F_o Membrane Domain of ATP Synthase from Bovine Heart Mitochondria: Purification, Subunit Composition, and Reconstitution with F_1-ATPase†

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ABSTRACT: The F_o membrane domain of the F_1F_o-ATP synthase complex has been purified from bovine heart mitochondria. The purification procedure involves the removal of peripheral membrane proteins, including F_1-ATPase, from submitochondrial particles with guanidine hydrochloride, followed by extraction of F_o and other membrane proteins from the stripped membranes in the presence of the detergent *n*-dodecyl β-D-maltoside. F_o was then purified by ion-exchange and dye ligand chromatography in the presence of the same detergent. Approximately 15 mg of pure F_o was recovered from 1.8 g of mitochondrial membrane protein. The purified F_o is a complex of nine different polypeptides. They are subunits a, b, c, d, e, F_6, and A6L characterized before in F_1F_o-ATPase preparations, and two new hitherto undetected subunits, named f and g. The sequences of subunits f and g have been determined. They are not related significantly to any known protein, but subunit f appears to contain a membrane-spanning α-helix. Proteins f and g are also present in approximately stoichiometric amounts in a highly purified preparation of intact F_1F_o-ATPase, and so it is concluded that they are authentic subunits of the bovine enzyme with unknown functions. Dibutyltin 3-hydroxyflavone, an inhibitor of F_1F_o-ATPase, also binds to the purified F_o in detergent and competes for binding with venturicidin. In the presence of F_1 and OSCP, the purified F_o was reassembled into the intact F_1F_o-ATPase complex. Therefore, this procedure provides a relatively abundant source of pure and functional F_o that is suitable for structural analysis.

An understanding of the molecular basis of ATP synthesis depends upon knowledge of the structure of ATP synthase (F_1F_o-ATPase) to atomic resolution. Crystals of bovine F_1-ATPase diffract X-rays to 2.8-Å resolution (Walker et al., 1990; Lutter et al., 1993a), and have been used to solve the structure of F_1 to 6.5-Å resolution (Abrahams et al., 1993), and recently to 2.8 Å (J. P. Abrahams, A. G. W. Leslie, and J. E. Walker, unpublished results). It may be possible to extend the structure of F_1 by cocrystallizing F_1 with polypeptides such as the oligomycin sensitivity conferred protein (OSCP)[1] that are present in the stalk connecting F_1-ATPase to the membrane domain F_o.

At present, very little is known about the structural organization of the polypeptides in F_o, particularly in the mitochondrial enzyme. Until now, mitochondrial F_o has never been purified to homogeneity, and its subunit composition has not been established with certainty. The first preparation of F_o was made by stripping F_1-ATPase from bovine heart mitochondrial membranes by sonication and treatment with urea (Kagawa & Racker, 1966a). This membrane-associated F_o was reconstituted successfully with F_1-ATPase and OSCP to produce a functional F_1F_o-ATPase (Kagawa & Racker, 1966b), but it was impure and contained many other respiratory components. Subsequent procedures for preparing mitochondrial F_o depended upon stripping away F_1 subunits by a variety of methods either from membranes or from preparations of F_1F_o-ATPase (Tzagoloff et al., 1968; Alfonzo et al., 1981; Galante et al., 1981; Guerrieri et al., 1989; McEnery et al., 1989). However, their removal was incomplete when sodium bromide or urea was employed in the stripping step (Tzagoloff et al., 1968; Glaser et al., 1980; Torok & Joshi, 1985; Pringle et al., 1990), and the samples of F_1F_o-ATPase themselves contained impurities (Galante et al., 1981; Glaser et al., 1980; Pringle et al., 1990). In some procedures, F_o was solubilized from stripped membranes with a detergent (Alfonzo et al., 1980; Galante et al., 1981; Guerrieri et al., 1989; McEnery et al., 1989), but other membrane proteins were solubilized along with F_o, and no procedures for their complete removal has been described hitherto. Recently, highly pure and monodisperse samples of bovine F_1F_o-ATPase have been made (Walker et al., 1991; Lutter et al., 1993b; Buchanan & Walker, 1993, 1994). Attempts to remove the F_1 subunits from these preparations under a variety of conditions resulted in the disruption of both the F_1 and F_o domains, possibly because there is little bound phospholipid to stabilize the F_o domain (J. E. Walker and I. R. Collinson, unpublished work).

In the procedure described below, bovine submitochondrial particles have been stripped with guanidine hydrochloride, which has been shown to be an effective reagent for complete removal of F_1 subunits from rat mitochondrial membranes (McEnery et al., 1989). Then the bovine F_o has been solubilized from the membranes and purified by a similar

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[1] Abbreviations: OSCP, oligomycin sensitivity conferral protein; BCA, bicinchoninic acid; PMSF, phenylmethanesulfonyl fluoride; HPLC, high-performance liquid chromatography; DCCD, *N,N*-dicyclohexylcarbodiimide; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; DES, diethylstilbestrol; DM, *n*-dodecyl β-D-maltoside.

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procedure to that described for obtaining pure, monodisperse F_1F_o-ATPase (Buchanan & Walker, 1993, 1994). This consists of the use of the detergent *n*-dodecyl β-D-maltoside to solubilize the F_o, followed by ion-exchange and dye ligand chromatography. The resultant purified bovine F_o is a complex of nine different polypeptides. Seven of them (subunits b, d, a, e, F_6, A6L, and c) have been characterized previously in F_1F_o-ATPase preparations (Walker et al., 1991), but two others (subunits f and g) are new subunits which have not been detected before. Neither of them is significantly related to any protein of known sequence, and both of them have been shown to be present in a pure preparation of F_1F_o-ATPase. The F_o preparation has been reconstituted with F_1-ATPase and OSCP to re-form an intact F_1F_o-ATPase complex. It is monodisperse and provides suitable material for crystallization trials and other biochemical experiments.

MATERIALS AND METHODS

Materials. *n*-Dodecyl β-D-maltoside was obtained from Calbiochem Novabiochem Ltd. (Nottingham, U.K.). Venturicidin was supplied by Sigma (St. Louis, MO). Stock solutions were made in methanol (10 mg/mL).

