigorously with 100 μl of *S. aureus* cell suspension treated as above. After collecting the cells by centrifugation, the supernatant was mixed with 100 μl antiserum and incubated at 4°C for 1 h. *S. aureus* cell suspension (100 μl) was then added to the antigen-antibody mixture, mixed and centrifuged. The result-

ing immunoprecipitate was washed five times with the immunoprecipitation buffer.

For electrophoretic analysis on SDS-polyacrylamide gels the immunoprecipitate was dissociated at 100°C for 2 min in 50 μl of 120 mM Tris-HCl buffer, pH 6.7, containing 2% SDS-mercaptoethanol, 14% (w/v) sucrose, 5 mM aminocaproic acid and 0.5 M phenylmethylsulphonyl fluoride. After removing the *S. aureus* cells by centrifugation the supernatant was analysed as previously described [8]. For two-dimensional electrophoretic studies, the immunoprecipitates were incubated for 15 min at room temperature, in a medium containing (final concentrations): 1% (w/v) SDS/8 M urea/1% (w/v) β-mercaptoethanol/4 mM ε-aminocaproic acid/4 mM paraaminobenzamidine-HCl/0.4 mM phenylmethylsulphonyl fluoride. This incubation releases about 98% of the antigen from the *S. aureus* cells [14]. *S. aureus* cells were removed by centrifugation and the supernatants, adjusted to pH 3.0 with 100 mM citric acid, were applied to the positive end of the isoelectric-focusing gel as previously described [11].

Enzymatic assays

Mitochondrial used for determination of enzyme activities were isolated in the presence of delipidated bovine serum albumin (2 mg/ml) by the snail enzyme method as previously described [15]. Published procedures were used to measure whole cell respiration, and to assay cytochrome oxidase [16], NADH-cytochrome *c* reductase [17], ATPase [18] and ATP-$^{32}P_i$ exchange [19] activities.

Results

Identifaction of subunit 6 of the mitochondrial ATPase in the two-dimensional map of the mitochondrial translation products of yeast Saccharomyces cerevisiae

Our laboratory has recently reported evidence which indicates that the *oli2* region of the mtDNA contains the structural gene for subunit 6 of the mitochondrial ATPase (apparent M_r 20 000 in our gel system). Thus, studies on a group of *oli2 mit*$^-$ mutants showed that mutations in the *oli2* region of mtDNA can lead to either a complete loss of subunit 6 (as shown by immunoprecipitation of the mitochondrial ATPase complex) or its replacement with shorter polypeptides which are either antigenically related to a subunit of the mitochondrial ATPase, or associated with an antigenic component of the enzyme complex [18,4].

One of the *oli2 mit*$^-$ mutants which lacked subunit 6 of the mitochondrial ATPase was subsequently used to identify this subunit in the two-dimensional electrophoretic map of the mitochondrial translation products of *Saccharomyces cerevisiae* [11]. Results of this study indicate that, under our experimental conditions, subunit 6 of the mitochondrial ATPase was resolved into two spots (corresponding to apparent M_r 20 000, apparent pI 6.9 and 7.1). This observation has been confirmed in the present study by two-dimensional electrophoretic analysis of two other *oli2 mit*$^-$ mutants (Figs. 1B and C). In both cases the *oli2* mutation results in the simultaneous disappearance of the two spots mentioned above. In one strain (Fig. 1C) the disappearance of these spots is accompanied by the appearance of two new mitochondrial translation products (apparent M_r 16 000 and 17 000, apparent p*I* 7.9 and 7.8).

Although one could not rule out the possibility that the two subunit 6 spots are due to technical artifact, one of the alternative explanations for this observation is that subunit 6 is composed of two slightly different polypeptides which are genetically linked; one, for example, could arise from post-translational modification of the other. This suggestion is supported by the recent finding that subunit 6 can occasionally be resolved into two bands when a purified ATPase preparation is analysed on a 4–17% SDS-polyacrylamide gel [20,21].

