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Biogenesis of Mitochondria: Defective Yeast H^+-ATPase Assembled in the Absence of Mitochondrial Protein Synthesis Is Membrane Associated[1]

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Abstract

We have investigated the extent to which the assembly of the cytoplasmically synthesized subunits of the H^+-ATPase[3] can proceed in a mtDNA-less (rho°) strain of yeast, which is not capable of mitochondrial protein synthesis. Three of the membrane sector proteins of the yeast H^+-ATPase are synthesized in the mitochondria, and it is important to determine whether the presence of these subunits is essential for the assembly of the imported subunits to the inner mitochondrial membrane. A monoclonal antibody against the cytoplasmically synthesized β-subunit of the H^+-ATPase was used to immunoprecipitate the assembled subunits of the enzyme complex. Our results indicate that the imported subunits of the H^+-ATPase can be assembled in this mutant, into a defective complex which could be shown to be associated with the mitochondrial membrane by the analysis of the Arrhenius kinetics of the mutant mitochondrial ATPase activity.

Key Words: H^+-ATPase complex; assembly defect; *Saccharomyces cerevisiae;* mitochondrial biogenesis; membrane association.

Introduction

The mitochondrial H^+-ATPase complex (ATP phosphohydrolase; EC 3.6.1.3) in yeast is assembled from three subunits which are synthesized within the organelle and about seven subunits imported from the extramitochondrial cytoplasm (Tzagoloff *et al.*, 1973, 1979; Orian *et al.*, 1981; Marzuki

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[3]H^+-ATPase, proton translocating ATPase; SDS, sodium dodecyl sulfate.

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et al., 1983). The mitochondrially synthesized subunits are part of the membrane sector of the enzyme complex (designated F_0) which is a proton channel, while five of the imported subunits form the hydrophilic F_1 sector, which catalyzes the synthesis of ATP *in vivo* when coupled with the translocation of H^+ through the F_0 sector (Criddle *et al.,* 1979).

In recent years, the synthesis of the nuclearly coded F_1 subunits of the H^+-ATPase complex in the extramitochondrial cytoplasm, and their import into mitochondria, have been the focus of detailed investigations. A great deal of information has now been accumulated on the nature of the precursors of some of these subunits, as well as on the details of the mechanism by which the precursors are imported into the mitochondria and processed into their mature forms (see Neupert and Schatz, 1981, Schatz and Butow, 1983, and Hay *et al.,* 1984 for recent reviews). The assembly pathway which follows the import of these subunits, however, is largely unknown.

One of the central questions in the study of the assembly of the H^+-ATPase is the mechanism by which the imported subunits become associated with the mitochondrial inner membrane. Since most, if not all, of the membrane sector proteins of the enzyme complex are synthesized in the mitochondria, it is important to determine whether the presence of these subunits is required for the assembly of the imported subunits to the inner mitochondrial membrane. There have been previous attempts to address this question by using petite mutants of yeast which are not capable of mitochondrial protein synthesis, as a result of deletions in their mtDNA (Tzagoloff *et al.,* 1973; Schatz, 1968). However, these investigations were largely inconclusive mainly because of the limitations inherent in the experimental techniques available at the time. In the present study we have investigated the extent to which the assembly of the cytoplasmically synthesized subunits of the H^+-ATPase can proceed in a mtDNA-less (*rho*°) strain of yeast, by using a monoclonal antibody against the cytoplasmically synthesized β-subunit of the complex. Our results show that the imported subunits of the F_1-sector are assembled in this mutant to form a complex within the mitochondria which has an ATPase activity. Although the defective H^+-ATPase complex do not contain the mitochondrially synthesized membrane sector proteins, the complex could be shown to be intimately associated with the mitochondrial membrane, such that the mutant mitochondrial ATPase activity is influenced by the fluidity of the membrane lipid.

Materials and Methods

Strains of Yeast

The strains of yeast used in this study were the respiratory competent strains J69-1B α *adel his* [*rho*$^+$], KD115 α *ole* [*rho*$^+$] and KD69-1B α *ole*

[*rho*$^+$], the *mit*$^-$ strains M11-28 α *adel his* [*oli*2 *mit*$^-$], KD11-28 α *ole* [*oli*2 *mit*$^-$], 2223 α *adel his* [*cyb*], the *rho*$^-$ strain G4 a *adel lys*2 *trp* [*rho*$^-$] and the *rho*° strains EJO α *adel his* [*rho*°], KDO α *ole* [*rho*°], and BT2-l a *karl*-1 *leu* [*rho*°].

The *mit*$^-$ mutant strain Mll-28, isolated from the wild-type strain J69-lB by $MnCl_2$ mutagenesis (Putrament *et al.,* 1973), has previously been characterized (Stephenson *et al.,* 1981). The mutation in strain Mll-28 maps in the *oli*2 region of the mtDNA, and results in a change in the apparent p*I* (Stephenson *et al.,* 1981) of the 20,000-dalton subunit of the H^+-ATPase complex (subunit 6 in Tzagoloff's nomenclature; Tzagoloff *et al.,* 1973). The alteration does not affect the assembly of this subunit into the H^+-ATPase, but leads to a defect in the assembly of the 7,600-dalton mitochondrially synthesized subunit proteolipid (subunit 9 in Tzagoloff's nomenclature) into the complex (Stephenson *et al.,* 1981).

Construction of Mutant Strains Used in This Study

Strains KD69-1B, KD11-28, and KDO were derived from strain KD115 which contains a nuclear mutation (*ole*) affecting the Δ^9 desaturase enzyme (Keith *et al.,* 1969) and is an unsaturated fatty acid auxotroph (Resnick and Mortimer, 1966). Strain KDO was derived by ethidium bromide treatment (Nagley and Linnane, 1972) of strain KD115. Strain KD69-1B contains the *ole* mutation and the mtDNA of the wild-type strain of J69-1B, and was constructed by cytoduction using the approach described below for strain KD11-28.

Strain KD11-28 was constructed from strains KDO and M11-28. Since both parental strains are of the same mating type, strain KD11-28 was constructed in two steps using the mtDNA-less strain BT2-1 which carries the *kar*1-1 mutation; haploid cells carrying the *kar*1-1 mutation form diploids at low frequencies and the majority of cells resulting from such a cross are heterokaryons which subsequently segregate haploid cells containing one of the parental nuclei and the mixed cytoplasm from both parents (Conde and Fink, 1976).

The mtDNA carrying the *mit*$^-$ mutation was first transferred from strain M11-28 to strain BT2-1 by crossing the two strains as described previously (Linnane and Nagley, 1978). After the selection for colonies which carry the BT2-1 nuclear marker (leucine), the leucine-requiring colonies were screened for the presence of the mtDNA from strain M11-28 by crossing to a *mit*-strain which is defective in cytochrome *b* synthesis as the result of a mutation in the *cob-box* region of the mtDNA (strain 2223).

One such strain, carrying the BT2-1 nuclear DNA and the mtDNA of strain M11-28, was used for the subsequent cross with strain KDO. Colonies having the nuclearly determined unsaturated fatty acid growth requirement were first selected. These were then screened for the presence of the *oli*2 *mit*$^-$

mutation by crossing to a petite deletion mutant G4, which retains a 1.8-kb segment of mtDNA including part of the wild-type *oli*2 gene (Linnane *et al.*, 1980). The presence of the original *oli*2 *mit*$^-$ mutation in strain KD11-28, which was finally selected from the above cross, was confirmed by the analysis of the ATPase activity in this mutant strain (see Table I).