Analytical Methods. Protein concentrations were determined by the BCA method (Pierce Chemicals, Rockford, IL) using bovine serum albumin as a standard. Polyacrylamide gels containing a 12–22% acrylamide gradient [acrylamide: bis(acrylamide) ratio 30:0.8, by weight] were prepared and run in the buffer system of Laemmli (1970). The gel system described by Schägger and von Jagow (1987) was also employed. Both types of gel were cast in the minigel (10 cm × 10 cm) format.

Preparation of Membranes. All manipulations concerning the isolation of bovine F_o were performed at 4 °C. Bovine heart mitochondria were prepared according to Smith (1967). They were resuspended in a buffer (210 mL) consisting of 20 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 5 mM 2-mercaptoethanol, and 0.001% PMSF. Submitochondrial particles were made from mitochondria by sonication as described before (Lutter et al., 1993a). The submitochondrial particles (ca. 1.8 g) were suspended in a buffer (120 mL; protein concentration 15 mg/mL) composed of 0.15 M potassium phosphate, pH 7.5, 1 mM ATP, 25 mM EDTA, 0.5 mM dithiothreitol, 5% ethylene glycol, and 0.001% PMSF. A solution of guanidine hydrochloride (3.3 M) in the same buffer was added to give a final concentration of 2.6 M and a protein concentration of 3 mg/mL. The suspension was stirred for 5 min, and then the guanidine was removed by dialysis for 3 h against 7 L of the same buffer. The stripped membranes were harvested by centrifugation at 142000*g*.

Solubilization of Bovine F_o. The stripped membranes were resuspended in a buffer composed of 20 mM Tris-HCl, pH 7.5, 50 mM sucrose, 1% *n*-dodecyl β-D-maltoside, 10% glycerol, 0.001% PMSF, and 1 mM EDTA to a final membrane protein concentration of 2.5 mg/mL. The membrane suspension was dispersed by sonication for 30 s at 40% power output with a Model W-385 Ultrasonic Processor (Heat Systems-Ultrasonics Inc., Farmingdale, NY) using a probe with a 2-cm tip diameter, then stirred for 2 h, subjected again to sonication under the same conditions, and finally centrifuged (142000*g*). The insoluble material was discarded.

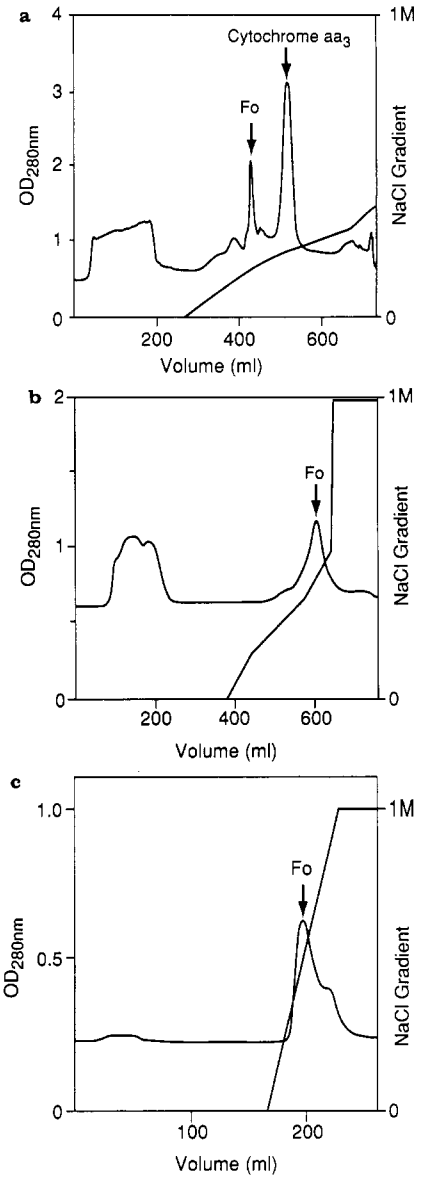

FIGURE 1: Purification of F_o from bovine heart mitochondria. (a) Chromatography of *n*-dodecyl β-D-maltoside extract of stripped membranes from bovine heart mitochondria on Q-Sepharose. (b) Chromatography of F_o-containing fractions from part a on S-Sepharose. (c) Chromatography of F_o-containing fractions from panel b on a reactive yellow 3 dye ligand column. See Materials and Methods and Figure 2 for further details.

Purification of Bovine F_o. The F_o was purified from the detergent-solubilized proteins in three chromatographic steps. First, the extract (ca. 400 mL) was applied to a Q-Sepharose high-performance column (10 cm × 5 cm i.d.; Pharmacia Ltd., Milton Keynes, U.K.) equilibrated in a buffer composed of 20 mM Tris-HCl, pH 7.5, 50 mM sucrose, 0.1% *n*-dodecyl β-D-maltoside, 10% glycerol, 0.001% PMSF, and 1 mM EDTA. The bound proteins, including F_o, were eluted with a NaCl gradient from 0 to 0.5 M. The partially pure F_o was detected by analysis of the fractions by polyacrylamide gel electrophoresis under denaturing conditions, and was found to have eluted at a salt concentration of about 0.2 M (see Figure 1a). The appropriate fractions were pooled and dialyzed overnight against 2 L of the column buffer lacking *n*-dodecyl β-D-maltoside. Second, the solution of partially purified F_o was applied to an S-Sepharose high-performance column (7.5 cm × 5 cm i.d.; Pharmacia) in the same column buffer as above, except that the 20 mM Tris-HCl, pH 7.5, was replaced by 20 mM MOPS adjusted to pH 7.0 with NaOH.

