ATP synthase from bovine heart mitochondria: reconstitution into unilamellar phospholipid vesicles of the pure enzyme in a functional state

Georg GROTH* and John E. WALKER†

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

A highly purified and monodisperse preparation of proton-translocating F_1F_o-ATPase from bovine heart mitochondria is an assembly of 16 unlike polypeptides. This preparation has been reconstituted in the presence of various detergents into unilamellar phospholipid vesicles. Incorporation of the enzyme into vesicles increases the ATP hydrolase activity of the enzyme by 10–20-fold, depending on the detergent, and the highest activities of ATP hydrolysis, 70 units/mg, were obtained by reconstitution from dodecylmaltoside or CHAPS. This activity is mostly sensitive to inhibitors that act on the F_o membrane sector of the complex. From the quenching of the pH-sensitive probe, 9-amino-6-chloro-2-methoxyacridine, it was shown that the reconstituted enzyme was able to form a transmembrane proton gradient in an ATP-dependent manner. By co-reconstitution of the enzyme with bacteriorhodopsin, it was demonstrated that in the presence of a light-induced proton gradient the enzyme can synthesize ATP from ADP and phosphate. Therefore, the characteristic biological functions of the F_1F_o-ATPase in mitochondria have been demonstrated with the purified enzyme. Thus, in terms of both its physical and biochemical properties, the purified enzyme fulfils important pre-requisites for formation of two- and three-dimensional crystals.

INTRODUCTION

An understanding of the mechanism of ATP synthesis in eubacteria, mitochondria and chloroplasts would be greatly aided by an atomic structure for the proton-translocating F_1F_o-ATP synthase. Progress has been made with the F_1 catalytic domain of the enzyme from bovine heart mitochondria, as its atomic structure has been established by X-ray analysis of crystals grown in the presence of both ADP and the ATP analogue, β,γ-imidoadenosine 5′-triphosphate (AMP-PNP) [1]. In this structure, the three catalytic β-subunits have different conformations and nucleotide contents: one of them contains AMP-PNP, the second contains ADP, and the third is distorted and empty. Therefore, the structure is in accord with a binding change mechanism of ATP synthesis, which proposes that at any instant the catalytic sites are in different states, but each of them passes through the same cycle of interconverting conformations [2]. Thus, although the three catalytic sites differ at the moment of capture in the crystal structure, they are equipotent. The structure of bovine F_1-ATPase also suggests that the different conformations of the three catalytic sites may be interconverted by the rotation of a central α-helical structure, and recent experiments with the bacterial enzyme support this idea [3,4]. However, there is still little understanding of how energy released by proton translocation through the membrane sector of the enzyme is transmitted to the catalytic sites of the enzyme via the stalk that links the F_1 and membrane domains, and, if the enzyme has a mechanism based on rotation, how such a rotation could be generated.

One approach to trying to provide a framework for answering these and related questions involves determining the atomic structure of the intact F_1F_o-ATPase, and with this objective in mind a procedure has been developed for the purification of the intact enzyme complex from bovine heart mitochondria. As discussed in the accompanying paper [5], this preparation fulfils important requirements for formation of three-dimensional crystals for X-ray crystallographic analysis of a membrane-bound complex, namely that it should be highly pure and monodisperse. In addition, it is helpful in attempting to grow two-dimensional crystals of F_1F_o-ATPase to be aware of the conditions under which the purified complex can be re-incorporated into membranes [6]. For any future structural analysis, it is also desirable that the purified enzyme should retain the ability to generate a proton gradient by using energy released from ATP hydrolysis, and that in the presence of a proton gradient, it should be able to synthesize ATP from ADP and inorganic phosphate. In the present paper, it is demonstrated that the pure bovine F_1F_o-ATPase complex fulfils these latter requirements.

MATERIALS AND METHODS

Materials

n-Dodecyl-β-D-maltoside was obtained from Calbiochem Novabiochem Ltd. (Nottingham, U.K.). The detergents CHAPS, CHAPSO, cholate, n-octyl-β-D-glucopyranoside and Triton X-100, the inhibitors venturicidin, oligomycin, *N*,*N*′-dicyclohexylcarbodiimide (DCCD) and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), and phospholipids were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Lauryldimethylamine oxide (LDAO) was supplied by Fluka (Dorset,

Abbreviations used: ACMA, 9-amino-6-chloro-2-methoxyacridine; BR, bacteriorhodopsin; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulphonate; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; LDAO, lauryldimethylamine oxide; MF_1F_o, mitochondrial F_1F_o-ATPase; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

* Present address: University of Düsseldorf, Department of Plant Biochemistry, D-40225 Düsseldorf, Universitätsstr. 1, Germany.