Growth Conditions

Unless otherwise stated, cells were grown in glucose-limited chemostat cultures at 28°C essentially as described by Marzuki and Linnane (1979), except that 1-liter capacity fermenters (modified LKB Biotec polyferm fermenters) with a working volume of 500 ml were used. Cells with the KD115 nuclear background were grown in a medium containing yeast extract (Difco Laboratories, Detroit, Michigan; 1% w/v), a salt mixture (Proudlock *et al.*, 1971), and glucose (1% w/v for respiratory-competent strains and 2% w/v for respiratory-deficient mutant strains). The medium was supplemented with various concentrations of Tween-80 (Selbys, Notting Hill, Victoria, Australia) as a source of unsaturated fatty acids. The fatty acid composition of Tween-80 was found to be typically as follows: 81% oleic acid ($C_{18:1}$), 6% palmitoleic acid ($C_{16:1}$), 2% myristoleic acid ($C_{14:1}$), and 11% saturated fatty acids. Other strains were grown in a medium containing yeast extract (Difco; 1% w/v), a salt mixture (Proudlock *et al.*, 1971), glucose (1% w/v for the wild-type strain J69-1B and 2% w/v for respiratory-deficient mutant strains), adenine (100 μg/ml), and histidine (50 μg/ml).

The following solid media were employed during the construction of the mutant strain KD11-28: (a) YEP medium consisting of yeast extract (1% w/v; obtained from Difco Laboratories, Detroit, Michigan) and peptone (1% w/v, from Oxoid Ltd., London), supplemented with glucose (2% w/v) or ethanol (1% v/v) and, if necessary, Tween-80 (1% w/v); (b) minimal medium consisting of yeast nitrogen base without amino acids (0.67% w/v; obtained from Difco) and glucose (2% w/v), supplemented with leucine (20 μg/ml); (c) KD medium consisting of yeast extract (0.1% w/v) and glucose (2% w/v) in a salt mixture (Proudlock *et al.*, 1971), supplemented with Tween-80 (1% w/v). Solid media prepared in the presence of Tween-80 contained 1.5% (w/v) agar; other media contained 1% (w/v) agar.

Estimation of Petite Frequency and Reversion Frequency

The frequency of petite and revertant cells in cultures was determined as described by Marzuki *et al.*, (1975a). The petite frequency of strain KD11-28 varied between 10 and 14% and that of strains KD69-1B and J69-1B varied between 1 and 3%. The reversion frequency of the *mit*$^-$ phenotype in strain KD11-28 was approximately 20×10^{-6}, while the frequency of *ole* revertants

in all cultures used in the experiments described in this paper was about 1×10^{-6}.

Isolation of Mitochondria

Intact mitochondria were used in the determination of the ATPase activity, and isolated from zymolase-prepared spheroplasts as described by Cobon *et al.* (1974). For immunoprecipitation studies, mitochondria were isolated by the method of Roberts *et al.* (1978), except that cells were disrupted by shaking manually in the presence of glass beads (4-g beads, 0.5 mm in diameter, to 1 ml cell suspension) in a small glass vial (20 ml capacity). The cell suspension was shaken for 1.5 min at a frequency of two strokes per second and an amplitude of 50 cm per stroke (Lang *et al.*, 1979).

Chloroform Extraction of F_1-ATPase

F_1-ATPase was extracted from mitochondria by a modification of the method of Takeshige *et al.* (1976). Mitochondria were resuspended at a concentration of 10 mg protein/ml in a 10 mM Tris-SO_4 buffer, pH 7.4, containing 250 mM sucrose, 1 mM EDTA, 2 mM ATP, and protease inhibitors (Ryrie and Gallagher, 1979) phenylmethylsulfonyl fluoride (PMSF, 0.5 mM), ϵ-aminocaproic acid (ACA, 5 mM), and *p*-aminobenzamidine hydrochloride (PAB, 5 mM) (all obtained from Sigma, St. Louis, Missouri). The temperature of the suspension was quickly brought up to 18°C and 0.5 vol of analytical grade chloroform (Univar, Ajax chemicals, Sydney, Australia) was added to the suspension. After vigorous mixing for 25 sec, the emulsion was broken by low-speed centrifugation and the aqueous layer collected. Residual chloroform was removed from the aqueous phase by stirring under a stream of nitrogen and the slightly turbid solution was centrifuged at 140,000 *g* at 18°C for 30 min to remove insoluble material. The resultant clear, yellow solution was brought to pH 7.5 with NaOH.

Imunoprecipitation of the ATPase Complex with Monoclonal Anti-β-subunit Antibody

A monoclonal anti-β-subunit antibody (designated RH48.6) was used in immunoprecipitation studies. The isolation, specificity, and other properties of this antibody have previously been described (Hadikusumo *et al.*, 1984).

Whole cell proteins were labeled with [^{35}S]sulfate (500 μCi/ml) in a low-sulfate medium (Douglas and Butow, 1976) at 28°C for 4 hr (Murphy *et al.*, 1980), and mitochondria were isolated as described earlier. The H^+-ATPase complex was immunoprecipitated from Triton extracts of the mitochondria (Tzagoloff and Meagher, 1971) using monoclonal antibody RH48.6 which was conjugated to Sepharose-4B as described previously (Hadikusumo

et al., 1984). Material which binds nonspecifically to Sepharose-4B beads was first adsorbed using CNBr-activated Sepharose-4B which had been treated with glycine to block the active sites on the beads. After the removal of Sepharose-4B beads, the resulting supernatant (50 μl) was incubated for 4 hr at 4°C with 50 μg of purified monoclonal antibody, coupled to CNBr-

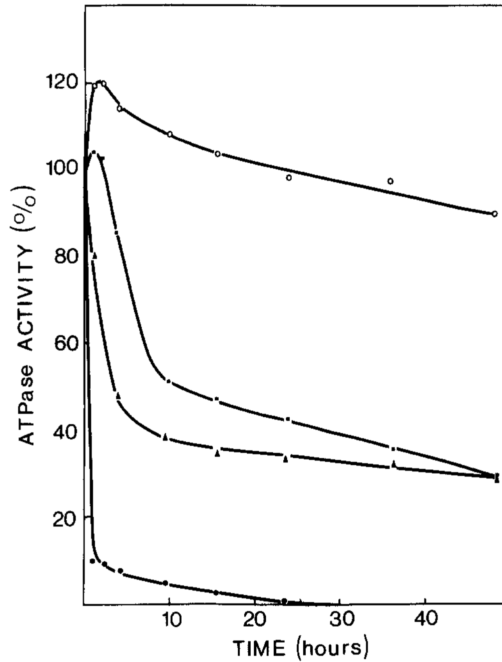

Fig. 1. The ATPase activity in yeast strains, which are defective in the synthesis or assembly of the membrane sector of the enzyme complex, is cold labile. Cells of the wild-type strain J69-1B and the mutant strains EJ0 and M11-28 were grown in glucose-limited chemostat cultures (Marzuki and Linnane, 1979). Intact mitochondria were isolated from these cells (Cobon *et al.*, 1974) and maintained at 4°C. At times indicated the ATPase activity in these mitochondria was assayed at 28°C (Roberts *et al.*, 1979). The ATPase activity was also assayed in a preparation of solubilized F_1-ATPase obtained by chloroform treatment of mitochondria from strain J69-1B as described under Materials and Methods. Results are expressed as the percentage of the specific activity (μmol ATP hydrolyzed/min/mg protein) measured at the beginning of the incubation at 4°C (T_0). The specific activity at T_0 in strain J69-1B (○) was 0.8, while that observed in strains M11-28 (■) and EJ0 (▲) were 0.38 and 0.35, respectively. The specific ATPase activity of the F_1-ATPase preparation (●) at T_0 was 33.5.

activated Sepharose-4B beads. The beads were collected by centrifugation in an Eppendorf centrifuge and washed at least three times (Hadikusumo *et al.*, 1984).