A mutation in the oli2 region of mtDNA causes a change in the apparent pI of the 20 000 dalton subunit(s) of mitochondrial ATPase complex

One of the *oli2 mit*$^-$ strains isolated in our laboratory displays a very interesting phenotype. The mutant, designated M11-28, retains some ability to grow on ethanol, albeit at a very low rate (doubling time 16 h) and yet shows no detectable alteration in the amount or the mobility of the mitochondrially made subunits of mitochondrial ATPase when either total mitochondrial translation products or mitochondrial ATPase isolated by immunoprecipitation using a rabbit specific antiserum against F_0F_1-ATPase were analysed by one-dimensional gel electrophoresis

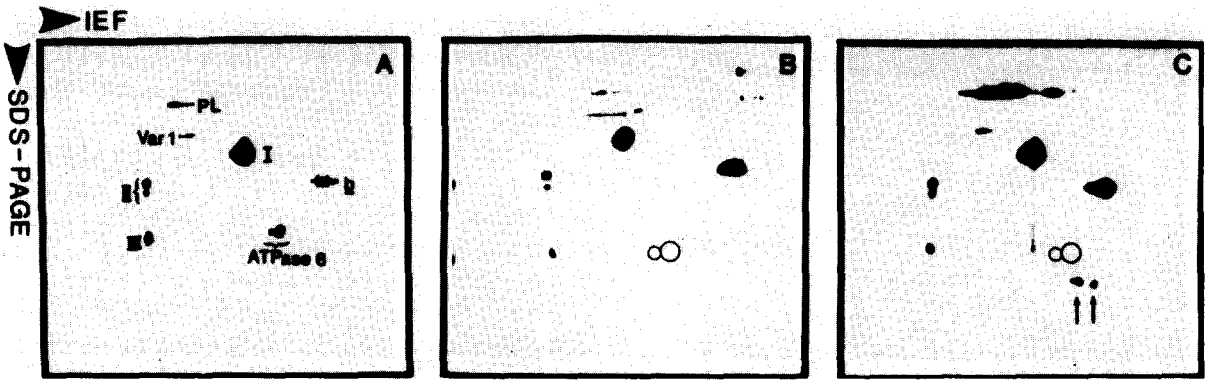
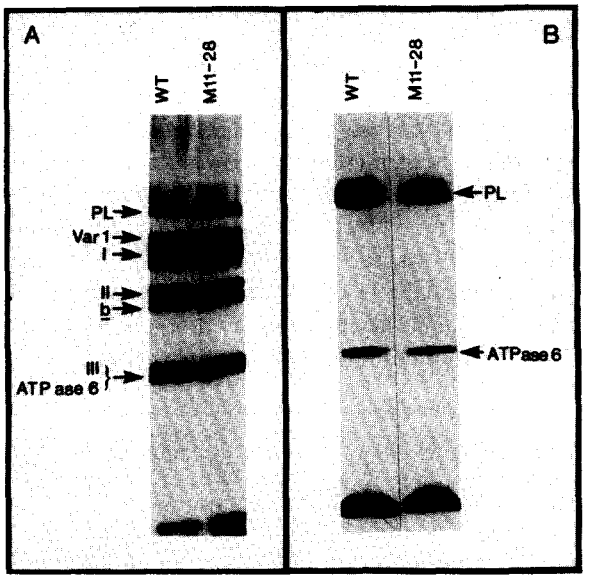
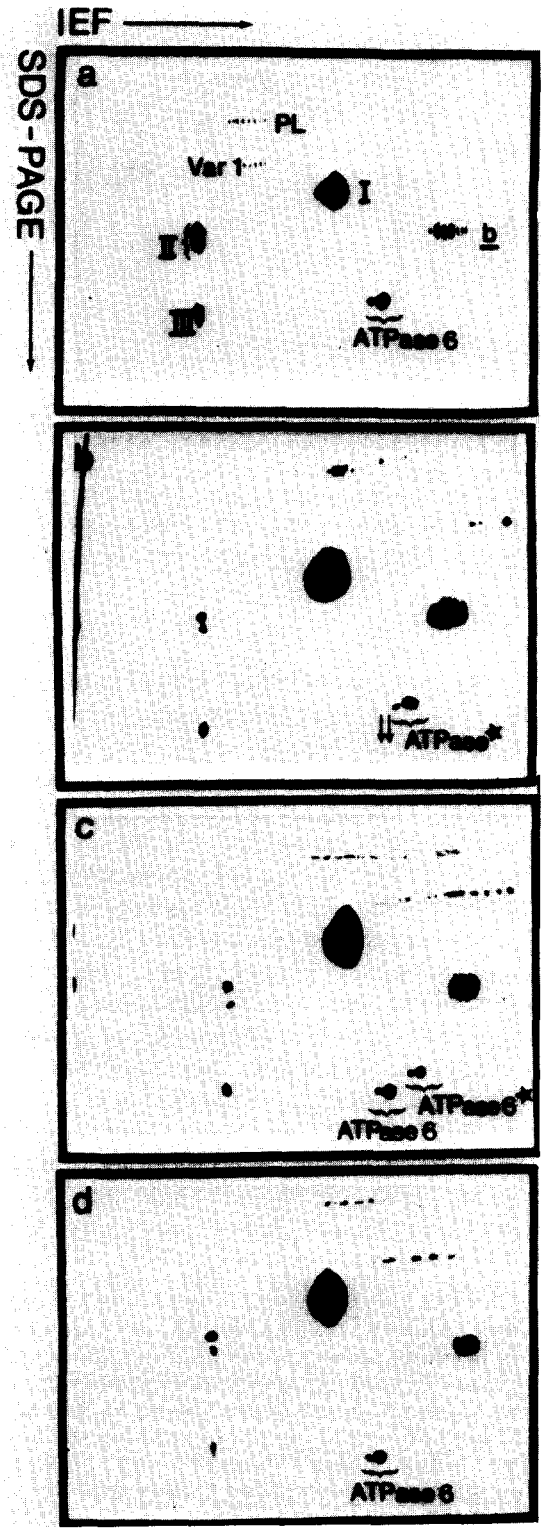


