F₁F₀-ATP synthase from bovine heart mitochondria: development of the purification of a monodisperse oligomycin-sensitive ATPase

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A new procedure for the isolation of ATP synthase from bovine mitochondria has been developed, with the primary objective of producing enzyme suitable for crystallization trials. Proteins were extracted from mitochondrial membranes with dodecyl- β -D-maltoside, and the ATP synthase was purified from the extract in the presence of the same detergent by a combination of ion-exchange and gel-filtration chromatography and ammonium sulphate precipitation. This simple and rapid procedure yields 20–30 mg of highly pure and monodisperse enzyme, evidently consisting of 14 different subunits, amongst them, in apparently

stoichiometric amounts with the established subunits, subunit e, a recently discovered subunit of unknown function. The enzyme preparation has an oligomycin-sensitive ATP hydrolysis activity, and so the F₁ domain is functionally associated with the membrane domain, F₀. In contrast with the N-termini of some of the subunits of bovine mitochondrial F₁-ATPase, those of the F₁F₀-ATP synthase are not degraded by proteolysis during the isolation procedure. This preparation therefore satisfies prerequisites for crystallization trials.

INTRODUCTION

Mitochondrial F₁F₀-ATP synthase employs the proton-potential chemical gradient across the inner mitochondrial membrane in respiring mitochondria to synthesize ATP from ADP and inorganic phosphate (Mitchell, 1961). F₁, the globular extra-membrane part of the ATP synthase where catalysis takes place, is connected via a stalk to the membrane domain, F₀, which contains a proton channel. Intact F₁F₀-ATP synthase can synthesize and hydrolyse ATP. It is cold-stable and can be inhibited by oligomycin and by dicyclohexylcarbodi-imide, which both interact with F₀. In contrast, F₁-ATPase, which is easily dislodged from F₀, is cold-labile and its ATP hydrolase activity is unaffected by oligomycin.

Several preparations of the enzyme from bovine heart mitochondria have been described (Serrano et al., 1976; Berden and Voorn-Brouwer, 1978; Galante et al., 1979; Hughes et al., 1982). They all contain substantial amounts of phospholipid and they are polydisperse. They also differ somewhat in purity, subunit composition and enzymic characteristics. Some of them have a diminished sensitivity to oligomycin and a reduced ATP synthase activity (Serrano et al., 1976; Stiggall et al., 1978; Galante et al., 1979), but they are cold-stable, indicating that no dissociation between F₁ and F₀ has occurred. The reduction in activities of the enzyme could be caused by removal of *bona fide* subunits or phospholipids, or by a conformational change in the enzyme induced by exposure to detergents (Laird et al., 1986a).

An understanding of the molecular basis of ATP synthesis depends upon knowledge of its structure to an atomic resolution. Crystal of bovine F₁-ATPase diffract to at least 3.0 Å resolution

(Walker et al., 1990; Lutter et al., 1993), and the structure of F₁ has been solved so far to 6.5 Å resolution from these crystals (Abrahams et al., 1993). An atomic-resolution structure now appears to be attainable. To extend this structural analysis to the entire ATP synthase, as described below, several years ago we set out to develop a procedure for the purification of mitochondrial F₁F₀-ATP synthase of appropriate quality for crystallization trials (Saraste et al., 1982). At the outset, it was considered that preparations of the enzyme isolated for this purpose should fulfil a number of criteria, as follows. (1) After being reconstituted into liposomes the isolated enzyme should be a proton-pumping ATP hydrolase. Inhibition of this hydrolytic activity by oligomycin would provide additional support for an intact proton channel, but, as the effect of the antibiotic is dependent on the phospholipids associated with the enzyme (Laird et al., 1986b; Dabbeni-Sala et al., 1989), the reduction or absence of sensitivity to the antibiotic would not necessarily prove that the enzyme was damaged. Therefore, for the purposes of crystallization, sensitivity to oligomycin was considered to be a desirable but not essential prerequisite. (2) The enzyme should be free from contamination both by mitochondrial proteins and by phospholipids. (3) The subunits of the enzyme should not be damaged, for example by proteolysis of their N termini. (4) The enzyme should be monodisperse and aggregated forms should be absent.

