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The definition of mitochondrial H^+ATPase assembly defects in *mit*$^-$ mutants of *Saccharomyces cerevisiae* with a monoclonal antibody to the enzyme complex as an assembly probe *

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***mit*$^-$ mutants with genetically defined mutations in the mitochondrial structural genes of the H^+-ATPase membrane subunits 6, 8 and 9 were analysed to determine the H^+-ATPase assembly defects that resulted as a consequence of the mutations. These include mutants which do not synthesize one of the membrane subunits and mutants which can synthesize these subunits, but in an altered form. Protein subunits which can still be assembled to the defective H^+-ATPase in these mutants were determined by immunoprecipitation using a monoclonal antibody to the β-subunit of the enzyme complex. The results suggest that the assembly pathway of the mitochondrially synthesized H^+-ATPase subunits involves the sequential addition of subunits 9, 8 and 6 to a membrane-bound F_1-sector. In addition to subunits of the F_0- and F_1-sectors, two other polypeptides (M_r = 18000 and M_r = 25000) are associated with the yeast H^+-ATPase. These polypeptides were not observed in the immunoprecipitates obtained from mutants in which the F_0-sector is not properly assembled.**

Introduction

The biogenesis of the mitochondrial H^+-ATPase (ATP synthetase, ATP phosphohydrolase, EC 3.6.1.3) has been the subject of intensive studies in recent years. This enzyme complex is a mosaic consisting of mitochondrially synthesized protein subunits, as well as subunits imported from the extramitochondrial cytoplasm, organized into two functionally distinct domains [1,2]. The F_1-sector contains the catalytic site for ATP synthesis, and consists of five subunits designated α, β, γ, δ and ϵ (apparent molecular weights 56000, 54000, 31000, 14000 and 12000, respectively, in yeast), all of which are synthesized in and imported from the extra-mitochondrial cytoplasm. The membrane F_0-sector is the proton channel of the enzyme complex [3] and assembled from three different mitochondrially synthesized subunits designated subunits 6, 8 and 9 (apparent molecular mass in SDS-polyacrylamide gel 20, 10 and 7.6 kDa, respectively) in yeast. In addition, two other cytoplasmically synthesized polypeptides (apparent M_r 18000 and 25000) consistently copurify with the yeast H^+-ATPase and thus suggested to

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be genuine subunits of the enzyme complex [4].

Recent studies on the biosynthesis of the enzyme complex have been focused mainly on the characterisation of the molecular structure of mitochondrial and nuclear genes coding for subunits of the enzyme complex, and on the elucidation of processes involved in the import of subunits which are synthesized in the extramitochondrial cytoplasm into the organelle. In contrast, very little is known about the assembly events that follow the import of the cytoplasmically synthesized subunits into the organelle, and the synthesis of the other subunits on the mitochondrial ribosomes. Information regarding these events can be deduced from the extent to which the assembly process can proceed in mutants in which one of the H^+-ATPase subunits is not synthesized or is synthesized in a grossly altered form.

As part of a general strategy to investigate the assembly of H^+-ATPase in yeast, a battery of monoclonal antibodies to various subunits of the enzyme complex [4] have been isolated in our laboratory. One of these monoclonal antibodies, directed against the β subunit of the F_1-sector, has previously been used to define the extent of H^+-ATPase assembly defects in a mitochondrial DNA-less mutant (*rho*^{0}) which cannot synthesize the three F_0-subunits [5]. In the absence of the F_0-sector, the F_1-subunits were found to be assembled into a catalytically active complex on the inner mitochondrial membrane.

In this communication, we report results of our investigation to define the assembly defects in *mit*$^-$ mutants that show a respiratory-deficient phenotype as a result of lesions in the mitochondrial structural genes for subunits 6, 8 and 9 of the F_0-sector. The mutational changes have been defined by DNA sequencing of the genes *oli*2 [6], *aap*1 [7] and *oli*1 [8], respectively. Our results suggest that subunit 9 plays a key role in the assembly or the stabilization of the F_0-sector, in that in the absence of this polypeptide, no subunits 6 or 8 were found to be associated with the defective H^+-ATPasee complex. Thus, subunit 9 is probably the first mitochondrially synthesized subunit to be assembled to the F_1-sector. The assembly of subunit 9 appears to be followed by subunit 8, because in the absence of this subunit the ATPase complex still contains subunit 9, but not subunit 6. Subunit 6 appears to be the last mitochondrially synthesized subunit to be assembled, since the absence of subunit 6 does not affect the assembly of subunit 8 and 9.

Materials and Methods

Yeast strains. Saccharomyces cerevisiae strain J69-1B (α *adel his*[*rho*$^+$]) is the wild-type parent of the *mit*$^-$ mutants used in the present study, all of which were derived by mutagenesis with manganese chloride [9]. The *mit*$^-$ strains, carrying mutations in the *oli*2, *aap*1 and *oli*1 region [6–8], are listed in Table I.