In some experiments, the S-Sepharose column was replaced by a column of Mono-S HR 16/10 (Pharmacia), with slight improvement in resolution. The F_o was bound to the column and was eluted on a gradient of NaCl (0–0.5 M) at 0.25 M (see Figure 1b). In the third step of the purification, the F_o-containing fractions were dialyzed as in the previous chromatographic step, and were then applied to a reactive yellow 3 dye ligand column (15 cm × 2.6 cm i.d.; Sigma Chemical Co. Ltd., Poole, Dorset, U.K.) packed in buffer of the same composition as used in the Q-Sepharose high-performance chromatography step. The F_o was bound to the dye column. It was eluted on a linear NaCl gradient (0–1.0 M) at 0.5 M (Figure 1c). The final recovery of F_o was about 15 mg. Purified F_o was concentrated by ultrafiltration on a YM 100 membrane (Amicon). It was stored at −20 °C at a protein concentration of 0.5–2.0 mg/mL in a buffer containing 20 mM Tris-HCl (pH 7.5), 50 mM sucrose, 0.1% *n*-dodecyl β-D-maltoside, 10% glycerol, 0.001% PMSF, 1 mM EDTA, and ca. 500 mM NaCl (from salt gradients).

Binding of Dibutyltin 3-Hydroxyflavone to F_o and F_1F_o-ATPase. The interaction of dibutyltin 3-hydroxyflavone bromide (Griffiths et al., 1993) with F_o in intact F_1F_o-ATPase and in F_o during its purification from stripped mitochondrial membranes was followed by fluorescence measurements. The experiments were conducted at 18–20 °C in a buffer (2 mL) consisting of 10 mM HEPES (pH 7.4), 0.25 M sucrose, and 0.5 mM EGTA. The samples at various concentrations (see Figure 3) were suspended in the buffer, and the reagent (usually 1 nmol) was added. The fluorophore was excited at 395 nm, and the emission was monitored at 450 nm with a Perkin Elmer LS5 spectrofluorometer. When maximal fluorescence emission had been reached, venturicidin (2 or 20 μg) and then tributyltin (10 nmol) were added, and the subsequent reductions in fluorescence were recorded.

Purification of Other Proteins. F_1F_o-ATPase and F_1-ATPase were purified from bovine heart mitochondria as described elsewhere (Buchanan & Walker, 1993, 1994; Lutter et al., 1993b). Bovine OSCP was obtained by overexpression in *Escherichia coli* (Studier et al., 1990). The inclusion bodies produced in the bacterium were dissolved in 30–50 mL of 6 M guanidine hydrochloride (protein concentration 0.25 mg/mL) and were dialyzed twice at 4 °C against a buffer (4 L) containing 10 mM MOPS–NaOH (pH 7.0), 200 mM NaCl, 5 mM 2-mercaptoethanol, 0.001% PMSF, and 1 mM EDTA. The resolubilized protein was applied to a Pharmacia Hi-Load S-Sepharose column (10 cm × 2.6 cm i.d.) equilibrated with the same buffer. The protein was eluted with a gradient of NaCl and eluted at a salt concentration of about 400 mM. The yield of purified OSCP was about 50 mg/L of bacterial culture. The purified protein was stored at −20 °C and dissolved at 10–35 mg/mL in 10 mM Tris-HCl (pH 8.0) and 5 mM 2-mercaptoethanol. The refolded protein has been demonstrated to be fully active in a reconstitution assay (M. J. van Raaij, S. Joshi, and J. E. Walker, unpublished results).

Reconstitution of the F_1F_o-ATPase Complex. Bovine F_1F_o-ATPase (8.2 mg), OSCP (0.5 mg), and F_o (3.0 mg) (approximate molar proportions of 1.5:1.5:1.0, respectively) were mixed in a buffer (2.0 mL) consisting of 20 mM Tris-HCl, pH 7.5, 0.1% *n*-dodecyl β-D-maltoside, 50 mM sucrose, 10% glycerol, 0.001% PMSF, 1 mM EDTA, and 8 mM dithiothreitol. The mixture was left at 25 °C for 45 min, and then passed through a column of Sephacryl S-300 (60 cm × 1.6 cm i.d.) in the same buffer, but lacking dithiothreitol and containing 100 mM NaCl.

Purification of Subunits of F_o and F_1F_o-ATPase. A sample (0.5 mL) of 6 M guanidine hydrochloride in 17 mM sodium acetate buffer, pH 4.0, was added to a solution (0.5 mL) of purified F_o (protein concentration 0.55 mg/mL). This solution was applied to a column of C_8 Aquapore RP-300 (7-μm particles, 300-Å pore size; 10 cm × 2.1 mm i.d.) equilibrated in 0.1% trifluoroacetic acid. The proteins were eluted with a linear gradient of acetonitrile. The subunits of F_1F_o-ATPase were fractionated in a similar manner.

Mass Spectrometric and N-Terminal Sequence Analysis of Subunits of F_o and F_1F_o-ATPase. Samples of subunits of F_o and F_1F_o-ATPase isolated by reverse-phase HPLC were introduced into a VG BIO-Q triple quadrupole mass spectrometer with electrospray ionization (VG Biotech, Altrincham, Cheshire, U.K.). Electrospray mass spectra were measured as described previously (Walker et al., 1992). The subunits of F_o and F_1F_o-ATPase were separated by polyacrylamide gel electrophoresis under denaturing conditions and transferred to a poly(vinylidene difluoride) membrane (Pilkington et al., 1991), and after being stained with Coomassie blue dye, their N-terminal sequences were determined (Fearnley et al., 1989). The bands containing subunits a and A6L were excised from the membrane, and submerged for 2 h at 25 °C in an anhydrous solution of 1.5 M HCl dissolved in methanol in order to remove their α-*N*-formyl groups. The reagent was evaporated off, and the residue, including the membrane, was transferred to the reaction chamber of the protein sequencer for N-terminal sequence analysis.

Sequence Analysis of Subunits f and g. Samples of subunits f and g, purified from F_o as described above, were digested with cyanogen bromide. The resulting fragments were separated by reverse-phase HPLC and on Schägger gels (Schägger & von Jagow, 1987) under similar conditions to those used for purifying the intact subunits, and were then subjected to automated Edman degradation. Segments of the resulting partial protein sequences were used for designing degenerate oligonucleotide primers and probes. These oligonucleotide mixtures were synthesized and were employed for generating partial cDNAs for each protein by the polymerase chain reaction, using total bovine heart cDNA as template as described before (Walker et al., 1992). These partial cDNAs were sequenced (Biggin et al., 1983). The partial DNA sequences were then extended to the 5′ and 3′ extremities of the complete cDNAs in two further polymerase chain reactions, as described previously (Walker et al., 1992). Thus, the total cDNA sequence of each subunit was obtained from the overlapping partial cDNAs. The protein sequences of subunits f and g encoded in the cDNAs were compared with the sequences in the PIR, SWISSPROT, and EMBL databases with the program FASTA (Lipman & Pearson, 1985). Hydrophobicity profiles were calculated with HYDROPLOT, a version of SOAP (Kyte & Doolittle, 1982).

