Purification and Properties of the Adenosine Triphosphatase Released from the Liver Mitochondrial Membrane by Chloroform

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1. Soluble ATPase (adenosine triphosphatase) activity is released when rat liver submitochondrial particles are shaken with chloroform, provided that ATP or glycerol is present in the suspending medium. The extraction is very rapid and appears to be complete. 2. The ATPase of the chloroform extract is about 50% pure and can be readily purified to a specific activity of 60–70 $\mu\text{mol/min}$ per mg of protein by $(\text{NH}_4)_2\text{SO}_4$ fractionation and column chromatography on Sephadex G-200. 3. The particulate and soluble ATPases have many similar properties, including their K_m values for ATP, activation by various metal ions, hydrolytic activity with other nucleotides and stimulation by bicarbonate ions. 4. Unlike the particulate enzyme, the soluble enzyme is cold-labile and insensitive to oligomycin. 5. The molecular weight indicated by the mobility of the soluble ATPase on Sepharose 6B is 360 000. 6. The soluble ATPase combines very readily with liver submitochondrial particles depleted of ATPase by salt extraction, and oligomycin-sensitivity is restored. Very little recombination of the enzyme occurs with chloroform-extracted particles. 7. The soluble enzyme contains orcinol-reactive material, suggesting that it may be a glycoprotein. The carbohydrate content was estimated to be 1–2% by weight. 8. It is concluded that the liver ATPase obtained by the chloroform extraction method of Beechey, Hubbard, Linnett, Mitchell & Munn [(1975) *Biochem. J.* **148**, 533–537] is similar to other preparations described previously and that this method is superior in simplicity and speed.

The ATPase activity of mitochondria and submitochondrial particles has long been thought to be due to a reversal of the terminal steps in ATP synthesis (Lardy, 1956). Evidence supporting this conclusion includes the specific inhibition of both reactions by oligomycin (Lardy *et al.*, 1958) and by an antibody prepared against a soluble mitochondrial ATPase preparation (Fessenden & Racker, 1966).

Beechey *et al.* (1975) introduced a simple and rapid method for the preparation of soluble ATPase from ox heart submitochondrial particles and suggested that the method could be usefully applied to other sources of mitochondrial ATPase. The value of the Beechey *et al.* (1975) method has been questioned by Gomez-Puyou & Gomez-Puyou (1977) on the grounds that the method yields a preparation of low specific activity, and by Drahota & Houstek (1977), who applied the method unsuccessfully to liver preparations.

The present paper describes a simple and rapid procedure for the extraction and purification of ATPase from liver submitochondrial particles which gives a preparation in good yield and having a specific

activity comparable with that of preparations obtained by more complex and time-consuming methods. We have compared the properties of the particulate ATPase with those of the soluble form of the enzyme obtained by the method of Beechey *et al.* (1975) and have also studied the binding of the soluble enzyme to submitochondrial particles deficient in ATPase activity. The purified enzyme contains orcinol-reactive material, supporting the suggestion of Andreu *et al.* (1978) that energy-transducing ATPases are glycoproteins.

Materials and Methods

Alcohol dehydrogenase, fructose 6-phosphate kinase, nucleotides, phosphoenolpyruvate (potassium salt) and pyruvate kinase were obtained from the Boehringer Corp., London W5 2TZ, U.K. Crystallized bovine plasma albumin was from Armour Pharmaceutical Co., Eastbourne, Sussex, U.K. Other reagents, including chloroform, Folin–Ciocalteu phenol reagent and orcinol, were from BDH, Poole, Dorset, U.K. AnalaR-grade reagents were used whenever possible. Orcinol was recrystallized from benzene before use. Oligomycin was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Blue Dextran, Sephadex G-200 and Sepharose 6B

Abbreviation used: ATPase, adenosine triphosphatase (EC 3.6.1.3).

were from Pharmacia Fine Chemicals, Uppsala, Sweden.