Growth conditions. Yeast cells used for the isolation of the H^+-ATPase complex were grown in glucose-limited chemostat cultures as described by Marzuki et al. [10]. The working volume of the cultures was either 500 ml (in modified LKB-Biotek polyferm fermentors) or 4 l (in LKB ultraferm fermentation systems). The growth medium contained a salt mixture [11], 1% Difco yeast extract and glucose (1% w/v for the wild-type strain and 2% w/v for the mutant strains). Cells used for the innoculum were grown to an exponential phase in batch cultures at 28° C and added to the chemostat culture medium to give an initial cell concentration of approx. 0.1 mg cell dry wt./ml. The cultures were grown under forced aeration (1–2 culture volumes per min) at a dilution rate of 0.1 h^{-1}. Foam formation was prevented by the intermittent injection of sterile silicone antifoam AF (Dow Corning Australia Pty. Ltd, Blacktown, N.S.W., Australia) into the culture. In all cases, the steady-state glucose level of the cultures was maintained below that which induces catabolite repression [10].

In vivo labelling of mitochondrial proteins. Mitochondrial translation products were labelled with [^{35}S]sulphate by a modified procedure of Douglas and Butow [12]. Cells grown to late logarithmic phase were harvested, resuspended to 2 mg cell dry wt/ml in low sulphate medium [12] containing 0.6% glucose, chloramphenicol (2 mg/ml; Boehringer Mannheim GmbH), adenine (100 μg/ml) and histidine (50 μg/ml), and incubated in a gyratory shaker for 2 h at 28° C. The

cells were harvested, washed three times with ice-cold water and resuspended to 12 mg cell dry wt./ml in low sulphate medium containing 2% glucose, cycloheximide (0.5 mg/ml), adenine (100 μg/ml) and histidine (50 μg/ml). After 10 min of incubation at 28°C, [^{35}S]sulphate was added to 500 μCi/ml and the incubation was continued for 1 h. For labelling of total mitochondrial proteins, yeast cells were harvested in late exponential phase, washed with ice-cold water and incubated with [^{35}S]sulphate (200 μCi/ml) in low sulphate medium [12] at 28°C for 4 h in the absence of antibiotics. Mitochondria were isolated from radioactively labelled cells in the presence of protease inhibitors [13], as described previously [14].

Immunoprecipitation. ^{35}S-labelled mitochondria were suspended in extraction buffer (4 mM Tris-acetate pH 7.5, containing 0.25% Triton X-100, 5 mM *para*-aminobenzamidine, 5 mM ϵ-aminocaproic acid and 0.5 mM phenylmethylsulphonylfluoride) at 6.25 mg protein/ml, and incubated at 0–4°C for 10 min. The solubilized mitochondrial suspension was centrifuged at 106 000 × *g* for 20 min and aliquots (50 μl) of the supernatant were incubated with a monoclonal antibody to the β subunit of the H$^+$-ATPase (designated RH 48), coupled to CNBr-activated Sepharose-4B beads as described previously [4]. Immunoprecipitates obtained were then subjected to polyacrylamide gel electrophoresis in the presence of SDS [13], and visualized by fluorography [15].

TABLE I

NATURE OF ALTERATIONS OF THE H$^+$-ATPase SUBUNITS 6, 8 AND 9 IN THE *oli*2, *aap*1 AND *oli*1 *mit*$^-$ MUTANT STRAINS USED IN THE PRESENT STUDY

Strain	Nature of mutation	Predicted length of mutated gene product (amino acid residues)	Reference
wild-type			
J69-1B	none	subunit 6: 259	6
		subunit 8: 48	7
		subunit 9: 76	8
*oli*2 *mit*$^-$ mutants			6
M10-7	frameshift [a]	subunit 6: 57	
Ma1	frameshift [a]	211	
M11-28	Ser_{241} → Phe Thr_{248} → Lys	259	
*aap*1 *mit*$^-$ mutant			7
M26-10	frameshift [a]	subunit 8: 18	
*oli*1 *mit*$^-$ mutants			8
38.6.1	frameshift [a]	subunit 9: 7	
5726	frameshift [a]	65	
2422	Arg_{39} → Met	76	

[a] Details of novel C-terminal sequences (with amino acid residue numbers) resulting from frameshift mutations are:
M10-7 (38– 57) IIFIMYYYCIISYYKFMSIN
Ma1 (210–211) FN
M26-10 (14– 18) MYGFY
38.61 (5– 7) FSS
5726 (57– 65) YQKTQVYSV
Amino acids underlined are the same as those in the wild-type strain.

Results

Structural defects in F_0 subunits of mit$^-$ mutants

Seven *mit*$^-$ mutants with lesions in the structural genes of subunit 6, 8 or 9 (the *oli*2, *aap*1 or *oli*1 genes, respectively) were selected for the present investigation (Table I). The mitochondrial mutations in these strains have been characterized by DNA sequence analysis [6–8], and shown to be of three types: (a) missense mutations which give rise to full-length products with one or more amino acid changes; (b) nonsense mutations which result in truncated products; and (c) frameshift mutations which result in products that have stretches of amino acid substitutions and generally lead to premature termination. Mitochondrial translation products of the mutant strains were analysed by SDS-polyacrylamide gel electrophoresis, in order to confirm the structural alteration in the H$^+$-ATPase subunits predicted by DNA sequencing. For this purpose, cells were 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.