As described below, most of these objectives have been attained. Early attempts at solubilization and purification in the presence of cholate yielded a particulate, aggregated enzyme, which remained polydisperse even when resolubilized in the presence of Triton X-100. Subsequently, by using dodecyl- β -D-maltoside to solubilize the enzyme, and by carrying out the

Abbreviations used: oscp, oligomycin-sensitivity conferral protein; PMSF, phenylmethanesulphonyl fluoride; BCA, bicinchoninic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine.

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purification steps in the presence of this detergent, a monodisperse ATP synthase has been isolated. The final preparation is evidently an assembly of 14 different and intact subunits. It retains the ATP hydrolase activity, which can be inhibited by oligomycin, and, after reconstitution into phospholipid vesicles, it pumps protons. This preparation is therefore suitable for attempting crystallization.

MATERIALS AND METHODS

Materials

Dodecyl- β -D-maltoside and ATP were obtained from Nova-biochem Ltd. (Nottingham NG7 1BR, U.K.). DEAE-cellulose DE-52 was supplied by Whatman Ltd. (Maidstone, Kent ME15 9XN, U.K.), TSK G4000 SW by Anachem Ltd. (Luton, U.K.) and Mono Q by Pharmacia Ltd. (Milton Keynes, U.K.). Asolectin, cardiolipin, phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, oligomycin and FCCP were purchased from Sigma Chemical Co. Ltd. (Poole, U.K.). Mitochondria were isolated according to Smith (1967), and were either stored at -20°C or were used immediately.

Protein analysis

Protein concentrations were estimated by the procedure of Bradford (1976), as modified by Macart and Gerbaut (1982), by the BCA method (Pierce and Warriner Ltd., Chester, U.K.), or according to Lowry et al. (1951).

PAGE was performed using 10–25% acrylamide gradients with 0.1% (w/v) SDS (Laemmli, 1970). Proteins were detected either by staining with Coomassie Blue G-250 or by silver-staining (Morrissey, 1981; Ansorge, 1985).

Isolation of F_1F_0 -ATPase

Two buffers (referred to as buffers A and B) were used in the purification procedures that are described in methods 1 and 2 below. These were made up as follows. Buffer A: 10 mM Tris/HCl, pH 7.5, 50 mM sucrose, 0.5 mM MgSO_4 , 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.1% (w/v) benzamidine/HCl, 0.1 mM phenylmethanesulphonyl fluoride (PMSF) and 0.02% (w/v) sodium azide; a modified form of buffer A contained 20 mM Tris/HCl, pH 8.0, 50 mM sucrose and 1 mM MgSO_4 . Buffer B: 20 mM Tris/HCl, pH 8.0, 50 mM sucrose, 50 mM NaCl, 1 mM MgSO_4 , 0.1 mM EDTA, 0.1% (w/v) benzamidine/HCl, 0.1% (w/v) PMSF and 0.02% (w/v) sodium azide.

Method 1

In our first preparations, whole mitochondria (1 g of protein) were extracted by stirring for 15 min at $0-4^{\circ}\text{C}$ in buffer A, and 0.2 M ammonium sulphate (adjusted to pH 7.5–8.0 with ammonium hydroxide), 2.5 mM ATP and sodium cholate (final concentration 1.875%, w/v) were added. Insoluble material was removed by centrifugation (4°C , 90 min, 280000 g), and the supernatant was applied at room temperature to a Sephacryl S-300 column (40 cm $\times$ 5 cm internal diameter) that had been equilibrated previously with buffer A containing sodium cholate (0.15%, w/v). The first peak, which emerged in the void volume of the column, was turbid and contained ATPase activity. The enzyme was salted out from these turbid fractions by the addition, at 0°C , of solid ammonium sulphate to 50% saturation. After 30 min, the precipitate was resuspended in 5–10 ml of buffer B

Figure 1 Chromatography of F_1F_0 -ATPase from bovine heart mitochondria in the presence of Triton X-100 according to method 1

The column (90 cm $\times$ 2.5 cm i.d.) consisted of Ultrogel AcA 34. The sample applied to the column was obtained by chromatography on Sephacryl S-300 in the presence of cholate (see Materials and methods section). (a) (●) Protein concentration; ATPase activity in the absence (○), and in the presence (×), of oligomycin. (b) SDS/PAGE of selected fractions. The positions of the subunits of the F_1 -ATPase are indicated at the side. The material recovered from the Sephacryl S-300 column before and after precipitation and resolubilization with Triton X-100 is designated (1) and (2) respectively.