Enzyme preparations

Rat liver mitochondria were isolated in 0.25M-sucrose containing 10mM-Tris/H₂SO₄ buffer, pH 7.4, by the method of Schneider (1953) and stored at -20°C for 1-7 days. Liver submitochondrial particles were prepared from thawed liver mitochondria by the method of Kielley & Bronk (1958), by using 0.03M-potassium phosphate buffer, pH 7.4. Before sonication, the mitochondria were washed once by dilution and centrifugation in phosphate buffer. The sonic particles obtained were washed once and homogenized in the sucrose/Tris solution to obtain a stock suspension containing 40-60mg of protein/ml. The particles were stored at -20°C for up to 1 month before use. In some experiments, the particles were extracted with 2M-NaCl by the method of MacLennan *et al.* (1968). The ATPase activity of the salt-extracted particles was about 10% of the activity of the stock particle preparation.

Extraction of ATPase by chloroform

All solutions used were maintained and centrifuged at 25°C. Submitochondrial particles, usually containing 5-8mg of protein/ml, were suspended in a solution containing 0.25M-sucrose, 10mM-Tris/H₂SO₄ buffer, pH 7.6, 1mM-EDTA and 4mM-ATP (STE-ATP solution). The suspension was vortex-mixed vigorously on a Whirlimixer (Fisons Scientific Apparatus, Loughborough, Leics., U.K.), and 0.5 vol. of chloroform was added rapidly dropwise. When chloroform addition was complete, vortex-mixing was continued for a further 20 s. The chloroform/water emulsion was broken by centrifugation in a bench centrifuge for 2 min. The upper aqueous layer was removed and diluted 1:1 with fresh STE-ATP solution and both the lower chloroform layer and insoluble material at the interface were discarded. Dilution of the aqueous extract at this stage appeared to increase the yield of soluble ATPase slightly and also protected the polycarbonate centrifuge tubes from damage by residual chloroform during the next centrifugation step. The diluted extract was centrifuged at 100000g for 20 min to yield a clear supernatant containing the ATPase and a tightly packed reddish-brown pellet. The supernatant was stored in a water bath at 25°C until use. The pellet was washed once by resuspension and centrifugation in ice-cold sucrose/Tris solution and homogenized in fresh solution to yield a preparation of washed chloroform-extracted submitochondrial particles.

Ammonium sulphate purification. The ATPase present in the aqueous extract was purified by (NH₄)₂SO₄

fractionation. All procedures were carried out at 25°C.

First (NH₄)₂SO₄ precipitation. Solid (NH₄)₂SO₄ (0.39 g/ml) was added slowly to a stirred sample of the aqueous extract. When all the (NH₄)₂SO₄ had been added and dissolved, the sample was left for 15 min and then centrifuged at 12000g for 10 min to sediment the finely dispersed protein precipitate. After centrifugation the supernatant was removed and discarded and the white sediment was redissolved in a volume of STE-ATP solution equal to one-quarter of the volume of the aqueous extract used. The solution was left for 15 min and then centrifuged for 4 min in an Eppendorf Microcentrifuge 3200 to remove a small amount of insoluble material.

Second (NH₄)₂SO₄ precipitation. The ATPase solution was reprecipitated again by a procedure similar to that used in the first precipitation step. After all the (NH₄)₂SO₄ had been added and dissolved, the sample was left for 15 min at 25°C and then transferred to a refrigerator at 2°C. When required for assay, the cold precipitate was resuspended by gentle swirling and a sample was centrifuged in the Microcentrifuge for 4 min. The sedimented ATPase was redissolved in an equal amount of STE-ATP solution at 25°C. This solution was re-centrifuged for 4 min to remove a small amount of insoluble material before assay. The enzyme remained stable and fully active for several months when stored at 2°C as the second (NH₄)₂SO₄ precipitate.

