Biochimica et Biophysica Acta, 975 (1989) 222–230
Elsevier

BBABIO 43022

Mitochondrial H^+-ATPase in mutants of *Saccharomyces cerevisiae* with defective subunit 8 of the enzyme complex

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(Received 2 December 1988)

Key words: Mitochondrial mutant; ATPase, H^+-; Membrane subunit; (Yeast mitochondria)

Mutants of *Saccharomyces cerevisiae* carrying defined lesions in the mitochondrial *aap1* gene, coding for membrane subunit 8 of the H^+-ATPase, have been investigated to examine the consequence of the mutations on the function and assembly of the enzyme complex. These include three *mit*$^-$ mutants, which cannot grow by oxidative metabolism due to their inability to synthesize full-length subunit 8, and three partial revertants of one of the mutants. The mutations in these strains have been previously characterized by DNA sequencing. The use of a monoclonal antibody to the β subunit of the H^+-ATPase as a probe of assembly defect revealed that the presence of subunit 8 is essential for the assembly of subunit 6 to the enzyme complex. Mitochondria isolated from the *mit*$^-$ mutants have negligible [$^{32}P_i$]ATP exchange activity and they exhibited ATPase activity which is not sensitive to inhibition by oligomycin, indicating a defective membrane F_0 sector. Normal assembly of subunit 8 (and subunit 6) was observed in the revertant strains, despite 8–9 amino-acid substitutions in the membrane-spanning region of the H^+-ATPase subunit 8 in two of the strains. The assembled complex, however, exhibited reduced [$^{32}P_i$]ATP exchange activity and low sensitivity to oligomycin, indicating that the product of the *aap1* gene is a functional subunit of the mitochondrial H^+-ATPase.

Introduction

The mitochondrial H^+-ATPase (ATP synthase, ATP phosphohydrolase, E.C. 3.6.1.3) is assembled from protein subunits synthesized within the mitochondria as well as those imported from the extramitochondrial cytoplasm (see Ref. 1 for a recent review). The F_1-sector of the enzyme complex contains the catalytic site for ATP synthesis and hydrolysis and consists of five different subunits designated α, β, γ, δ and ϵ (in 3 : 3 : 1 : 1 : 1 stoichiometry), all cytoplasmically synthesized. The F_0 sector of the H^+-ATPase, which is an integral part of the mitochondrial inner membrane, constitutes the H^+ channel of the enzyme complex and is suggested to link a transmembrane proton gradient, generated by the electron transport chain, to the synthesis of ATP on the F_1 sector.In the yeast *S. cerevisiae*, the membrane F_0 sector has been shown to be assembled from three mitochondrially synthesized subunits [2]: subunit 6 (in Tzagoloff nomenclature [3], with an apparent M_r of 20 000 in SDS-polyacrylamide gel electrophoresis), subunit 8 (apparent M_r 10 000) and subunit 9 (a proteolipid, M_r 7600).

As part of our general strategy to elucidate the processes involved in the assembly of the F_0 sector of the H^+-ATPase [4,5], we have recently studied a large series of *mit*$^-$ mutants with genetically defined mutations in the mitochondrial structural genes for subunit 6 (the *oli2* gene) [6] and subunit 9 (the *oli1* gene) [7,8], to characterize the defective H^+-ATPase complex in these mutants. These studies have revealed the central role played by subunit 9 in the assembly of the F_0 sector [4,5,7,8], as well as in the assembly of other respiratory enzyme complexes [7], and in the involvement of subunit 6 in the coupling of oxidative phosphorylation [6].

The role of subunit 8 in the assembly of a functional F_0 sector has been now investigated by using a similar strategy. The H^+-ATPase assembly and functional defects were studied in a series of mutants of *S. cerevisiae* with lesions in the *aap1* gene [9,10], which codes for this subunit. The nature of the mutations in these strains has been previously determined by DNA sequencing [10] and the strains studied include *mit*$^-$ mutants, which cannot grow by oxidative metabolism due to their inability to synthesize full-length subunit 8, and partial revertants derived from one of the *mit*$^-$ mutants. Here we report results of this investigation, which represent the only detailed information on the biochemical properties of H^+-ATPase subunit 8 mutants documented to date.

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Materials and Methods

Yeast strains

The *mit*$^-$ strains employed in the present study were derived from the wild-type strain J69-1B (α *ade1 his5 [rho*$^+$*]*) following mutagenesis with $MnCl_2$ [11] and selected for their inability to grow on non-fermentable substrates such as ethanol [9,10]. The revertant strains were isolated as spontaneous revertants from one of these *mit*$^-$ mutants (strain M26-10). The nature of the mutations in these strains has been previously determined [9,10]. 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, London, U.K.) and agar (15 g/l) and subcultured at least every 3 months.