RESULTS

Purification of F_o from Bovine Mitochondria. The treatment of bovine heart submitochondrial particles with 2.6 M guanidine hydrochloride proved to be an effective way of removing the subunits of F_1-ATPase from the membranes. Neither the α nor the β subunits of the enzyme could be detected in a detergent extract prepared from the stripped membranes under conditions that are effective for solubilizing them from untreated membranes [see Figure 2b]. The most prominent component in the extract was a protein with an apparent molecular mass of about 30 kDa, which has been

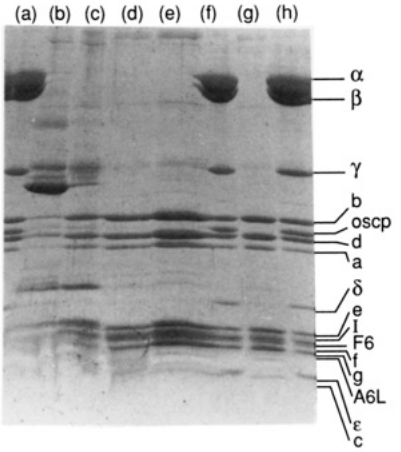

FIGURE 2: Protein compositions of fractions containing bovine F_o during its purification. The samples (see Figure 1) were analyzed by polyacrylamide gel electrophoresis under denaturing conditions (Laemmli, 1970) and stained with Coomassie blue dye. Lanes (a), (f), and (h), F_1F_o-ATPase purified from bovine heart mitochondria (Buchanan & Walker, 1993, 1994); lane (b), 1% *n*-dodecyl β-D-maltoside extract of stripped submitochondrial particles; lane (c), partially pure F_o from Q-Sepharose column; lane (d), F_o after S-Sepharose column; lanes (e) and (g), F_o after dye ligand chromatography, with 3 times more material loaded in lane (e) than in lane (g). The identities of the various subunits are indicated at the right-hand side.

identified as the ADP/ATP translocase (I. R. Collinson and J. E. Walker, unpublished results). Substantial quantities of cytochrome aa_3 were also present in the extract. The bands in the polyacrylamide gel attributable to F_o subunits (see subunits b and d, for example) were quite weak at this stage. The ADP/ATP translocase and cytochrome aa_3 were both removed from the F_o by anion-exchange chromatography on Q-Sepharose [see Figures 1a and 2c]. The former protein did not bind to the anion exchanger, whereas cytochrome aa_3 bound more strongly than F_o. After this stage of the purification, the bands of F_o subunits were more pronounced [see Figure 2c], but substantial quantities of impurities remained. Most of these impurities were removed by cation-exchange chromatography [Figures 1b and 2d]. The subsequent dye ligand chromatography (Figure 1c) was effective in removal of traces of other more minor impurities that remained after the cation-exchange step [Figure 2e]. From 1.8 g of mitochondrial membranes about 15 mg of pure F_o was recovered. For comparison, the yield of F_1-ATPase from the same amount of membranes is about 40 mg (Lutter et al., 1993). When the molecular masses of F_1 and F_o are taken into account, this corresponds to about 24 mg of F_o. The relatively lower yield of F_o is probably a consequence, at least in part, of the larger number of chromatographic steps that are needed to purify it.

During the purification of F_o, its interaction with the inhibitor dibutyltin 3-hydroxyflavone was monitored. These measurements (summarized in Figure 3 and Table 1) showed that the binding activity was retained during the purification. It is also clear that the binding of the inhibitor is reversed by both venturicidin and tributyltin, confirming previous work that the binding sites for the three inhibitors overlap (Griffiths et al., 1993).

In the presence of 1 M sodium chloride in F_o buffer, the freshly prepared F_o is monodisperse, as judged by gel filtration chromatography through Sephacryl S-300. On prolonged storage of the complex at −20 °C, or at a salt concentration of 0.1 M, up to 50% of a dimeric form of the complex is also present.

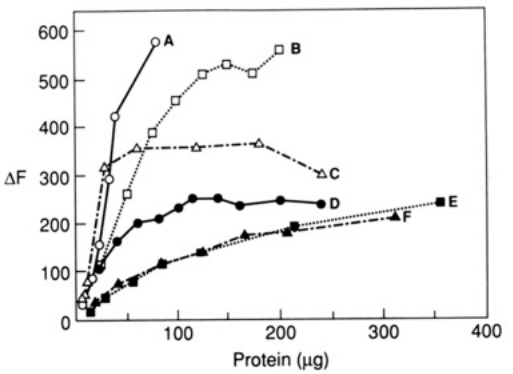

FIGURE 3: Fluorescence enhancement on binding of dibutyltin 3-hydroxyflavone to F_o from bovine heart mitochondria. The following samples were tested: A, F_1F_o-ATPase reconstituted from purified F_o, F_1, and OSCP; B, F_1F_o-ATPase purified from mitochondria; C, partially pure F_o obtained after chromatography on Q-Sepharose and Mono S; D, extract of submitochondrial particles with 1% *n*-dodecyl β-D-maltoside; E, bovine heart mitochondria; F, submitochondrial particles. One nanomole of reagent was added in each experiment, except for C, where half that amount was employed (in which case the fluorescence shown in the figure was halved). For further details, see Materials and Methods.

Table 1: Competition of Binding of Dibutyltin 3-Hydroxyflavone to F_o and F_1F_o-ATPase by Venturicidin and Tributyltin Acetate

			residual ΔF (%) after		
			venturicidin		tributyltin
sample	protein (μg)	ΔF^a	2 μg	20 μg	10 nmol
F_1F_o (purified)	200	516	94	62	9
F_1F_o (purified)	100	412	82	53	14
F_1F_o (purified)	50	256	80	63	26
F_1F_o (reconstituted)	80	548	57	16	4
1% DM extract	100	213	57	31	15
F_o (QS)[b]	95	377	71	36	2
F_o (QSY)[c]	70	300	61	23	5

[a] ΔF, change in fluorescence emission. [b] Purified on Q-Sepharose and Mono S. [c] Purified on Q-Sepharose, Mono S, and reactive yellow dye.