Enzyme assays

ATPase activity was measured at 30°C, in 4ml disposable plastic tubes. The reaction mixture (0.3 ml) contained 0.05M-Tris/H₂SO₄ and 0.03M-NaHCO₃ (Ebel & Lardy, 1975), at pH 7.4 or 8.5 as indicated in the text. For assays at fixed substrate concentration, the mixture also contained 2mM-MgCl₂ (or other metal ion; see the Results section) and 5mM-ATP. The reaction was started by the addition of enzyme (10μg of submitochondrial-particle protein or 0.2-2.0μg of soluble ATPase), and the mixture was incubated for 5 min before the addition of 0.3 ml of 10% (w/v) trichloroacetic acid. The P_i content of the whole reaction mixture was measured by the method of Lowry & Lopez (1946). Corrections were made for the P_i content of reagent and enzyme, by using samples in which trichloroacetic acid was added before the enzyme. For the estimation of K_m and V_{max} values, the Tris/H₂SO₄/NaHCO₃ buffer contained equimolar concentrations of MgCl₂ and ATP in the range 0.25-2.5mM. The reaction was started by the addition of enzyme (2.0μg of submitochondrial-particle protein or 0.2-1.0μg of soluble enzyme), and the mixture was incubated for 10 min before the addition of trichloroacetic acid. In some experiments,

the reaction mixture also contained 2mM-phosphoenolpyruvate and 6 μ g of pyruvate kinase. This enzyme system is often used in ATPase assays to remove ADP, a product inhibitor of the ATPase reaction (Kielley & Kielley, 1953). It had no significant effect on the ATPase activity measured under the conditions specified above, but in agreement with Pullman *et al.* (1960) it maintained linear rates of ATP hydrolysis during more prolonged incubation periods.

One unit of ATPase activity is defined as the amount of enzyme that catalyses the hydrolysis of 1 μ mol of ATP/min under the assay conditions. Specific activity is expressed as units/mg of protein.

Protein was measured in clean glass test tubes by a modification of the method of Sutherland *et al.* (1949). The protein samples (0.4ml) were mixed with 0.1ml of 10% (w/v) sodium deoxycholate and 2.5ml of alkaline CuSO₄ solution and left at room temperature (about 20°C) for 10min. Each sample was then mixed with 0.25ml of Folin-Ciocalteu phenol reagent (diluted 1:1 with water) and incubated at 37°C for 30min before measurement of the A_{660} of the sample. Bovine plasma albumin, dissolved in the same buffer solution as the protein samples (usually STE-ATP), was used to construct a standard curve.

Binding of soluble ATPase to submitochondrial particles

Salt-extracted particles or chloroform-extracted particles (4.2mg of protein in 0.5ml) were mixed with 1.0ml of soluble ATPase or cold-inactivated ATPase (second (NH₄)₂SO₄ precipitate, 0.12mg of protein) and 20 μ l of 0.2M-MgCl₂. Control samples were also prepared, consisting of particles mixed with buffer containing no soluble ATPase or ATPase mixed with buffer containing no particles. The samples were incubated at 30°C for 10min before 10 μ l portions were withdrawn for the assay of ATPase activity at fixed substrate concentration in buffer containing 3 μ l of ethanol with or without 3 μ g of oligomycin. Ethanol alone inhibited both particulate and soluble ATPase activity by about 7%.

The remainder of the samples containing particles mixed with soluble ATPase were diluted by the addition of 6ml of a solution containing 0.25M-sucrose, 10mM-Tris/H₂SO₄, 0.6mM-EDTA, 2.7mM-MgCl₂ and 2.7mM-ATP, pH7.6, and centrifuged at 100000g for 60min. The supernatants were discarded and the sediments resuspended separately in 10ml of 0.25M-sucrose containing 10mM-Tris/H₂SO₄, pH7.6, and re-centrifuged at 100000g for 60min. The washed sediments were resuspended to a final volume of 1.5ml in sucrose/Tris solution. The particles were maintained at 20°C throughout this procedure. Samples were then taken for ATPase assay at 30°C.

Molecular-weight estimation

This was carried out by the method of Andrews (1964), by using gel-filtration chromatography of standard proteins (Lambeth & Lardy, 1971) and ATPase at 25°C. Sepharose 6B, equilibrated with STE-ATP solution, was packed in a 0.9cm column to a bed height of 49cm. The effluent volume of each protein was measured individually. Each protein sample (0.5mg), dissolved in 0.5ml of STE-ATP solution containing 10% (v/v) glycerol, was applied to the top of the column and the effluent was collected in six-drop fractions (0.17ml) by using an automatic fraction collector with a drop-counting attachment. The flow rate was 3.8ml/h. An elution diagram was plotted by measuring the protein and enzymic activity in the collected fractions. The effluent volume corresponding to the maximum concentration of each enzyme was estimated by extrapolating both sides of the elution peak to an apex (Andrews, 1964).