Growth conditions

Cells used for the study of the synthesis and assembly of mitochondrial translation products were grown aerobically at 28° C in 75 ml liquid batch cultures. The growth medium contained yeast extract (10 g/l), a salts mixture [12], glucose (10 g/l for the wild-type strain, 20 g/l for the *mit*$^-$ mutants) and the auxotropic requirements of the strains (adenine, 100 mg/l, histidine, 50 mg/l). Cultures were harvested early in the stationary phase of growth. To minimize the effects of catabolite repression, cells used for studies of mitochondrial respiratory functions were grown to a steady-state in glucose-limited chemostat cultures in modified LKB ultraferm fermentors, under conditions in which the steady-state glucose level of the cultures was maintained below that which induces catabolite repression [13]. All cultures were routinely checked for petite mutants.

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

For the analysis of mitochondrial translation products, cells were grown in 75 ml liquid batch cultures. These products were labelled in vivo for 60 min at 28° C in a low-sulphate medium containing [^{35}S]sulphate and cycloheximide [14], which specifically inhibits cytoplasmic protein synthesis. After labelling, the cells were washed and resuspended in a 12 mM Tris buffer (pH 7.2) containing 0.33 M mannitol/0.27 M sorbitol/0.67 mM EDTA. Mitochondria were isolated from mechanically ruptured cells [15]. The products of mitochondrial protein synthesis were electrophoretically separated on SDS-polyacrylamide gels [16] and visualized by autoradiography.

Immunoprecipitation of H$^+$-ATPase

For immunoprecipitation of the H$^+$-ATPase, mitochondria were isolated as described above from cells labelled with [^{35}S]sulphate in the presence of cycloheximide, except that the buffer used to resuspend the cells before rupture contained the proteinase inhibitors, *p*-aminobenzamidine-HCl (10 mM), ϵ-amino-*N*-caproic acid (10 mM) and phenylmethylsulphonyl fluoride (2 mM). The H$^+$-ATPase complex was immunoprecipitated from Triton extracts of mitochondria essentially as described previously [17], using Sepharose-conjugated monoclonal antibody RH 48 which recognizes an epitope on the β subunit. After an incubation of 1 h at 4° C with the monoclonal antibody, the antigen-antibody complex attached to the beads was washed three times in a 5 mM Tris acetate buffer (pH 7.4) containing 0.1% Triton X-100 and 0.1% bovine serum albumin. Mitochondrial translation products associated with the immunocomplex, which represent assembled F_0 sector subunits, were visualized by fluorography [18] after electrophoresis [16].

Assays of mitochondrial enzyme activities

Cells were grown to a steady-state in glucose-limited chemostat cultures [13] and intact mitochondria isolated from spheroplasts were prepared by zymolyase digestion of the cell wall [19]. Delipidated bovine serum albumin was added (2 mg/ml) to the isolation buffer prior to rupture of the spheroplasts and in all subsequent steps, ensuring the isolation of coupled mitochondria. The activity of the oligomycin-sensitive mitochondrial ATPase was determined as detailed in Ref. 20. ATP-[^{32}P]P_i exchange activity was determined essentially as described by Choo et al. [6]. Published procedures were employed to determine the activities of NADH-cytochrome *c* reductase [21] and cytochrome *c* oxidase [22].

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

Results

Description of mutants

A total of six mutant strains were analysed in the present study, three *aap1 mit*$^-$ and three partial revertants of one of these *mit*$^-$ strains. The mutations in these strains have been previously characterized by DNA sequencing [9,10], and the mutational alterations to the H$^+$-ATPase subunit 8 predicted from the sequencing data are shown in Table I. All three *aap1 mit*$^-$ strains have frame-shift mutations, leading to premature termination during the synthesis of subunit 8. In one of the *mit*$^-$ mutants (strain M31) the frame-shift mutation resulted in a possible product of only two amino acids. The predicted truncated product in the other two (M68-5 and M26-10) was 18 amino-acid residues long, com-

TABLE I

Predicted amino acid sequence of defective H^+-ATPase subunit 8 in aap1 mutants

Summarized from Macreadie et al. [10]. Underlined residues are those that differ from the wild-type sequence.

Strain	General phenotype	Predicted amino acid sequence of subunit 8
J69-1b	wild-type	MPQLVPFYFM NQLTYGFLLM ITLLILFSQF FLPMILRLYP SRLFISKL
M31	*mit*$^-$ mutant	MP
M68-5	*mit*$^-$ mutant	MPQLVPFYFM NQLTYGFY
M26-10	*mit*$^-$ mutant	MPQLVPFYFM NQLMYGFY
M26-10R1	revertant	MPQLVPFYFM NQLMYGFTLM ITLLILFSQF FLPMILRLYP SRLFISKL
M26-10R7	partial revertant temperature-sensitive	MPQLVPFYFY ESINMWFTLM ITLLILFSQF FLPMILRLYP SRLFISKL
M26-10R13	partial revertant temperature-sensitive	MPQLVPFIFY ESINMWFTLM ITLLILFSQF FLPMILRLYP SRLFISKL

pared to the wild-type subunit 8 of 48 amino acids. Strain M26-10 has in addition a base substitution in the *aap1* gene which leads to Thr-14 → Met amino-acid replacement.

