Large-scale chromatographic purification of F_1F_o-ATPase and complex I from bovine heart mitochondria

Susan K. BUCHANAN* and John E. WALKER†

The Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, U.K.

A new chromatographic procedure has been developed for the isolation of F_1F_o-ATPase and NADH:ubiquinone oxidoreductase (complex I) from a single batch of bovine heart mitochondria. The method employed dodecyl β-δ-maltoside, a monodisperse, homogeneous detergent in which many respiratory complexes exhibit high activity, for solubilization and subsequent purification by ammonium sulphate fractionation and column chromatography. A combination of anion-exchange, gel-filtration, and dye-ligand affinity chromatography was used to purify both complexes to homogeneity. The F_1F_o-ATPase preparation contains only the 16 known subunits of the enzyme. It has oligomycin-sensitive ATP hydrolysis activity and, as demonstrated elsewhere, when reconstituted into lipid vesicles it is capable of ATP-dependent proton pumping and of ATP synthesis driven by a proton gradient [Groth and Walker (1996) Biochem. J. **318**, 351–357]. The complex I preparation contains all of the subunits identified in other preparations of the enzyme, and has rotenone-sensitive NADH:ubiquinone oxidoreductase and NADH:ferricyanide oxidoreductase activities. The procedure is rapid and reproducible, yielding 50–80 mg of purified F_1F_o-ATPase and 20–40 mg of purified complex I from 1 g of mitochondrial membranes. Both preparations are devoid of phospholipids, and gel filtration and dynamic light scattering experiments indicate that they are monodisperse. Therefore, the preparations fulfil important prerequisites for structural analysis.

INTRODUCTION

The F_1F_o-ATPase in bovine heart mitochondrial membranes is a multisubunit assembly of 16 unlike proteins [1,2] with a total molecular mass of approx. 550 kDa. It synthesizes ATP from ADP and inorganic phosphate by employing the transmembrane proton potential gradient generated by electron transport to drive the reaction forward [3]; for reviews, see refs. [4–7]. This membrane-bound enzyme is composed of a globular domain, called F_1, which catalyses the synthesis of ATP, connected by a slender stalk to an intrinsic membrane part, known as F_o. The F_o domain transports protons through the membrane, converting energy from the proton potential gradient, which is coupled to ATP synthesis in F_1. The atomic structure of the separate bovine F_1 domain, which retains ATP hydrolase activity, suggests that the coupling mechanism could involve the rotation of an α-helical structure in the γ-subunit relative to the surrounding structure, which consists of three α-subunits alternating with three β-subunits [8]. Recent experiments support this rotary catalytic mechanism [9,10], but how rotation might be generated by proton transport and other important details of the coupling mechanism remain poorly understood.

Complex I (NADH:ubiquinone oxidoreductase) is another large protein complex in the inner membrane of mitochondria that helps to generate the proton potential gradient used by the F_1F_o-ATPase to make ATP; for reviews, see refs. [11,12]. It does so by coupling energy, derived from transfer of two electrons from NADH to ubiquinone-10, to the translocation of four protons from the matrix into the intermembrane space [13–18]. The bovine heart enzyme is composed of at least 43 different polypeptides, including seven hydrophobic proteins encoded in mitochondrial DNA with a combined molecular mass in excess of 900 kDa [12]. It also contains one flavin mononucleotide, at least one binuclear iron–sulphur centre, and at least three tetranuclear iron–sulphur centres [19–24]. All of the prosthetic groups of the enzyme appear to be in an extensive globular domain made of about 15 polypeptides, which is attached to a large intrinsic membrane sector, where the site of the ubiquinone binding is found. Treatment of the bovine enzyme with *N*,*N*-dimethyldodecylamine *N*-oxide dissociates the complex into two subcomplexes called Iα and Iβ. Subcomplex Iα contains the globular subunits and a few hydrophobic subunits, and retains all of the prosthetic groups and catalytic activity and redox centres; subcomplex Iβ consists of hydrophobic polypeptides and has no known catalytic activity [25]. A simpler active water-soluble globular subcomplex of about 15 subunits, called Iλ, can be prepared from bovine complex I by a similar procedure [26].

In order to be able to study the structure and function of these complex enzymes by biochemical and biophysical techniques, it is desirable that suitable procedures for their purification on a preparative scale should be developed. The resulting enzyme preparations should be highly pure and exhibit the same enzymic properties as those found in the enzymes in inner mitochondrial membranes. Additionally, if the enzyme is to be used for making two- and three-dimensional crystals, the procedure should employ a homogeneous, monodisperse detergent, and the isolated enzymes themselves should be monodisperse and have low phospholipid contents, as phospholipids can contribute to enzyme heterogeneity. Substantial steps have been made already towards satisfying these requirements, and procedures have been developed for making pure and active forms of both bovine F_1F_o-ATPase [27] and bovine complex I [25]. The purification procedure described here has been developed from these earlier methods, and it enjoys a number of advantages. First, it allows both enzymes to be purified in the same experiment, with savings in effort and in consumption of mitochondria. Secondly, it gives a 5-fold higher yield of pure, monodisperse F_1F_o-ATPase than previously obtained [27]. As demonstrated in an accompanying paper, after reconstitution into phospholipid vesicles the preparation is highly active in both ATP-dependent proton pumping

* Present address: Howard Hughes Medical Institute, Southwestern Medical Center at Dallas, 5323 Harry Hines Blvd., Dallas, TX 75235-9050, U.S.A.
† To whom correspondence and reprint requests should be sent.

and in ATP synthesis driven by a proton gradient [28]. Thirdly, the complex I preparation is obtained in a similar yield to that obtained before [25], but the enzyme is significantly purer. All of the known subunits of complex I associated with earlier preparations are also in the new preparation. An additional cost benefit is that the lifetime of a Mono-Q anion-exchange column used in the purification process is greatly extended by the new procedure. The new preparation of bovine complex I contains 43 subunits. It can transfer electrons from NADH to both ferricyanide and decylubiquinone, and the latter activity can be inhibited by rotenone.