Polyacrylamide Gel Electrophoresis

Subunits of the H^+-ATPase complex were analyzed on polyacrylamide slab gels in the presence of 0.1% w/v sodium dodecyl sulfate (SDS) as described by Murphy *et al.*, (1980) with the following modifications: (a) The dimensions of the separating gel used were 11.5 cm × 10 cm × 0.15 cm and electrophoresis was carried out at 15 mA for approximately 4 hr. (b) The buffer solution used to solubilize immunoprecipitates did not contain β-mercaptoethanol. (c) Radioactively labeled polypeptides were visualized by fluorography as described by Chamberlain (1979).

Arrhenius Kinetics of ATPase Activity

ATPase activity in isolated mitochondria or in chloroform extracts of the mitochondria was assayed as described by Roberts *et al.*, (1979) at temperatures ranging from 4 to 36°C. Before starting the reaction, mitochondria were first incubated in the assay mixture at room temperature for 5 min to ensure lysis of the mitochondria. The mixture was then incubated for a further 10 min at the specified temperature, followed by the addition of ATP to start the reaction. Chloroform extracts, which were prepared in the presence of ATP and EDTA to stabilize the F_1-ATPase, were centrifuged through Sephadex G25 (Pharmacia chemicals) in a 1-ml syringe for 2 min in a bench centrifuge immediately before each assay, to remove excess ATP and EDTA.

The mitochondrial ATPase of strains KDO and KD11-28 is cold-labile (as that of strains EJO and M11-28; see Fig. 1). Since the mitochondria were kept at 4°C during the measurement of the Arrhenius kinetics to minimize proteolytic degradation, the ATPase activity of the two mutant strains observed at each temperature was corrected for the loss of activity due to cold inactivation.

Unsaturated Fatty Acid Analysis

The unsaturated fatty acid composition of the whole cell lipids was analyzed as described by Marzuki *et al.* (1975a) except that the extracted fatty acids were methylated with methanoic HCl (the final concentration of acid being approximately 1 M) and analyzed by gas–liquid chromatography in a Packard Becker Gas chromatograph 417 on 10% Apiezon L, 80–100 mesh (Applied Science Laboratories, Pennsylvania).

Results

Defective Mitochondrial H^+-ATPase in mtDNA-less (rho°) Strain of Yeast

A number of earlier investigations have indicated that yeast cells can partially assemble the mitochondrial H^+-ATPase in the absence of mitochondrial protein synthesis, as mitochondrial ATPase activity can be detected in petite mutants of yeast (Tzagoloff *et al.*, 1973) which as the result of the deletion of the mitochondrial rRNA and/or tRNA genes do not have mitochondrial protein synthetic activity. Significant level of mitochondrial ATPase activity was also detected in the mtDNA-less (*rho*°) mutant investigated in the present study (Table I). However, despite the fact that the mutant strain has been grown in glucose-limited chemostat cultures to minimize catabolite repression, the ATPase activity in the *rho*° petite mutant was found to be lower than that of the wild type strain. Thus, the activity was only 0.33 μmol ATP hydrolized/min/mg protein in strain EJO as compared to 0.78 ATP hydrolized/min/mg protein in strains J69-1B. In addition, unlike the ATPase activity in the wild type strains which was more than 90% inhibited by oligomycin, an inhibitor of the H^+-ATPase proton channel activity, the activity in the mutant strain was found to be insensitive to this inhibitor (Table I).

It was also observed that the mitochondrial ATPase activity in the mutant strain was cold-labile. Thus, when intact mitochondria isolated from strain EJO were incubated at 4°C, the ATPase activity decreased rapidly and reached a minimal level after 8 hr (Fig. 1). In contrast, the ATPase activity of the wild-type strain J69-1B remained almost constant over the same period of time (Fig. 1). The loss of ATPase activity in the mutant strain, however, occurred more slowly than that in a preparation of F_1-ATPase, obtained by chloroform extraction of the complex from the mitochondrial membranes. This observation indicates that the defective ATPase complex in strains EJO is different from free F_1-ATPase (Fig. 1).

Table I. ATPase Activity in Yeast Strains Containing Defective H^+-ATPase[a]

Strain	ATPase activity	
	Specific activity (μmol ATP/min/mg protein)	Oligomycin sensitivity (% inhibition)
J69-1B (*rho*$^+$)	0.78	96
EJ0 (*rho*°)	0.33	0
M11-28 (*oli2 mit*$^-$)	0.37	18

[a]Cells of the wild-type strain J69-1B and the mutant strains EJ0 (*rho*°) and M11-28 (*oli2 mit*$^-$) were grown in glucose-limited chemostat cultures, mitochondria were isolated, and the ATPase activity was measured as described in Materials and Methods. The sensitivity of the enzyme activity to inhibition by oligomycin was measured in the presence of 10 μg/ml of the inhibitor.

We have also included in our analysis an *oli*2 *mit*⁻ mutant strain in which the assembly of subunit 9 is impaired (strain KD11-28; Stephenson *et al.*, 1981) in order to obtain a better understanding of the interaction between partially assembled ATPase and the membrane lipid. The mitochondrial ATPase in this mutant strain exhibited similar properties to that of the rho° strain in that the specific ATPase activity was only about 50% of that of the wild-type strain (Table I), and exhibited a low degree of sensitivity to inhibition by oligomycin. The mitochondrial ATPase activity in the mutant strain KD11-28 was also cold-labile as shown in Fig. 1, but the ATPase of strain KD11-28 is significantly more cold-stable than that of free F_1-ATPase, as the activity in the rho° strain.

The Defective H^+-ATPase from the rho° Strain of Yeast Contains the Five Subunits of the F_1-sector

In agreement with the conclusion of the earlier studies (Tzagoloff *et al.*, 1973), the above observation indicates that some of the cytoplasmically synthesized subunits of the H^+-ATPase are capable of forming the catalytic center of the enzyme complex in the absence of the mitochondrially synthesized membrane sector subunits. However, although it has been shown that all five F_1-subunits are synthesized in the absence of mitochondrial protein synthesis (Tzagoloff *et al.*, 1973), it has been very difficult in the past to identify the subunit composition of the defective H^+-ATPase assembled under this condition. Although it has not been determined in yeast, in the H^+-ATPase of the thermophilic bacterium PS3, only the α and the β subunits are necessary for the reconstitution of the ATPase activity *in vitro* (Kagawa *et al.*, 1979). The other subunits are presumably involved in the interaction between this catalytic unit and the membrane F_0 sector of the enzyme complex.