The three revertant strains were derived from strain M26-10. Two of these revertants (strains M26-10R7 and M26-10R13), were found to be temperature-sensitive in that they grow only very slowly at the restrictive temperature of 36 °C. In the non-temperature-sensitive strain M26-10R1, two amino-acid replacements remain in the subunit 8 sequence following the restoration of the *aap1* gene reading frame, of which the first one is the Thr-14 → Met substitution carried over from the mutant strain M26-10. The second difference is a consequence of the reopening of the M26-10 reading frame at position 18 by a T nucleotide insertion in the coding sequence [10], resulting in a threonine at position 18 rather than the leucine found in the wild-type (Table I).

The two temperature-sensitive revertants have significant alterations in their subunit 8 amino-acid sequences. In both cases, this arises from nucleotide insertions early in the *aap1* gene, which, while restoring the reading frame, have resulted in eight altered amino-acid residues (residues 10–16 and 18) in strain M26-10R7. Similar amino-acid substitutions were observed in strain 26-10R13, but an additional Tyr-8 → Ile substitution was also found (Table I).

H^+-ATPase subunit 8 in the mutant strains

To confirm that the three *mit*$^-$ mutants investigated in this study were lacking in the H^+-ATPase subunit 8, as predicted from the DNA sequence of their *aap1* genes, the mitochondrial translation products of these mutants were analysed by SDS-polyacrylamide gel electrophoresis. Although the DNA sequence indicates that the product of the *aap1* gene is only 5.8 kDa in size, the H^+-ATPase subunit 8 in the wild-type strain exhibits the mobility of a 10 kDa protein in the SDS-polyacrylamide gel (Fig. 1a). The predicted truncated products (18 residues in strains M26-10 and M68-5, 2 residues in M31) could not be observed in the display of mitochondrial translation products (Fig. 1b–d), as expected from the detection limit of the gel system used. Subunits 6 (apparent M_r 20 000) and 9 (M_r 7600) of the H^+-ATPase were found to be synthesized in all three *aap1 mit*$^-$ mutants analysed, although better resolving gels showed that the amount of subunit 6 synthesized might be slightly reduced.

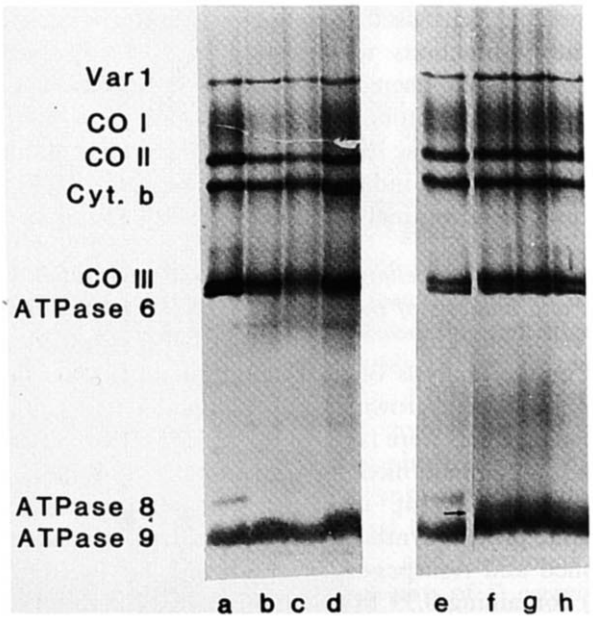

Fig. 1. Mitochondrial translation products in the *aap* mutant strains. Mitochondrial translation products in the various yeast strains were labelled with [^{35}S]sulphate and mitochondria isolated as described in Materials and Methods. Following electrophoretic separation in 12% polyacrylamide gels (23 cm long, 15 mA), mitochondrial translation products were visualized by autoradiography. The figure shows the translation products in the wild-type strain J69-1B (lanes a and e), in the *mit*$^-$ strains M26-10 (lane b), M31 (lane c), M68-5 (lane d), and in the revertant strains M26-10R1 (lane f), M26-10R7 (lane g), M26-10R13 (lane h). The altered subunits 8 in the revertant strains, which show faster mobilities than that of the wild-type strain, are indicated by an arrow. Cyt b, cytochrome *b*.

TABLE II

Steady-state growth yields of the aap1 mit⁻ mutants

Cells were grown in glucose-limited chemostat cultures at 28° C at a dilution rate of 0.1 h^{-1}. Results shown are the mean ± S.D. of at least five determinations.

Strain	Genotype	Growth yield (g cell dry weight/ mol glucose)	Petite frequency (% total population)
J69-1b	wild-type	121 ± 6	6 ± 3
M31	*aap1 mit⁻*	27 ± 3	65 ± 10
M26-10	*aap1 mit⁻*	27 ± 3	60 ± 8
EJ0	rho⁰	23 ± 3	100

As the *aap1* gene-reading frame has been restored in the three revertant strains, a full-length 48 amino-acid residue product is expected. The restored subunit 8 in the revertant strains, however, showed a significantly faster mobility than that of the wild-type strain, and could only be resolved from subunit 9 in longer gels (for example, Fig. 1e–h), or in relatively high polyacrylamide gel concentrations. The amount of the altered subunit 8, as well as subunits 6 and 9, synthesized appeared to be comparable to that of the wild-type strain.