MATERIALS AND METHODS

Materials

Dodecyl β-δ-maltoside and ATP were obtained from Novabiochem Ltd., Nottingham, U.K. NADH, phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase, decylubiquinone, phosphatidylcholine, phosphatidylethanolamine, cardiolipin, molybdenum oxide phospholipid reagent, and Reactive Yellow 3 dye matrix were purchased from Sigma Chemical Company Ltd., Poole, Dorset, U.K. Superose 6 HR 10/30, Hiload 26/60 Sephacryl S-300 HR, Hiload Q-Sepharose 26/10 High Performance, and Mono-Q HR 10/10 columns were supplied by Pharmacia Ltd., Milton Keynes, U.K.

Protein analysis

Protein concentrations were estimated by the bicinchoninic acid method [29] in the presence of dodecyl maltoside, using BSA as standard. PAGE was performed in 12–22 % acrylamide gels with 0.1 % (w/v) SDS [30]. Proteins were detected with Coomassie Blue G250 dye.

Enzyme activity measurements

ATP hydrolase activities were measured by monitoring the decrease in absorbance at 340 nm using an NADH-linked ATP-regenerating system [31]. One unit of activity of the enzyme catalyses the hydrolysis of 1 μmol of ATP per min at 30 °C. The NADH:ferricyanide oxidoreductase activity of complex I was measured from the decrease in absorbance at 410 nm over 30 s, in a buffer containing 40 mM potassium phosphate, pH 7.5, 1 mM potassium ferricyanide and 500 μM NADH. One unit of this activity of the enzyme catalyses the oxidation of 1 μmol of NADH per min at 30 °C. The NADH:decylubiquinone oxidoreductase activity was measured by monitoring the decrease in absorbance at 340 nm over 30 s in a buffer consisting of 40 mM potassium phosphate, pH 7.5, 100 μM decylubiquinone, egg yolk phosphatidylcholine (0.1 mg/ml), and 500 μM NADH. One unit of activity of this enzyme catalyses the oxidation of 1 μmol of NADH per min at 30 °C.

Isolation of F_1F_o-ATPase and complex I

Three buffers were employed in the chromatographic purification procedure described below. The S-300 buffer contained 20 mM Tris/HCl, pH 8.0, 100 mM sodium chloride, 0.1 % dodecyl maltoside, 50 mM sucrose, 10 % glycerol, 2 mM magnesium sulphate, 0.001 % PMSF and 0.02 % sodium azide. Buffers A and B were identical to S-300 buffer except that sodium azide was omitted from both, and 1.1 M sodium chloride was added to the latter. Unless otherwise indicated, all procedures, including chromatography, were performed at 4 °C.

Bovine heart mitochondria prepared according to the method of Smith [32], were stored at −20 °C. They were thawed, washed once in cold buffer containing 50 mM sodium dihydrogen phosphate, pH 8.0, 100 mM sucrose and 0.5 mM EDTA, and then centrifuged (17000 ***g***, 45 min). The pellet was resuspended at a protein concentration of 8.5 mg/ml in a buffer consisting of 20 mM Tris/HCl, pH 8.0, 10 % glycerol and 0.5 mM EDTA. To this suspension were added 20 mM magnesium sulphate, 20 mM ATP (buffered to pH 8.0) and 0.001 % PMSF. Dodecyl maltoside was added dropwise from a 10 % stock solution in water to a final concentration of 1.0 %, and the solution was stirred for 15 min and then centrifuged (31000 ***g***, 45 min).

The supernatant, which contained solubilized respiratory complexes, was fractionated with ammonium sulphate by adding sodium cholate (from a 20 % solution in water) to a final concentration of 1.6 %, and cold, saturated ammonium sulphate (neutralized with ammonia) to a final concentration of 40 %. The solution was centrifuged (31000 ***g***, 10 min), the supernatants pooled and the sodium cholate concentration was adjusted to 1.6 %. Then ammonium sulphate was added as before to a final concentration of 60 %. The pellets obtained after centrifugation contained F_1F_o-ATPase and complex I, and the supernatant was enriched in cytochrome *c* oxidase. The precipitated proteins were redissolved in a buffer consisting of 20 mM Tris/HCl, pH 8.0, 100 mM sodium chloride, 1.0 % dodecyl maltoside, 50 mM sucrose, 10 % glycerol, 2 mM magnesium sulphate, 0.001 % PMSF and 0.02 % sodium azide. The solution was desalted and delipidated by passage through a Hiload 26/60 Sephacryl S-300 HR gel-filtration column equilibrated with S-300 buffer.

The respiratory complexes were resolved on a Hiload 26/10 Q-Sepharose High Performance anion-exchange column equilibrated in buffer A. After sample application, the column was eluted at a flow rate of 2 ml/min with a linear gradient of sodium chloride from 100 to 500 mM in buffer B over a period of 200 min. The F_1F_o-ATPase and complex I eluted at sodium chloride concentrations of approx. 200 mM and 300 mM, respectively. Then the enzymes in these fractions were purified to homogeneity separately, as follows.