Fig. 1. Mitochondrial translation products of a wild-type and two *oli2 mit*$^-$ strains resolved by two-dimensional polyacrylamide gel electrophoresis. Cells were labelled with $^{35}SO_4$ in the presence of cycloheximide [8] and the mitochondrial translation products were electrophoresed in two dimensions as previously described [11]. (A) strain J69-1B (wild-type); (b) strain M10-7 (*oli2 mit*$^-$); (C) strain M13-20 (*oli2 mit*$^-$). Products which are missing in the mutant strains are indicated by open circles. Thin arrows indicate new translation products observed in the mutants; PL, oligomer of the proteolipid subunit of the mitochondrial ATPase; var 1, var 1 protein, I, II and III, subunits I, II and III of cytochrome oxidase. *b*, cytochrome *b* apoprotein; ATPase 6, subunit 6 of mitochondrial ATPase; IEF, isoelectric focusing (first dimension); SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis (second dimension).



Fig, 2. Mitochondrially synthesized subunits of the mitochondrial ATPase from the *oli2 mit*$^-$ strain M11-28. Mitochondrial translation products were labelled in vivo with $^{35}SO_4{}^{2-}$ in the presence of cycloheximide [8]. Mitochondria were isolated [10] and Triton extracts of the mitochondria were prepared. The Triton extracts were challenged with rabbit specific antiserum against purified yeast F_0F_1-ATPase as described in Methods. Total mitochondrial translation products and mitochondrially synthesized proteins associated with the immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis as previously described [8], and were visualized by fluorography [12]. (A) total mitochondrial translation products of the wild-type (J69-1B) and mutant strain (M11-28); (B) mitochondrial translation products associated with the immunoprecipitates; PL, oligomer of the proteolipid subunit of the mitochondrial ATPase; var 1, var 1 protein; I, II and III, subunits I, II and III of cytochrome oxidase; *b*, cytochrome *b* apoprotein; ATPase 6, subunit 6 of the mitochondrial ATPase.