† To whom correspondence and reprint requests should be sent.

U.K.). ATP Bioluminescence Assay Kits CLS II, lactate dehydrogenase (EC 1.1.1.27), pyruvate kinase (EC 2.7.1.40) and phosphoenolpyruvate were obtained from Boehringer Mannheim Ltd. (Lewes, U.K.).

Protein analysis

Protein concentrations were determined by the bicinchoninic acid assay (Pierce Chemicals, Rockford, IL, U.S.A.) and by the Bio-Rad protein assay (Bio-Rad, Herts., U.K.), using BSA as a standard. The subunit composition of purified mitochondrial F_1F_o-ATPase (MF_1F_o) was analysed in SDS/PAGE gradient gels (10–25% acrylamide) in a buffer system described by Laemmli [7]. After electrophoresis, proteins were detected by silver staining [8].

Purification of MF_1F_o

The purification of MF_1F_o from bovine heart mitochondria is described elsewhere [5]. Samples of the purified enzyme (1–2 mg/ml) were stored at −20 °C in a buffer containing 20 mM Tris, pH 8.0, 200 mM sucrose, 100 mM sodium chloride, 3 mM magnesium sulphate, 2 mM GTP, 20% (w/v) glycerol and 8% (w/v) glycine.

Preparation of liposomes

Large unilamellar phospholipid vesicles were prepared from phosphatidylcholine and phosphatidic acid by reverse-phase evaporation [9]. Liposomes with a uniform size distribution were obtained by successive filtration through two polycarbonate filters (0.4 and 0.2 μm pore size; Millipore Corporation, Bedford, MA 011730, U.S.A.). Stocks of liposomes were stored at 4 °C at a phospholipid concentration of 18 mg/ml in a buffer containing 20 mM Tricine, pH 8.0, 40 mM sodium sulphate, 20 mM succinate and 5 mM 2-mercaptoethanol.

Reconstitution of MF_1F_o into liposomes

A mixture of the preformed phospholipid vesicles (4 mg/ml), detergent, magnesium sulphate (2.5 mM), GTP (2 mM) and MF_1F_o was kept at room temperature for 60 min. Then the detergent was removed by stepwise addition of Biobeads SM2 [10].

ATP hydrolysis by reconstituted liposomes

The ATPase activity of MF_1F_o reconstituted into phospholipid vesicles was determined by a coupled assay in which ADP production was linked to NADH oxidation [11]. The reaction was started by addition of 0.1 ml of phospholipid vesicles (4 mg/ml) containing reconstituted MF_1F_o (0.016 mg/ml) to a reaction mixture (2.9 ml) consisting of 50 mM Tris-acetate, pH 7.4, 1 mM magnesium chloride, 2 mM phosphoenolpyruvate, 2 mM ATP, 0.2 mM NADH, 15 units/ml of lactate dehydrogenase and 50 units/ml of pyruvate kinase. Oxidation of NADH at 37 °C was monitored spectrophotometrically at 340 nm.

ATP–P_i exchange activity of reconstituted MF_1F_o

ATP–P_i exchange was measured by incubating the vesicles (0.4 ml) containing the reconstituted enzyme at a protein to lipid ratio of 1:10 (w/w) in a medium (600 μl) containing 50 mM Tris-acetate, pH 7.4, 10 mM magnesium chloride, 3 mM ATP, 5 mM ADP and 10 mM phosphate ($[^{32}P]P_i$, 10 μCi). The reaction was stopped at various times by the addition of 3 M perchloric acid (110 μl). The amount of $[^{32}P]P_i$ incorporated into ATP was determined by precipitating the remaining inorganic phosphate as the phosphomolybdate complex, and by measuring the residual radioactivity in the supernatant [12].

ATP-induced transmembrane proton gradient in reconstituted liposomes

ATP-dependent proton translocation by MF_1F_o was monitored by fluorescence quenching of 9-amino-6-chloro-2-methoxyacridine (ACMA) [13]. Proteoliposomes containing reconstituted mitochondrial ATPase (0.1 mg) were resuspended in buffer containing 20 mM Tricine, pH 8.0, 40 mM sodium sulphate, 20 mM succinate and 5 mM magnesium chloride at a protein to lipid ratio of 1:10 (w/w), and at a final phospholipid concentration of 4 mg/ml. The final reaction volume was 0.5 ml. Formation of the transmembrane proton gradient was started by the addition of 3 mM ATP (15 μl).