The sodium chloride concentrations of fractions containing F_1F_o-ATPase were adjusted to 100 mM and applied at 20 °C to a Reactive Yellow 3 dye column equilibrated with buffer A. The eluate was concentrated by ultrafiltration through an Amicon YM100 membrane to about 5 ml, and applied to a Hiload 26/60 Sephacryl S-300 HR gel-filtration column equilibrated with S-300 buffer. The flow rate of buffer was 0.3 ml/min. Fractions of the eluate (3 ml) that contained F_1F_o-ATPase were pooled and concentrated by ultrafiltration through a YM100 membrane.

The sodium chloride concentrations of the fractions that contained complex I were adjusted to 100 mM, and were applied to a Mono-Q HR 10/10 anion-exchange column equilibrated in buffer A. The column was washed first with one column volume of buffer A, and then with a 'saw-tooth' gradient of 10 % buffer B, 0 % B, 11 % B, 0 % B and 12 % B. Complex I was eluted with a linear gradient of sodium chloride from 12 to 40 % in buffer B. Peak fractions were pooled, concentrated to about 5 ml by ultrafiltration through a YM100 membrane, and applied to a Hiload 26/60 Sephacryl S-300 HR gel-filtration column equilibrated with S-300 buffer. The flow rate was 0.3 ml/min, and fractions (3 ml) that contained complex I were pooled and concentrated by ultrafiltration through a YM100 membrane.

Phospholipid analysis

Phospholipids were extracted twice from 30 mg of each purified complex [33,34]. Briefly, protein was precipitated with ammonium sulphate and the precipitate was resuspended in 20 mM Tris/HCl, pH 8.0 (1 ml). The suspension was extracted

with methanol/chloroform (2:1, v/v) for 2 h and centrifuged. The pellet was re-extracted with methanol/chloroform/water (2:1:0.8, by vol.) and centrifuged. To the combined extracts were added chloroform and water (1:1, v/v), and the mixture was shaken and centrifuged. The chloroform phase was withdrawn and evaporated to dryness, and the lipid residue was redissolved in chloroform/methanol (1:1, v/v; 100 μl).

Identification of phospholipids was performed by TLC on precoated, grooved, silica gel 60 plates in chloroform/methanol/water (65:25:4, by vol.) [35], using phosphatidylcholine, phosphatidylethanolamine and cardiolipin standards. A better separation of detergent from phospholipid was achieved by two-dimensional TLC [36] on precoated silica gel 60 plates. The solvent described above was used in the first dimension, and the second solvent was chloroform/methanol/25% aqueous ammonia (65:35:5, by vol.). Phospholipids were revealed by spraying with 1 M sulphuric acid and heating at 100 °C for 20 min, or by spraying with the molybdenum oxide reagent.

Molecular mass estimation by gel filtration

A Pharmacia Superose 6 HR 10/30 gel-filtration column was calibrated with aldolase (158 kDa), catalase (232 kDa), ferritin (440 kDa), and thyroglobulin (669 kDa) both in an aqueous buffer (containing 50 mM potassium phosphate, pH 7.5 and 100 mM sodium chloride) and in a detergent-containing buffer (S-300 buffer, described above). The void volume was determined with Blue Dextran. Samples of F_1F_o-ATPase and complex I were run individually at the same flow rate used for the standard proteins, and the partition coefficients, K_{av}, were determined from the equation

$$K_{av} = (V_e - V_o)/(V_t - V_o)$$

where V_e is the elution volume for the protein, V_o is the column void volume, and V_t is the total bed volume. Values of K_{av} for the protein standards were plotted against the logarithm of the corresponding molecular mass. Molecular masses for F_1F_o-ATPase and complex I were calculated from the standard curve.

Dynamic light-scattering measurements

Dynamic light-scattering measurements were made with a Biotage dp-801 Molecular Size Detector, kindly lent by Protein Solutions Ltd., Hertford, U.K. The instrument used a 780 nm, 30 mW solid-state laser for excitation, and scattered light was detected at 90 ° to the incident radiation by an avalanche photodiode. The intensity fluctuations of the scattered light were correlated to the Stokes radii of the protein molecules with the Stokes–Einstein relationship:

$$R_H = (\boldsymbol{k}T)/(6\pi n D_T)$$

where R_H is the Stokes radius, $\boldsymbol{k}$ is Boltzman's constant, T is the absolute temperature, n is the solvent viscosity, and D_T is the translational diffusion coefficient. The distribution of Stokes radii for each protein sample gave an estimate of particle size distribution. All measurements were made on freshly prepared protein samples in S-300 buffer (see below) and with reference to the scattering distribution of S-300 buffer alone.

RESULTS AND DISCUSSION

Purification of bovine F_1F_o-ATPase and complex I

First, cytochrome oxidase was separated from the other respiratory complexes by ammonium sulphate fractionation, and then the F_1F_o-ATPase and complex I were purified by chromatography in the presence of dodecyl maltoside. Most of the lipids and hydrophobic proteins precipitated at an ammonium sulphate concentration of 40% satn., F_1F_o-ATPase and complex I precipitated at 60% satn., and the bulk of cytochrome oxidase remained in the soluble fraction. Proteins in the 40–60% fraction were desalted and delipidated by gel filtration. Because all fractions containing F_1F_o-ATPase and complex I were pooled, this step did not increase the purity of the proteins, but it served the important functions of lowering the dodecyl maltoside concentration quickly and of separating the majority of phospholipids from the detergent-solubilized enzymes, thereby prolonging the lifetimes of columns used in subsequent steps.