Growth characteristics of the aap1 mutants

All three *aap1* mutants were isolated for their lack of ability to grow on non-fermentable substrates such as ethanol. This deficiency in oxidative metabolism is quantitatively apparent from the low steady-state growth yields of the strains when grown in glucose-limited chemostat cultures. The steady-state growth yields of the *mit⁻* mutants (around 27 g cell dry weight/mol glucose) were only slightly higher than that of a mtDNA-less (*rho⁰*) petite strain (23 g cell dry weight/mol glucose), compared to the growth yield of the wild-type strain of around 120 g cell dry weight/mol glucose (Table II). The mutant strains were found to be rather unstable during growth, with petite frequencies in the range of 50–70% being observed in the glucose-limited cultures (Table II).

The growth characteristics of the three revertant strains were examined in more detail to determine the extent of their phenotypic reversion, in batch cultures with glucose (10 g/l) as an energy source. As two of the revertants have previously been shown to be temperature-sensitive when grown on solid media [9], the growth studies were carried out at 18 and 36° C, as well as at the optimal growth temperature of 28° C. The wild-type strain J69-1B exhibited the characteristic biphasic growth pattern, the first phase of which represents the fermentative growth of the cells on glucose, followed after a short lag period by an oxidative growth phase, utilizing ethanol produced during the fermentative growth (Fig. 2). The lag period between the fermentative and the oxidative growth phases was longer at 18 and 36° C when compared to that at 28° C. In addition, the cell doubling time during growth on ethanol increased from 4 h at 28° C to 5.5 h and 8.5 h at 18 and 36° C, respectively. The *mit⁻* mutant strain M26-10 showed a similar fermentative growth phase, but no oxidative growth was apparent (data not shown).

As expected, the revertant strain that has only two amino-acid residue changes (strain M26-10R1) grew as the wild-type strain J69-1B (Fig. 2). The other two revertants (M26-10R7 and M26-10R13), however, were quite different in their growth characteristics. While the growth rates of these two revertants during the fermentative phase were found to be comparable to that of the wild-type, both strains showed a significantly longer lag period between the two growth phases at 28 and 36° C. Strain M26-10R7 grew as the wild-type strain at 18° C, but exhibited longer doubling times at 28 and 36° C

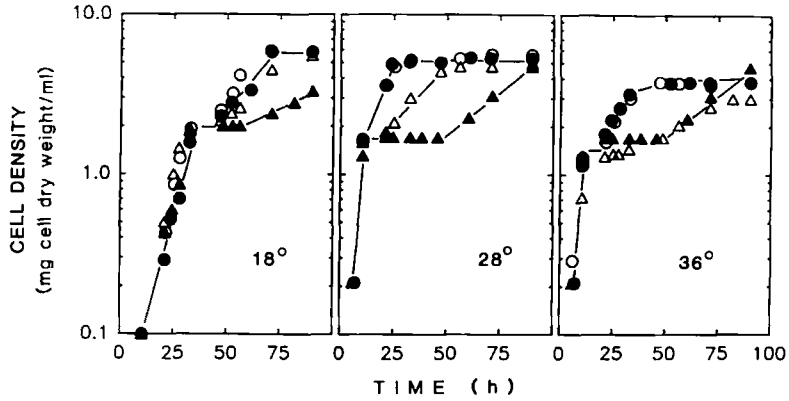

Fig. 2. Growth characteristics of the revertants of the *aap1 mit⁻* strain M26-10. Cells were grown in 600 ml cultures in fluted 2 L Erlenmeyer flasks in the presence of glucose (10 g/l) as an energy source at the temperatures indicated. Aliquots (5 ml) were taken at intervals and cell density was determined as previously described [12]. The growth curves for the wild-type strain J69-1B (○), and the revertant strains M26-10R1 (●), M26-10R7 (△) and M26-10R13 (▲) are shown. The *aap1 mit⁻* strain M26-10 did not show any growth in the oxidative phase of the growth curve in agreement with results shown in Table II.

(7.5 and 26 h compared to 4 and 8.5 h for the wild-type strain, respectively) indicating the partial nature of the phenotypic reversion. The additional Tyr-8 → Ile substitution in strain M26-10R13 is presumably critical for the normal function of subunit 8, as this strain grew by oxidative metabolism only slowly at the three temperatures tested. At 28 and 36 °C, the strain took 1–2 days to adapt from the fermentative to the oxidative growths, then only grew at a doubling time of more than 25 h during the oxidative phase (Fig. 2). It is significant that all revertant strains showed an appreciably lower petite frequency of less than 20% as compared to their parental *aap1* mutant strain M26-10.

Assembly of the mitochondrial H⁺-ATPase in the aap1 mutants

The extent to which the assembly of the H⁺-ATPase can still proceed in the absence of subunit 8 was investigated by using a monoclonal antibody to the β subunit of the enzyme complex (antibody RH48 [17]) as an assembly probe; only those subunits which are assembled to the enzyme complex would be associated with the β subunit when immunoprecipitated with the monoclonal antibody [4,17]. For the purpose of this study, mitochondrial translation products in the yeast strains examined were first labelled with [^{35}S]sulphate in the presence of the cytoplasmic protein synthesis inhibitor, cycloheximide, allowing the incorporation of the isotope into mitochondrial translation products only [14].