Measurement of sugar content

A column (0.9cm $\times$ 50cm) of Sephadex G-200 was equilibrated for several days at 30°C with 10mM-Tris/H₂SO₄ buffer (pH7.6) containing 1mM-EDTA, until the blank colours obtained by the orcinol method were identical when using samples of the reservoir buffer and samples of the effluent buffer leaving the column. Soluble ATPase [second (NH₄)₂SO₄ fraction containing about 3mg of protein] was freed of sucrose and ATP by passage of the enzyme through the column. The effluent was collected in 48-drop fractions (1.4ml) by using an automatic fraction collector with a drop-counting attachment. The flow rate was 5.4ml/h. A portion (0.2ml) of each fraction containing the protein peak eluted shortly after the void volume was diluted 1:1 (v/v) with Tris/EDTA buffer containing 8mM-ATP and assayed for ATPase activity. The remaining 1.2ml of each fraction was analysed for protein and sugar content. Sugar was measured by the method of Winzler (1955), by adding 2.5ml of orcinol/H₂SO₄ reagent to each 0.3ml sample. The ethanol precipitation step was omitted. A galactose/mannose solution (10 μ g of each sugar/ml) in Tris/EDTA buffer was used as a standard solution. No colour was obtained with ATPase blank samples prepared without orcinol in the H₂SO₄ reagent.

Results

Extraction of ATPase by chloroform

When liver submitochondrial particles were suspended in the presence of ATP and extracted with 0.5vol. of chloroform, about 40% of the particle ATPase activity was recovered in the chloroform extract. In the absence of ATP, lower amounts of

ATPase were obtained, particularly when low particle protein concentrations were used (Fig. 1). At each particle protein concentration used, equal amounts of protein were recovered in the chloroform extract in the presence or absence of ATP, and amounted to 6–9% of the particle protein. Thus the ATPase extracted in the presence of ATP had a considerably higher specific activity. In agreement with the results of Beechey *et al.* (1975), only about 2% of the original ATPase activity was recovered in the chloroform-extracted submitochondrial particles prepared in either the presence or absence of ATP. The chloroform-extracted particles recovered by high-speed centrifugation contained about 40% of the original particle protein. Since the high-speed supernatant contained 6–9% of the original protein, approx.

Fig. 1. Effect of ATP on the chloroform-induced release of ATPase

Samples of submitochondrial particles containing 2.7–13.7 mg of protein (specific activity 4.2 units/mg) were suspended in 2 ml of 0.25 M-sucrose/10 mM-Tris/ H_2SO_4 buffer/1 mM-EDTA, pH 7.6, with or without 4 mM-ATP. Each sample was extracted with 1.0 ml of chloroform. The soluble ATPase fraction was obtained by centrifugation and assayed at fixed substrate concentration as described in the Materials and Methods section. ○, △, No ATP present; ●, ▲, with ATP.

50% of the particle protein shaken with chloroform must have been present in the discarded lower chloroform layer after bench centrifugation (see the Materials and Methods section).

When ATP was omitted, the amount of ATPase recovered in the chloroform extract was increased by adding 10% (v/v) glycerol to the particle-suspending medium before extraction. The amount of ATPase recovered in the presence of glycerol and ATP was similar to that obtained with ATP alone, and glycerol was therefore omitted in subsequent experiments. The increased yield of ATPase obtained in the presence of ATP or glycerol is probably due to the stabilizing effect of these reagents on soluble mitochondrial ATPase (Selwyn, 1967).

