EXISTENCE OF TWO DIFFERENT SPECIES OF MITOCHONDRIALLY TRANSLATED PROTEOLIPIDS IN ATPase COMPLEX OF YEAST MITOCHONDRIA

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1. Introduction

The first subunit composition of the mitochondrial ATPase complex of *Saccharomyces cerevisiae* was proposed in [1]. The F_1 part of the ATPase is a water-soluble enzyme composed of 5 non-identical subunits; all of these subunits are made on the cytoplasmic ribosomes [2]. The membrane-embedded F_0 part of the ATPase complex was shown to be constituted of at least 4 subunits, some of which are made on mitochondrial ribosomes [1–3]. For instance, the well-characterized mitochondrial markers *oli*-1 and *pho*-2 have been shown to be in the structural gene of subunit 9 [4,5]. Furthermore the primary structure of this subunit [5,6] and the sequence of the corresponding mitochondrial gene [7,8] have been elucidated. This subunit is a proteolipid known to bind the DCCD, and inhibitor of the ATPase complex [9] and seems involved in mitochondrial membrane proton translocation [10].

From initial observations [11] a proteolipid having affinity for P_i was extracted from yeast mitochondria by organic solvents [12]. This proteolipid is mitochondrially translated and its participation in phosphate transport has been suggested [12,13]. This P_i binding proteolipid is, however, different from the DCCD-binding protein [13]. However, a chloroform extract able to bind P_i was obtained directly from the ATPase complex [12].

Here, we show that the immunoprecipitated yeast ATPase complex contains two different species of mitochondrially translated proteolipids.

Abbreviations: ATPase complex, oligomycin-sensitive adenosine triphosphatase complex; F_1, oligomycin-insensitive ATPase complex, also termed coupling factor 1; F_0, hydrophobic membrane sector required to confer oligomycin sensitivity on F_1; DCCD, dicyclohexylcarbodiimide; PMSF, phenylmethylsulfonylfluoride; P_i, inorganic phosphate; SDS, sodium dodecylsulfate; TEMED, *N,N,N',N'*-tetramethylethylene-diamine

2. Materials and methods

[^{14}C]DCCD (50 mCi/mmol) was obtained from the CEA-Saclay. $^{35}SO_4^{2-}$ (25–40 Ci/mg) was obtained from the Radiochemical Centre, Amersham.

Cells of the diploid yeast strain 'Yeast foam' were grown aerobically with 2% galactose as carbon source [14]. Mitochondria were prepared by the manual shaking method in the presence of 1 mM PMSF [15]. In vivo labeled mitochondria were obtained from small scale experiments using $^{35}SO_4^{2-}$ as label, in the presence or absence of cycloheximide [13,16]. Mitochondria were labeled with [^{14}C]DCCD (20 nmol/mg protein, 4°C, 4 h) as in [13]. The procedure for the purification of F_1 was as in [17]. Antisera were prepared using F_1 according to [18]. The ATPase complex was obtained from immunoprecipitation with the F_1 antiserum as in [19]: sonicated mitochondria were extracted for 10 min at 4°C with 0.75% Triton X-100 (w/v) then pelleted at 160 000 × *g* for 20 min. The ATPase complex was precipitated from the supernatant by addition of antiserum. After 6 h at 4°C the immunoprecipitate was pelleted, washed and kept at −20°C. The immunoprecipitate was dissolved with 1% SDS prior to counting or with the electrophoresis sample buffer prior to gel analysis.

A modification of the discontinuous SDS system of Laemmli [20] was used for slab electrophoresis. The separating gel was 250 × 145 × 1 mm in dimensions and contained 15% acrylamide, 0.2% methylene–bisacrylamide, 0.1% SDS, 0.4 M Tris (pH 8.9). For polymerization 20 μl TEMED and 200 μl 10% $(NH_4)_2S_2O_8$ were added per 40 ml. The stacking gel contained 5% acrylamide, 0.25% methylene–bisacryl-