Co-reconstitution of bacteriorhodopsin (BR) and MF_1F_o into liposomes

Phospholipid vesicles were prepared in the presence of purple membranes at a lipid to protein ratio of 10:1 (w/w; [14]). Vesicles were kept in the dark during the preparation, and were stored at 4 °C at a final BR concentration of 2 mg/ml in a buffer consisting of 20 mM Tricine, pH 8.0, 40 mM sodium sulphate, 20 mM succinate and 5 mM 2-mercaptoethanol. Bovine MF_1F_o was incorporated into these liposomes (BR-liposomes) by detergent-mediated reconstitution using the zwitterionic detergent CHAPS. BR-liposomes were incubated at room temperature for 10 min in the presence of CHAPS (1.5 mg/ml). Then magnesium sulphate (2.5 mM), GTP (2 mM) and MF_1F_o (0.016 mg/ml) were added. After 60 min, the detergent was absorbed by Biobeads SM2 added in 40 mg amounts. A total of 160 mg of Biobeads was required for complete removal of the detergent. The liposomes were separated from the Biobeads and stored at 4 °C in the dark.

ATP synthesis in co-reconstituted liposomes

Liposomes containing reconstituted BR and MF_1F_o at a ratio of 100:1 (w/w) were suspended in a medium consisting of 20 mM Tricine, pH 7.8, 40 mM potassium sulphate, 20 mM sodium sulphate, 10 mM succinate, 5 mM magnesium sulphate, 10 mM potassium dihydrogen phosphate and 0.75 mM ADP. The stirred mixture was illuminated with a Kodak projector S-AV 2010 at a distance of 10 cm. UV light was removed from the illuminating beam with a plastic filter. The vial containing the reaction mixture (1.5 ml) was placed in a transparent water bath at 37 °C and the reaction was started by illumination. Samples of the reaction mixture (150 μl) were withdrawn at various intervals, and the reaction was quenched by adding the samples to trichloroacetic acid (40 g/l; 150 μl). Samples were titrated with 1 M Tris to pH 7.0, and ATP was estimated by a luciferin–luciferase assay with an ATP Bioluminescence Kit CLS II.

Electron microscopy

Liposomes were adsorbed on carbon-coated grids that had been flow-discharged in pentylamine vapour. The grid was washed with three drops of distilled water, stained with uranyl formate (0.75%, w/v; pH 4.25), and blotted with filter paper. Specimens were examined in a Philips CM 12 electron microscope operated at 120 kV.

RESULTS

Subunit composition of bovine MF_1F_o-ATP synthase

The subunit composition of purified bovine MF_1F_o [5] was verified by SDS/PAGE. On a silver-stained gel (see Figure 1) subunits α, β, γ, δ and ϵ of the membrane associated F_1-ATPase domain could be clearly identified, together with subunits a, b, d and OSCP (components of the F_o integral membrane domain) and the associated stalk domain. Another group of partially resolved proteins with lower molecular masses is also apparent, and it is known from other experiments to contain subunits e, I, f, g, F_6 and A6L [15]. Conditions have not yet been established for complete resolution of these bands on SDS/PAGE gels. Finally, at the bottom of the gel the hydrophobic subunit c can be identified near to the ϵ-subunit. However, no band was observed on the gel that could be attributed to factor B, a 28 kDa polypeptide that has been reported to be an essential subunit of mitochondrial ATPases required to catalyse ATP–P_i exchange [16]. A striking feature of the gel is that all of the bands that were detected can be attributed to known subunits of bovine F_1F_o-ATPase, demonstrating the high purity of the enzyme preparation.

Solubilization of phospholipid vesicles

The solubilization of the phospholipid vesicles by stepwise addition of various detergents was monitored from changes in the turbidity of the liposome solution [9]. The resulting absorption curves at 560 nm, obtained in the presence of the zwitterionic detergents CHAPS, CHAPSO and LDAO, of the non-ionic detergents n-dodecyl-β-D-maltoside, n-octyl-β-D-glucopyranoside and Triton X-100, and of the anionic detergent cholate, are shown in Figures 2(A)–2(G). During solubilization, at least two distinct phases can be distinguished. In the cases of Triton X-100, sodium cholate and LDAO, solubilization of the vesicles follows the three-stage model described by Lichtenberg et al. [17]. In the first phase, which is related to minor changes in turbidity, detergent-saturated vesicles form. In the second phase, where solubilization of the vesicles takes place, remarkable changes in turbidity are found. In the third phase, where the turbidity is low, solubilization of the vesicles is complete. With CHAPS and CHAPSO, only two phases were observed. They are equivalent to phases two and three observed with Triton X-100, sodium cholate and LDAO. With n-dodecyl-β-D-maltoside and n-octyl-β-D-glucopyranoside, detergent-saturated vesicles form in an extended first phase, as before. In a second phase, characterized by an increase in absorption, it has been suggested that micellar aggregation occurs [18]. Finally, in the third phase, the vesicles are solubilized completely.