When the monoclonal antibody (coupled to CNBr-activated Sepharose-4B beads) was used to immunoprecipitate the enzyme complex from a Triton X-100 extract [17] of the wild-type strain J69-1B, three bands were observed following the electrophoretic separation of the immunoprecipitate in SDS-polyacrylamide gel [16] and the visualization of mitochondrial translation products by fluorography [18] (Fig. 3a, c). These bands correspond to subunit 6 (apparent M_r in SDS-polyacrylamide gel 20000), subunit 8 (apparent M_r 10000) and subunit 9 (apparent M_r 7500) [17]. Only the subunit 9 band, on the other hand, could be observed in the precipitate from the *aap1 mit⁻* mutants (Fig. 3b). This protein was present in apparently normal amounts. Subunit 6, which could be shown to be synthesized in the mutant strains, was not detected in the gel display of the immunoprecipitate from the *aap1 mit⁻* mutants, suggesting that subunit 8 is needed for the assembly of subunit 6 into the H⁺-ATPase complex.

In order to investigate whether the altered subunit 8 in the revertant strains can be assembled into the mitochondrial H⁺-ATPase, the enzyme complexes from these strains were also immunoprecipitated with the monoclonal antibody. Despite the extensive amino-acid changes in the N-terminal end of the H⁺-ATPase subunit 8 in two of the revertant strains, the altered subunit

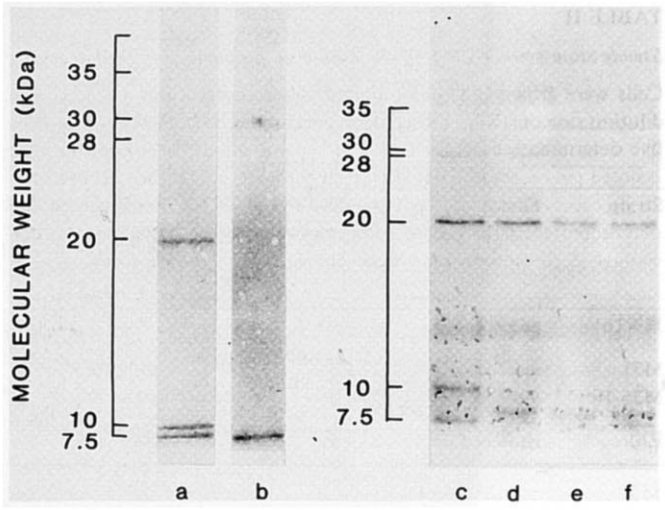

Fig. 3. Mitochondrially synthesized subunits associated with the H⁺-ATPase in the *aap1* mutants. Mitochondria isolated from cells labelled in vivo with [^{35}S]sulphate for mitochondrial translation products (Fig. 1) were solubilized with Triton X-100 (Materials and Methods). H⁺-ATPase was immunopurified from the Triton extracts with an anti-β subunit monoclonal antibody (RH 48) coupled to Sepharose-4B beads [17]. Mitochondrially synthesized proteins associated with the H⁺-ATPase were visualized by fluorography [18], following electrophoresis on 15% polyacrylamide gels in the presence of SDS. (a) and (c) are the wild-type strain J69-1B, and (b) the *aap1 mit⁻* strain M26-10. The other two *aap1 mit⁻* strains, M31 and M68-5, showed identical electrophoretic patterns to (b). (d), (e) and (f) are the revertant strains M26-10R1, M26-10R7 and M26-10R13, respectively.

8 appeared to be assembled to the enzyme complex (Fig. 3d–f); all three mitochondrially synthesized F_0 subunits were found to be associated with the immunoprecipitated H⁺-ATPase. As also observed in the electrophoretic gel display of the total mitochondrial translation products (see Fig. 2), the assembled subunit 8 in the revertant strains showed significantly faster electrophoretic mobilities than the wild-type protein and almost comigrated with subunit 9.

Impairment in the function of the H⁺-ATPase in the aap1 mutants

To investigate the defect in the function of the H⁺-ATPase as the consequence of the absence of subunit 8, or in the presence of an altered form of this subunit, the ATPase activity was measured in mitochondria isolated from the mutant strains. In all three *mit⁻* mutant strains examined, this activity was found to be reduced but still significant, ranging from 40% (in strain M31) to 57% (in strain M26-10) of the wild-type activity (Table III). The ATPase activity in these mutants, however, was insensitive to inhibition by oligomycin. Thus, while this inhibitor inhibited the ATPase activity in the wild-type strain by about 80%, the activity in the mutant strains was inhibited by only 10–20% (Table III). This value is only slightly higher than the 5% inhibition observed in a mtDNA-less *rho⁰* strain which lacks mitochondrial protein synthesis [6].

TABLE III

The ATPase and ATP-[^{32}P]P_i exchange activities in mitochondria of the aap1 mutant strains

All yeast 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 from spheroplasts. The concentration of oligomycin used was 20 μg/ml. The ATPase activities are the mean ± S.D. of at least five determinations. The ATP-[^{32}P]P_i exchange is expressed as the percentage of the rate in the wild-type.