When experiments similar to that of Fig. 1 were repeated on ten different particle preparations, the chloroform extract was found to contain an average of 37.2% of the ATPase units present in the particle preparation before extraction (range 28.2–45.3%). Various factors in the extraction procedure were varied in an attempt to improve that yield of ATPase, without success. Similar yields of enzyme were obtained by using 0.1, 0.2 or 0.5 vol. of chloroform for extraction, or by using 0.5 vol. of chloroform and vortex-mixing for 0, 10 or 60 s after the chloroform

Fig. 2. Effect of temperature on soluble ATPase activity

Samples of a chloroform extract containing soluble ATPase (0.13 mg of protein/ml; specific activity 28.8 units/mg) were incubated at various temperatures. Portions (10 μ l) were withdrawn from each sample at the times indicated and assayed for ATPase activity at 30°C by using a fixed substrate concentration.

Table 1. *Purification of ATPase*
ATPase activity was assayed at fixed substrate concentration.

Purification stage	Protein concn. (mg/ml)	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Submitochondrial particles	5.1	16	81.6	459	5.6	100	—
Chloroform extract	0.23	32	7.2	188	26.1	41.0	4.7
First $(\text{NH}_4)_2\text{SO}_4$ precipitate	0.48	8	3.8	171	45.6	37.3	8.1
Second $(\text{NH}_4)_2\text{SO}_4$ precipitate	0.31	8	2.5	153	62.3	33.4	11.1

Fig. 3. *Sephadex G-200 column chromatography of soluble ATPase preparations*

Chloroform extract (a) or second $(\text{NH}_4)_2\text{SO}_4$ precipitate (b) was chromatographed in descending fashion on a column (0.9 cm $\times$ 45 cm) of Sephadex G-200 previously equilibrated with STE-ATP solution. The column was loaded with either 0.8 ml of chloroform extract (0.27 mg of protein containing 6.5 units of ATPase) or 1 ml of second $(\text{NH}_4)_2\text{SO}_4$ precipitate (0.31 mg of protein containing 19.5 units of ATPase). The flow rate was 6.9 ml/h; fractions (1.37 ml) were collected and analysed for ATPase activity and protein content. The arrows indicate the points of maximal elution of Blue Dextran (1) and 2,4-dinitrophenol (2). ∇ , ATPase activity; Δ , protein content.

showed that slightly higher specific activity (30–35) was obtained in the chloroform extract when sucrose was omitted from the particle suspension medium before extraction.

The chloroform-released liver ATPase was cold-inactivated, in contrast with the cold-stability of the ox heart enzyme isolated by Beechey *et al.* (1975). The liver enzyme lost some activity even on incubation at 20°C in the presence of ATP, and a more extensive inactivation set in at lower temperatures commonly encountered in some U.K. laboratories during winter (Fig. 2).

Further purification of the ATPase

The chloroform-released enzyme could be readily purified either by an $(\text{NH}_4)_2\text{SO}_4$ -precipitation procedure (see the Materials and Methods section and Table 1) or by passage of the chloroform extract through a column of Sephadex G-200 (Fig. 3). With a few preparations, almost all the units of ATPase activity present in the chloroform extract were recovered in the second $(\text{NH}_4)_2\text{SO}_4$ precipitate. It seems probable that losses of activity during $(\text{NH}_4)_2\text{SO}_4$ fractionation in other preparations occurred through incomplete sedimentation and recovery of the finely dispersed protein precipitate. During column chromatography, the ATPase present in the chloroform extract or obtained by $(\text{NH}_4)_2\text{SO}_4$ fractionation was eluted from the column as a single peak of activity shortly after the void volume (Fig. 3). The specific activity throughout the peak was almost constant, suggesting the presence of a single major protein species. This conclusion was supported by analysis of the enzyme by polyacrylamide-gel electrophoresis, which indicated the presence of one major high-molecular-weight protein and three faintly staining proteins of much lower molecular weight (D. D. Tyler & P. R. Webb, unpublished work). The specific activity observed with several preparations was in the range of 60–70 units/mg of protein, which is similar to or higher than the values

addition had been completed. These observations suggest that the extraction of ATPase by chloroform is remarkably rapid and complete. Later experiments

obtained by Catterall & Pedersen (1971) and by Drahota & Houstek (1977), using more elaborate methods of isolation of the enzyme.