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amide, 0.1% SDS, 0.125 M Tris (pH 6.8). Polymerization was initiated by addition of 7.5 μl TEMED and 75 μl 10% $(NH_4)_2S_2O_8$/15 ml. The running buffer contained 0.1% SDS, 0.38 M glycine, 0.05 M Tris (pH 8.3). Samples for electrophoresis were dissociated for 2 min at 60°C with the following sample buffer: 2% SDS, 20% glycerol, 0.3 M mercaptoethanol, 0.02% bromophenol blue, 0.08 M Tris (pH 6.8). Electrophoresis was at 12 mA current constant for 15–17 h at room temperature. The gel slab was fixed with 10% acetic acid for 2 h and stained with Coomassie brilliant blue [21]. The slab was dried according to [22] and autoradiography of the plate was performed by contact with a Kodirex film which was prepared according to standard procedures. Then the film was scanned with a Vernon densitometer. Serum albumin, egg albumin, pepsin, trypsin, β-lactoglobulin and lysozyme were used as standards.

The proteolipids were extracted from immunoprecipitates by incubation for 2 h at 50°C with chloroform:methanol (1:2). The extract was evaporated to dryness and solubilized with the sample buffer and analyzed as above.

ATPase activity was measured according to [23]. Protein was determined by the method in [24].

3. Results and discussion

To prepare an antiserum to yeast ATPase, F_1 was extracted and purified according to [17]. The specific activity of the enzyme preparation was 180 μmol ATP hydrolyzed . min^{-1} . mg $protein^{-1}$. The purity of the enzyme was checked by subunit analysis on 15% polyacrylamide gel electrophoresis in the presence of SDS. Fig.1 shows that the F_1 preparation contained essentially 3 bands corresponding to the α, β and γ subunits as in [17].

An immunoprecipitate of the mitochondrial Triton extract of ^{35}S-labeled yeast cells was analyzed on a 15% polyacrylamide gel electrophoresis. The densitometric scan of the autoradiograph shows a typical pattern of the oligomycin sensitive ATPase complex with 11 subunits (fig.2A). In agreement with [25] the M_r-values of the α, β and γ bands were lower than the values previously reported (respectively, 50 000, 48 000 and 30 200 in fig.2). An unidentified band of ~60 000 M_r was also observed [25]. The 12 300 and 11 700 M_r bands were attributed, respectively, to the subunits δ and ε. In agreement with [25], other bands of 25 000 and 19 000 M_r were resolved.