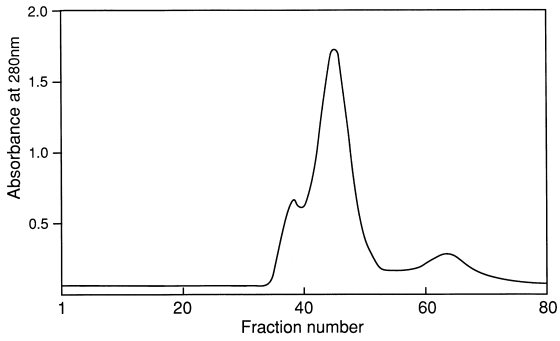

Figure 1 Chromatography of partially purified F_1F_o-ATPase from bovine heart mitochondria on Sephacryl S-300

For details, see the Materials and methods section. The shoulder eluting before the main peak of monodisperse F_1F_o-ATPase contained aggregated enzyme, and the subsequent small peak contained low-molecular-mass impurities.

The F_1F_o-ATPase and complex I were separated from each other and from most of the other mitochondrial proteins by chromatography of delipidated material on Q-Sepharose. The F_1F_o-ATPase eluted first at 150–200 mM sodium chloride, and was completely resolved from other peaks. It was followed in the next peak by small amounts of cytochrome oxidase (at 230–260 mM sodium chloride), which immediately preceded a large peak of complex I (at 260–340 mM sodium chloride). Two subsequent peaks (at 260–400 and 450–500 mM sodium chloride) contained cytochrome bc_1.

The F_1F_o-ATPase was purified further on a Reactive Yellow 3 dye-ligand column, which bound most of the lower-molecular-mass impurities, including a protein with an apparent molecular mass of 30 kDa (ADP/ATP translocase), but not the F_1F_o-ATPase. By passage of the F_1F_o-ATPase through a gel-filtration column, small amounts of aggregated F_1F_o-ATPase were removed in a shoulder preceding the main peak of the monodisperse enzyme. Low-molecular-mass impurities were present in a small subsequent peak (Figure 1).

The complex I from the Q-Sepharose column was purified further on a Mono-Q column, which separated away a slight contamination of F_1F_o-ATPase. In a final step, residual cytochrome oxidase and other minor impurities were removed on Sephacryl S-300. The impurities were in a low shoulder after the main peak (see Figure 2).

The Q-Sepharose chromatography of the delipidated crude extract of mitochondria indicates that the purification procedure could be extended to yield cytochrome bc_1 and cytochrome oxidase in addition to F_1F_o-ATPase and complex I. Some initial attempts have been carried out that confirm this possibility, but further developmental work is required to make these procedures robust and reliable. The impetus for doing so has been diminished by the determination of the structure of cytochrome oxidase from mitochondria [37] using material made by extant methods.

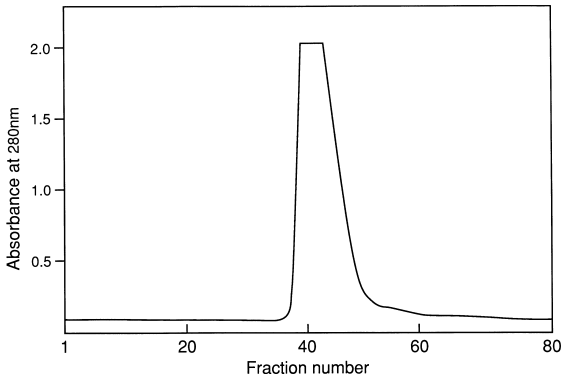

Figure 2 Chromatography of partially purified complex I from bovine heart mitochondria on Sephacryl S-300

For details, see the Materials and methods section. The main peak contained purified complex I, and the small subsequent shoulder contained cytochrome oxidase.

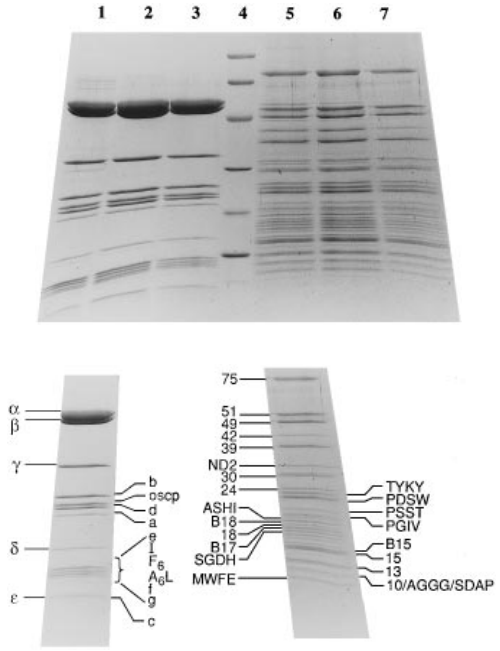

Figure 3 Subunit compositions of the new preparations of F_1F_o-ATPase and complex I

The proteins were separated by SDS/PAGE and stained with Coomassie Blue G250 dye. Upper panel: lane 1, the F_1F_o-ATPase fraction eluted from the Q-Sepharose column, exhibiting high-molecular-mass impurities and a weakly staining band at approx. 30 kDa; lane 2, the F_1F_o-ATPase complex after chromatography on a Reactive Yellow 3 dye column; lane 3, purified F_1F_o-ATPase, obtained after chromatography on a Sephacryl S-300; lane 4, molecular-mass markers of 77, 66, 45, 30, 17, and 12 kDa; lane 5, the complex I-containing fraction eluted from the Q-Sepharose, exhibiting faint contaminations of F_1F_o-ATPase, cytochrome oxidase, and transhydrogenase; lane 6, the complex after chromatography on a Mono-Q, using a shallow gradient of sodium chloride to remove most of the contaminating F_1F_o-ATPase; lane 7, the purified enzyme, obtained after gel filtration. Lower panel: identification of the 16 subunits of bovine F_1F_o-ATPase (left) and some of the 43 subunits of bovine complex I (right).