also containing Triton X-100 (1.5%, w/v) and 1 M KCl. Any insoluble material remaining after 10 min at room temperature was removed by centrifugation (10 min, 19000 g, room temperature), and the supernatant was layered onto an Ultrogel AcA 34 column (90 cm $\times$ 2.5 cm internal diameter) pre-equilibrated with buffer B containing Triton X-100 (0.1%, w/v). The first peak to emerge from this column contained the ATPase activity (see Figure 1). The recovery of protein was 9% of total

Figure 2 Chromatography of partially purified F_1F_0 -ATPase from bovine heart mitochondria on Ultrogel AcA 22 in the presence of Triton X-100 according to method 2

(a) Shows the analysis of fractions by SDS/PAGE. The extreme right-hand lane contains F_1F_0 -ATPase after the removal of contaminants by red-dye-ligand matrix. The positions of various subunits of ATP synthase are indicated on the right. Trace amounts of subunit e (not marked) can be seen in the region of the gel immediately above F_0 . Subunits A6L and a are hydrophobic subunits encoded by overlapping genes in mitochondrial DNA (Fearnley and Walker, 1986); I is the ATPase inhibitor protein.

mitochondrial protein as determined by the procedure of Lowry et al. (1951).

Method 2

Whole mitochondria were extracted with modified buffer A containing sodium cholate (3%, w/v, final concentration), and ammonium sulphate was added to a final concentration of 20% (w/v) saturation. The resulting precipitate was removed by centrifugation, and lipid aggregates in the supernatant were filtered off in a sintered-glass funnel. This solution was fractionated on a Sephacryl S-300 column as in method 1. The proteins in the ATPase-containing fractions were precipitated with solid ammonium sulphate (45%, w/v, saturation), and redissolved in modified buffer A containing 0.2 M KCl, 10% (v/v) glycerol, 3% (w/v) Triton X-100, 7.1 mM 2-mercaptoethanol and 0.001% (w/v) PMSF. The resulting clear solution was layered onto an Ultrogel AcA 22 column (140 cm $\times$ 5 cm internal diameter) equilibrated with modified buffer A containing 0.2 M NaCl, 10% (v/v) glycerol, 0.1% (w/v) Triton X-100 and 0.001% (w/v) PMSF. The second peak to emerge contained the purified F_1F_0 -ATPase (see Figure 2). Usually, 20–30 mg of F_1F_0 -ATPase were obtained from 1 g of mitochondrial protein. High-molecular-mass contaminants were removed by batchwise treatment with Procion Red HE3B dye immobilized on Sepharose (Figure 2),

whereas most of the F_1F_0 -ATPase activity remained in the supernatant. The same buffer that was employed in the gel filtration on Ultrogel AcA 22 was also used in this final step.