Subunit Composition of Bovine F_o. After separation of the subunits of the F_o preparation on polyacrylamide gels in the presence of dodecyl sulfate, six bands in two separate clusters of three were found to stain strongly with Coomassie blue dye [see Figures 2e, 2f, and 4]. In addition, two weakly staining bands could be seen, one immediately below the lower cluster and the other close to the bottom of the gel. These latter proteins are the hydrophobic subunits A6L and c, respectively, as was confirmed by protein analysis (see below). Both are intrinsic membrane components of bovine ATP synthase, and are known to stain weakly with Coomassie blue dye (Fearnley & Walker, 1986; Walker et al., 1991). There is probably 1 copy of A6L per complex and 6–12 copies of subunit c. An additional difficulty with subunit c is that it forms oligomers which often persist in the presence of SDS, and run at various positions in PAGE–SDS. The faintly staining band between subunits a and e in Figure 4 (right-hand lane) is an example of an oligomer of subunit c.

The migration positions of the upper strongly staining triplet corresponded with those of subunits b, d, and a. In other gels, subunit a, which like subunits c and A6L is also hydrophobic, stained less strongly with the dye. It was clear that the OSCP, an important component of the stalk region of ATP synthase that migrates between subunits b and d in samples of the intact F_1F_o-ATPase, was not present in the F_o preparation (see Figure 4). The migration positions of the components of the lower triplet corresponded with those of subunits e, F_6, and the ATPase inhibitor protein in F_1F_o-ATPase. The

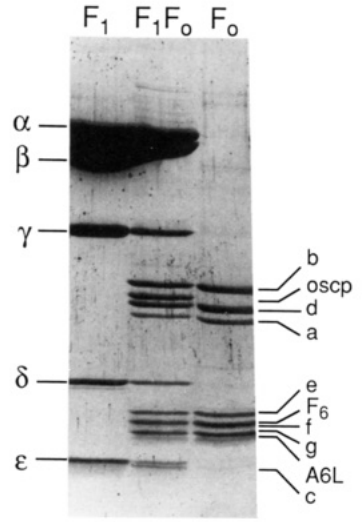

FIGURE 4: Comparison by gel electrophoresis of subunit compositions of F_1-ATPase, F_1F_o-ATPase, and F_o from bovine heart mitochondria. Note that band c did not stain with Coomassie blue dye. Its presence in the indicated position was determined by N-terminal sequence analysis. For further experimental details, see the legend to Figure 2 and Materials and Methods.

Table 2: Identification of Subunits of F_o from Bovine Heart Mitochondria

subunit	molecular mass (daltons) from sequence	molecular mass (daltons) by es-ms	N-terminal sequences
a	24815[a]	nd	MNXNLF[c]
b	24670	24671.9	PVPPL
d	18603[b]	18604.5	none
F_6	8958	8959.2	NKELD
e	8189	8189.4	VPPVQ
f	10209[b]	10209.2	none
g	11328[b]	11328.0	none
A6L	7964[a]	7964.1	MPQLD[c]
c	7608	nd	DIDTA

[a] Including *N*-formyl group; es-ms, electrospray mass spectrometry; nd, not determined. [b] Including *N*-α-acetyl group. [c] After treatment with anhydrous methanolic HCl.

presence of a band in F_o with the same apparent molecular weight as the ATPase inhibitor subunit, which is thought to bind to the β-subunit of F_1-ATPase, was initially surprising. The identities of subunits b, F_6, e and c were readily confirmed by N-terminal sequence analysis, as were those of a and A6L after removal of their α-*N*-formyl residues (see Table 2). No N-terminal sequence was obtained from the remaining bands. The N-terminal alanine residue of subunit d has been shown previously to be acetylated (Walker et al., 1987).

In order to investigate the unidentified components of F_o, its subunits were fractionated by HPLC (Figure 5a), and characterized by electrospray ionization mass spectrometry and N-terminal sequencing. Subunits b, F_6, and e were again recognized from their N-terminal sequences, and their presence and those of subunits d and A6L were confirmed by mass spectrometry. Two other prominent peaks labeled f and g in the HPLC trace (Figure 5a) could not be identified at this stage. Both had modified N-terminals, and their molecular masses (10 209 and 11 328 daltons, respectively) did not correspond with that either of the inhibitor protein (molecular mass 9581 daltons) or of any other known component of bovine F_1F_o-ATPase and the bovine respiratory complexes. This conclusion was confirmed by partial protein sequences determined from cyanogen bromide digests of both proteins. Therefore, subunits f and g were new and hitherto undetected subunits of the enzyme.

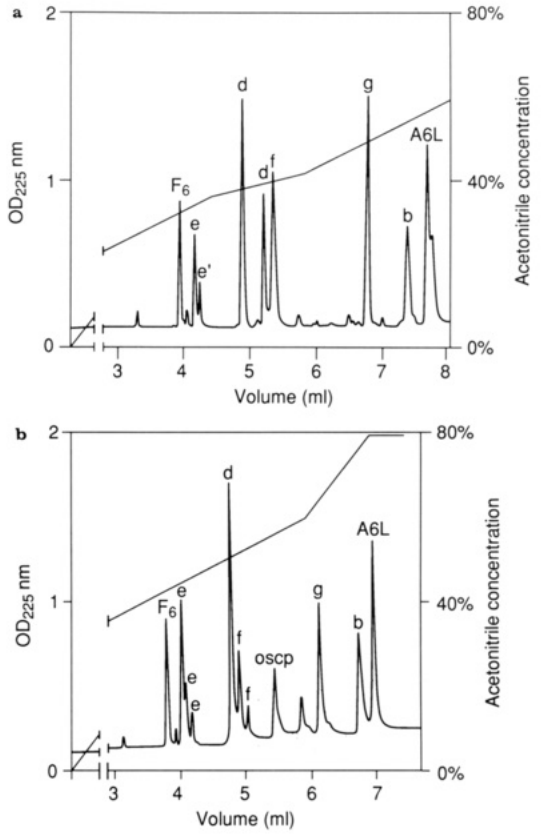

FIGURE 5: Separation of the subunits of F_o and F_1F_o-ATPase from bovine heart mitochondria. Part a, F_o; part b, F_1F_o-ATPase. For further details concerning the chromatography, see Materials and Methods. The subunits were identified by N-terminal sequence analysis and by electrospray mass spectrometry (see Table 2); e′ and e″ denote degraded forms of subunit e lacking one and two amino acids, respectively, from the N-terminus. In part b, F_1 subunits were recovered together in low yield in the unmarked peak between subunits OSCP and g. The hydrophobic subunits a and c were not recovered in either part a or part b.