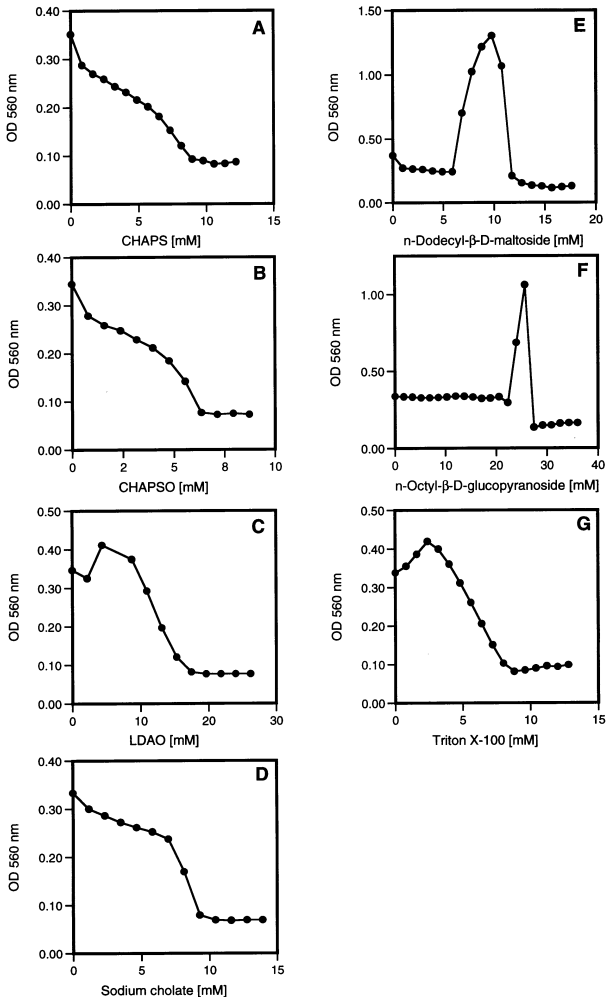

Figure 2 Changes in turbidity of phospholipid vesicles prepared by reversed-phase evaporation upon addition of various detergents

Phospholipid vesicles were resuspended in buffer containing 20 mM Tricine, pH 8.0, 40 mM sodium sulphate, 20 mM succinate and 5 mM 2-mercaptoethanol, at a final phospholipid concentration of 4 mg/ml. Then various detergents were added stepwise from stock solutions. After detergent equilibration, the turbidity (OD) of the phospholipid vesicles was measured at 560 nm.

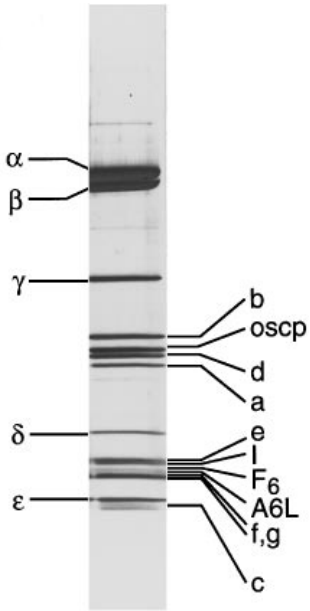

Figure 1 Subunit composition of F_1F_o-ATPase from bovine heart mitochondria

The gel was silver stained (for other details, see the Materials and methods section). The positions of the subunits of the F_1-ATPase, and of the stalk and membrane sector are shown on the left and right, respectively.

ATP hydrolysis of MF_1F_o reconstituted into phospholipid vesicles

The effects of detergents on the ATPase activity of reconstituted

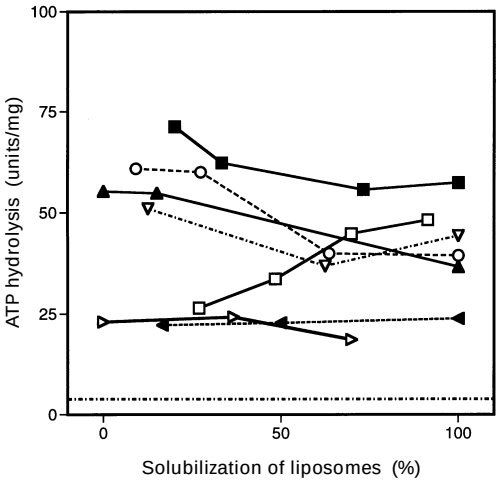

Figure 3 ATPase activity of MF_1F_o at various stages of solubilization of the phospholipid vesicles