We have used one of the monoclonal antibodies recently isolated in our laboratory, which is specific for the β subunit of the H^+-ATPase, to determine the extent to which the assembly of the H^+-ATPase complex can proceed in the petite mutant of yeast. This antibody has been shown to be very effective in immunoprecipitating intact H^+-ATPase complex from Triton extracts of wild type mitochondria (Hadikusumo *et al.*, 1984). The immunoprecipitate obtained from the wild-type strain contains ten different polypeptides (Marzuki *et al.*, 1983; Hadikusumo *et al.*, 1984), nine of which are apparent in Fig. 2. Five of these polypeptides were identified as subunits of the F_1 sector of the H^+-ATPase (α, β, γ, δ, and ϵ; apparent M_r 56,000, 54,000, 31,000, 14,000, and 12,000, respectively; Hadikusumo *et al.*, 1984). Three of the other subunits, with apparent molecular weights of 20,000, 10,000, and 7,500, are the mitochondrially synthesized subunits 6, 8, and 9 of the membrane F_0 sector of the enzyme complex. The wild-type immunoprecipitate also contains two other polypeptides (apparent M_r 18,000 and 25,000; Hadikusumo *et al.*,

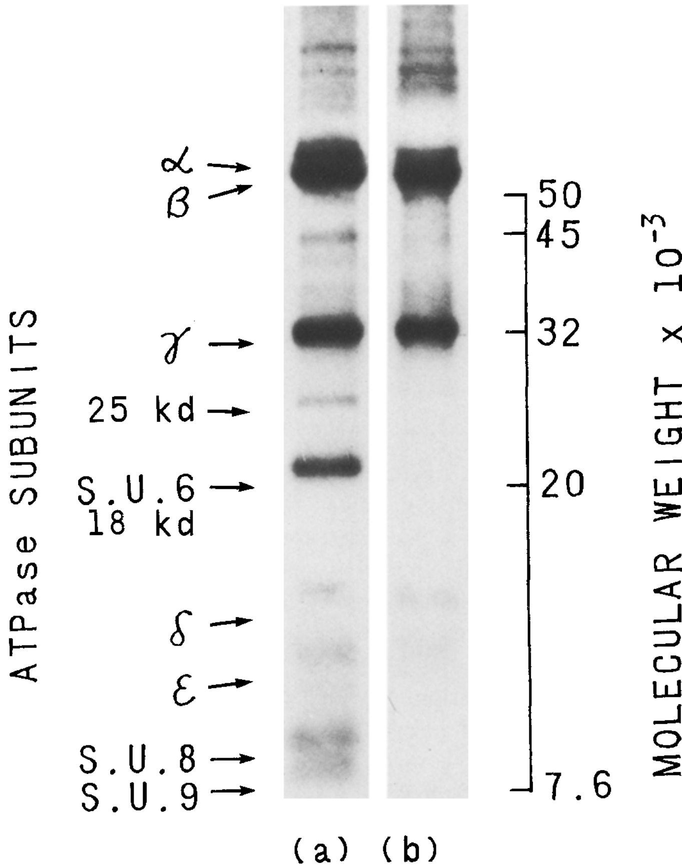

Fig. 2. Immunoprecipitation of the defective H^+-ATPase complex from the *rho*° strain EJ0. Cells of the wild-type strain J69-1B and the *rho*° mutant strain EJ0 were labeled with [^{35}S]sulfuric acid as described in Materials and Methods. Mitochondria were isolated from these cells by the methods of Roberts *et al* (1978) and a Triton extract of these mitochondria was prepared. The H^+-ATPase complex was then immunoprecipitated by using a monoclonal anti-β-subunit antibody as previously reported (Hadikusumo *et al.*, 1984). The immunoprecipitate was analyzed on a 13.4% w/v polyacrylamide gel in the presence of 0.1% w/v SDS, and visualized by fluorography (Chamberlain, 1979). Shown are the autoradiographs of immunoprecipitates obtained from (a) mitochondria of strain J69-1B, and (b) mitochondria of strain EJ0. Subunits of the H^+-ATPase were identified as described previously (Hadikusumo *et al.*, 1984).

1984). The mobility of the 18,000-dalton polypeptide, however, varies slightly in different gels and frequently comigrates with the 20,000-dalton subunit 6 (Marzuki *et al.,* 1983; Hadikusumo *et al.,* 1984). The identity of these polypeptides has not been established as yet, but since they reproducibly copurify with the monoclonal anti-β-antibody immunoprecipitate, it appears

that they are genuine subunits of the yeast H^+-ATPase. It is possible, for example, that the 18,000-dalton polypeptide is in fact the OSCP (oligomycin sensitivity conferring protein), which in yeast has been reported to have a similar apparent molecular weight (Tzagoloff *et al.*, 1973).

The defective H^+-ATPase complex immunoprecipitated from the *rho°* petite strain was found to contain the five F_1-sector subunits, as shown in Fig. 2. It appears, therefore, that the F_1-sector of the yeast mitochondrial H^+-ATPase can be assembled in the absence of mitochondrially synthesized subunits of the complex. However, the F_1-sector proteins might only represent the minimum composition of the complex in the mutant strain. The defective ATPase complex in the *rho°* strain is probably less stable than that of the wild-type strain, as indicated by its relative cold lability. Thus, while the monoclonal anti-β-subunit antibody has been shown to precipitate the assembled subunits of the wild-type H^+-ATPase complex reproducibly, a possibility exists that the absence of the 25,000- and 18,000-dalton polypeptides in the immunoprecipitate of the petite mitochondria is simply due to the dissociation of these two subunits during washing of the immunoprecipitate. The two subunits might bind less tightly to the F_1-sector in the absence of the mitochondrially synthesized F_0 subunits.

Manipulation of the Membrane Fatty Acid Composition of Strains Used in the Study of the Arrhenius Kinetics of ATPase Activity

The approach used in the present study to investigate the interaction between the partially assembled ATPase complex of the mutant strains and the mitochondrial membranes is dependent on the ability to manipulate the membrane lipid composition of yeast strains employed. We have previously shown that such manipulations can be performed in a strain of yeast (strain KD115) which has an auxotropic requirement for unsaturated fatty acid (Marzuki *et al.*, 1975a). The mutant strain carries the *ole* mutation affecting the activity of the Δ^9 fatty acid desaturase and resulting in the inability of the mutant to desaturate palmitic and stearic acids. New strains of yeast were therefore constructed, containing the *ole* mutation and the mitochondrial mutations required for the present investigation (see Materials and Methods). These include a respiratory-competent strain KD69-1B, a *rho°* strain KDO and strain KD11-28 which carry an *oli*2 *mit*$^-$ mutation which has previously been shown to prevent normal assembly of ATPase subunit 9.