Method 3

Membranes were prepared from mitochondria from one heart, as described previously (Finel et al., 1992). They were collected by centrifugation (18000 g, 20 min, at 4 °C) and then resuspended in a Potter homogenizer with 20 mM Tris/HCl, pH 7.5, 1 mM EDTA and 10% (v/v) glycerol. The final volume of the homogenate was 50–100 ml and the protein content was about 12.5 mg/ml (determined by the BCA procedure). The composition of the buffer was adjusted to that of the DE-52 elution buffer (see below) by addition to this homogenate of succinate (from a 1 M sodium succinate stock solution), NaCl (from a 5 M stock solution), ATP (solid) and 2-mercaptoethanol. Dodecyl- β -D-maltoside (from a stock of 10%, w/v) was added to a final concentration of 1.2% (w/v), and the protein content was brought to 10 mg/ml with DE-52 elution buffer. The membrane suspension was stirred for 10 min at 0–4 °C, and then insoluble material was removed by centrifugation (49000 g, 15 min, 0–4 °C). The supernatant was either stored at –20 °C or used immediately. One-quarter to one-third of this solution was layered onto a DE-52 column (15 cm $\times$ 2.7 cm internal diameter) that had been pre-equilibrated with buffer consisting of 20 mM Tris/HCl, pH 7.5, 1 mM EDTA, 0.1% (w/v) dodecyl- β -D-maltoside, 10 mM succinate, 35 mM NaCl, 10% (v/v) glycerol, 10 mM 2-mercaptoethanol and 2 mM ATP. The F_1F_0 -ATPase was found in the unretarded material. Any turbid fractions were discarded. Non-turbid fractions were pooled and stored at 4 °C. Occasionally a precipitate formed during storage and was removed by centrifugation and discarded. Cholate was added to the pooled fractions from a 10% (w/v) stock solution to a final concentration of 2% (w/v). The F_1F_0 -ATPase was precipitated by the addition of solid ammonium sulphate to 45% (w/v) saturation, and the precipitate was resolubilized in a minimal volume (300–500 μ l) of a buffer containing 20 mM Tris/HCl, pH 8.0, 50 mM sucrose, 1 mM $MgSO_4$, 0.5% (w/v) dodecyl- β -D-maltoside, 10% (v/v) glycerol, 0.2 M KCl, 0.001% (w/v) PMSF and 7.1 mM 2-mercaptoethanol. Any insoluble material was removed by centrifugation. The supernatant was layered onto a TSK G4000 SW column equilibrated in a buffer consisting of 20 mM Tris/HCl, pH 8.0, 50 mM sucrose, 1 mM $MgSO_4$, 0.05% (w/v) dodecyl- β -D-maltoside, 0.001% (w/v) PMSF, 10% (v/v) glycerol, 0.1 M NaCl and 2 mM 2-mercaptoethanol, delivered at a flow rate of 4 ml/min. The F_1F_0 -ATPase eluted in the first peak (see Figure 3a). The fractions containing F_1F_0 -ATPase were applied at a flow rate of 1 ml/min to a Mono Q column (HR10/10) equilibrated with the same buffer as was used with the TSK column. The F_1F_0 -ATPase eluted at a NaCl concentration of approx. 0.2 M (see Figure 3b).

Enzyme activity measurements

One unit of ATPase is defined as the amount that hydrolyses 1 μ mol of ATP/min at 37 °C. ATP hydrolase activities were determined by the colorimetric estimation of phosphate released from ATP (Serrano et al., 1976). Samples (50 μ l) of enzyme solutions were added to a solution (500 μ l) of 20 mM Tris/sulphate, pH 8.0, 50 mM NaCl and 2 mM $MgCl_2$. When necessary, phospholipids (10 mg/ml; asolectin, phosphatidylethanolamine and phosphatidylinositol in chloroform; cardiolipin and phosphatidylcholine in ethanol) were dispersed in the assay mixture by sonication, the final concentration of the organic

Figure 3 Purification of F_1F_0 -ATPase from bovine heart mitochondria in the presence of dodecyl- β -D-maltoside using method 3

(a) and (b) show the gel filtration on TSK G4000 SW and ion-exchange chromatography on Mono Q with the corresponding SDS/PAGE analyses respectively. (c) is a comparison by silver staining of an SDS-polyacrylamide gel of the enzyme recovered from (a) and (b), marked (1) and (2) respectively. The subunits present in the F_1 -ATPase domain of ATP synthase are indicated down the right-hand side; the positions of other subunits of the enzyme are shown on the left (for explanation, see the legend to Figure 2).

solvent being at most 1.0% (v/v). After 2 min at 37 °C, ATP hydrolysis was started by the addition of a solution (20 μ l) containing 0.1 M ATP and 40 μ M phosphoenolpyruvate dissolved in 0.2 M Tris buffer, pH 7.8, and pyruvate kinase (4 μ g). The reaction was stopped after a further 5 min by the addition of 1 ml of 0.5% (w/v) ammonium molybdate in 0.35 M sulphuric acid, followed by water (2 ml), 10% (w/v) ascorbic acid (50 μ l) and, if the solution was cloudy, 10% (w/v) SDS (100 μ l). The colour developed within 30 min. The phosphate released was calculated from the absorption at 750 nm. Inhibition of enzyme activity by oligomycin was determined by adding the antibiotic (100 μ M in methanol; final concentration 1.5 μ g/ml) to the assay solution immediately after the enzyme.