Subunit compositions of the purified complexes

The progress of purification at various stages was monitored by analysis of the polypeptide compositions of the complexes (see Figure 3, upper panel). The F_1F_o-ATPase obtained from the Q-Sepharose column was already highly purified, and contained only slight amounts of contaminating high-molecular-mass aggregates, and polypeptides at about 30 and 35 kDa (Figure 3, upper panel, lane 1). The latter proteins were removed by the Reactive Yellow 3 dye matrix (Figure 3, upper panel, lane 2), and the former by gel filtration (Figure 3, upper panel, lane 3).

The purified F_1F_o-ATPase contains its 16 constituent polypeptides; they are subunits α, β, γ, δ and ϵ from the F_1 domain, and subunits a, b, c, d, e, f, g, OSCP, A6L and F_6 from the F_o and stalk domains (Figure 3, lower panel). The ATPase inhibitor protein, I, is also present in apparently stoichiometric amounts. No contaminating proteins were detected by silver staining samples of the pure enzyme fractionated by denaturing PAGE [28]. The subunit composition of the purified enzyme was confirmed by a combination of N-terminal sequence analysis and electrospray ionization MS (results not shown).

The complex I recovered from the Q-Sepharose column contained small amounts of F_1F_o-ATPase and cytochrome oxidase, and a weakly staining band at about 80 kDa could also be seen (Figure 3, upper panel, lane 5). The F_1F_o-ATPase impurities (see bands in lane 5 at about 53 and 50 kDa corresponding to the α- and β-subunits) were removed almost entirely by chromatography on Mono-Q (Figure 3, upper panel, lane 6). Other remaining impurities were removed by gel filtration (Figure 3, upper panel, lane 7). Some of the subunits in the purified bovine complex I are indicated in Figure 3 (lower panel) (see refs. [12] and [25], for further details). A noticeable feature of the preparation, also observed in an earlier preparation [25], is that the 42 kDa subunit is diminished in intensity in comparison with other bands. Otherwise the subunit composition at this level of analysis is very similar to that of preparations of the enzyme made by conventional methods [38]. Subsequently, the subunit composition of the chromatographically purified bovine complex I and of a conventional preparation [38] have been scrutinized by protein sequence analysis, electrospray MS and with the use of antibodies that recognize specific subunits of the complex. A total of 43 subunits has been recognized in the pure preparation. All of them were also present in the conventional preparation, as well as contaminants that have been recognized as transhydrogenase, ADP/ATP translocase, and subunits of F_1F_o-ATPase, cytochrome bc_1 and cytochrome c oxidase (J. M. Skehel, I. M. Fearnley and J. E. Walker, unpublished work). None of these contaminants has been detected in the preparation made by the chromatographic method.

Table 1 Purification of F_1F_o-ATPase from bovine heart mitochondria

For details of the ATPase assay, see the Materials and methods section.

Preparation	Total units of ATPase activity	Total yield (activity) (%)	Protein (mg)
Mitochondrial extract	306	100	1054
Q-Sepharose anion exchange	280	92	123
Reactive Yellow 3 dye matrix	243	79	101
Sephacryl S-300 gel filtration	170	56	57

Catalytic activities

A representative purification of F_1F_o-ATPase is summarized in Table 1. The ATPase activity of the purified enzyme, at 3 units per mg of protein, is within the range of hydrolytic activities reported for F_1F_o-ATPases prepared chromatographically previously [27]. Activities of up to 15 units per mg of protein have

Table 2 Purification of complex I from bovine heart mitochondria

Preparation	Total units of activity*	Total yield (activity) (%)	Protein (mg)
Mitochondrial extract	10604	100	1054
Q-Sepharose	2554	24	76
Mono-Q	1624	15	35
Sephacryl S-300	1328	13	21

* NADH:ferricyanide oxidoreductase activity was measured as described in the Materials and methods section.

Table 3 Estimation of molecular masses of the complexes by gel filtration

Protein	V_e* (ml)	K_{av}†	Molecular mass
Aldolase	16.00	0.487	158000
Catalase	15.80	0.474	232000
Ferritin	14.64	0.400	440000
Thyroglobulin	12.84	0.285	669000
F_1F_o-ATPase	13.00	0.295	755357
Complex I	11.88	0.223	1279589

* V_e, the elution volume.