In confirmation of this finding, the presence of subunits f and g in a pure sample of F_1F_o-ATPase was demonstrated by the fractionation of its subunits by HPLC under similar conditions to those employed for the fractionation of the subunits of F_o. Peaks eluting in the same positions as subunits f and g in F_o were also present in the F_1F_o-ATPase (see Figure 5b). Both proteins had modified N-terminals, and their molecular mass values determined by mass spectrometry were 10 209 and 11 328 daltons, the same values, respectively, as determined for subunits f and g isolated from F_o.

The molar ratios of subunits f and g relative to other subunits of F_o and F_1F_o-ATPase have not been measured accurately. However, it is clear from Figure 5a,b and also from other similar experiments (not shown) that both subunits are present in approximately equal amounts, and that their levels are comparable with that of F_6, for example. Therefore, it is tentatively proposed that there may be one of each subunit per F_o and F_1F_o complex.

Sequences of Subunits f and g. The complete protein sequences of subunits f and g were determined by making use of the partial protein sequences determined from fragments isolated from cyanogen bromide digests. In subunit f, two such sequences were obtained, following methionines-25 and -61 in the completed sequences (see Figure 6). Two pairs of oligonucleotide mixtures of 17mers, based on the protein sequences 1F and 1R, and 2F and 2R, were then used as primers in polymerase chain reactions with total bovine cDNA as template, as described before (Walker et al., 1992). Partial

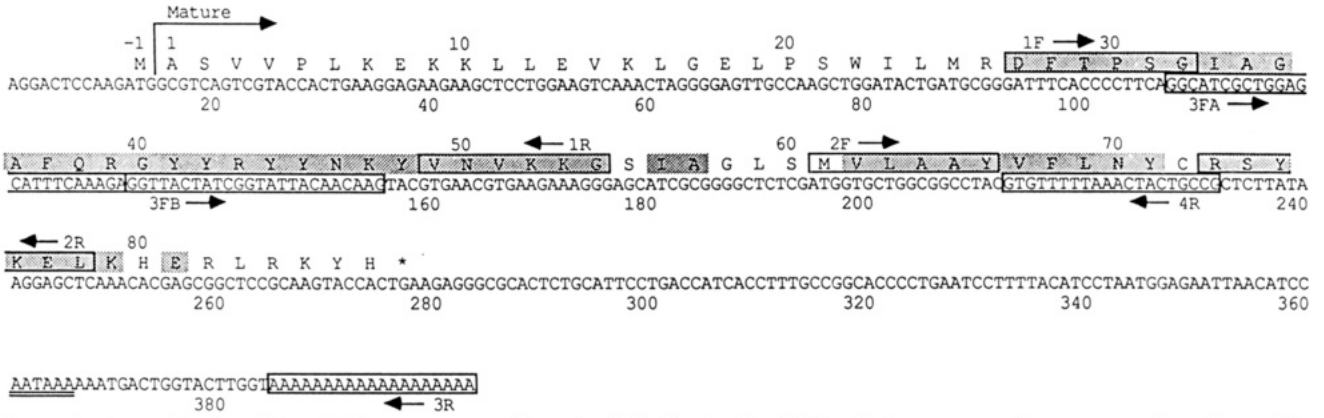

FIGURE 6: Determination of the cDNA sequence encoding subunit f of bovine F_o. N-Terminal sequences of two cyanogen bromide peptides produced by cleavage after amino acids 25 and 61 are shaded. In the former, arginine-26 was not identified. Boxed protein sequences 1F and 1R and 2F and 2R were used as the basis of synthetic oligonucleotide mixtures, which were employed as primers in two independent polymerase chain reactions with total bovine heart cDNA as template, to amplify partial cDNAs coding for the two segments of protein sequence. In 2F, the presence of methionine-61 was assumed from the cyanogen bromide cleavage site. Unique oligonucleotide primers were made from boxed nucleotide sequences 3FA, 3FB, 3R, and 4R (see Results). The DNA sequence shown in the figure is compiled from the overlapping partial cDNAs. The doubly underlined nucleotide sequence is a polyadenylation signal.