To measure the ATP/ P_i exchange activity of the enzyme, it was first reconstituted into liposomes by one of several procedures, the one following being representative. Phospholipids (200 mg) were dispersed under nitrogen at 0 °C by sonication in 25 mM Tricine (pH 7.0), 2 mM $MgCl_2$, 10 mM potassium bicarbonate, 1 mM 2-mercaptoethanol with 1% (w/v) dodecyl- β -D-maltoside and 1% (w/v) cholate. This suspension was clarified by centrifugation, and the resulting solution of phospholipids was combined with protein (1 mg/ml) at lipid:protein ratios of

50:1, 25:1 and 10:1. The mixture was left for at least 2 h at room temperature, and then dialysed for 16 h at room temperature against a buffer consisting of 25 mM Tricine (pH 7.0), 2 mM $MgCl_2$, 10 mM potassium bicarbonate and 1 mM 2-mercaptoethanol.

ATP/ P_i exchange activity was assayed as described by Joshi et al. (1979). For this assay, the enzyme was either incorporated into liposomes as described above, or was added to the phosphate assay buffer with appropriate phospholipids and 2 mg/ml BSA and 2 mM 2-mercaptoethanol.

Electron microscopy

Electron micrographs were taken with Philips EM 301 and EM 400 microscopes operated at 80 kV. Specimens were stained either with 1% (w/v) potassium phosphotungstate or with 1% (w/v) uranyl acetate.

Phospholipid analysis

Phospholipids were extracted twice from preparations containing identical amounts of enzymic activity with 20 volumes of

chloroform:methanol:concentrated ammonia solution (7:1:5, by vol.). After each addition, the suspension was shaken vigorously for 3 min and then centrifuged to separate the phases. The organic phases were pooled and dried *in vacuo* at room temperature. The residue was redissolved in chloroform:methanol (2:1, v/v; approx. 5 ml), and washed twice with 0.2 volumes of 10 mM CaCl_2 . The organic phase was evaporated off and the residue was redissolved in chloroform (approx. 50 μl). Phospholipids were analysed on Kieselgel 60 thin-layer plates (supplied by Merck, 20 cm $\times$ 20 cm) that had been sprayed previously with a solution of 0.4 M boric acid and dried at room temperature for 1 h and then at 120 $^{\circ}\text{C}$ for 2 h. The extracts and a sample (5 μl) of a solution of phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol and cardiolipin (each at a concentration of 0.5 mg/ml) were applied to the plates, and chromatographed in chloroform:methanol:water:ammonia (90:54:5.5:5.5, by vol.). The plates were dried in air, and phospholipids detected with iodine vapour.

RESULTS

Isolation of the F_1F_0 -ATPase complex

The first preparation of F_1F_0 -ATP synthase, made using method 1 (see the Materials and methods section), was contaminated with a variety of other proteins. A number of the known subunits of ATPase [α , β , γ , δ , ϵ and oligomycin-sensitivity conferral protein (oscp)] could be recognized by SDS/PAGE, but the precise subunit composition of the complex was not determined. It contained highly aggregated and distinctive pinnatilobate micellar assemblies (lobes attached to pinnate structures; see Figures 4a and 4b), in which the hydrophobic subunits of the enzyme, together with bound phospholipids and probably detergents, form a core to which the hydrophilic F_1 -ATPase domain is attached. The specific activity of this enzyme preparation (measured in the presence of 1 mg/ml aroclorin) was 7–9 units/mg of protein, and up to 38 % of this activity could be inhibited with oligomycin.

After several modifications to the initial procedure (see method 2), purer preparations were obtained in which 14 different bands were detected by SDS/PAGE followed by staining with Coomassie Blue dye (Walker et al., 1991). However, subunit ϵ was not present in stoichiometric amounts in all preparations made by method 2. An additional diffuse high-molecular-mass band, revealed by silver staining, was removed by absorption on immobilized Procion Red HE3B dye (Figure 2).

From its gel filtration behaviour, and from its appearance in electron micrographs (not shown), the material made by method 2 appeared to be monodisperse. Its ATP hydrolase activity was unaffected by cold treatment, and it was almost completely dependent upon exogenous phospholipids (aroclorin). Its specific activity was 3–5 units/mg of protein in the absence of bicarbonate, and twice this value in the presence of bicarbonate. Up to 60 % of the ATP hydrolase activity could be inhibited by oligomycin, the effect being dependent upon the amount of added aroclorin. The maximal effect of oligomycin was found when aroclorin stimulated up to 30–50 % of the maximal ATP hydrolase activity (not shown). However, no ATP/ P_i exchange activity could be demonstrated with this reconstituted enzyme.