The membrane unsaturated fatty acid content of the newly constructed strains was maintained at reduced steady-state levels by growing cells in glucose-limited chemostat cultures in the presence of defined amounts of Tween-80 as a source of unsaturated fatty acids, as described previously (Marzuki and Linnane, 1975a). It has been shown that under these growth conditions, the fatty acid composition of the mitochondrial membrane is similar to that of the total cell. The membrane unsaturated fatty acid content

Table II. Membrane Unsaturated Fatty Acid Content of Strains KD69-1B, KD0, and KD11-28 Used in the Present Study[a]

Strain	% UFA	Fatty acid composition (g/100 g total fatty acid)										
		C_{12}	C_{14}	$C_{15:1}$	C_{15}	$C_{16:1}$	C_{16}	$C_{18:1}$	C_{18}	C_{8-14}	C_{16+18}	$C_{16:1+18:1}$
KD115	75	0.4	0.9	n.d.	0.9	7.9	17.8	66.5	4.5	1.6	22.3	74.4
KD69	30	2.5	4.1	1.2	9.3	7.7	46.2	20.0	6.9	6.6	53.1	27.7
	34	2.8	6.4	0.62	2.2	7.4	42.9	24.1	11.0	9.2	53.9	31.5
	37	n.d.	3.9	1.1	0.75	8.4	58.4	27.3	n.d.	3.9	58.4	25.6
KD0	58	1.1	2.5	1.1	4.3	5.1	21.9	51.0	10.9	3.5	32.8	56.1
	60	1.8	1.8	0.4	0.6	3.6	24.1	54.7	11.5	3.6	35.5	52.3
	61	0.5	1.9	0.4	1.0	4.1	22.0	54.2	13.3	2.4	35.3	58.3
KD11-28	48	n.d.	2.7	1.5	0.9	9.6	33.7	33.1	14.7	2.7	48.4	42.7
	56	1.6	4.2	1.3	1.3	5.7	22.6	45.9	9.2	5.7	31.7	51.6
	57	n.d.	6.1	0.6	1.5	5.5	28.2	46.8	17.5	6.1	45.7	51.2

[a]Cells of strains KD69-1B, KD11-28, and KD0 were grown in glucose-limited chemostat cultures, supplemented with 4000, 1000, 800, or 600 μg Tween 80/ml as previously described (Marzuki *et al.*, 1975a), and the whole cell fatty acid composition was analyzed. The composition of fatty acid in whole cells has been shown to be representative of that of mitochondrial membranes (Marzuki *et al.*, 1975a). n.d. = not detectable.

in cells of strain KD115 was 75% of the total fatty acid when the growth medium was fully supplemented with Tween-80 (Marzuki *et al.*, 1975a; Table II). In this study cells of strains KD69-1B, KD11-28, and KDO were grown at various reduced levels of supplementation of Tween-80, so that the membrane unsaturated fatty acid content ranged from 30 to 60% of the total fatty acid (Table II). The membrane unsaturated fatty acid was found to consist mainly of oleic acid ($C_{18:1}$) which is the major component found in Tween-80 (see Materials and Methods). The amount of palmitoleic acid ($C_{16:1}$) was low and that of fatty acids with a chain length shorter than 14 carbon atoms was negligible (Table II).

The Defective H^+-ATPase Complex Assembled in the rho° Strain Is Associated with the Mitochondrial Membrane

We have further investigated whether the ATPase complex in the *rho*° mutant is functionally associated with the mitochondrial membrane by analyzing the Arrhenius kinetics of the ATPase activity in the mutant strain. The Arrhenius kinetics (plot of log activity vs. reciprocal of the absolute temperature) of a number of membrane-related phenomena have been reported to show a discrete discontinuity, due to an increase in the enzyme activation energy below the temperature at which the discontinuity occurs (Raison, 1973). Although the details of the molecular mechanism for this discontinuity are still not well understood, it is generally accepted that it is due to an alteration in the conformation of the membrane-associated proteins, occurring as a result of a phase transition in the membrane lipid from a

liquid-crystalline to a gel phase as the temperature decreases (Raison, 1973). Therefore, the analysis of Arrhenius kinetics of the enzyme activity in the *rho*° strain provides a convenient tool to investigate the interactions between the partially assembled H^+-ATPase and the membrane lipid in this mutant.

The Arrhenius kinetics of the ATPase activity in mitochondria of strain KD69, isolated from cells in which the membrane unsaturated fatty acid content was 34% of total fatty acid, showed a discrete discontinuity at 23°C with activation energies of 13.6 and 25 kcal/mol for the temperature ranges of 11–23°C and 23–37°C, respectively (Fig. 3). When the experiment was repeated using mitochondria in which the membrane unsaturated fatty acid content was 37 and 30% of total fatty acid, discontinuities were again observed (Table III). As shown in a previous study on the Arrhenius kinetics of the ATPase activity in strain KD115 (Marzuki *et al.,* 1975b), there is a correlation between the membrane unsaturated fatty acid content and the temperature at which the discontinuity occurred (Table III, Fig. 5). Thus the temperature of discontinuity was 21 and 28°C for mitochondria containing 37 and 30% of their total fatty acid as unsaturated acids, respectively. The possibility that the discontinuity in the Arrhenius plots of the ATPase activity was due to an effect on the transport mechanism for substrates involved in the ATPase reaction can be discounted, as the assay mixture used here does not provide enough osmotic support to keep the mitochondria intact. The examination of the Arrhenius kinetics of the ATPase activity of F_1-ATPase, solubilized from mitochondria of strain KD69-1B, further confirms the previous conclusions that the discontinuity in the Arrhenius plot of the ATPase activity is due to the interaction between the ATPase complex and the membrane lipid. F_1-ATPase preparation obtained from the above KD69-1B cells, containing 34% of their total fatty acids as unsaturated fatty acids, did not show the discontinuity observed in the mitochondrial preparation (Fig. 3).

The Arrhenius kinetics of the mutant strains KDO and KD11-28 were analyzed exactly as described for the respiratory-competent strain KD69-1B. Both mutants exhibited biphasic Arrhenius kinetics (Fig. 3). In strain KDO the discontinuity occurred at 19°C in mitochondria containing 58% of the total fatty acids as unsaturated acids, with activation energies of 4.9 and 14.4 kcal/mole above and below the temperature of discontinuity, respectively. In strain KD11-28, a discontinuity was observed at 20.5°C in mitochondria containing 48% of the total fatty acids as unsaturated acids with activation energies of 3.7 and 19.6 kcal/mole above and below the breakpoint, respectively (Table III). When the experiment was repeated using mitochondria from the strains KDO and KD11-28 in which the unsaturated fatty acid content was around 60 and 56% respectively (Table III), a discontinuity in the Arrhenius plots was again observed. Furthermore, as the unsaturated fatty