Three *oli*2 *mit*$^-$ mutants, strains M10-7, Ma1 and M11-28, were analysed. DNA-sequencing data [6] predict that mutant strains M10-7 and Ma1 have truncated subunit-6 polypeptide of 57 and 211 amino acids, respectively, while strain M11-28 has a full-length polypeptides with two amino acid substitutions (Ser_{241} → Phe and Thr_{248} → Lys; Table I). Consistent with this prediction no full-length subunit-6 band can be observed in the gel display

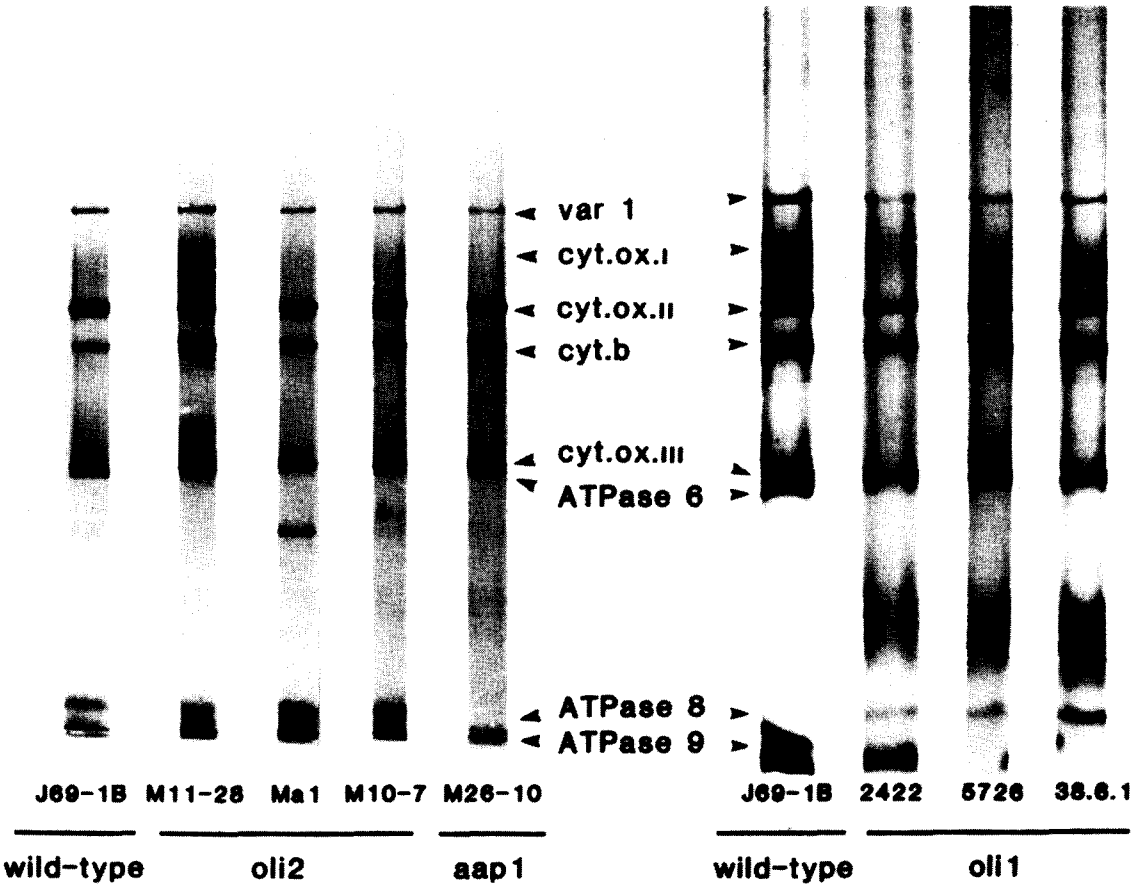

Fig. 1. SDS polyacrylamide gel electrophoresis of mitochondrial translation products of the mutant strains used in the present study. Chemostat-grown cells were labelled with [^{35}S]sulphate in the presence of cycloheximide [13]. Mitochondria were isolated from these cells and electrophoresed on polyacrylamide slab gels in the presence of SDS [13]. Labelled products were detected by autoradiography. cyt. ox I and cyt. ox II denote cytochrome oxidase subunits I and II, respectively; cyt. *b* denotes apocytochrome *b*; ATPase 6, ATPase 8 and ATPase 9 denote subunits 6, 8 and 9 of the H^+-ATPase, respectively.

of the mitochondrial translation products of strains Mal1 and M10-7 (Fig. 1). In strain Mal, the predicted truncated subunit 6 can be seen as a mitochondrial translation product with a mobility of a 16 kDa protein. However, no truncated subunit 6 can be observed in the gel display of strain M10-7.

One possible explanation for this observation is that the mutant polypeptide, predicted to have a molecular weight of about one-fifth of the normal ATPase subunit 6, might have run to the bottom of the gel, or might have co-migrated with subunit 8 or 9; the migration of SDS-protein complexes in the lower molecular-weight range is unpredictable [16], especially for hydrophobic proteins. In strain M11-28, subunit 6 could not be observed in the gel display (Fig. 1). However, we have previously shown by two-dimensional gel electrophoretic analysis that the H^+-ATPase subunit 6 in this mutant has an altered p*I* [17], which is consistent with the introduction of the positive charge predicted by DNA sequence data (Table I). This altered subunit 6 has a slightly slower electrophoretic mobility in SDS-polyacrylamide gels as compared to that of the wild-type strain (data not shown), and therefore comigrates with subunit III of the cytochrome oxidase (Fig. 1).