Method 2 suffered from a number of drawbacks. The major difficulty was that the Ultrogel AcA 22 column used in the purification procedure frequently became blocked, probably because of the presence of lipids in the partially purified samples that were applied to the column. The use of Triton X-100, a detergent that is considered to be unfavourable for the formation

of three-dimensional crystals, was a further disadvantage. Therefore, dodecyl- β -D-maltoside was used in place of Triton X-100, and the pellet obtained with 45 % (w/v) ammonium sulphate in method 2 was resolubilized in a minimal volume (usually 300–500 μl) of modified buffer A containing 0.5 % (w/v) dodecyl- β -D-maltoside, 10 % (v/v) glycerol, 0.2 M KCl, 0.001 % (w/v) PMSF and 7.1 mM 2-mercaptoethanol. This material was chromatographed by gel filtration on TSK G4000 SW, followed by anion-exchange chromatography on Mono Q, as described under method 3. This procedure gave improved enzyme (see Figure 3). Eventually, the method was refined to form method 3, in which additional improvements in quality and yield of enzyme were obtained by preparing mitochondrial membranes in the presence of KCl, and by then extracting them in the presence of dodecyl- β -D-maltoside instead of cholate. This extraction step also solubilized substantial quantities of complexes I, III and IV, which were bound to DEAE-cellulose DE-52, whereas F_1F_0 -ATPase did not bind. Minor contaminants in the F_1F_0 -ATPase were removed by gel filtration on TSK G4000, and a final remaining impurity, a diffuse high-molecular-mass protein that was detected by SDS/PAGE and silver staining, was eliminated by chromatography on Mono Q. It is evident from the SDS/PAGE analyses of the fractions from both columns that the relative staining of the subunits is similar in all fractions (see Figure 3), and that there is no tendency for any of the subunits to purify away from the others, indicating that all of the subunits are bound together in a single stable complex. This conclusion is also supported by the cold stability of the preparation. About 20–30 mg of F_1F_0 -ATPase were purified by method 3 from the mitochondria isolated from one bovine heart. The characteristics of this preparation are described below.

Characterization of the present F_1F_0 -ATPase preparation

The F_1F_0 -ATPase made by method 3 appears to be a complex of 14 different proteins. No phospholipids were detected in the final complex in substantial amounts, whereas the enzyme which did not bind to the DE-52 column contained substantial amounts of cardiolipin and phosphatidylethanolamine (results not shown). As described elsewhere (Walker et al., 1991), the 14 subunits have been identified by protein chemical methods. None of the N-terminals of the subunits are degraded, whereas the N-terminals of the α , β and δ subunits is degraded in preparations of F_1 -ATPase from bovine heart mitochondria. The main difference between the preparations made by methods 2 and 3 is that the preparation using method 3 appears to contain stoichiometric amounts of subunit ϵ , whereas this subunit is depleted or completely absent from the preparation using method 2.

Two pieces of evidence indicate that F_1F_0 -ATPase made by method 3 is monodisperse. Firstly, the elution profiles of the enzyme both from the Mono Q and from the TSK G4000 SW matrices are symmetrical. Secondly, electron micrographs of negatively stained specimens of the enzyme contain mostly single F_1F_0 particles, and none of the higher aggregated pinnatilobate forms can be seen (Figure 4c).

The specific ATP hydrolase activity of the final preparation was 3–7 units/mg, a similar value to those measured on earlier preparations made using methods 1 and 2. Chromatography on DE-52 results in a marked drop in the specific activity (Table 1). Analysis by SDS/PAGE revealed that virtually no F_1F_0 -ATPase was retarded on this matrix, and thus the drop in activity may be due to the removal of molecules associated with the F_1F_0 -ATPase, such as phospholipids. Further purification had little effect on the level of activity, but the activity became more dependent on exogenous phospholipids. It also appears from

Figure 4 For legend see facing page.