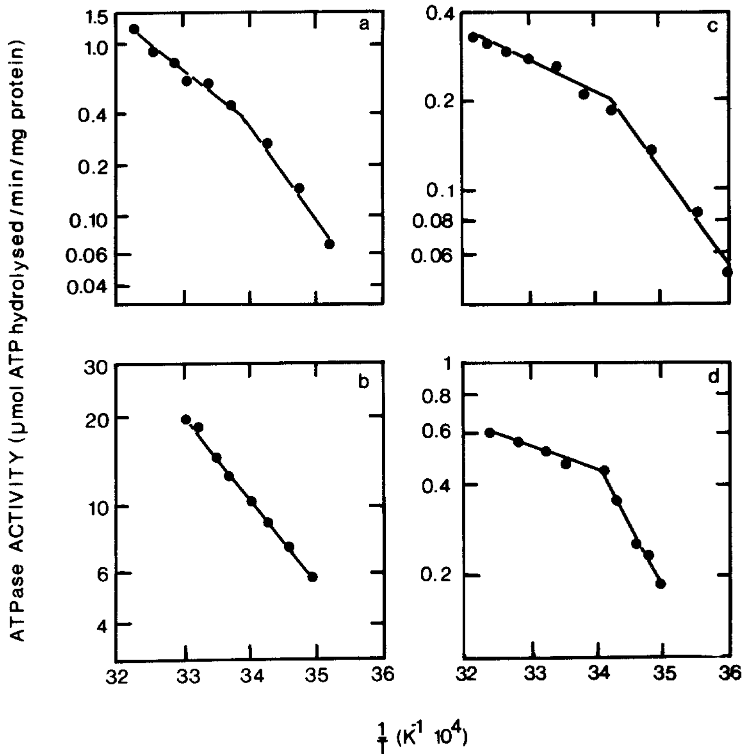

Fig. 3. Arrhenius plots of the ATPase activity in the respiratory-competent strain KD69-1B, the *rho*° strain KD0, and the *oli2 mit*$^-$ mutant strain KD11-28. Cells were grown in glucose-limited chemostat cultures at various levels of Tween-80 as a source of unsaturated fatty acids as described in Materials and Methods. Mitochondria were isolated from these cells, maintained at 4°C, and the mitochondrial ATPase activity assayed over the range of 4 to 36°C. The ATPase activity was also assayed of an F_1-ATPase preparation obtained by chloroform extraction of mitochondria from the respiratory-competent strain. The chloroform extract was maintained at room temperature and, under this condition the ATPase activity of the preparation was found to be stable as previously reported (Takeshige *et al.*, 1976). Arrhenius plots of the ATPase activity were constructed and, in the case of strains KD0 and KD11-28, the results were corrected for the loss of activity due to cold inactivation (similar to strains EJ0 and M11-28; see Fig. 1). Arrhenius plots shown are of (a) mitochondria of the respiratory-competent strain KD69-1B, containing 34% of total fatty acids as unsaturated acids; (b) a chloroform extract of mitochondria of the respiratory-competent strain KD69-1B above (34% of total fatty acids as unsaturated acids); (c) mitochondria of the *oli2 mit*$^-$ mutant strain KD11-28, containing 48% of total fatty acids as unsaturated acids; (d) mitochondria of the mtDNA-less *rho*° strain KD0, containing 58% of total fatty acids as unsaturated acids.

Table III. Summary of Data from the Analysis of the Arrhenius Kinetics of ATPase Activity in Strains KD69-1B, KD11-28, and KD0[a]

Strain	Membrane unsaturated fatty acid content (% of total fatty acid)	Temperature of discontinuity (°C)	Activation energy (kcal/mol)	
			E_I	E_{II}
KD69-1B	30	28.0	12.7	20.5
	34	23.0	13.6	25.0
	37	21.0	8.7	15.2
KD0 (rho°)	58	19.0	4.9	14.4
	60	16.0	7.4	18.5
	61	15.0	8.2	20.1
KD11-28 (*oli2 mit*$^-$)	48	20.5	3.7	19.6
	56	16.0	6.1	12.3
	57	16.0	6.8	13.4

[a] Mitochondria were isolated from cells grown in glucose-limited chemostat cultures as described in Materials and Methods. Arrhenius plots of the mitochondrial ATPase activity were constructed and the activation energies were determined from the slopes of the plots. The membrane unsaturated fatty acid content was determined as described in the methods. E_I = Activation energy above temperature of discontinuity. E_{II} = Activation energy below temperature of discontinuity.

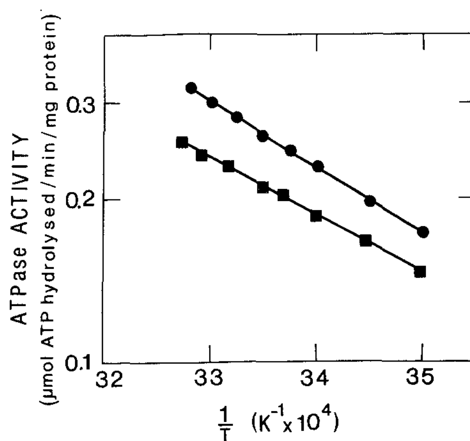

Fig. 4. Arrhenius plots of the ATPase activity released by sonication from mitochondria of the mtDNA-less strain KD0. Mitochondria were isolated from cells of strain KD0 grown in glucose-limited chemostat cultures as described in Materials and Methods. The mitohondria were then subjected to ultrasonic disruption as described in Table IV. After the removal of broken mitochondria, the ATPase activity in the supernatant fraction was assayed at the indicated temperatures, and corrected for the loss of activity due to cold inactivation as in Fig. 3. Arrhenius plots were constructed for the ATPase activity released from mitochondria of strain KD0 with unsaturated fatty acid content of 61 (●) and 58% (■).

acid content of the mitochondria increased, the temperature of discontinuity was reduced in both strain KDO and strain KD11-28.

The above results strongly suggest that the defective H^+-ATPase complex in the mutants analyzed is associated with the inner mitochondrial membrane, and that the presence of products of mitochondrial protein synthesis is not required for the association of the subunits imported from the cytoplasm to the membrane. The data, however, do not rule out the possibility that the measured activities may reflect the influence of temperature on lipid associated with defective ATPase complex which is nonmembrane associated. We have, therefore, subjected mitochondria of strain KDO from the above experiment to ultrasonic disruption with a Branson Sonifer. Results of this experiment confirm our conclusion that the defective ATPase complex in the mtDNA-less strain KDO is membrane associated, but in addition also indicate that in the absence of the mitochondrially synthesized membrane sector proteins, the defective complex does not physically bind as tightly to the mitochondrial membrane. Thus, as shown in Table IV, more than 90% of the ATPase activity was released from the mitochondria of strain KDO (with unsaturated fatty acid contents of 58, 61, and 78%), under sonication conditions which released less than 10% of the activity from the wild type mitochondria. In addition, a significant proportion (about 30%) of the ATPase activity could not be recovered after sonication of the mutant mitochondria, further suggesting that the defective ATPase complex is less

Table IV. The Tightness of the Association between the Defective ATPase Complex in the mtDNA-less Strain KD0 and the Mitochondrial Membrane[a]

Strain	Membrane unsaturated fatty acid content (% of total fatty acid)	Total ATPase activity (μmol ATP hydrolyzed/min)		
		Mitochondria	Sonicated preparation	
			Pellet	Supernatant
KD69-1B	38%	19.5	17.2	1.3
KD0	78%	11.0	0.3	7.5
KD0	61%	7.1	0.3	4.8
KD0	58%	8.4	0.2	5.7

[a]Mitochondria were isolated from cells of strains KD69-1B and KD0 grown in glucose-limited chemostat cultures as described in Materials and Methods, and resuspended at a concentration of 10 mg protein/ml in 10 mM Tris-SO_4 buffer, pH 7.4, containing 250 mM sucrose, 1mM EDTA, 2 mM ATP, and protease inhibitors as described in Materials and Methods. The mitochondrial suspensions (48 mg) were then disrupted with a Branson sonifier equipped with a microtip at 18°C. The sonication was carried out for 15 sec at position 5 on the output control. Broken mitochondria were pelleted by centrifugation in a Beckman Type 50 Ti rotor at 105,000 × g for 30 min at 18°C, and resuspended in a Tris-acetate buffer, pH 7.5. The ATPase activity in the supernatant and in the broken mitochondrial fraction was determined at 28°C as described in Materials and Methods. In all cases the activity was found to be more than 93% sensitive to inhibition by the specific F_1-inhibitor.

stable than that of the wild-type strain. The Arrhenius plots of the ATPase activity released from strain KDO mitochondria (unsaturated fatty acid contents 58 and 61%) did not show the discontinuities observed before the sonication (Fig. 4 as compared to Fig. 3 and Table III), indicating that the biphasic Arrhenius kinetics of the mitochondrial ATPase activity are not due to the effect of temperature on lipid associated with nonmembrane-associated ATPase.