Strain	ATPase		ATP-[^{32}P]P_i (% of wild-type)
	spec. act. (μmol/min per mg protein)	inhibition by oligomycin (%)	
J69-1b	0.72 ± 0.1	80	100
M31	0.29 ± 0.11	16	1
M68-5	0.32 ± 0.14	10	1
M26-10	0.41 ± 0.15	20	1
M26-10R1	0.74 ± 0.15	77	65
M26-10R7	0.70 ± 0.12	80	23
M26-10R13	0.50 ± 0.16	55	7

All three revertants of the *aap1* mutant M26-10 showed similar levels of ATPase activity to the wild-type strain (Table III). However, strain M26-10R13 showed a reduced sensitivity to oligomycin (about 55% compared to 76–80% for the wild-type and the other two revertants). This observation suggests that the Tyr-8 → Ile substitution, which is the only difference between the revertant strain M26-10R7 and strain M26-10R13, may somehow affect the binding of oligomycin to the F_0 sector of the enzyme complex, possibly as the consequence of a conformational change in this subunit. Alternatively, the reduced sensitivity of the ATPase activity to oligomycin could also be due to a partial defect in the coupling of the ATPase activity of the F_1 sector and the proton translocation through the H^+ channel of the F_0 sector.

The ATP synthetase activity in the mutant strains was also investigated by measuring the ATP-[^{32}P]P_i exchange reactivity in isolated mitochondria. The activity observed for strain M26-10R1 was around 65% of that of the wild-type, but the other two revertants showed severe reduction in this activity (23 and 7% of the wild-type activity for strains M26-10R7 and M26-10R13, respectively).

The mitochondrial respiratory activity in the aap1 mutants

Mit$^-$ mutations in the structural genes of the H^+-ATPase subunit 9, and to a lesser extent of subunit 6, have been shown to have pleiotropic effects on the assembly of the mitochondrial respiratory enzyme complexes, in particular the cytochrome *c* oxidase. In order to establish whether the functional deficiency caused by the absence of subunit 8 is also pleiotropic, the mitochondrial respiratory activities in the mutant strains were determined. Mitochondrial respiration of the three *aap1 mit*$^-$ strains was found to be significantly reduced to levels of only around 8% (for strain M31) to 12% (for strain M68-5) of the wild-type rate (Table IV). The residual respiratory activity in the mutant strains was not stimulated by the addition of ADP, indicating a lack of respiratory control (data not shown). Similarly, when the uncoupler CCCP (carbonyl cyanide-*m*-chlorophenylhydrazone), which allows free translocation of protons across the mitochondrial membrane, was added, no stimulation of the mutants' respiratory activity was apparent (Table IV). In contrast, the wild-type activity was stimulated by around 220% by the addition of CCCP. Although the mutant strains showed relatively high petite frequencies, it could not fully account for the severe reduction in the respiration activity.

TABLE IV

Mitochondrial respiratory activities of the aap1 mutants

Yeast cells were grown in glucose-limited chemostat cultures at 28° C at a dilution rate of 0.1 h^{-1}, and intact mitochondria were isolated from spheroplasts. Specific activities for cytochrome oxidase were tabulated as percentages of the wild-type rates. Concentration of inhibitors used were CCCP 50 μM, antimycin A 10 μg/ml and KCN 3 μM.

Strain	Mitochondrial respiration		NADH cytochrome *c* reductase		Cytochrome *c* oxidase	
	spec. act. (nmol O_2/min per mg protein)	stimulation by CCCP (%)	spec. act. (μmol/min per mg protein)	inhibition by antimycin A (%)	spec. act. (% wild-type)	inhibition by KCN (%)
J69-1b	130 ± 20	220	0.37 ± 0.15	90	100	98
M31	11 ± 4	0	0.07 ± 0.04	76	5	100
M68-5	16 ± 5	0	0.09 ± 0.05	72	20	100
M26-10	12 ± 5	0	0.10 ± 0.05	66	15	100
M26-10R1	106 ± 21	160	0.33 ± 0.12	82	96	94
M26-10R7	70 ± 16	85	0.19 ± 0.08	70	60	94
M26-10R13	65 ± 12	90	0.16 ± 0.08	73	38	93

Consistent with the low levels of the respiratory activity observed, both the NADH-cytochrome *c* reductase and the cytochrome *c* oxidase activities were found to be significantly reduced in the mutant strains. The NADH-cytochrome *c* reductase activity in the three mutant strains was around 18–27% of the wild-type activity, but the cytochrome *c* oxidase activity in strain M31 was only about 5% of that in the wild-type strain. The other two strains possessed 15 and 20% of the wild-type level of the cytochrome oxidase activity.

The mitochondrial respiratory activity in the revertant strain M26-10R1 was found to be not significantly different from that of the wild-type, but with reduced CCCP stimulation. In contrast, the two partial revertants showed only about half of the wild-type respiratory, NADH-cytochrome *c* reductase and cytochrome *c* oxidase activities (Table IV). The respiratory activity in these two strains was only slightly stimulated by the uncoupler CCCP, but the NADH-cytochrome *c* reductase and the cytochrome *c* oxidase activities were inhibited by antimycin A and KCN, respectively, at near wild-type levels. Thus, despite the apparently normal assembly of the H^+-ATPase, the presence of an extensively altered subunit 8 in these strains still affects the assembly, or the activity of the respiratory chain. The decrease in the activity of both NADH-cytochrome *c* reductase and cytochrome *c* oxidase could contribute to the significantly longer generation time of these two strains when grown in ethanol, in addition to the primary defect in the ATP synthase activity described in the previous section.