Table 1 Effect of phospholipids (asolectin) and oligomycin on ATP hydrolysis during isolation of F_1F_0 -ATPase

Asolectin (10%, w/v, in chloroform) was dispersed by sonication at a 1:100 dilution in the assay buffer before the addition of the enzyme. Oligomycin (100 μ M in methanol) was added at a 1:100 dilution and incubated with the enzyme for 10 min at 37 °C before the assay. See the Materials and methods section for further details.

Preparation	Total protein (%)	Total activity* (%)	Oligomycin sensitivity† (%)	Stimulation by asolectin (%)
Membrane extract	100	100	75	—
DE-52 breakthrough	75	18	80	1.3
TSK	9	17	16	2.9
Mono Q	7	9	24	1.5

* Hydrolase activity determined in the presence of asolectin.

† Oligomycin sensitivity was tested in the absence of exogenous phospholipids.

preliminary experiments that the effects of phospholipids on the ATP hydrolase activity are mimicked in part by dodecyl- β -D-maltoside (results not shown), which could explain the lower phospholipid dependency of the final preparation. This effect requires further study. The phospholipid-dependent preparations showed a diminished sensitivity to oligomycin. We have not been able to demonstrate either ATP/ 32 P_i exchange or ATP synthesis with this preparation, whereas in control experiments submitochondrial particles had a considerable ATP/ 32 P_i exchange activity. It is possible that this indicates that the reconstituted enzyme is insufficiently coupled. The proton-pumping activities of reconstituted F_1F_0 -ATPase isolated by a procedure derived from method 3 have been studied recently by various methods, including by pH changes external to the reconstituted vesicles and by the use of uncouplers. These experiments show that this recent preparation is a proton-pumping ATPase (S. K. Buchanan, H. S. van Walraven and J. E. Walker, unpublished work).

DISCUSSION

Crystallization of membrane proteins is far from being a routine procedure (see Kühlbrandt, 1988; Garavito and Picot, 1990; Garavito, 1991; Michel, 1991). Before suitable conditions for growing three-dimensional crystals of complex proteins can be found, a wide range of conditions will probably have to be surveyed. These experiments demand large amounts of pure protein. For example, of the order of 10 g of F_1 -ATPase purified from bovine heart mitochondria was used in establishing suitable crystallization conditions (Lutter et al., 1993). It is also desirable that the protein should be as pure as possible, and the membrane proteins should be in a monodisperse state.

Several procedures have been described before making F_1F_0 -ATPase from bovine heart mitochondria (Serrano et al., 1976; Berden and Voorn-Brouwer, 1978; Galante et al., 1979; Hughes et al., 1982), but none of these methods is suitable for making

enzyme for crystallization trials. All the preparations from previous methods contain substantial amounts of phospholipids, they are polydisperse, and other contaminating proteins are also present (Ludwig et al., 1980). Some of these contaminants were seen in Coomassie-Blue-stained SDS/polyacrylamide gels migrating with apparent molecular masses greater than the α -subunit of ATPase, others migrated below the β -subunit, and a prominent band was detected at about 30 kDa. In the region above the α -subunit in the preparation of Serrano et al. (1976), transhydrogenase, the large subunit of succinate dehydrogenase and a member of the acyl-CoA dehydrogenase family (Matsubara et al., 1989) have all been identified by protein sequencing, and malate dehydrogenase and creatine kinase have been found below the β -subunit (S. Chiaramonte, M. J. Runswick, J. M. Skehel, K. Sorgato and J. E. Walker, unpublished work). The 30 kDa band contains substantial amounts of ADP/ATP translocase (Freisleben and Zimmer, 1986). Preparations of rat liver F_1F_0 -ATPase also appear to contain contaminants with similar sizes to those in the bovine heart preparations (McEnery et al., 1984; Yoshihara et al., 1991).