Only one *aap*1 *mit*$^-$ mutant (M26-10) has been used in the present study, as mutants with full-length subunit 8 have not been isolated to date [7].

The truncated subunit 8 in this strain is predicted to be of 18 amino acid residues in length (Table I). This short peptide is not observed on the display of mitochondrial translation products of the *mit*$^-$-mutant strain (Fig. 1).

Two *oli*1 *mit*$^-$ mutants used in the present study are predicted to have truncated subunit 9 of 7 (strain 38.6.1) and 65 (strain 5726) amino acids in length (Table I). The altered subunit 9 was not observed in the display of mitochondrial translation products of both strains (Fig. 1). A short peptide of 7 amino acid residues (strain 38.6.1) is not expected to be seen on our gel system. However, it is of interest to note that the truncated subunit 9 of 65 amino acid residues (compared to 76 amino acid residues in the wild-type strain) was not seen in strain 5726; the reason for this is not clear at present. Also analysed was an *oli*1 *mit*$^-$ strain 2422 which has a full-length subunit 9 with a single amino acid substitution (Table I). This substitution occurs in a highly conserved region, in which a positively charged Arg_{39} is replaced by a non-polar methionine residue. The mutant subunit 9 showed a slightly faster electrophoretic mobility as compared to that of the wild-type strain (Fig. 1).

The H^+-ATPase subunit 6 migrated very close to cytochrome oxidase subunit III in Fig. 1. More extensive studies of *oli*1 *mit*$^-$ mutants in our laboratory, however, have shown that the amount of subunit 6 synthesised in mutants that do not synthesise a full-length subunit 9 is significantly reduced [18,19]. The amounts of subunit 6 observed in these mutants varied slightly between experiments and is approx. 20–30% of that in the wild-type strain [19].

The structural alterations in subunits 6, 8 or 9 in the *mit*$^-$ mutant strains have been shown to

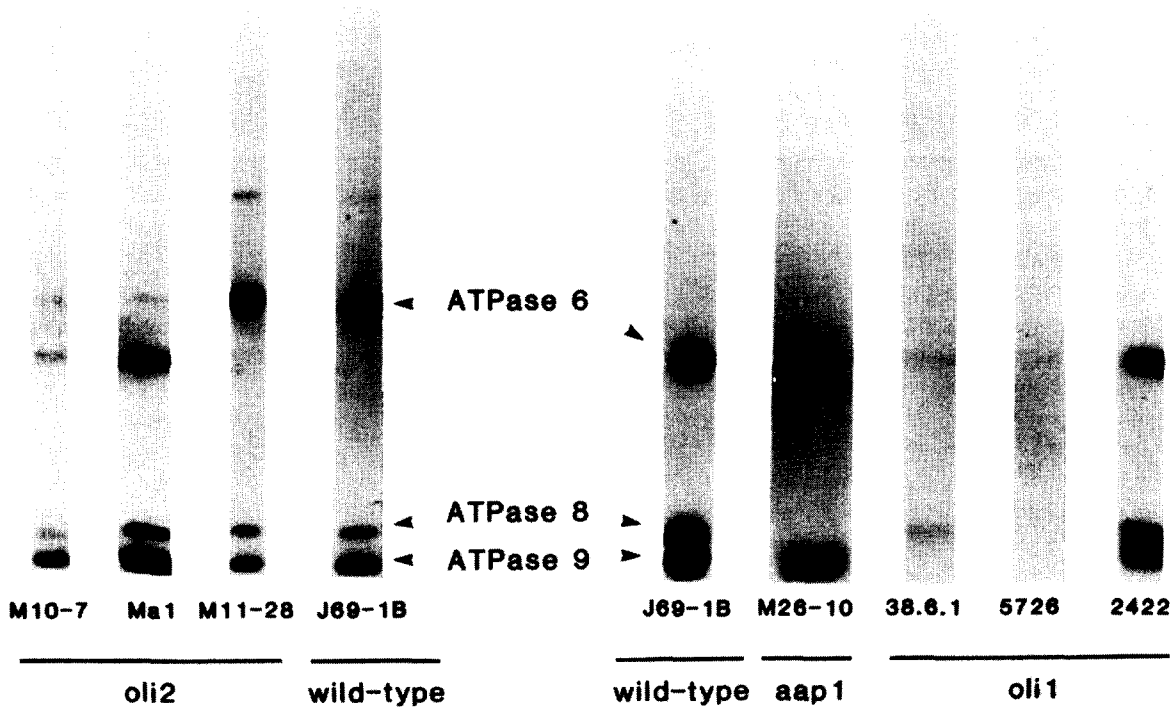

Fig. 2. Mitochondrially synthesized subunits associated with H^+-ATPase immunoprecipitated from *mit*$^-$ mutant strains. Mitochondrial translation products of chemostat grown cells were labelled in vivo with [^{35}S]sulphate in the presence of cycloheximide [13]. Mitochondria were isolated [14] and Triton X-100 extracts of the mitochondria were prepared [4]. The Triton extracts were incubated with monoclonal anti-β antibody, RH 48 [4] (conjugated to Sepharose-4B beads) as described in Materials and Methods. Mitochondrially synthesized proteins associated with the immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis [13] and were visualized by fluorography [15]. ATPase 6, ATPase 8 and ATPase 9 denote ATPase subunits 6, 8 and 9, respectively.