Properties of the ATPase

The kinetic properties of the enzyme at various stages of purification are summarized in Table 2. The K_m for ATP was similar in the particulate and soluble forms of the enzyme, suggesting that the configuration of the catalytic centre of the enzyme was largely unaffected during extraction and purification. The apparent loss of enzyme activity occurring when the ATPase was released from the membrane by chloroform (Table 1) could therefore not be attributed to a change in the affinity between the ATPase and ATP. Calculations of the percentage yield of ATPase activity, based on V_{max} values, also showed an apparent loss (about 50%) of ATPase activity after release of the enzyme in soluble form. Both forms of the enzyme showed higher activity at alkaline pH than at neutral pH (Table 2). At pH 8.5, at a fixed substrate concentration, the addition of 30mM-NaHCO₃ stimulated particulate ATPase acti-

vity 1.5-fold, that of the chloroform-released enzyme 1.8-fold, and that of the second (NH₄)₂SO₄ precipitate 1.9-fold. Also at pH 8.5, with both particulate and soluble ATPase, activity was increased when the ATP/Mg²⁺ ratio was increased from 1 to 2.5, indicating an apparent activation of the enzyme by ATP. This activation is of doubtful biological significance, since it was not observed at the more physiological pH value of 7.4.

Both the particulate and soluble forms of ATPase catalysed the hydrolysis of other nucleotides, especially purine nucleotides. At fixed substrate concentration, the relative rates of hydrolysis were as follows (ATP = 100): submitochondrial particles: GTP, 38; ITP, 36; UTP, 7; second (NH₄)₂SO₄ precipitate: GTP, 47; ITP, 47; UTP, 22. The most marked change in activity during purification was a relative increase in UTPase activity. Neither the particulate nor the soluble ATPase liberated any P_i when ADP was used as substrate.

The particulate and soluble ATPase activities were activated by a number of different metal ions. At fixed substrate concentration, the relative rates of ATP hydrolysis (Mg²⁺ = 100) were: particulate ATPase, no metal ion added, 2.3; Ca²⁺, 11.8; Zn²⁺, 34.6; Co²⁺, 47.5; Fe²⁺, 58.2; and for the chloroform extract ATPase activity, no metal ion added, 4.6; Ca²⁺, 12.9; Fe²⁺, 52.0; Zn²⁺, 61.9; Co²⁺, 75.1. With the soluble enzyme, the relative power of activation by the various metal ions was very similar to that described by Selwyn (1968) for activation of the soluble ox heart enzyme.

Recombination of soluble ATPase

The purified soluble ATPase recombined very readily with submitochondrial particles depleted of ATPase by salt washing, but only to a limited extent with chloroform-extracted particles (Table 3). Similar results were obtained with the ATPase present in the chloroform extract instead of the

Table 2. *Kinetic constants for ATPase at various stages of purification*

Assays were performed on particulate and soluble ATPase preparations derived from the same stock preparation of liver mitochondria.

Purification stage	At pH 7.4		At pH 8.5	
	K_m (mM)	V_{max} (units/mg)	K_m (mM)	V_{max} (units/mg)
Submitochondrial particles	0.26	3.8	0.37	5.0
Chloroform extract	0.41	34.2	0.46	42.8
Second (NH ₄) ₂ SO ₄ precipitate	0.35	57.6	0.42	72.0

Table 3. *Recombination of soluble ATPase*

The samples were incubated and assayed for ATPase activity as described in the Materials and Methods section. Soluble ATPase was cold-inactivated by incubation for 16h at 2°C.

Enzyme	Oligomycin	...	Total ATPase activity (units)		Sedimentable ATPase activity (units)	
			—	+	—	+
Soluble ATPase			7.0	7.0	—	—
Cold-inactivated soluble ATPase			0	0	—	—
Salt-extracted particles			2.1	0	—	—
Salt-extracted particles + soluble ATPase			8.7	0.76	6.5	0.56
Salt-extracted particles + cold-inactivated ATPase			1.6	0.32	—	—
Chloroform-extracted particles			0.7	0.7	—	—
Chloroform-extracted particles + soluble ATPase			7.7	7.9	1.3	1.3

Fig. 4. *Estimation of molecular weight of ATPase*
The log (molecular weight) of standard proteins is plotted against their effluent volumes. The standard proteins used and their molecular weights were: ●, rabbit muscle fructose 6-phosphate kinase (385000); ■, rabbit muscle pyruvate kinase (237000); ▲, yeast alcohol dehydrogenase (150000). The molecular weight of the ATPase (○) is indicated from the effluent volume of the enzyme.