(Fig. 2). However, when mitochondrial translation products from strain M11-28 were analysed by two-dimensional gel electrophoresis, a significant shift in the apparent p*I* values of the two spots associated with subunit 6 of mitochondrial ATPase can be detected (Fig. 3B). Confirmation that the mutation in strain M11-28 has caused a charge modification in subunit 6 of the mitochondrial ATPase was obtained when labelled mitochondria isolated from this strain and its parental strain J69-1B were mixed, solubilized and co-electrophoresed on a two-dimensional polyacrylamide gel. The two-dimensional gel pattern shows clearly (Fig. 3C) that the only spots which do not superimpose are those of the subunit 6 of the mitochondrial ATPase. The mutant subunits, indicated by a star in Fig. 3C, have higher apparent p*I* values as well as slightly lower mobilities than those of the wild-type. The difference in mobility is in fact



so small that it cannot be detected by one-dimensional SDS-acrylamide gel electrophoresis because of the interference by the co-migrating subunit III of cytochrome oxidase (Fig. 2).

To ensure that the charge alteration in subunit 6 of mitochondrial ATPase is due to a mutation in the *oli2* region, mutant M11-28 was crossed to a *rho*$^-$ strain which has previously been shown to retain a short segment of mtDNA containing part of the structural gene of subunit 6 [4]. Analysis of the mitochondrial translation product of a diploid progeny which grows normally on ethanol shows subunit 6 spots which have the wild-type characteristics with respect to both charge and molecular weight (Fig. 3d).

Enzymatic properties of mutant M11-28

Data from the above electrophoretic studies indicate that although the mutation in the structural gene of subunit 6 of the mitochondrial ATPase in strain M11-28 has cuased only a minor alteration of the subunit, this alteration has apparently led to the inability of the mutant to grow by oxidative metabolism. Since very little is known about the role played by subunit 6 of the membrane sector of the mitochondrial ATPase in the function of the enzyme complex, it is of interest to compare the most relevant biochemical characteristics of the mutant strain and its wild-type parent. As expected, no significant difference in the spectral properties of the mutant and the parental strains can be detected (Table I). A lower mitochondrial respiration rate was, however, observed in the mutant strain, perhaps due to a

Fig. 3. Charge alteration in subunit 6 of the mitochondrial ATPase from the *oli2 mit*$^-$ strain M11-28. Mitochondrial translation products of the wild-type and the mutant strains were labelled in vivo and resolved in two dimensions as described in Methods. (a), Mitochondria (50 μg protein) of the wild type strain J69-1B; (b), mitochondria (50 μg protein) of mutant M11-28. The positions of the two wild-type subunit 6 spots are indicated by arrows; (c), mitochondria of the wild type (25 μg protein) and the mutant M11-28 (25 μg protein), mixed and co-electrophoresed; (d). mitochondria (50 μg) of a respiratory-competent, oligomycin-sensitive diploid progeny obtained from a cross between strain M11-28 and a *rho*$^-$ strain G4 (see Methods). PL, proteolipid; var 1, var 1 protein; I, II and III, subunits I, II and III of cytochrome oxidase; *b*, cytochrome *b* apoprotein. ATPase 6, subunit 6 of mitochondrial ATPase; IEF, isoelectric focusing; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