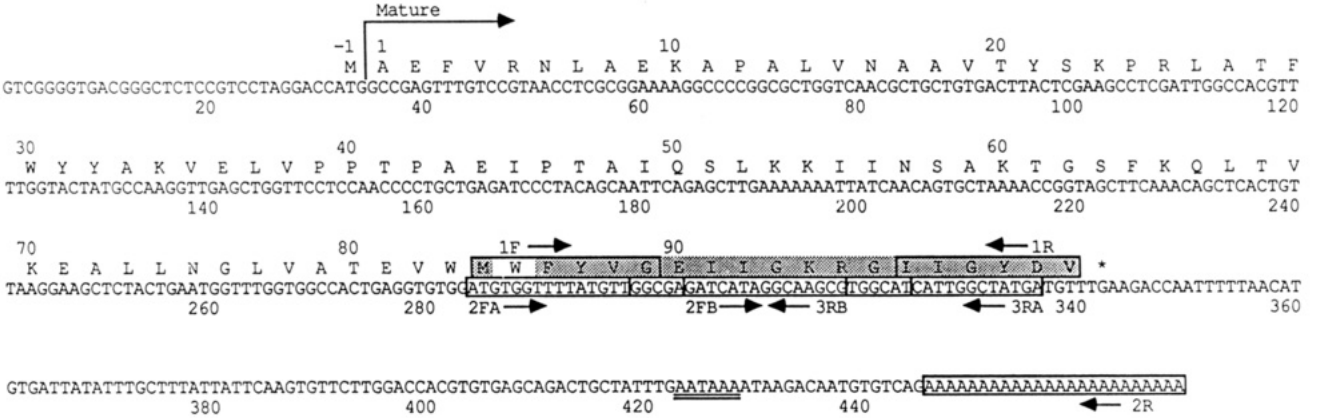

FIGURE 7: Determination of the cDNA sequence encoding subunit g of bovine F_o. The N-terminal sequence produced by cyanogen bromide cleavage after methionine-84 is shaded. Residue 85 was wrongly identified as homoserine (methionine). In consequence, the mixed oligonucleotide primer based on 1F was made with ATG rather than the correct sequence TGG in these positions, and was employed in the initial polymerase chain reaction with a second mixed primer based on 1R. Boxed nucleotide sequences contain the sequences of unique oligonucleotide primers 2FA, 2FB, 3RB, and 3RA. The doubly underlined sequence is a polyadenylation signal.

cDNAs representing these segments of protein sequences were isolated with the aid of mixed oligonucleotide probes based on amino acids 33–38 and 66–71, respectively. Unique oligonucleotides based on DNA sequences 3FA and 3FB determined between primers 1F and 1R were then used with primer 3R to generate a partial cDNA extending to the 3′ extremity of the complete cDNA. In a similar experiment, unique primer 4R was employed with oligo(dC) as forward primer using bovine cDNA to which oligo(dG) had been added to the 5′ end with terminal transferase. In this way, the total cDNA sequence was generated from the four overlapping partial cDNAs.

The cDNA sequence encoding subunit g was obtained by a similar route (summarized in Figure 7). Mixed oligonucleotide primers 1F and 1R were used to generate the first partial cDNA representing the known segment of protein sequence (shaded in Figure 7). This initial sequence was recognized by hydridization with a third mixed oligonucleotide based on amino acids 90–95. Nested oligonucleotide primers 2FA and 2FB were used with reverse primers 2R to extend the sequence to the 3′ end of the cDNA, and nested primers 3RA and 3RB were used with oligo(dC) to obtain a cDNA extending to the 5′ end of the cDNA.

The cDNA sequences of subunits f and g encode mature proteins of 87 and 102 amino acids, respectively. The molecular weights measured by mass spectrometry are both 42 mass units greater than the values calculated from the protein sequence. Therefore, it appears that both proteins are N-acetylated. In both cases, the codon for the N-terminal alanine residue is preceded by an ATG codon. In subunit g, this must be the translational initiator since the cDNA sequence to the 5′ side of the ATG codon contains an in-phase TGA "stop" codon, and in subunit f, it is likely to have the same function, although this is not absolutely certain. Therefore, it appears that neither subunit has a processed N-terminal extension to direct the protein into the mitochondrion. The mitochondrial targetting information must therefore be in the mature protein, as it is also in a large number of other proteins in the inner mitochondrial membrane [see Walker et al. (1992)].

Reconstitution of Purified F_o with F_1-ATPase and OSCP. The purified bovine F_o was mixed with purified OSCP and F_1-ATPase (approximate molar proportions 1.0:1.5:1.5, respectively). This material was then passed through a Sephacryl S-300 gel filtration column. Analysis of the fractions from this column (Figure 8) by gel electrophoresis showed that the first peak contained material that in terms of its subunit composition appeared to be indistinguishable from authentic purified F_1F_o-ATPase, except that the reconstituted enzyme did not contain any of the ATPase inhibitor protein, since this

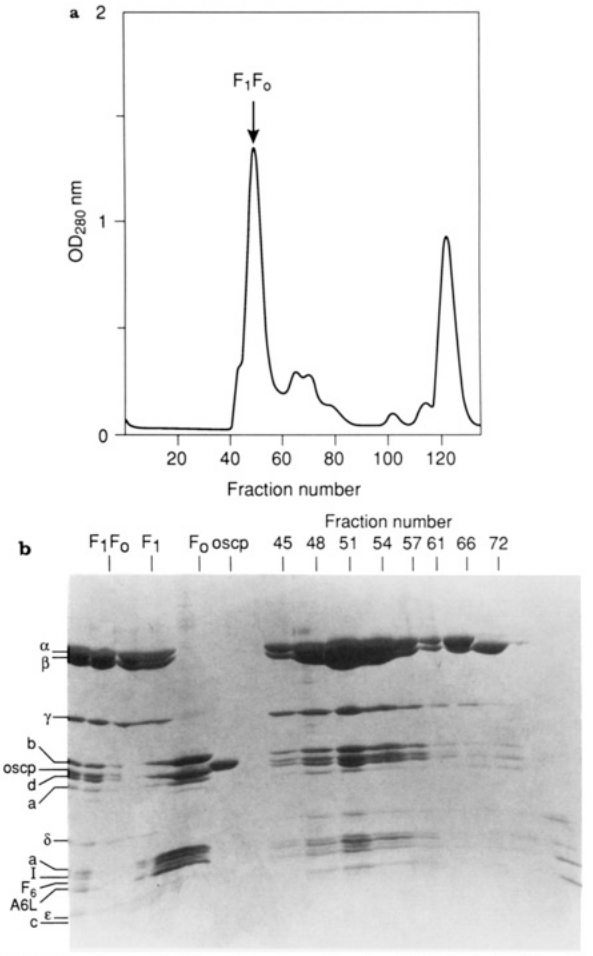

FIGURE 8: Reconstitution of bovine mitochondrial F_1F_o-ATPase from F_1-ATPase, OSCP, and purified F_o. (a) Gel filtration chromatography of reconstituted material on a Sephacryl S-300 column (60 cm × 1.5 cm i.d.). The volume of the fractions was 1 mL. (b) Analysis by gel electrophoresis under denaturing conditions of fractions from part a.

subunit is removed during the purification of F_1-ATPase. Therefore, the purified F_o retains the ability to bind F_1-ATPase in the presence of the OSCP (see Figure 8), as has been demonstrated previously with unpurified F_o in mitochondrial membranes (Kagawa & Racker, 1966b). Moreover, no free F_o was recovered in this experiment, and therefore all of it was reconstituted into the F_1F_o-ATPase complex. In a control experiment in which OSCP was omitted, as expected, there was no association between F_1 and F_o. The reconstituted F_1F_o-ATPase bound the dibutyltin 3-hydroxyflavone more strongly than a sample of highly purified F_1F_o-ATPase (see Figure 3, part A). The effect of oligomycin on the activity of the reconstituted F_1F_o-ATPase complex has not yet been studied.