Reconstitution of MF_1F_o was performed as described in the Materials and methods section in a medium consisting of 20 mM Tricine, pH 8.0, 40 mM sodium sulphate, 20 mM succinate, 3 mM magnesium sulphate, 2 mM GTP, 5 mM 2-mercaptoethanol and various concentrations of detergent. After 60 min, detergents were removed by stepwise addition of Biobeads SM2, and the ATPase activity was estimated according to the method of Chang and Penefsky [11]. The stage of solubilization of the vesicles was identified from measurements of turbidity (see Figure 2). Before reconstitution into phospholipid vesicles, the basal activity of MF_1F_o was 3.8 units/mg ( ). Detergents: CHAPS (○), CHAPSO (▽), n-dodecyl-β-D-maltoside (■), LDAO (▷), n-octyl-β-D-glucopyranoside (▲), Triton (□), sodium cholate (◀).

bovine MF_1F_o were studied at various solubilization stages of the preformed phospholipid vesicles. With the zwitterionic detergents CHAPS and CHAPSO, and with the non-ionic detergents n-dodecyl-β-D-maltoside and n-octyl-β-D-glucopyranoside, high ATP hydrolytic activity was found to be associated with the proteoliposomes made at a concentration of detergent corresponding to detergent-saturated vesicles (Figure 3). With vesicles made in the presence of Triton X-100, high ATPase activity was observed only when almost completely solubilized vesicles were employed. Low catalytic activities were observed when either LDAO or cholate was used in the reconstitution procedure, but even so, the observed activity was 5-fold higher than the activity of the non-reconstituted enzyme (see Figure 3).

Inhibition of ATPase activity of reconstituted MF_1F_o by inhibitors that bind to MF_o

In order to eliminate the possibility that the high ATPase activity observed after reconstitution of MF_1F_o into liposomes was caused by contaminating water-soluble F_1-ATPase, the effects of DCCD, oligomycin and venturicidin on the activity of the reconstituted enzyme were examined. In each case, the addition of the inhibitor decreased the ATPase activity by about 75% (see Figures 4A–4C). This value is similar to values reported for bovine F_1F_o-ATPase [19,20], and supports the view that the ATP hydrolysis observed after reconstitution of mitochondrial ATPase into liposomes is catalysed by F_1F_o-ATPase integrated into the membrane vesicles rather than by free F_1-ATPase.

Formation of transmembrane proton gradients in proteoliposomes by reconstituted MF_1F_o

Reconstitution of proton-transporting activity of the membrane-incorporated F_1F_o-complex was followed by measuring the ATP-induced quenching of the fluorescence of the pH-sensitive probe ACMA. The distribution of ACMA across the liposome membrane is affected by the internal and external pH values of the proteoliposome [21], and proton transport into the proteoliposomes acidifies the internal compartment, and so quenches the fluorescence of the probe [22]. As shown in Figure 5, the fluorescence of liposomes containing ACMA and MF_1F_o was quenched after addition of ATP. This ATP-induced quenching of fluorescence was abolished by the ATPase inhibitors oligomycin and venturicidin, which act on F_o, and by the uncoupler nigericin (see Figures 5A–5C). Therefore, the quenching of fluorescence was caused by the ΔpH generated across the membrane of the vesicle by MF_1F_o that had been incorporated into their membranes.

ATP–P_i exchange activity of reconstituted MF_1F_o

ATP–P_i exchange activity of mitochondrial ATPase is considered to be a characteristic of vesicles formed from inner mitochondrial membranes. Therefore, the ATP–[^{32}P]P_i exchange activity of proteoliposomes made from purified F_1F_o-complex and liposomes was investigated. Exchange activities of 5–10 nmol/min