TABLE I

ENZYMATIC PROPERTIES OF STRAIN M11-28

Mitochondrial respiration was measured polarographically at 30°C in 2.0 ml of 10 mM Tris-phosphate buffer, pH 7.4, containing 0.6 M sucrose, 1.2 mM K_2 EDTA and 31 μM carbonylcyanide *m*-chlorophenylhydrazone (CCCP). The protein concentration was 0.4 mg/ml and the reaction was started by the addition of 10 μl absolute ethanol. Whole cell spectra of sodium dithionite reduced cells were recorded by using a starch cell as a blank. Cytochrome oxidase activity was inhibited by 3 mM KCN by at least 90%. NADH-cytochrome reductase activity was inhibited by Antimycin (10 μg/ml) by at least 85%. ATP-$^{32}P_i$ exchange activity was inhibited by oligomycin (20 μg/ml) and CCCP (250 μM) by at least 95%. The mitochondrial respiratory enzyme activities are expressed as Mean ± S.D. of between three and 16 determinations. wt, wild-type.

Strain	Mitochondrial Respiration Rate (uncoupled, nmol O_2/min mg mitochondrial protein)	Whole cell Cytochromes		Cytochrome *c* Oxidase (μmol/min per mg)	NADH-Cytochrome c Reductase (μmol/mol per mg)	ATPase (μmol/min per mg)	ATP-$^{32}P_i$ exchange (μmol/min per mg)
		b	aa_3				
J69-1B (wt)	41.4 ± 1.5	+	+	1.01 ± 0.26	0.81 ± 0.20	0.74	177
M11-28	19.5 ± 2.2	+	+	0.48 ± 0.16	0.68 ± 0.21	0.6	<5

secondary effect of the *oli2 mit*$^-$ mutation on the activity of the cytochrome oxidase complex. A similar effect has previously been observed to be associated with other mutations in various regions of the mitochondrial genome [4,8,19]. Mitochondria isolated from strain M11-28 all have enzymatic activities which are dependent on mitochondrial cytochromes. In agreement with the mitochondrial respiration data, the cytochrome-oxidase activity in the mutant strain was found to be lower than that of the wild-type strain.

The mutant ATPase activity was also found to be comparable to that of the wild-type strain. The most striking feature of strain M11-28 phenotype, however, is the extremely low sensitivity of its mitochondrial ATPase activity to oligomycin inhibition. Thus, a comparative analysis of the oligomycin inhibition curves of the mitochondrial ATPase activities of the parental and mutant strains (Fig. 4) shows that while the ATPase activity of the wild-type mitochondria is more than 80% inhibited by 2 μg oligomycin/mg protein, the ATPase activity of the mutant strain is only slightly inhibited (less than 20%) at concentrations of as high as 14 μg inhibitor/mg protein.

The oligomycin sensitivity of the mitochondrial ATPase activity is a parameter frequently used to assess the degree of assembly of the membrane sector (F_0) to the catalytic portion (F_1) of the ATPase enzyme complex; the F_1 sector retains the hydrolytic activity of the ATPase complex in the absence of the