When yeast cells were labeled in the presence of

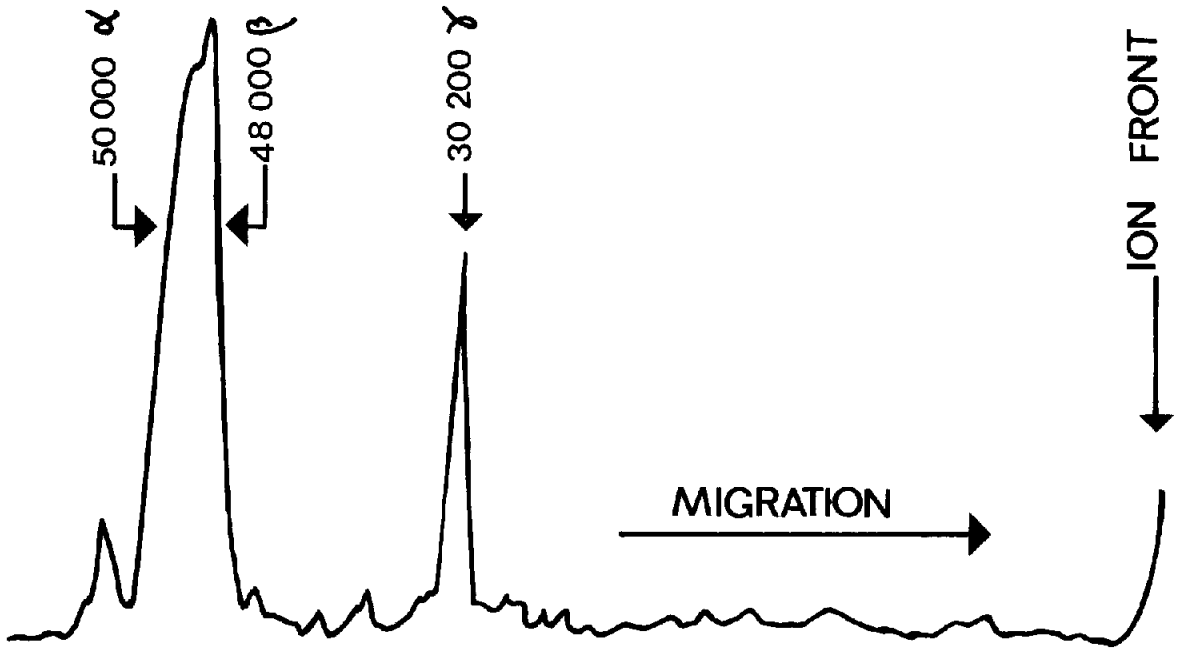

Fig.1. Densitometric scan of SDS–polyacrylamide gel electrophoresis of F_1. Pure F_1 was prepared by the chloroform:methanol technique according to [17]; 16 μg F_1 were dissociated and applied to a 15% polyacrylamide slab gel. The slab was stained and read by densitometry.