Discussion

The aap1 gene product is a functional subunit of the mitochondrial H^+-ATPase

Although, following our earlier suggestion [9], the *aap1* gene has been confirmed by direct protein sequencing [23] to code for a 48 amino-acid-long mitochondrial translation product with the electrophoretic mobility of a 10 kDa polypeptide in SDS-polyacrylamide gel, the evidence for the functional association of this protein with the mitochondrial H^+-ATPase has been actually very limited. Results of the present study, therefore, are of particular significance, as they provide the experimental evidence to support the contention that the *aap1* gene product is indeed a functional subunit of the mitochondrial H^+-ATPase.

Analysis of the *aap1 mit*$^-$ mutants, which due to frame-shift mutations in the *aap1* gene cannot synthesize the 10 kDa polypeptide, confirms our previous suggestion that this polypeptide is necessary for the assembly of functional H^+-ATPase [9]. More important, however, are the results of the analysis of partial revertants of one of these *mit*$^-$ strains, as the restoration of the subunit 8 reading frame in these revertant strains has resulted in the synthesis of full-length subunits 8 with a number of amino-acid substitutions. The altered subunits 8 could be shown to be assembled to the H^+-ATPase complex by immunoprecipitation experiments employing a monoclonal antibody to the β subunit of the enzyme complex as an assembly probe. The assembled H^+-ATPase complex, however, was functionally defective as evidenced from the low oligomycin sensitivity of the ATPase activity in strain M26-10R13, and the significantly lower ATP-[^{32}P]P_i exchange activity observed, in particular in the two strains in which the amino-acid sequence of subunit 8 has been significantly altered.

Of the yeast F_0 subunits, the proteolipid subunit 9 is believed to be directly involved in the proton-channel activity of the enzyme complex [24]. Subunit 6 has been also suggested to be involved in the proton channel and in the coupling of oxidative phosphorylation [6]. Results of the present investigation indicate that the integrity of subunit 8 is also essential for the coupling of oxidative phosphorylation. The precise role of subunit 8 in this process, however, remains to be elucidated.

An inorganic phosphate binding property was observed in a purified preparation of a protein thought to be subunit 8 [25] when it was isolated from mitochondria and from purified H^+-ATPase complex. The mammalian equivalent of the yeast H^+-subunit 8 has been isolated from beef heart and sequenced [28] and shown to be coded for by the mitochondrial A6L gene [27]. An inorganic phosphate binding activity has been reported for a hydrophobic bovine mitochondrial protein [26], which is presumably subunit 8 according to its amino-acid composition. More recently, antibodies against the H^+-ATPase subunit 8 from rat liver mitochondria have been shown to inhibit the ATP-P_i exchange activity in an energy-dependent fashion [29]. These findings are consistent with our suggestion that the H^+-ATPase subunit 8 may have an essential role in the energy transduction mechanism of the mitochondrial oxidative phosphorylation.

Functionally important structural features of subunit 8

The results of the present study on the defective H^+-ATPase complex in the partial revertant strains have also provided information on certain structural features of subunit 8 which might be functionally important. Thus, since strain M26-10R1 has the growth characteristics of the wild-type strain, the two amino-acid substitutions in this strain, methionine at residue 14 and threonine at residue 18, are alternate 'wild-type' amino acids. The partial revertants M26-10R7 and M26-10R13, on the other hand, have four and five amino-acid substitutions, respectively, with different chemical characteristics from that of the wild-type, and these substitutions are presumably responsible for the