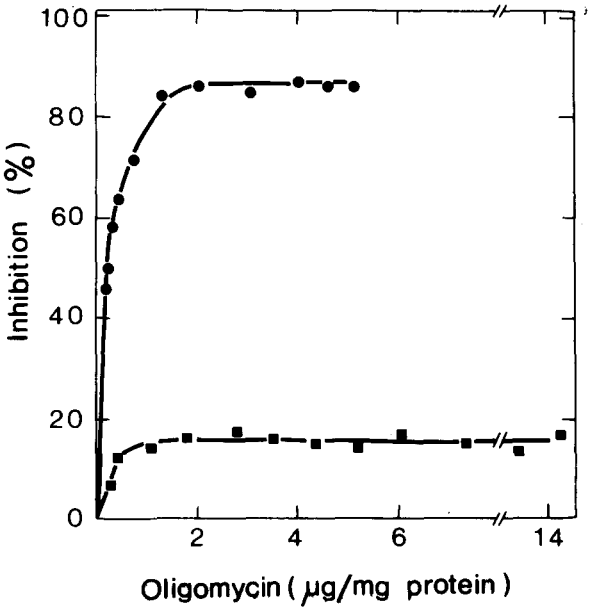


Fig. 4. Mitochondrial ATPase activity in strain M11-28 is insensitive to inhibition by oligomycin. Cells were grown [8] and mitochondria were isolated [10] from the wild-type strain J69-1B and the *oli2 mit*$^-$ strain M11-28. ATPase activity of the isolated mitochondria was determined at different concentrations of oligomycin. •, wild-type strain J69-1B; ▪, *oli2 mit*$^-$ strain M11-28.

F_0 subunits but is no longer sensitive to oligomycin. The inability of the mitochondrial ATPase from strain M11-28 to be inhibited by oligomycin might, therefore, be due to a defective association between the F_1 and F_0 components of the ATPase complex. That the mutation in strain M11-28 has affected the assembly of the mitochondrial ATPase complex is further substantiated by the fact that in contrast to the wild-type, the respiratory activity of the mutant cells is not stimulated by uncouplers of oxidative phosphorylation such as carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (data not shown). Furthermore, mitochondria of the mutant strain show no detectable ATP-$^{32}P_i$ exchange activity (Table I).

The modification in subunit 6 of the mitochondrial ATPase in strain M11-28 affects the assembly of the proteolipid into the enzyme complex

Is the defect in the assembly of the mitochondrial ATPase complex in strain M11-28 due to the fact that subunit 6, modified by the *mit*$^-$ mutation, does not assemble into the enzyme complex? To answer this question Triton extracts of mitochondria from strain M11-28 and its wild-type parent, radioactively labelled in vivo for 1 h as described in Methods, were challenged with a rabbit antiserum against F_1-ATPase. Comparative electrophoretic analysis of the immunoprecipitates from the mutant and the wild-type strains shows clearly that in strain M11-28 the altered subunit 6 is immunoprecipitated to the same extent as that of the wild type (Fig. 5, left). It appears, therefore, that the mutation in the structural gene of subunit 6 in strain M11-28 does not affect the assembly of this subunit to the F_1-ATPase.

The above results, however, do not provide any information concerning the state of assembly of the proteolipid subunit 9 into the enzyme complex, because after 1 h of labelling in the presence of cycloheximide, very little of the newly synthesized proteolipid was found to be assembled into the mitochondrial ATPase of the wild-type strain. Only when the in vivo labelling time was extended to 3 h was the association of the newly synthesized subunit 9 with pre-existing subunits of F_1 observed in the wild-type strain (Fig. 5, right). The mutant strain was therefore re-examined using a 3 h labelling period, The result, shown in Fig. 5, confirms that the altered subunit 6 is assembled in the mutant strain to the same extent