The isolation procedure described in this paper takes about 2 days to perform and yields about 20–30 mg of highly pure enzyme from bovine heart mitochondria in a form that is suitable for attempting crystallization. This enzyme is very pure, and almost no impurities can be detected, even by silver staining after SDS/PAGE. From its chromatographic behaviour and from its appearance in electron micrographs, the enzyme appears to be monodisperse. It appears to contain 14 different protein subunits, 13 of which are well-established subunits of the enzyme, but the other, known as subunit e, had not been observed previously as a component of the complex, and it has no counterparts in chloroplast or in bacterial F_1F_0 -ATPases. Under some conditions it is partially or totally lost from the complex, but its presence in the enzyme after several chromatography steps in both low and higher ionic-strength buffers containing dodecyl- β -D-maltoside suggests that subunit e and the other subunits form a stable complex. On removal of the F_1 subunits from the submitochondrial particles, subunit e remains firmly associated with the membrane fraction, and it is present in purified F_0 (I. Collinson and J. E. Walker, unpublished work). It is therefore considered to be an authentic component of the F_1F_0 -ATPase from bovine heart mitochondria. A protein of similar sequence (58 identical amino acids out of a total of 70) has been found recently in the F_1F_0 -ATPase complex from rat liver mitochondria (Higuti et al., 1992), adding further support to this view.

The N-terminal regions of the subunits of the purified enzyme are intact, and, in contrast to the α , β and γ subunits in F_1 -ATPase from bovine heart mitochondria, they are not degraded (Walker et al., 1991). Such degradation could disrupt packing of molecules in a crystalline array and could lead to disorder within the crystal. For example, the quality of some three-dimensional crystals of F_1 -ATPase is dramatically improved by the removal of the slightly degraded N-terminal regions of the α - and β -subunits under conditions that do not affect the ATP hydrolase activity (R. Lutter, R. J. Todd and J. E. Walker, unpublished work).

Phospholipids interacting with F_0 , the intrinsic membrane domain of the F_1F_0 -ATPase, influence catalytic events in F_1 , the

Figure 4 Electron micrographs of negatively stained samples of purified F_1F_0 -ATPase

(a) Samples isolated according to method 1; (b) collection of pinnatlobate aggregates from (a) and similar samples; (c) material made according to method 3. The magnifications were (a) about $\times 200\,000$, (b) about $\times 200\,000$, and (c) $\times 300\,000$.

extramembraneous part of the enzyme (Laird et al., 1986b). A random population of phospholipids in association with the membrane domain could therefore cause conformational heterogeneity in F_1 , which would be undesirable in crystallization experiments. However, the preparation of F_1F_0 -ATPase made according to method 3 is devoid of phospholipids, and so this potential problem is averted. Some of the membrane-associated activities of the enzyme may depend to some extent on the presence of specific phospholipids. For example, the sensitivity of the ATPase activity to oligomycin is approx. 25% of that of the starting preparation, and the ATP synthase activity after reconstitution into liposomes, measured as $ATP^{32}P_i$ exchange, is completely absent from the preparation. Loss of oligomycin-sensitivity and of ATP synthase activity of F_1F_0 -ATP synthase during its purification have been noted before (Stiggall et al., 1978; Galante et al., 1979). One possible explanation is that essential phospholipids or an essential subunit (such as factor B; Joshi et al., 1979) have been removed during purification (for additional comments, see Walker et al., 1991). Therefore, the preservation of all of the enzymic activities and the simultaneous fulfilment of the other criteria required for crystallization may not be possible.

The ATP hydrolase activity of the final enzyme preparation is similar to those reported for other preparations (Serrano et al., 1976; Stiggall, et al., 1978). The retention of this activity is important, as it allows the occupancy of the ligand-binding sites in F_1F_0 -ATPase to be modulated. Several studies have indicated significant conformational changes in the F_1F_0 -ATPase when the occupancy of the various ligand-binding sites is changed (for example, see Gogol et al., 1990). Careful control of the occupancy of the nucleotide-binding sites of the F_1 -ATPase has proved to be an important factor in the production of crystals that are suitable for structure determination by X-ray crystallography (Lutter et al., 1993), and it is likely to be important also in crystallizing the F_1F_0 -ATPase.

Note added in proof (received 19 July 1993)

Recently it has been found that F_0 purified from bovine heart mitochondria contains two subunits named f and g, that have not been observed before. Both subunits are also present in preparations of F_1F_0 -ATPase made by a procedure that is similar, but not identical, to Method 3 (I. R. Collinson, S. K. Buchanan and J. E. Walker, unpublished work). Therefore this preparation of F_1F_0 -ATPase is a complex of 16 different subunits. It is intended to re-examine other preparations of the enzyme for the presence of subunits f and g.

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