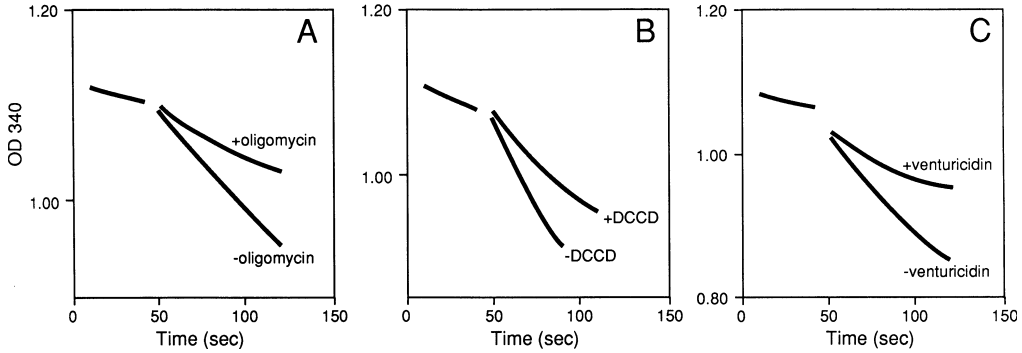

Figure 4 Effect of inhibitors of the activity of reconstituted MF_1F_o

ATP hydrolysis of reconstituted MF_1F_o was measured as described in the Materials and methods section by monitoring spectral changes (OD) at 340 nm associated with the oxidation of NADH. The reaction was started by the addition of reconstituted MF_1F_o. Activities were measured both in the absence and presence of 200 μM DCCD, 40 μM oligomycin and 20 μM venturicidin.

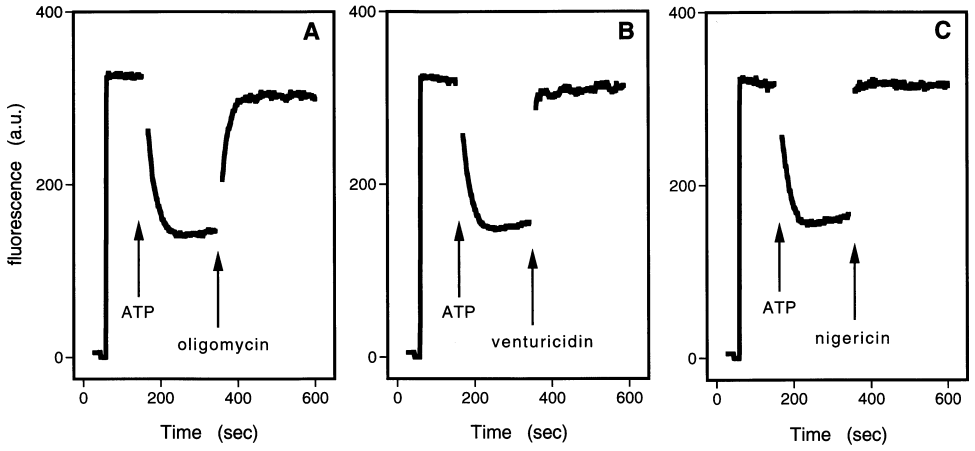

Figure 5 ATP-induced generation of a transmembrane proton gradient in proteoliposomes monitored by fluorescence quenching of the pH-sensitive probe ACMA

Phospholipid vesicles containing reconstituted MF_1F_o at a protein to lipid ratio of 1:10 (w/w) were resuspended in a buffer consisting of 20 mM Tricine, pH 8.0, 40 mM sodium sulphate, 20 mM succinate, 5 mM 2-mercaptoethanol and 1 mM magnesium sulphate. Fluorescence was monitored after addition of 10 μM ACMA. The wavelengths of excitation and emission were 400 nm and 490 nm, respectively. After addition of 3 mM ATP, the quenching of fluorescence indicated that a transmembrane proton gradient had been formed. Addition of (**A**) 60 μM oligomycin, (**B**) 10 μM venturicidin, or (**C**) 10 μM nigericin, indicated by arrows, inhibited the proton gradient generated by MF_1F_o.

Table 1 Effects of inhibitors and ionophores on the ATP–P_i exchange activity of reconstituted bovine MF_1F_o

Addition	Concentration (μM)	ATP–P_i exchange (nmol/mg per min)
Control*	–	13.2 ± 1.9
None	–	20.5 ± 0.5
DCCD	100	12.7 ± 0.9
Oligomycin	50	13.2 ± 2.1
FCCP	10	12.1 ± 2.5

* Addition of reconstituted vesicles to an incubation medium containing 0.3 M perchloric acid.

Table 2 ATP–P_i exchange and ATPase activities of various preparations of MF_1F_o

ATP–P_i exchange (nmol/mg per min)	ATP hydrolysis (μmol/mg per min)	Comments	Ref.
70	9.3	ATPase complex purified by LDAO extraction	[37]
1600	5.4	ATPase complex purified with α-lysophosphatidylcholine*	[39]
20–25	20.0	ATPase complex extracted with Triton X-100	[20]
87	11.3	Cholate extract of inner mitochondrial membranes†	[13]
350	10.0	Complex V preparation; contaminated with complexes I, II and III	[19]
190	20.0	Purified complex V preparation; contaminated with 2–3% transhydrogenase, ADP/ATP translocase	[25]
150‡	15.0	ATPase complex; some contamination by ADP/ATP translocase and respiratory components (< 15% NADH-dehydrogenase, cytochromes *b*, *c*, c_1)	[24]
10	50–75		This study