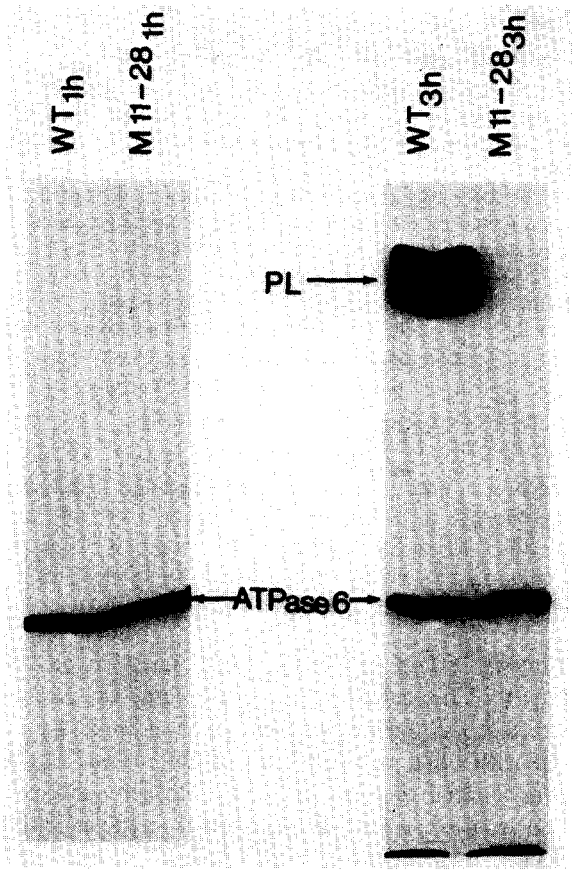


Fig. 5. SDS-polyacrylamide gel analysis of anti F_1-ATPase immunoprecipitates from the wild-type strain J69-1B and the mutant strain M11-28 mitochondria. Cells were labelled with $^{35}SO_4^{2-}$ in the presence of cycloheximide for 1 or 3 h. Mitochondria were prepared and mitochondrial ATPase precipitated from Triton extracts of the mitochondria by using a rabbit specific antiserum against purified F_1-ATPase. PL, proteolipid; ATPase 6, subunit 6 of the mitochondrial ATPase.

as in the wild-type. However, virtually no coprecipitation of the newly synthesized proteolipid subunit of mitochondrial ATPase with the anti F_1-ATPase is observed in strain M11-28. We conclude, therefore, that under our conditions of immunoprecipitation there is no tight association of the proteolipid to the F_1 subunits in the mutant M11-28.

Discussion

The data reported in this paper indicate that in yeast strain M11-28, a mutation in the structural gene

of subunit 6 of the mitochondrial ATPase does not alter the association of this polypeptide to the F_1-subunits but results in a defective assembly of subunit 9 into the enzyme complex. This defect appears to slow down rather than completely prevent the assembly of the functional F_0F_1-ATPase complex because the mutant retains the capacity to grow by oxidative metabolism, albeit at an extremely low rate. The simplest interpretation of these data would be that the alteration in subunit 6 of the mitochondrial ATPase, although minor, leads to the incorrect folding of subunit 6, which might easily slow the access of subunit 9 to its binding site, located on the subunit 6 polypeptide. This interpretation is in agreement with results of recent cross-linking studies which suggest that subunit 6 interacts in a specific manner with the F_1-sector as well as subunit 9 of the mitochondrial ATPase [20,22]. Thus, mutant M11-28 represents a particular case of incorrect assembly of the mitochondrial ATPase in which an F_1-subunit 6 (altered) complex is temporarily 'separated' from the slowly assembling subunit 9.

The F_1-ATPase associated to the altered subunit 6 is still capable of performing its hydrolytic activity at a rate comparable to that in the wild-type. However, the ATP hydrolysis reaction catalysed by the F_1-subunit 6 (altered) complex is almost completely insensitive to oligomycin. It has been previously suggested [22] that the binding site for oligomycin is on the proteolipid subunit of the mitochondrial ATPase complex, If so, the insensitivity of the mutant ATPase activity to the inhibitor might well be due to the inability of the F_1-subunit 6 (altered) complex, accumulated in the mitochondria because of the slow assembly of the proteolipid subunit, to bind oligomycin.

As expected, the slow assembly of subunit 9 into the mitochondrial ATPase complex of strain M11-28 has a more drastic effect on the ATP synthetic activity. The mutant mitochondria lacked a detectable ATP-$^{32}P_i$ exchange reaction and this effect is probably responsible for the significantly low growth rate of the mutant strain by oxidative metabolism.

A final observation which emerged from the present study is that subunit 6 and subunit 9 of the mitochondrial ATPase do not integrate simultaneously into the enzyme complex but follow a well-defined time sequence with subunit 9 being assembled after subunit 6. This observation has been confirmed by several pulse-chase experiments (Orian, J., unpublished results) and work is in progress in our laboratory to determine the precise time sequence of assembly of the mitochondrial ATPase subunits.

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