Discussion

The results of the present study indicate that in the absence of the mitochondrially synthesized subunits, the subunits of the H^+-ATPase which are imported from the cytoplasm can be assembled within the mitochondria, into a complex consisting of at least the five F_1 subunits of the enzyme complex. In a mtDNA-less (*rho°*) strain, this partially assembled H^+-ATPase could be shown to be associated with the mitochondrial membrane by the analysis of the Arrhenius kinetics of the mutant mitochondrial ATPase activity. Thus, as in the Arrhenius kinetics of the activity in the respiratory-competent strain, the Arrhenius plots of the mitochondrial ATPase activity in the mtDNA-less mutant exhibited a discontinuity typical of membrane-associated functions (Raison, 1973). The temperature at which the discontinuity occurred was determined by the unsaturated fatty acid content of the mitochondrial membrane. Furthermore, it is interesting to note that the dependence of the discontinuity temperature on the membrane unsaturated fatty acid content in the mutant strain KDO and in the newly constructed respiratory-competent strain KD69 was similar to that of strain KD115 which has previously been extensively investigated (Marzuki *et al.*, 1975b). This is demonstrated in Fig. 5 in which the temperatures at which the discontinuities occur are plotted against the membrane unsaturated fatty acid content of the cells from which mitochondria were isolated. Thus, it appears that the association between the partially assembled complex and the mitochondrial membrane is intimate enough to be affected by a perturbation in the membrane lipid.

The observed Arrhenius kinetics of the ATPase activity represent the rate-limiting step in the various partial reactions involved in this activity, catalyzed presumably by a number of different subunits of this complex enzyme. It appears therefore that the imported subunits of the ATPase complex are associated in the mutant strain with the mitochondrial membrane in such a way that the rate-limiting step of the ATP hydrolytic reaction is influenced by the lipid environment. Since the mitochondrially synthesized subunits of the membrane sector of the ATPase complex are absent in the

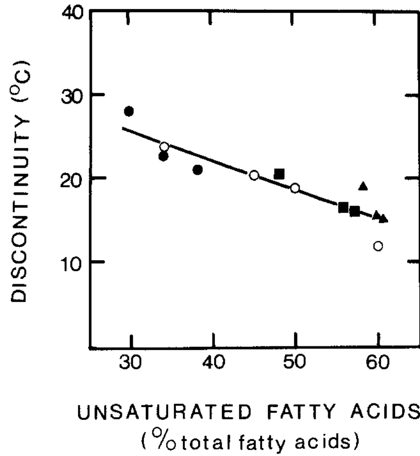

Fig. 5. The effect of membrane unsaturated fatty acid content on the discontinuity in the Arrhenius plots of the mitochondrial H^+-ATPase activity. The temperatures at which the discontinuities occur in the Arrhenius plots of the mitochondrial ATPase activity in the respiratory-competent strain KD69-1B (●), the mtDNA-less *rho°* strain KD0 (▲), and the *oli2 mit⁻* strain KD11-28 (■) are as in Table III. Data for strain KD115 (○) is reproduced from Marzuki *et al* (1975b).

mtDNA-less strain KDO, it appears that some of the cytoplasmically synthesized components of the complex can interact directly with the lipid bilayer. It is possible that the same interaction may also occur in the wild-type strain, prior to the assembly of the mitochondrially synthesized subunits of the membrane sector to the enzyme complex, and this interaction might be an important step in the assembly pathway of the ATPase complex. The importance of membrane phospholipid composition in the binding of beef-heart and chloroplast F_1-ATPase to membrane has previously been reported (Livine and Racker, 1969).

The exact nature of the association between the cytoplasmically synthesized subunits and the membrane lipid in the mutant strain cannot be determined as yet. The approach used in this study failed to show any difference in the Arrhenius kinetics of the ATPase activity in the *rho°* strain which do not synthesize the three mitochondrially coded membrane sector subunits and that in the *mit⁻* strain KD11-28 which, in a previous study, has been shown to contain an altered subunit 6 preventing normal integration of subunit 9 into the complex (Stephenson *et al.,* 1981). A comparison of preparations of strains KDO and KD11-28 containing similar levels of cellular

unsaturated fatty acids (57% unsaturated acids in strain KD11-28 and 58% in strain KDO) shows that the activation energies above and below the temperature of the discontinuity are of the same order of magnitude in the two mutant strains (Table III). It is not possible to directly compare these activation energies with those of the respiratory-competent parental strain which tends to have lower unsaturated fatty acid content because of its ability to grow oxidatively as well as fermentatively in the chemostat cultures, and therefore maintained at higher steady-state cell densities. The activation energy of the ATPase activity appears to vary slightly between experiments, and it has been shown in a more extensive study (Marzuki *et al.*, 1975a) that the activation energy of several mitochondrial inner membrane enzyme complexes, including that of the H^+-ATPase, is influenced by the unsaturated fatty acid content of the membrane. When the above factors are taken into consideration, however, it appears that the activation energies of the mitochondrial ATPase activity in the mutant strains observed at the various membrane unsaturated fatty acid levels are also of the same order of magnitude as that of the respiratory-competent strain KD69-1B (Table III). Since the activation energy is a measure of the energy required to overcome the rate-limiting step of the reaction, the addition of mitochondrially synthesized subunits of the H^+-ATPase to the enzyme complex does not appear to significantly affect the rate-limiting step of the ATP-hydrolysis reaction.

Attempts to study the Arrhenius kinetics of mutants lacking subunit 6 or subunit 8 of the H^+-ATPase were also made. However, a number of problems are associated with these studies. (a) During the construction of the required strains it was observed that the frequency of the petite mutation in strains containing the mtDNA of the above *mit*$^-$ mutants and the nuclear DNA of strain BT2-1 (which carries the *kar*1-1 mutation) was much higher than that of the original *mit*$^-$ mutant strains. (b) The frequency of petite mutation would be further increased if strains containing the *mit*$^-$ mutations were grown at reduced fatty acid supplementation (Marzuki *et al.*, 1974). The Arrhenius kinetics of the ATPase activity of such mutant strains, therefore, could not be analyzed.

The sequence of assembly of the individual subunits of the H^+-ATPase complex following import and processing is not known. Our results, however, clearly indicate that the sequence of assembly of the yeast mitochondrial H^+-ATPase is very different from that of *E. coli* H^+-ATPase which has been extensively studied. While in yeast, the assembly of the F_1 sector is not coupled to that of the F_0 sector, in *E. coli* the assembly of the F_1 sector and the F_0 sector is mutually dependent (Gibson, 1982; Cox *et al.*, 1981). Thus, the assembly of the *E. coli* H^+-ATPase is initiated by the integration of two of the F_0 subunits (M_r 24,000 and 8,000) into the membrane, which is followed by the assembly of one β and one α subunit. The subsequent addition of the third

F_0 subunit (M_r 18,000) completes the assembly of the F_0 sector, and the F_1-ATPase is then completed by the addition of the other subunits of the F_1 sector.