Enzymes were from *pig heart and †rat liver; ‡Without additional F_1 and OSCP, the value was 50 nmol/mg per min.

per mg were observed that could be inhibited almost completely by either the F_o-directed inhibitors, DCCD or venturicidin, or by the uncoupler FCCP. Therefore, the exchange is related to membrane-integrated F_1F_o (see Table 1). In comparison with ATP–P_i exchange activities reported before (see Table 2), the observed activities are rather low. In the past, ATP–P_i exchange activities have been stimulated by the addition of BSA to the assay medium [23], or by the addition of F_1 after reconstitution [24,25]. Neither of these effects was studied here.

Light-driven ATP synthesis in proteoliposomes containing both MF_1F_o and BR

The ability of reconstituted MF_1F_o to synthesize ATP in the presence of a transmembrane proton gradient was assessed by co-reconstitution of the enzyme with the light-driven proton pump, BR. Under appropriate conditions, the illumination of these proteoliposomes with white light should generate a steady-state ΔpH, which could then be used by a functional MF_1F_o to synthesize ATP. On illumination, ATP was synthesized at an intial rate of 57 nmol of ATP/mg per min (see Figure 6). In the steady state, reached after about 10 min, the corresponding value was 30 nmol of ATP/mg per min. Both values are more than one order of magnitude higher than those reported for reconstituted bovine MF_1F_o [25,26], and are comparable with activities reported by Kagawa [27] for F_1, F_o-ATPase from the thermophilic bacterium, *Bacillus* P53 (TF_1F_o) reconstituted into phospholipid vesicles, and by Racker [28] for proteoliposomes containing mitochondrial F_1-ATPase associated with partially purified hydrophobic proteins (F_o), purple membranes and soybean phospholipids. In the steady-state phase of ATP synthesis, about 25% of the activity was inhibited by oligomycin or DCCD.

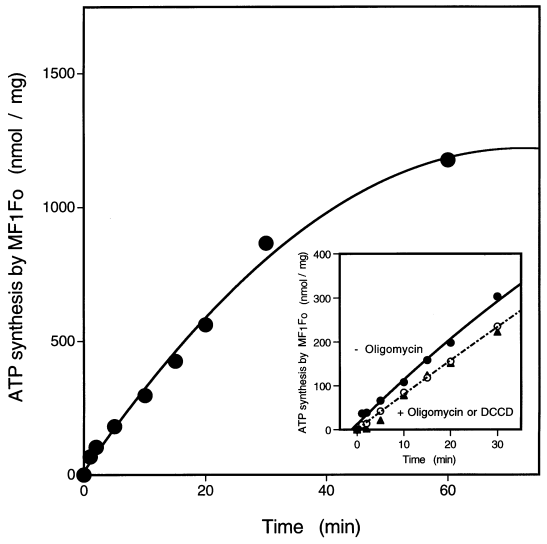

Figure 6 Light-driven ATP synthesis in liposomes containing MF_1F_o and BR

Proteoliposomes containing MF_1F_o and BR (see the Materials and methods section) were suspended in a medium containing 20 mM Tricine, pH 7.8, 40 mM potassium sulphate, 20 mM sodium sulphate, 10 mM succinate, 5 mM magnesium sulphate and 10 mM potassium dihydrogen phosphate. ADP (final concentration 0.75 mM) was added, and ATP synthesis was initiated by illuminating the reaction vial. The insert shows the inhibition of ATP synthesis by 50 μM oligomycin (○) and by 100 μM DCCD (▲).

Addition of the uncoupler nigericin quenched the catalytic reaction by about 40 %. However, a more efficient inhibition (up to 90 %) was found during the initial phase of the reaction by both F_o-specific inhibitors and uncouplers (see the insert in Figure 6).

Electron microscopic examination of MF_1F_o reconstituted into liposomes

The MF_1F_o reconstituted into liposomes was examined by electron microscopy of samples of proteoliposomes that had been negatively stained with uranyl formate. Several F_1F_o particles with their characteristic mushroom-like structure had been incorporated into the membrane of the liposomes.