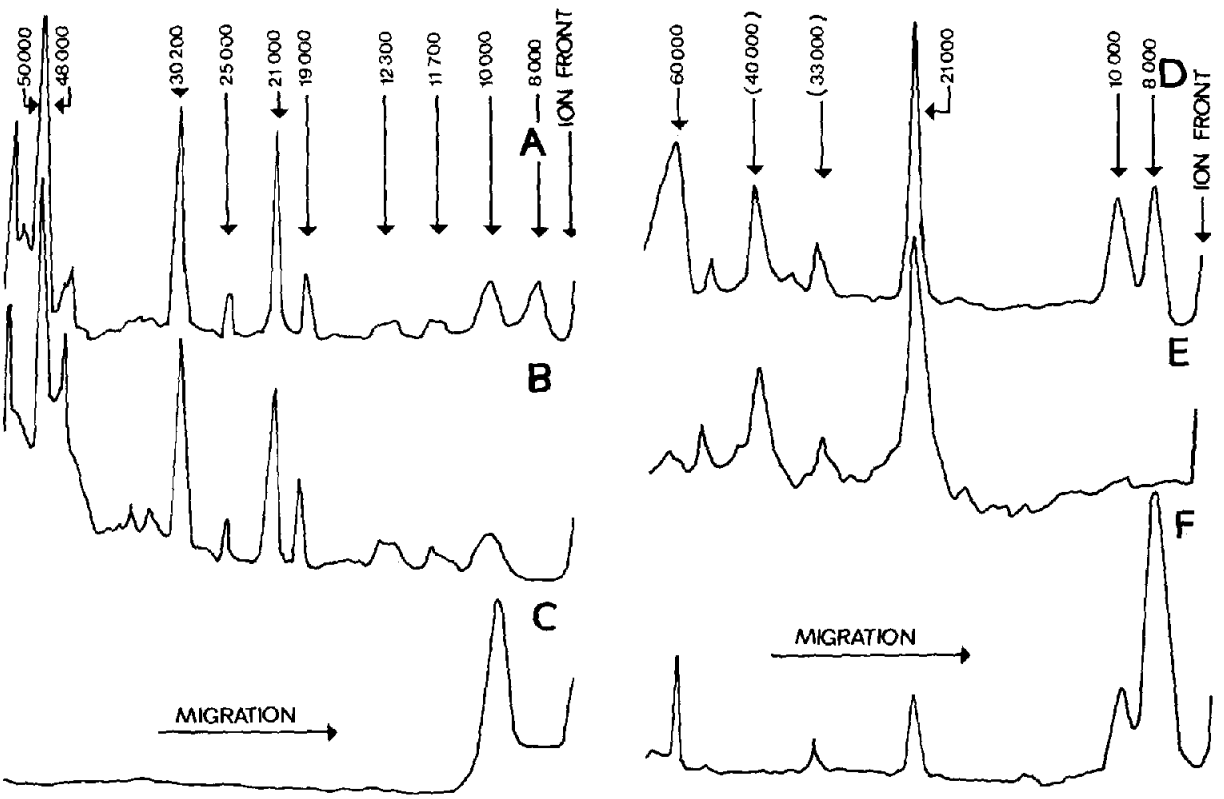

Fig.2. Densitometric scans of autoradiographs of immunoprecipitated ATPase complex. As indicated, cells were labeled in vivo for 90 min with 1 mCi $^{35}SO_4^{2-}$ in the presence or absence of 0.6 mg cycloheximide/ml [16]. Mitochondria were isolated and solubilized with 0.75% Triton X-100. Immunoprecipitates of the ATPase complex were solubilized directly in the sample buffer, heated for 2 min at 60°C, applied to a slab gel and electrophoresed. M_r-Values for the radioactive peaks were determined by comparison with relative mobilities of protein standards run simultaneously on identical gels: (A) ATPase complex immunoprecipitate from uniformly labeled mitochondria; (B) ATPase complex immunoprecipitate from uniformly labeled mitochondria; before immunoprecipitation, mitochondria were incubated with unlabeled DCCD (20 μg/mg protein); (C) before immunoprecipitation, unlabeled mitochondria were incubated with [^{14}C]DCCD (15 μg/mg protein, 54 mCi/mmol). The immunoprecipitated ATPase complex was incubated for 2 h at 50°C with 1 ml chloroform:methanol (1:2) and the extract was electrophoresed; (D) immunoprecipitated ATPase complex from in vivo ^{35}S-labeled mitochondria in the presence of cycloheximide. The immunoprecipitated ATPase complex labeled as in (D) was extracted with 1 ml chloroform:methanol (1:2) for 2 h at 50°C; (E) insoluble residue upon extraction; (F) chloroform:methanol extract.

cycloheximide, an inhibitor of the cytoribosomal translated proteins, the immunoprecipitate of the mitochondrial Triton extract shows essentially 4 bands in gel electrophoresis (fig.2D):

(i) A high M_r label protein which had been reported as an oligomer of the proteolipid [26]. This band was detected only when the yeast cells were labeled in the presence of cycloheximide since under these conditions the α and β subunits which migrated in the same region were not labeled.

(ii) A subunit of 21 000 M_r, called subunit 6 in the nomenclature of [1].

(iii,iv) Two low M_r proteins (10 000 and 8000, respectively). These subunits were both extracted by chloroform:methanol (1:2) as well as the oligomer (fig.2E). The relative amount of the 8000 M_r band was largely increased in the chloroform:methanol extract (fig.2F) suggesting that the high M_r band was an oligomer of this subunit as proposed [26]. In order to identify the DCCD-binding protein (or subunit 9 in the nomenclature of [1]) unlabeled mitochondria were incubated with [^{14}C]DCCD, then the immunoprecipitate was prepared and analyzed as above.

Only one labeled band migrating between the 10 000 and 8000 M_r bands was observed (fig.2C). When ^{35}S-labeled mitochondria were incubated with unlabeled DCCD, the 8000 M_r band disappeared and only one band migrated in the region including the 10 000 M_r and the [^{14}C]-DCCD bands. These results suggest that DCCD bound only to the 8000 M_r subunit and moved the band towards the high M_r side of the gel. It becomes clear, therefore, that the 10 000 M_r band is a mitochondrially translated proteolipid different from the subunit 9. The fact that the 10 000 M_r proteolipid is a component of the ATPase complex is suggested by the purity of the preparation; only a slight contamination by the subunits 1 and 2 of the cytochrome oxidase (40 000 M_r and 33 000 M_r, respectively) [27] was shown on fig.2D. However, the ratio (mol/mol ATPase) of this subunit was calculated in assuming that 1 ATPase complex contained 6 subunits 9. On this basis, the estimation made in the chloroform:methanol extract (fig.2F) gives one 10 000 M_r subunit/ATPase complex.

Using reverse-phase, high-pressure liquid chromatography, the proteolipid fraction isolated from the beef heart mitochondrial complex was reported to be heterogeneous [28]. However, as shown here, the proteolipid mixture was also resolved using a modification of the slab gel electrophoresis in [20]. Under our conditions (section 2) the pH difference which was imposed between the running gel and running buffer was essential to separate the low M_r species, otherwise these species migrated with the ion front. In [28,29] it was suggested that one proteolipid could be the P_i binding proteolipid. The possibility that the 10 000 M_r band was the P_i-binding proteolipid [12,13] is under investigation.

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