result in their inability to grow by oxidative metabolism [18–21]. These mutants strains retained significant, although reduced, ATPase activity, but the activity is insensitive to inhibition by oligomycin, indicating that the coupling between the F_1-sector and the proton channel of the enzyme complex is defective, or that the F_1-sector is associated with an abnormal F_0-sector.

Mitochondrially synthesized subunits associated with defective H^+-ATPase in mit$^-$ mutants

A monoclonal antibody to the β-subunit of the F_1-sector (RH 48) [4] was used to define the F_0-subunits associated with the H^+-ATPase in the above *mit*$^-$ mutants. For this purpose, cells were labelled with [^{35}S]sulphate in the presence of cycloheximide, allowing the incorporation of [^{35}S]sulphate into mitochondrial translation products only, and the H^+-ATPase was immunoprecipitated from Triton extracts of the mitochondria, as described in Materials and Methods. The results are shown in Fig. 2 and summarized in Table II.

In the wild-type strain J69-1B, all three protein subunits of the F_0-sector, subunits 6, 8 and 9, coprecipitated with the β-subunit indicating that the labelling conditions allowed the newly synthesized subunits to be assembled to the H^+-ATPase. In the *oli*2 *mit*$^-$ strain Ma1, the truncated subunit 6 (16 kDa) was found to be still associated with the H^+-ATPase together with subunits 8 and 9, suggesting that the C-terminal region beyond residue 211, is not required for the assembly of subunit 6 to the enzyme complex. In mutant strain M11-28 (two amino acid substitutions), subunit 6 was found to be assembled even though the F_0-sector is non-functional as indicated by the inability of the mutant strain to grow by oxidative metabolism [17].

TABLE II

SUMMARY OF THE ASSEMBLY DEFECTS OF MITOCHONDRIALLY SYNTHESIZED SUBUNITS OF THE H^+-ATPase IN *oli*1, *oli*2 AND *aap*1 *mit*$^-$ STRAINS

Strain	Subunits synthesized			Subunits assembled		
	6	8	9	6	8	9
Wild-type						
J69-1B	++	++	++	+	+	+
*oli*2 *mit*$^-$						
M10-7	–	++	++	–	+	+
Ma1	++ [a]	++	++	+ [a]	+	+
M11-28	++ [b]	++	++	+ [b]	+	+
*aap*1 *mit*$^-$						
M26-10	++	–	++	–	–	+
*oli*1 *mit*$^-$						
2422	++	++	++ [b]	+	+	+ [b]
5726	+	++	–	–	–	–
38.6.1	+	++	–	–	–	–

[a] Truncated subunit 6.

[b] Full length subunit 6 or 9 with altered electrophoretic mobility.

A 16 kDa polypeptide was unexpectedly observed in the immunoprecipitate of strain M10-7 (with predicted truncated subunit 6 of 57 amino acid residues), even though no such mitochondrial translation product was detected in the display of the mitochondrial translation products (Fig. 2). The significance of this observation is not clear at present, but the observation was reproducible in at least four independent experiments. In strains Ma1 and M10-7, trace amounts of a polypeptide with an apparent M_r of 20000 could also be detected in the immunoprecipitates (Fig. 2), but this was identified as contaminating cytochrome oxidase subunit III on a high-resolution SDS-polyacrylamide gel system that separate this protein from subunit 6 of H^+-ATPase (data not shown). All three *oli*2 *mit*$^-$ mutants showed the presence of both subunits 8 and 9 in the immunoprecipitates, including strain M10-7 which does not even have a truncated subunit 6 assembled. This result suggests that subunit 6 is not required for the assembly of subunit 8 and 9 to the F_1-sector of the mitochondrial H^+ATPase.

The analysis of the immunoprecipitate of the *aap*1 *mit*$^-$ strain M26-10 (no detectable subunit 8 synthesized) revealed that while subunit 9 was present in the immunoprecipitate, subunit 6 could not be detected (Fig. 2). The presence of subunit 8, therefore, appeared to be necessary for the assembly of subunit 6, or for its stabilisation in the enzyme complex, but not for the assembly of subunit 9.

Subunit 9 appeared to be the key subunit in the assembly, or the stabilisation, of the F_0-sector, because the absence of subunit 9 affects the association of both subunits 6 and 8 to the F_1-sector. In the *oli*1 *mit*$^-$ strains that do not synthesize

subunit 9, no subunits 6 and 8 (strain 5726) or only trace amount of these subunits (strain 38.6.1) were detected in the immunoprecipitates, even though approximately the same amount of H^+-ATPase immunoprecipitate was loaded in each well (Fig. 2). In contrast, the altered full-length subunit 9 in strain 2422 was found to be assembled to the H^+-ATPase and its presence in the enzyme complex has allowed the assembly of subunits 6 and 8.