† The partition coefficient, K_{av}, was calculated according to $K_{av} = (V_e - V_o)/(V_t - V_o)$, where V_t is the total column volume (24 ml) and V_o is the column void volume determined with Blue Dextran (8.4 ml). For further details, see the Materials and methods section.

been reported for the enzyme prepared by conventional methods [39], but they were measured in the presence of added phospholipids. The composition of boundary and bulk-phase phospholipids and detergents influences both ATP hydrolase activity and oligomycin sensitivity in preparations of mitochondrial F_1F_o-ATPase [40,41]. The strong effects of detergents in mixed micelles containing the reconstituted purified enzyme are demonstrated in the accompanying paper [28]. By use of appropriate conditions of reconstitution, the enzyme's ATP hydrolase activity can be stimulated 10–20-fold, and activities up to 70 units/mg were observed. It has also been shown that the reconstituted enzyme can couple ATP hydrolysis to proton pumping. In addition, by co-reconstitution of the enzyme and bacteriorhodopsin into phospholipid vesicles, it has been shown that in the presence of a proton gradient generated by the light-stimulated proton-pumping activity of bacteriorhodopsin that ATP can be formed from ADP and inorganic phosphate. In other words, the catalytic activities of F_1F_o-ATPase in mitochondria are also associated with the purified enzyme.

A representative purification of complex I is described in Table 2. The specific activity of the final preparation, measured with ferricyanide as electron acceptor, was 74 units per mg of protein, and is comparable with that described by Finel et al. [25]. The enzyme complex was also capable of transferring electrons from NADH to decylubiquinone at a rate of 1.23 μmol of NADH/min per mg of protein, and up to 99 % of the NADH:decylubiquinone oxidoreductase activity of complex I was inhibited by rotenone. The rotenone-sensitive reduction of this ubiquinone analogue by NADH indicates that at least some of the physiological activity of complex I has been preserved in the new preparation. The line shapes of EPR spectra of the iron–sulphur clusters in the preparation of Finel et al. [25] were broadened slightly. Whether the complete removal of phospholipids has significantly affected any of the iron–sulphur clusters in the new preparation remains to be established by further study. As has now been done with the new preparation of the F_1F_o-ATPase [28], the effects of relipidation on the activity of the new preparation of complex I will also need to be established in future reconstitution experiments.

Phospholipid analysis

Phosphatidylcholine (40 %), phosphatidylethanolamine (35 %), and cardiolipin (18 %) are the predominant phospholipids in the inner membranes of bovine heart mitochondria [42–44]. Various amounts of standard samples of these phospholipids were run on one-dimensional TLC plates. For each phospholipid, the limit of detection with sulphuric acid was 1 μg/lane. In order to detect the minimal quantity of phospholipid bound to proteins (assuming one phospholipid bound per protein complex), it would be necessary to extract phospholipids from 10 mg of each purified complex. Therefore, to allow for handling losses, 30 mg each of F_1F_o-ATPase and complex I were extracted, and 20 μl of the concentrated lipid extracts were run separately on two-dimensional TLC plates to give better separation of phospholipids from co-extracted detergent. No phospholipids were detected in either extract. Therefore, any phospholipid remaining bound to the complexes would be present in sub-stoichiometric amounts.

Molecular mass estimation by gel filtration

Although the sequences of all of the identified subunits of bovine F_1F_o-ATPase and 42 of the 43 known subunits of complex I have been determined ([2,12,45]; J. M. Skehel and J. E. Walker, unpublished work), their subunit stoichiometries have not been competely established. Therefore, the exact molecular masses of the complexes cannot be calculated at present. The relationship between elution volume and molecular mass by size-exclusion chromatography is less accurate for membrane proteins than for globular proteins [46]. Nonetheless, this method provides an estimate of particle size distribution from the chromatogram peak shape, and, especially for large complexes, it can indicate whether the complex is a monomer, dimer, or higher aggregate. In the present experiments, a Superose 6 HR 10/30 column was used because the high number of theoretical plates per meter gives it a high resolving power over a wide molecular mass range. The column was calibrated with four soluble proteins, and the void volume was measured with Blue Dextran. The elution times of each protein were identical in aqueous phosphate/saline buffer and in S-300 buffer containing detergent. The elution times for F_1F_o-ATPase and complex I, determined in S-300 buffer in separate experiments, corresponded to monomeric complexes with molecular masses of 755 and 1279 kDa, respectively (see Table 3). Both values include bound detergent, and they are incompatible with dimeric or higher species. As the peak was symmetrical in both cases, the enzyme preparations are probably monodisperse.

Dynamic light-scattering measurements

The distribution of particle size in each preparation was examined by dynamic light scattering, which allows the fluctuation of scattered light by molecules in solution to be analysed. The scattered light intensity is proportional to the square of the molecular mass. In a solution of monomers, the distribution of particle sizes fits a narrow unimodal Gaussian curve, whereas solutions containing mixtures of particle sizes often result in broad unimodal, bimodal, or more complex curves [47]. Soluble

Table 4 Determination of monodispersity of the purified complexes by dynamic light scattering

The intensity fluctuations of the scattered light were correlated to the Stokes radii of the scattering molecules by the equation described in the Materials and methods section. The distribution of Stokes radii for each sample gave an estimate of particle size distribution. All measurements were made on freshly prepared protein samples in S-300 buffer and were referenced to the scattering distribution of S-300 buffer alone.

Sample	Radius* (nm)	Polydispersity†	p/r‡	Baseline§	Distribution
F_1F_o-ATPase	13.3	3.853	0.29	1.000	Broad unimodal
Complex I	17.0	3.862	0.23	1.001	Broad unimodal
S-300 buffer	5.8	3.262	0.56	1.016	Multimodal
1% Dodecyl D-maltoside	3.6	0.771	0.21	1.000	Broad unimodal

* The hydrodynamic radius was determined from the fluctuation in scattered intensity using the Stokes–Einstein equation, as described in the Materials and methods section.