DISCUSSION

After detergent-mediated reconstitution into unilamellar phospholipid vesicles saturated with detergent, the purified bovine MF_1F_o was highly active in ATP hydrolysis. Depending on the detergent used for reconstitution, its activity was 10–20 times higher than that of the purified delipidated enzyme [5]. It is unlikely that the enhancement of activity was caused by the binding of specific lipids to the protein complex. Lipids common to all biological membranes, such as phosphatidylcholine and phosphatidic acid were employed in the reconstitution experiments, and cardiolipin, which is confined to mitochondrial membranes, was not used. It is more likely that the ATP hydrolytic activity of the bovine enzyme was activated by the pH gradient formed across the vesicular membrane by the proton pumping activity of the reconstituted enzyme energized by ATP hydrolysis. Such activation effects have been found with vesicular preparations of F_1F_o-ATPase from chloroplasts, where the pH gradient across the membrane activates both ATP synthesis and ATP hydrolysis by the enzyme [29–32]. The pH gradient can be generated either by the electron transport chain or by the F_1F_o-ATPase itself, as shown with the enzyme from *Rhodobacter capsulatus* [33]. In order to observe the effects of pH gradients on the activity of the enzyme, it is important that the transmembrane pH gradient formed by the reconstituted MF_1F_o is maintained. Therefore, it is essential to employ vesicles with a low passive ion flux via leaks, and this is a characteristic feature of the vesicles prepared by reversed-phase evaporation that were employed in the present study [34]. About 60 s after the addition of substrate to the vesicular MF_1F_o, the rate of ATP hydrolysis diminished (see Figure 4). This effect could be caused either by the inhibition of the ATP synthase by high ΔpH [35], or it could be due to the binding of ADP to a high-affinity catalytic site and the formation of an inactive state of the enzyme [36].

In addition to its ATP hydrolytic capability, the reconstituted MF_1F_o complex had ATP–P_i exchange activity and was capable of ATP-dependent proton pumping (see Figure 5 and Table 1). Therefore, the preparations of MF_1F_o used in the reconstitution experiments contain all of the subunits that are required for a functional and catalytically active enzyme. The sensitivity of these activities towards inhibitors that act on MF_o and towards uncouplers demonstrates that the MF_1F_o complex is properly integrated into the membrane. The inhibition and uncoupling effects were almost complete, as has been reported with earlier preparations of MF_1F_o [13,19,37].

From a comparison of the ATP–P_i exchange and ATP hydrolase activities of different preparations of MF_1F_o (see Table 2), it is evident that high ATP hydrolytic activity is associated with low exchange activity and vice versa. Therefore, it is reasonable to suggest that ATP hydrolysis and ATP–P_i exchange are catalysed by different states of the membrane-integrated MF_1F_o complex. It is known that factors such as free metal ions and ADP, which favour the inhibition of the ATP synthase, increase the ratio of ATP–P_i exchange:ATP hydrolysis [38]. In the experiments described above, the conditions used in the ATP–P_i exchange experiments were similar to those used for examining ATP synthesis and hydrolysis. Thus, the somewhat low exchange activity might be a consequence of the experimental conditions favouring ATP hydrolysis and synthesis and being suboptimal for ATP–P_i exchange, rather than indicating a partially dysfunctional enzyme.

The functional integrity of the proton pathway in the membrane sector of the reconstituted preparation of MF_1F_o, and of the coupling of ATP hydrolysis to proton transport, were demonstrated qualitatively by the use of the pH-sensitive fluorescence probe ACMA. The fluorescence changes were induced by ATP hydrolysis and they were blocked by the addition of inhibitors acting on MF_o and by uncouplers.

Finally, by co-incorporation of MF_1F_o and the light-driven proton pump, BR, into phospholipid vesicles, the MF_1F_o was shown to be capable of ATP synthesis. Again, this ATP synthetic activity was affected by the inhibitors DCCD and oligomycin that act on F_o, and by the uncouper, nigericin. However, only 25 % of this activity was inhibited, whereas Deisinger et al. [26] reported an inhibition of 95 % of the ATP synthetic activity of bovine MF_1F_o co-reconstituted with BR. However, 50 % of this previously reported inhibition can be attributed to the solvent ethanol used for dissolving the inhibitors, and after correction the true value is 35–40 %. It should also be noted that the catalytic activities observed in these earlier experiments were almost one order of magnitude lower than those reported in the present work, and therefore that it is difficult to determine the effects of inhibitors accurately with such a low basal activity. In the present work, the observed partial inhibition might arise because the inhibitors, which are lipophilic, are impeded in

binding to their inhibitory sites in F_o by the high density of proteins incorporated in the membrane. This interpretation is supported by the finding that inhibition of MF_1F_o by DCCD was increased to 50% by preincubation of MF_1F_o with the inhibitor (results not shown). It is also conceivable that incomplete inhibition might be caused by competition for binding of the inhibitor between BR and MF_1F_o.

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