DISCUSSION

Crystallization of membrane proteins is difficult, and before undertaking the extensive trials that are usually required for obtaining suitable crystals, it is desirable that the proteins should be as pure as possible and be monodisperse. In the past, several procedures have been described for preparing F_o, the membrane domain of ATP synthase, from the inner membranes of mitochondria. The resulting preparations retained the proton-transporting properties associated with F_o, and could be reassembled with F_1 and OSCP to restore the original complex. However, none of them is suitable for crystallization trials, since they are all polydisperse and contain substantial amounts of phospholipids and contaminating proteins. The method described above for preparing F_o combines an effective procedure for stripping F_1-ATPase and other peripheral membrane proteins from mitochondria (McEnery et al., 1989), together with a detergent extraction and chromatographic method that has been devised for making highly pure F_1F_o-ATPase from bovine heart mitochondria (Buchanan & Walker, 1993, 1994). This preparation is devoid of any unidentified major protein contaminants, as shown by silver staining of the gels in Figure 4. If it is freshly prepared and at an appropriate salt concentration, it behaves as a mondisperse complex in gel filtration experiments. Therefore, the preparation fulfills important criteria of suitability for crystallization trials. Since the F_1F_o-ATPase prepared in the same detergent and by a similar chromatographic procedure has no associated phospholipids (Buchanan & Walker, 1994), it is likely that the F_o is similarly devoid of phospholipids, although its phospholipid content has not yet been measured.

Two important characteristics of the purified bovine F_o are described in this paper. They are its subunit composition and its in vitro reassembly with OSCP and F_1-ATPase to form F_1F_o-ATPase. A third important property, the proton-translocating properties of the preparation, will be described elsewhere (H. S. van Walraven, I. R. Collinson, and J. E. Walker, in preparation).

The subunit composition of the purified F_o complex has been examined in two independent ways. First, the subunits have been separated on polyacrylamide gels under denaturing conditions, and stained sequentially with Coomassie blue dye and then with silver. The main difficulties here are that hydrophobic proteins (such as subunits c and A6L, and also a) stain much more weakly than other more hydrophilic subunits, although they are present in equivalent molar quantities. They can, however, be detected by silver staining. The problem with subunit c is particularly severe as it also tends to be present on gels in more than one oligomeric form. Therefore, it cannot be entirely excluded that one or more hydrophobic subunits remains to be detected, although no extra major band was revealed by the silver stain, and the additional weakly silver staining bands were attributed as being background contaminants.

The second way in which the complex has been examined is by HPLC fractionation of the subunits. All of the peaks in the HPLC trace were analyzed by protein sequencing and by electrospray ionization mass spectrometry, leading to the detection of subunits f and g. No additional proteins that are not known components of F_o were found in these experiments. The difficulty here is that unknown hydrophobic subunits could bind so strongly to the HPLC column that they are not recovered, as indeed happens with subunits a and c. Therefore, it is difficult to be absolutely certain from these experiments that no additional subunits of bovine F_o or of F_1F_o-ATPase remain to be discovered. In the past, it has been suggested that a protein known as factor B is a component of the bovine system (Sanadi et al., 1982). Despite extensive searches using protein chemical techniques, we have never detected factor B in bovine F_1F_o-ATPase (Walker et al., 1991), and it does not seem to be present in the F_o preparation either. We are currently developing immunological reagents to try and resolve this matter.

It is unlikely that it will be possible to establish the final subunit composition of bovine F_1F_o-ATPase with absolute certainty until it can be reconstituted in an active form from its purified constituent subunits. The in vitro reconstitution of subunits b, d, OSCP, and F_6 with F_1-ATPase into a nine-subunit complex is a significant step toward this goal (Collinson

et al., 1994). On the basis of existing information, purified bovine F_o appears to be a complex of nine different polypeptides. All of them are present in the highly purified bovine F_1F_o-ATPase, which also contains OSCP, the inhibitor protein, and the five subunits of F_1-ATPase. Therefore, bovine heart F_1F_o-ATPase is probably an assembly of 16 different proteins, which is 2 more than had been detected in previous studies (Walker et al., 1991). The two new subunits, name f and g, have modified N-terminals and have apparent molecular weights close to those of subunits F_6, e, A6L, and the inhibitor protein, from which they are difficult to resolve on polyacrylamide gels. For these reasons, their presence in F_1F_o-ATPase was overlooked in the past. The sequences of subunits f and g are unrelated to any known sequence, and they offer no clues about their functions. The hydrophobic profile of subunit f (not presented) shows that it has one particularly hydrophobic region from amino acids 55–71 that is likely to form a membrane-spanning α-helix. There are no such regions in subunit g. It remains to be demonstrated that subunits f and g are present in other preparations of bovine F_1F_o-ATPase, and that there are homologues in other mammalian mitochondrial ATPases. Similar reservations were expressed when subunit e was first discovered, but it is now a firmly established component of the bovine ATPase complex. It has been found in several preparations, and a rat homologue has been identified (Higuti et al., 1992).

The proton-translocating properties of the purified F_o will be described elsewhere, but the pure F_o described in this paper has lost its proton specificity and will also transport K^+ ions in a voltage-dependent manner (H. Miedema, H. S. van Walraven, I. R. Collinson, J. E. Walker, and A. H. de Boer, unpublished work).

Apart from its proton-transporting activity, the purified bovine F_o retains a second important property of the F_o in mitochondria, namely, its ability to reassociate with F_1-ATPase in the presence of OSCP. Many of the properties of the reconstituted F_1F_o-ATPase remain to be studied, but its dibutyltin 3-hydroxyflavone binding properties closely resemble those of purified F_1F_o-ATPase.

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