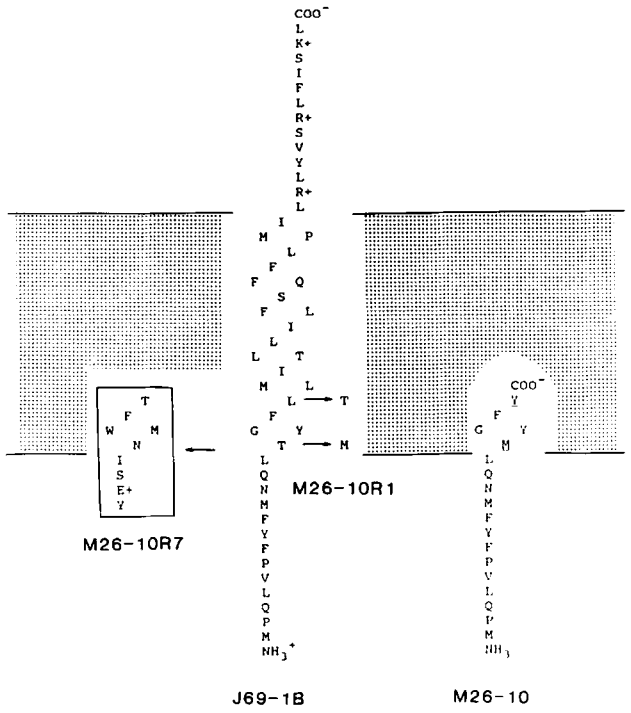

Fig. 4. Proposed model for the integration of the H^+-ATPase subunit 8 in the *aap1* mutants. This model is based on the secondary-structure prediction for subunit 8 [23] that suggests the central region to be an α-helix and likely to be membrane spanning. The amino-acid sequence of the wild-type strain J69-1B is shown, together with the amino-acid substitutions in the revertant strains M26-10R1 and M26-10R7 (indicated by arrows). The amino-acid sequence of strain M26-10R13 is identical to that of strain M26-10R7, except for the additional Tyr-8 → Ile substitution. The amino-acid sequence of the truncated subunit 8 in the *mit*$^-$ strain M26-10 is also shown, with the altered amino-acid residues underlined.

reduced ATP-[^{32}P]P_i exchange activity of the H^+-ATPase complex.

Prediction of the secondary structure of the H^+-ATPase subunit 8 [23] suggests that the central hydrophobic region of the protein (residues 14–35) is an α helix, which presumably traverses the lipid bilayer of the mitochondrial inner membrane. The N-terminal and the C-terminal segments appear to be a β sheet and a random coil respectively, protruding from the membrane lipid. The observation that membrane proteins are usually inserted from their N-termini during integration into membrane lipid, and that basic amino acids are more often found on the matrix side of the mitochondrial inner membrane suggests an orientation for the H^+-ATPase subunit 8, with the C-terminus directed into the mitochondrial matrix (Fig. 4).

As the altered subunit 8 polypeptides in the three revertants are assembled into the H^+-ATPase complex, it is likely that these proteins are similarly integrated into the membrane, with a single central transmembrane segment (Fig. 3). However, the major amino-acid changes preceding residues 19 in the partial revertants M26-10R7 and M26-10R13, which include a charged Glu-11 and two other hydrophilic residues (Ser-12 and Thr-18), would have resulted in a significant alteration in the conformation of the mutants subunit 8 in this region, part of which is in the membrane-spanning sector of the protein molecule.

Assembly of the F_0 sector of the H^+-ATPase

The failure to detect subunit 6 in the assembled H^+-ATPase in *aap1 mit*$^-$ mutants studied here is of particular interest, as this subunit was shown to be synthesized in all three strains examined, although in reduced amounts. The absence of subunit 6 in the immunoprecipitated complex suggests that the physical presence of subunit 8 is required for the assembly of subunit 6 into the enzyme complex.

A more extensive study on the assembly of mitochondrially synthesized subunits of the H^+-ATPase has been carried out recently in our laboratory by defining the assembly defects in various *oli1, oli2* and *aap1 mit*$^-$ mutants which lack subunit 9, 6 or 8 of the H^+-ATPase, respectively, or synthesize these subunits in a grossly altered form [32]. Subunit 9 was found to be the key subunit for the assembly of the F_0 sector, as, in its absence, only trace amounts of subunits 6 and 8 were detected in the immunopurified defective complex [7,30]. As evidence from the results of the present study, the assembly of subunit 9 is apparently followed by subunit 8, as *aap1 mit*$^-$ mutants which lack this subunit can assembly subunit 9 but not subunit 6. The final mitochondrially synthesized subunit of the F_0 sector to be assembled appears to be subunit 6, as in the absence of this subunit (in *oli2 mit*$^-$ mutants) both subunits 9 and 8 could be normally assembled [6,30]. A wider range of *aap1 mit*$^-$ mutants with characterized amino acid substitutions in the H^+-ATPase subunit 8 is required to define in detail the amino-acid residues critical for the assembly of this subunit to the H^+-ATPase complex, and for the subsequent assembly of subunit 6.

Pleiotropic effect of the aap1 mutations on the assembly of the respiratory chain

Also of particular significance was the observation that the assembly of the respiratory chain, in particular the cytochrome oxidase complex, was affected in the *aap1 mit*$^-$ strains. A similar effect has also been observed in *mit*$^-$ strains where subunit 6 [6] and 9 [7] of the H^+-ATPase F_0 sector are not synthesized or synthesized in altered forms. The various apoprotein subunits of the cytochrome oxidase complex have been shown to be normally synthesized even in the subunit 9 mutants, in which the secondary effect on the respiratory chain assembly is most severe [7]. Thus, this pleiotropic effect is presumably due to a defect in heme *a* synthesis, or the attachment of the heme groups to the apoprotein. It was proposed that the defective assembly of the H^+-ATPase complex might have resulted in the derange-

ment of the inner mitochondrial membrane in such a way that protoheme synthesis and/or heme *a* binding to the apoproteins of cytochrome oxidase are affected [7].

Acknowledgments

We wish to thank Professors A.W. Linnane and H.B. Lukins for the use of the yeast mutant strains, and for helpful discussions. The contribution of Dr. Richard Hadikusumo in the immunological study of the assembly defect is gratefully acknowledged. This work was supported by Grant 82/15245 from the Australian Research Grant Scheme.

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