The sequence of assembly in the yeast H^+-ATPase can be further studied by determining the extent to which the assembly process can proceed in mutant strains of yeast in which specific subunits of the ATPase complex cannot be synthesized. A number of mutant strains of yeast in which the cytoplasmically synthesized subunits of the H^+-ATPase are absent have been described. However, in the mutant strains which have been characterized so far, the mutations appear to be pleiotropic in that they affect the synthesis of more than single subunits of the H^+-ATPase (Todd *et al.,* 1979). In contrast, mutant strains of yeast in which the mitochondrially synthesized subunit 6 (Roberts *et al.,* 1979), subunit 8 (Macreadie *et al.,* 1982, 1983), or subunit 9 (B. Jean-Francois, S. Marzuki, and A. W. Linnane, unpublished results) is specifically affected are available and have been extensively characterized. Work is in progress in our laboratory to determine the extent to which the assembly of the H^+-ATPase can proceed in the absence of each of these subunits. Our preliminary results have already indicated an assembly pathway involving sequential addition of the mitochondrially synthesized subunits to the F_1-sector, leading to the formation of a functional H^+-ATPase complex (Marzuki *et al.,* 1983).

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References

Chamberlain, I. P. (1979). *Anal. Biochem.* **98,** 132–135.
Cobon, G. S., Crowfoot, P. D., and Linnane, A. W. (1974). *Biochem. J.* **144,** 265–275.
Conde, J., and Fink, G. R. (1976). *Proc. Natl. Acad. Sci. USA* **73,** 3651–3655.
Cox, G. B., Downie, J. A., Langman, L., Senior, A. E., Ash, G., Fayle, D. R. H., and Gibson, F. (1981). *J. Bacteriol* **148,** 30–42.
Criddle, R. S., Johnston, R. F., and Stack, R. J. (1979). *Curr. Top. Bioenerg.* **9,** 89–145.
Douglas, M. G., and Butow, R. A. (1976). *Proc. Natl. Acad. Sci. USA* **73,** 1083–1086.
Gibson, F. (1982). *Proc. R. Soc. London* **215,** 1–18.
Hadikusumo, R. G., Hertzog, P. J., and Marzuki, S. (1984). *Biochim. Biophys. Acta* **765,** 258–267.
Hay, R., Bohni, P., and Gasser, S. (1984). *Biochim. Biophys. Acta* **779,** 65–87.

Kagawa, Y., Sone, N., Hirata, M., and Yoshida, M. (1979). *J. Bioenerg. Biomembr.* **11,** 39–78.
Keith, A. D., Resnick, M. R., and Haley, A. B. (1969). *J. Bacteriol.* **98,** 415–420.
Lang, B., Burger, G., Doxiadis, I., Thomas, D. Y., Bandlow, W., and Kaudewitz, F. (1979). *Anal. Biochem.* **77,** 110–121.
Linnane, A. W., and Nagley, P. (1978). *Arch. Biochem. Biophys.* **187,** 277–289.
Linnane, A. W., Astin, A. M., Beilharz, M. W., Bingham, C. G., Choo, W. M., Cobon, G. S., Marzuki, S., Nagley, P., and Roberts, H. (1980). In *The Organization and Expression of the Yeast Mitochondrial Genome* (Kroon, A. M., and Saccone, C., eds.), Elsevier, Amsterdam, pp. 136–143.
Livine, A., and Racker, E. (1969). *J. Biol. Chem.* **244,** 1332–1338.
Macreadie, I. G., Choo, W. M., Novitski, C. E., Marzuki, S., Nagley, P., Linnane, A. W., and Lukins, H. B. (1982). *Biochem. Int.* **5,** 129–136.
Macreadie, I. G., Novitski, C. E., Maxwell, R. J., John, U., Ooi, B. G., McMullen, G. L., Lukins, H. B., Linnane, A. W., and Nagley, P. (1983). *Nucleic Acids Res.* **11,** 4435–4451.
Marzuki, S., and Linnane, A. W. (1979). *Methods Enzymol.* **56,** 568–577.
Marzuki, S., Hall, R. M., and Linnane, A. W. (1974). *Biochem. Biophys. Res. Commun.* **67,** 372–378.
Marzuki, S., Cobon, G. S., Haslam, J. M., and Linnane, A. W. (1975a). *Arch. Biochem. Biophys.* **169,** 577–590.
Marzuki, S., Cobon, G. S., Crowfoot, P. D., and Linnane, A. W. (1975b). *Arch. Biochem. Biophys.* **169,** 591–600.
Marzuki, S., Hadikusumo, R. G., Choo, W. M., Watkins, L., Lukins, H. B., and Linnane, A. W. (1983). In *Mitochondria 1983. Nucleo-Mitochondrial Interactions* (Schweyen, R. J., Wolf, K., and Kaudewitz, F., eds.), de Gruyter, Berlin, pp. 535–549.
Murphy, M., Choo, K. B., Macreadie, I., Marzuki, S., Lukins, H. B., Nagley, P., and Linnane, A. W. (1980). *Arch. Biochem. Biophys.* **203,** 260–270.
Nagley, P., and Linnane, A. W. (1972). *J. Mol. Biol.* **66,** 181–193.
Neupert, W., and Schatz, G. (1981). *Trends Biochem. Sci.* **6,** 1–4.
Novitski, C. E., Macreadie, I. G., Maxwell, R. J., Lukins, H. B., Linnane, A. W., and Nagley, P. (1984). *Curr. Genet.* **8,** 135–146.
Orian, J. M., Murphy, M., and Marzuki, S. (1981). *Biochim. Biophys. Acta* **652,** 234–239.
Proudlock, J. W., Haslam, J. M., and Linnane, A. W. (1971). *J. Bioenerg.* **2,** 327–349.
Putrament, A., Baranowska, H., and Prazmo, W. (1973). *Mol. Gen. Genet.* **126,** 357–366.
Raison, J. K. (1973). *Bioenergetics* **4,** 285–309.
Resnick, M. A., and Mortimer, R. K. (1966). *J. Bacteriol.* **92,** 597–600.
Roberts, H., Choo, W. M., Smith, S., Marzuki, S., Linnane, A. W., Porter, T. H., and Fokers, K. (1978). *Arch. Biochem. Biophys.* **191,** 306–315.
Roberts, H., Choo, W. M., Murphy, M., Marzuki, S., Lukins, H. B., and Linnane, A. W. (1979). *FEBS Lett.* **108,** 501–504.
Ryrie, I. J., and Gallagher, P. (1979). *Biochim. Biophys. Acta* **545,** 1–14.
Schatz, G. (1968). *J. Biol. Chem.* **243,** 2192–2199.
Schatz, G., and Butow, R. A. (1983). *Cell* **32,** 316–318.
Stephenson, G., Marzuki, S., and Linnane, A. W. (1981). *Biochim. Biophys. Acta* **653,** 416–422.
Takeshige, K., Hess, B., Bohm, M., and Zimmerman-Telschow, H. (1976). *Hoppe-Seyler Z. Physiol. Chem.* **357,** 1605–1622.
Todd, R. D., McAda, P. C., and Douglas, M. G. (1979). *J. Biol. Chem.* **254,** 11134–11141.
Tzagoloff, A., and Meagher, P. (1971). *J. Biol. Chem.* **246,** 7328–7336.
Tzagoloff, A., Rubin, M. S., and Sierra, M. F. (1973). *Biochim. Biophys. Acta* **301,** 71–104.
Tzagoloff, A., Macino, G., and Sebald, W. (1979). *Annu. Rev. Biochem.* **48,** 419–441.