Subunit composition of the defective H^+-ATPase complex in mit$^-$ mutants

The experiments described above were designed to examine the mitochondrially synthesized subunits of the H^+-ATPase of the *mit*$^-$ mutants. It was of interest to determine the extent to which the mutations in these strains have affected the association of the cytoplasmically synthesized subunits to the β-subunit. For this purpose, cells were labelled with [^{35}S]sulphate for 4 h in the absence of any antibiotics (as described in Materials and Methods), and assembled F_0F_1-ATPase subunits were immunoprecipitated with the monoclonal anti-β-subunit antibody and analysed by electrophoresis in SDS-polyacrylamide gels. The precipitate of the wild-type strain was found to consist of ten polypeptides (Fig. 3), the identity of which has previously been investigated [4,21]: five are subunits of the F_1-sector, three constitute the F_0-sector, and the other two subunits with molecular masses of 18 kDa (P18) and 25 kDa (P25) have not been characterized as yet.

All *mit*$^-$ mutants used in this study showed the presence of the five F_1-subunits in the immunoprecipitate (Fig. 3), indicating that the F_1-subunits of the *mit*$^-$ mutants are assembled irrespective of the defect in the F_0-sector. The association of the P18 and P25 polypeptides with the enzymes complex appeared to be dependent on

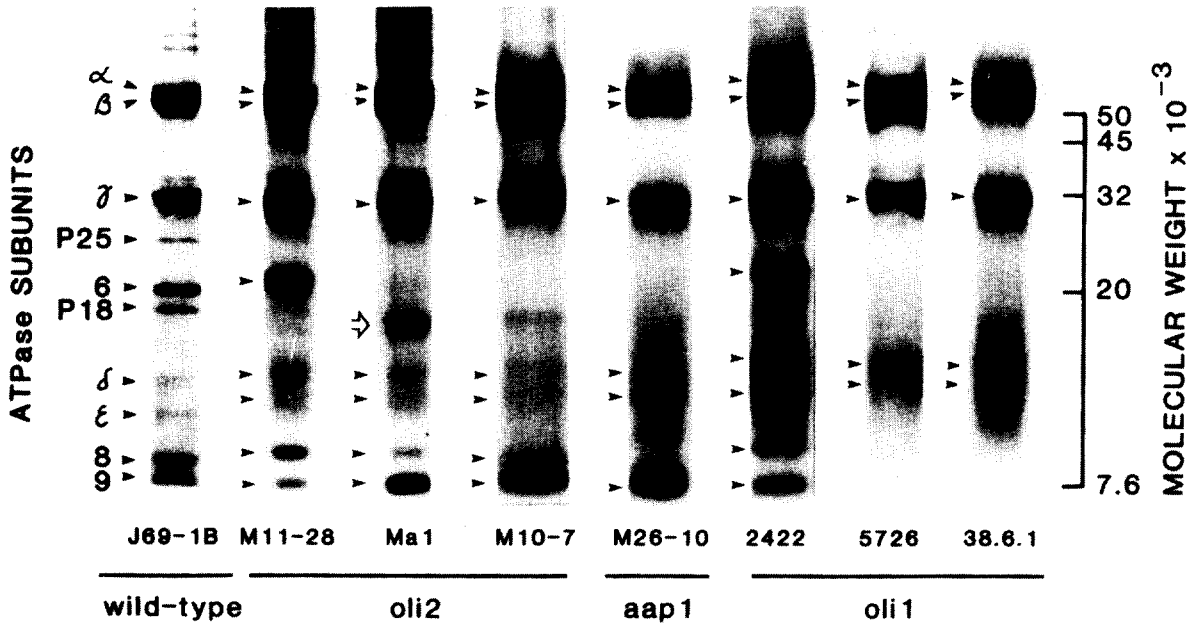

Fig. 3. Subunit composition of the mitochondrial H^+-ATPase immunoprecipitated from *mit*$^-$ mutant strains. *Mit*$^-$ mutants and wild-type strain were grown in glucose-limited chemostat cultures at 28° C. Cells were labelled in vivo with [^{35}S]sulphate for 4 h in the absence of protein synthesis inhibitors to allow the synthesis of both nuclearly and mitochondrially coded subunits of the H^+-ATPase. H^+-ATPase was immunoprecipitated from Triton X-100 extracts of mitochondria isolated from these mutants and analysed as described in the legend to Fig. 2. α, β, γ, δ and ϵ denote subunits α, β, γ, δ and ϵ of the F_1-sector of the H^+-ATPase. P18 and P25 denote two polypeptides with molecular weights of 18 and 25 kDa, respectively, considered to be true subunits of the enzyme complex [4]. ATPase 6, ATPase 8, ATPase 9 denote subunits 6, 8 and 9 of the F_0-sector. Open arrow indicates the truncated subunit-6 in strain Ma1.

the proper assembly of the F_0-sector. When any one of the F_0-subunits was not present, the P18 and P25 polypeptides were not observed in the immunoprecipitates of the *mit*$^-$ mutants (Fig. 3). Some P18 and P25 might be associated with the H^+-ATPase complex immunoprecipitate from strains 2422, Mal and M11-28 in which all subunits of the F_0-sector were assembled, despite of the defect in their respective mutated gene products. However, the amount of P18 and P25 polypeptides in these mutant strains was significantly reduced and variable, and in some experiments either one or both of these subunits could not even be detected.