† The polydispersity value is a measure of the width of the Gaussian curve when the data are fitted to a unimodal or bimodal distribution.

‡ The polydispersity to radius ratio indicates whether the data can be best fitted by a narrow or broad unimodal distribution, a bimodal distribution, or a more complex curve. A narrow unimodal curve is characterized by zero polydispersity, a broad unimodal distribution by a p/r ratio of less than 30%, and a bimodal or multimodal distribution by a p/r ratio of around 50% (when attempting to fit the data to a unimodal Gaussian curve).

§ The baseline is an estimate of goodness of fit of the data to a Gaussian curve. A narrow unimodal distribution will have a baseline of 1.000, the baseline for a broad unimodal distribution will be between 1.000 and 1.002, and a multimodal distribution (or a unimodal peak with a shoulder) will have a baseline over 1.005.

proteins with narrow or broad unimodal particle size distributions are said to have a greater probability of forming three-dimensional crystals than proteins with bimodal or trimodal distributions [48].

The size distributions for the preparations of F_1F_o-ATPase and complex I fitted best to unimodal Gaussian curves (see Table 4). The values of the ratio of polydispersity to particle radius, which indicates the width of the curve, suggested that both solutions were monodisperse (the polydispersity value should be less than 30% of the radius to reflect a unimodal particle distribution composed of monomers). As S-300 buffer alone was found to have a ratio of polydispersity to particle radius of 56%, the width of the curves for the protein solutions contained contributions from the buffer. An aqueous 1% solution of dodecyl maltoside had a narrow unimodal size distribution, consistent with uniform micellar size. The polydispersity of the detergent micelles in S-300 buffer may be due to micellar interactions with glycerol, or sodium chloride, or both [47] and accounts for the observed polydispersity values for the F_1F_o-ATPase and complex I preparations. However, the particle size distributions suggested unimodal curves even with this large buffer contribution. Therefore, both preparations appear to be monodisperse, and fulfil one of the requirements for crystallization experiments. The Stokes radius value for F_1F_o-ATPase agrees broadly with electron microscopy measurements on F_1F_o-ATPase prepared by a related chromatographic method [27].

Conclusions

The new purification procedure for F_1F_o-ATPase and complex I provides large yields of homogeneous, monodisperse enzymes in a detergent which is suitable for crystallization experiments; for a review see ref. [49]. The physiological activities of the native F_1F_o-ATPase have been preserved in the purification procedure, since ATP-driven proton pumping activity and ATP synthesis driven by a proton gradient can be demonstrated after re-incorporation of the pure enzyme into phospholipid vesicles [28]. Complex I prepared by the new method transfers electrons from NADH to either ferricyanide or decylubiquinone and the NADH:decylubiquinone oxidoreductase activity is up to 99% inhibited by rotenone. However, the precise extent of the conservation of the physiological activity of the pure enzyme remains to be established, although all of the known subunits of the enzyme are present. The preparations of both complexes are monomeric and contain no detectable phospholipids. Because the procedure is rapid and highly reproducible, it is well suited to providing enzymes which are consistently pure in amounts necessary for two-dimensional and three-dimensional crystallization trials. The monodisperse detergent, dodecyl maltoside, has been used previously in chromatographic purification procedures for cytochrome bc_1 [50] yielding pure and highly active enzyme, and has been shown to yield optimal rates of electron transfer and superior enzyme stability for cytochrome oxidase [51]. The successful purification of F_1F_o-ATPase and complex I in dodecyl maltoside suggests that this detergent may also be useful for the purification of other mitochondrial membrane proteins, and that it may be possible to purify all four major respiratory enzymes from the same membrane extract.

S.K.B. was supported by a long-term EMBO fellowship. We are grateful for the helpful comments of Drs. R. Lutter and M. Finel when this work was initiated, and to Protein Solutions Ltd. for the loan of a dynamic light-scattering instrument.