The examination of F_0-subunits associated with the immunoprecipitates (Fig. 3) confirms the earlier observations (Fig. 2). Thus subunits 8 and 9 were observed in the *oli*2 *mi*$^-$ mutant strains Mal and M11-28 that assembled altered subunit 6, and in strain M10-7 in which subunit 6 was not detected. In the *aap*1 *mit*$^-$ mutant M26-10, only subunit 9 was present in immunoprecipitate. In the *oli*1 *mit*$^-$ mutants in which subunit 9 is not assembled (strains 38.6.1 and 5726), no subunit 6 and 8 could be detected, while in strain 2422 (which contains the predicted full-length subunit 9) all three F_0-subunits were present in the immunoprecipitate.

Discussion

Results of the present study show that certain subunits of the F_0-sector are no longer associated with the F_1-ATPase in *mit*$^-$ mutants of the yeast *Saccharomyces cerevisiae*, even though these subunits are still synthesized by the mutant strains (Table II). A recent study in our laboratory, employing a mitochondrial DNA-less *rho*^{0} yeast mutant strain, indicates that in the absence of the mitochondrially synthesized F_0-subunits, the cytoplasmically synthesized subunits of the F_1-sector of the H^+-ATPase are still imported into the mitochondria and assembled into a catalytically active membrane bound complex [5]. The pattern of assembly defects observed in the various *mit*$^-$ mutants examined in the present study might reflect the sequence of assembly of the mitochondrially synthesized F_0-subunits to the F_1-sector.

The most extreme situation observed was in subunit 9-less *mit*$^-$ mutants, in which no subunit 6 or 8 were found to be associated with the enzyme complex, indicating that the presence of subunit 9 is critical for the interaction of the F_0 subunits with the F_1-sector. In the absence of subunit 9, the other subunits of the F_0-sector either cannot be assembled to the F_1-sector, or are only loosely associated to the enzyme complex and subsequently lost during the isolation of the ATPase complex. Thus, it appears that the proteolipid subunit 9 of the H^+-ATPase is the key component in the assembly pathway of the enzyme complex.

Following the same argument, a tentative assembly pathway of the F_0-sector has been deduced from the assembly defects observed in the other *mit*$^-$ mutants (Fig. 4). It is proposed in this pathway that subunit 9 is the first mitochondrially synthesized subunit to be assembled to the F_1-sector. The assembly of subunit 9 is followed by subunit 8 because in the absence of this subunit

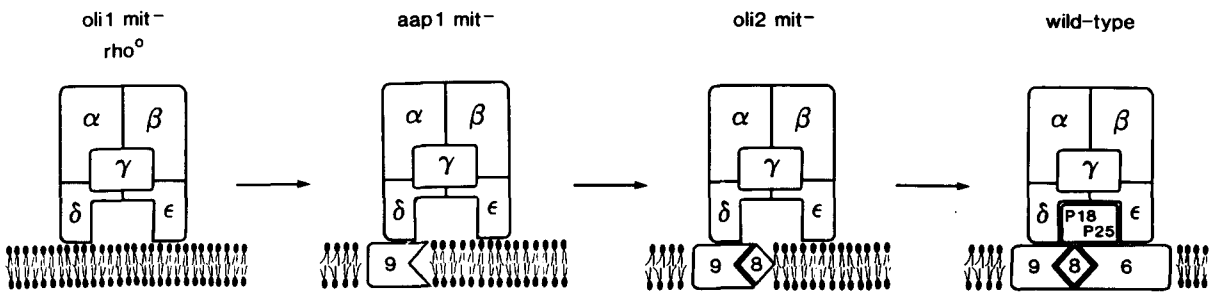

Fig. 4. Proposed assembly pathway for the F_0 sector of the yeast H^+-ATPase. H^+-ATPase assembly defects in the *oli*1, *aap*1 and *oli*2 *mit*$^-$ mutants were deduced from results presented in Fig. 2, Fig. 3 and Table II. In the *rho*^{0} strain the cytoplasmically synthesized F_1 subunits have been shown to be assembled into a membrane bound and catalytically active complex [5].

(in the *aap*1 mutant), the ATPase complex still contains subunit 9 but not subunit 6. The last mitochondrial translation products to be assembled is subunit 6, since in its absence (in *oli*2 *mit*$^-$ mutants) both subunits 8 and 9 are still associated with the F_1-sector (Table II).

The proposed pathway of assembly shown in Fig. 4 represents a first attempt to elucidate the mechanism of assembly of the F_0-sector of the mitochondrial H^+-ATPase, and still lacks many of the essential details. The conformation and refinement of this proposed pathway would require the isolation of more monoclonal antibodies, directed against other subunits of the enzyme complex. These antibodies, for example, are needed for the quantitation of the various subunits associated with the defective H^+-ATPase complex in the *mit*$^-$ mutants, essential to establish the fine detail of the assembly pathway such as whether all copies of subunit 9 [2] are required before subunits 8 and 6 can be assembled.

The proposed assembly pathway depicted in Fig. 4 is different from that originally suggested for *Escherichia coli* [22,23]. In *E. coli*, the assembly of the F_1-sector has been proposed to be tightly coupled to that of the F_0-sector. Assembly begins with the integration of F_0-subunit *a* (equivalent to subunit 6 in yeast mitochondria), and/or subunit *c* (equivalent to the mitochondrial subunit 9) into the plasma membrane. This F_0-subunit(s) appears to provide the binding site for the first copy of the β-subunit onto the plasma membrane. Assembly proceeds with the sequential addition of one α-subunit, F_0-subunit *b* (no yeast equivalent), and a second β-subunit. It was suggested that an important feature of this simultaneous assembly process between the F_1- and F_0-sector is that an unfavourable open proton channel would not occur at any time during assembly [22,23]. Results of a more recent study, however, indicate that the F_1-subunits can also be assembled into a catalytically functional complex in *E. coli*, in the absence of the F_0-subunits [24], similar to our observation in yeast [5]. Furthermore, it has been suggested more recently, that *E. coli* F_0-subunits can in fact be assembled into a functional proton channel in the absence of the F_1-subunits [25]. It is still not certain whether this is also the case in yeast mitochondria, although it has recently been reported that a functional proton channel is not present in mitochondria isolated from a subunit β-less mutant [26].

Some of the apparent differences in the proposed assembly events that lead to the formation of a functional H^+-ATPase complex in *E. coli* [22,23] and in the yeast mitochondria might simply be a manifestation of the difference in experimental approaches used. Implicit in the *E. coli* studies is the assumption that all H^+-ATPase subunits that are membrane bound are committed to assembly. It is obvious from the results presented here (summarized in Table II) that at least for the yeast mitochondrial H^+-ATPase, this assumption might not be valid (e.g., strain 38.6.1, 5726, M26-10). The use of subunit-specific antibodies to immunoprecipitate the assembled subunits of the H^+-ATPase is obviously essential in defining assembly defects in mutant strains.

The identity of the P18 and P25 polypeptides which are always associated with the immunoprecipitate of the enzyme complex from the wild-type strain J69-1B [4,21], and with the H^+-ATPase complex isolated and purified on glycerol or sucrose gradients (Refs. 27–29; see also Meltzer, S., unpublished data) is not fully established as yet. A protein with an apparent molecular weight of 24 kDa has been shown to cross-link to pig heart [30] and bovine heart [31] mitochondrial oligomycin-sensitivity-conferring protein (OSCP), indicating an integral location of this protein in the H^+-ATPase complex. It is possible that the yeast P18 polypeptide is the OSCP, since it has a similar molecular mass to that previously reported of around 18 kDa [32].

Our results suggest that the assembly of P18 and P25 is conditional on the assembly of the F_0-sector; both of these polypeptides were not observed in the immunoprecipitate in which one or more of the three F_0 subunits were not assembled. In the case of the P25 polypeptide at least, the absence of this subunit does not appear to be due to a defect in its synthesis. Western immunoblot analysis of the Triton X-100 extracts of mitochondria isolated from the *oli*2 *mit*$^-$ mutants (strains M10-7, Ma1 and M11-28), with an anti-P25 monoclonal antibody previously described [4], showed the presence of normal amounts of this subunit in the mutant mitochondria (data not

shown). Since the P18 and P25 polypeptides only copurify with the β subunit when all three F_0-subunits are assembled into a functional sector, it is likely that the assembly of the F_0-subunits to the F_1-sector is followed by P18 and P25.

The present study has been extended to the analysis of an extensive collection of mutants with defined structural changes in subunits 9 and 6. The correlation between assembly and functional defects in the H^+-ATPase of *mit*$^-$ mutants with the mutations in their structural genes has allowed molecular dissection of these F_0 subunits in order to define functionally important structural features. Thus, we have assigned a critical role to the C-terminal arm of subunit 9 and to a glycine residue at position 18 in the assembly of this polypeptide [18]. In addition, analysis of *oli*2 *mit*$^-$ mutants have shown that while the two conserved hydrophobic regions at the C-terminal end of subunit 6 (identified by the comparison of H^+-ATPase subunit 6 sequence in different organisms) are essential for its function, they do not appear to be required for the assembly of this subunit [33]. More mutants are being analysed to characterize further the functional domains on the mitochondrially synthesized subunits of the mitochondrial H^+-ATPase proton channel.

The order of events involved in the assembly of the F_1-sector prior to the pathway depicted in Fig. 4 is still not known. Detailed information has now been assembled on how the three largest subunits of the F_1-sector are synthesized on the cytoplasmic ribosomes as larger precursors, the transport of these precursors across the inner mitochondrial membrane and their processing into mature subunits [34–37]. In order to understand the assembly events that follow their import into the mitochondria, mutants which lack various cytoplasmically synthesized H^+-ATPase subunits need to be analysed, to determine the extent to which the assembly of the F_1- and F_0-sectors of the enzyme complex can proceed in the absence of each of the F_1 subunits.

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