REFERENCES

1 Walker, J. E., Fearnley, I. M., Lutter, R., Todd, R. J. and Runswick, M. J. (1990) Phil. Trans. R. Soc. **326**, 367–378
2 Collinson, I. R., Runswick, M. J., Buchanan, S. K., Fearnley, I. M., Skehel, J. M., van Raaij, M. J., Griffiths, D. E. and Walker, J. E. (1994) Biochemistry **33**, 7971–7978
3 Mitchell, P. (1961) Nature (London) **191**, 144–148
4 Boyer, P. D. (1989) FASEB J. **3**, 2164–2178
5 Senior, A. E. (1990) Annu. Rev. Biophys. Biophys. Chem. **19**, 7–41
6 Fillingame, R. H. (1990) Bacteria **12**, 345–391
7 Penefsky, H. S. and Cross, R. L. (1991) Adv. Enzymol. **64**, 173–214
8 Abrahams, J. P., Leslie, A. G. W., Lutter, R. and Walker, J. E. (1994) Nature (London) **370**, 621–628
9 Aggeler, R., Haughton, M. A. and Capaldi, R. A. (1995) J. Biol. Chem. **270**, 9185–9191
10 Duncan, T. M., Bulygin, V. V., Zhou, Y., Hutcheon, M. L. and Cross, R. L. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 10964–10968
11 Weiss, H., Friedrich, T., Hofhaus, G. and Preis, D. (1991) Eur. J. Biochem. **197**, 563–576
12 Walker, J. E. (1992) Q. Rev. Biophys. **25**, 253–324
13 Rottenberg, H. and Gutman, M. (1977) Biochemistry **16**, 3220–3227
14 De Jonge, P. C. and Westerhoff, H. V. (1982) Biochem. J. **204**, 515–523
15 Wikström, M. (1984) FEBS Lett. **169**, 300–304
16 Scholes, T. A. and Hinkle, P. C. (1984) Biochemistry **23**, 3341–3345
17 Lemasters, J. J. (1984) J. Biol. Chem. **259**, 13123–13130
18 Brown, G. C. and Brand, M. D. (1988) Biochem. J. **252**, 473–479
19 Ohnishi, T., Asakura, T., Wohlrab, H., Yonetani, T. and Chance, B. (1970) J. Biol. Chem. **245**, 901–902
20 Orme-Johnson, N. R., Orme-Johnson, W. H., Hansen, R. E., Beinert, H. and Hatefi, Y. (1971) Biochem. Biophys. Res. Commun. **44**, 446–452
21 Ohnishi, T. (1975) Biochim. Biophys. Acta **387**, 475–490
22 Ohnishi, T. (1979) in Membrane Proteins in Energy Transduction (Capaldi, R. A., ed.), pp. 1–87, Dekker, New York
23 Albracht, S. P. J., Dooijewaard, G., Leeuwerik, F. J. and van Swol, B. (1977) Biochim. Biophys. Acta **459**, 300–317
24 Kowal, A. T., Morningstar, J. E., Johnson, M. K., Ramsey, R. R. and Singer, T. P. (1986) J. Biol. Chem. **261**, 9239–9245
25 Finel, M., Skehel, J. M., Albracht, S. P. J., Fearnley, I. M. and Walker, J. E. (1992) Biochemistry **31**, 11425–11434
26 Finel, M., Majander, A. S., Tyynela, J., De Jong, A. M. P., Albracht, S. P. J. and Wikström, M. (1994) Eur. J. Biochem. **226**, 237–242
27 Lutter, R., Saraste, M., van Walraven, H. S., Runswick, M. J., Finel, M., Deatherage, J. F. and Walker, J. E. (1993) Biochem. J. **295**, 799–806

28 Groth, G. and Walker, J. E. (1996) Biochem. J. **318**, 351–357
29 Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and Klenck, D. C. (1985) Anal. Biochem. **150**, 76–85
30 Laemmli, U.K. (1970) Nature (London) **227**, 680–685
31 Chang, T. M. and Penefsky, H. S. (1973) J. Biol. Chem. **248**, 2746–2754
32 Smith, A. L. (1967) Methods Enzymol. **10**, 81–86
33 Bligh, E. G. and Dyer, W. J. (1959) Can. J. Biochem. Physiol. **31**, 911–917
34 Kates, M. (1982) in Techniques of Lipidology: Isolation, Analysis, and Identification of Lipids (Burdon, R. H. and van Knippenberg, P. H., eds.), p. 106, Elsevier, Oxford
35 Reiss-Husson, F. (1992) in Crystallization of Nucleic Acids and Proteins: A Practical Approach (Ducruix, A. and Giegé, R., eds.), pp. 179–184, IRL Press, Oxford
36 Nussberger, S., Dörr, K., Wang, D. N. and Kühlbrandt, W. (1993) J. Mol. Biol. **234**, 347–356
37 Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-Itoh, K., Nakashima, R., Yaono, D. and Yoshikawa, S. (1995) Science **269**, 1069–1074
38 Hatefi, Y., Haavik, A. G. and Griffiths, D. E. (1962) J. Biol. Chem. **237**, 1676–1680
39 Galante, Y. M., Wong, S. Y. and Hatefi, Y. (1979) J. Biol. Chem. **254**, 12372–12378
40 Laird, D. M., Parce, J. W., Montgomery, R. I. and Cunningham, C. C. (1986) J. Biol. Chem. **261**, 14851–14856
41 Dabbeni-Sala, F., Vázquez-Laslop, N., Fachinetti, A., Devars, S. and Dreyfus, G. (1989) Biochem. Biophys. Res. Commun. **158**, 1013–1020
42 Fleischer, S., Brierley, G. and Klouwen, H. (1961) J. Biol. Chem. **236**, 2936–2941
43 Daum, G. (1985) Biochim. Biophys. Acta **822**, 1–42
44 Schlame, M., Beyer, K., Hayer-Hartl, M. and Klingenberg, M. (1991) Eur. J. Biochem. **199**, 459–466
45 Walker, J. E., Lutter, R., Dupuis, A. and Runswick, M. J. (1991) Biochemistry **30**, 5369–5378
46 Le Maire, M., Aggerbeck, L. P., Monteilhet, C., Andersen, J. P. and Moller, J. V. (1986) Anal. Biochem. **154**, 525–535
47 Bloomfield, V. A. (1981) Annu. Rev. Biophys. Bioeng. **10**, 421–450
48 Zulauf, M. and D'Arcy, A. (1992) J. Crystal Growth **122**, 102–106
49 Kühlbrandt, W. (1988) Q. Rev. Biophys. **21**, 429–477
50 Ljungdahl, P. O., Pennoyer, J. D. and Trumpower, B. L. (1986) Methods Enzymol. **126**, 181–191
51 Robinson, N. C., Neumann, J. and Wiginton, D. (1985) Biochemistry **24**, 6298–6304

Received 26 March 1996; accepted 30 April 1996