(NH_4) $_2\text{SO}_4$ enzyme. Recombination of the soluble enzyme to salt-extracted particles restored the oligomycin-sensitivity of the enzyme, whereas the low ATPase activity of the chloroform-extracted particles was oligomycin-insensitive. The ATPase activity of the soluble ATPase and salt-extracted particles when mixed together was approximately equal to the sum of their separate ATPase activities (Table 3). Added Mg^{2+} , which inhibited the release of ATPase activity from submitochondrial particles during chloroform extraction, increased the amount of ATPase recombining with salt-extracted particles by about 2-fold.

An estimate of the mol.wt. of the ATPase gave a value of 360000 (Fig. 4), which is in good agreement with previous estimates of the liver enzyme mol.wt. [355000 (Lambeth & Lardy, 1971); 384000 (Catterall & Pedersen, 1971)].

Presence of orcinol-reactive material

The ATPase contained orcinol-reactive material (Fig. 5), which indicated an average sugar content of 1.6 μg of galactose/mannose equivalents/100 μg of protein (range 1.0–2.4 $\mu\text{g}/100\mu\text{g}$, for five different ATPase preparations). This value corresponds to a content of 32 mol of galactose/mannose/mol of ATPase, or approx. 0.9 mol of sugar/100 mol of amino acid. The latter value may be compared with

Fig. 5. *Sugar content of ATPase*
Fractions of the ATPase obtained after passage of the enzyme through a column of Sephadex G-200 were analysed for ATPase activity (▲), sugar content (●) and protein content (○). For other details, see the Materials and Methods section. ATP began to be eluted from the column in fraction 19.

the value of 3 mol/100 mol of amino acid obtained by Andreu *et al.* (1978) with the chloroplast coupling-factor ATPase (CF_1).

Ribose gave a more intense orcinol colour at 420 nm compared with the hexoses galactose, mannose and glucose. Assuming that all the orcinol colour is due to the presence of ribose in the enzyme, the sugar content may be calculated to be 21 mol of ribose/mol of ATPase. It appears to be unlikely that the orcinol-reactive material in the enzyme is solely due to the presence of ribose in adenine nucleotide tightly bound to the enzyme, because the A_{280}/A_{260} ratio of the enzyme samples emerging from the column was in the range 1.27–1.4, which suggests the presence of no more than 3 mol of tightly bound adenine nucleotide/mol of ATPase (Chang & Penefsky, 1973). According to Pedersen (1975), the content of bound nucleotide in the isolated liver enzyme is less than 2 mol/mol of ATPase. Furthermore, the spectrum of the orcinol-reactive material obtained either by using the enzyme or standard hexose solutions showed a minor band at 500 nm in addition to the major peak at 420 nm, whereas standard ribose solutions gave a single peak at 420 nm.

Some loss of activity was observed with the enzyme used for the sugar determinations (Fig. 5). This may have occurred during the period during which the enzyme samples stood in the fraction collector at room temperature in the absence of ATP. Comparable losses of activity were observed by Chang & Penefsky (1973) after chromatography of ox heart ATPase on Sephadex G-50.

Discussion

The properties of the chloroform-released liver ATPase indicate that it closely resembles the enzymes isolated by Lambeth & Lardy (1971) and by Catterall & Pedersen (1971). Similar conclusions have been made by Beechey *et al.* (1975) and by Spitzberg & Blair (1977), showing the identity between the chloroform-released ATPase of ox heart submitochondrial particles and the coupling factor or F_1 protein isolated by Pullman *et al.* (1960). The yield of liver ATPase activity released by chloroform is only approx. 40%. A similar loss of ATPase units during extraction of the particulate enzyme into soluble form by sonication was observed by Catterall & Pedersen (1971). When a more prolonged sonication treatment was used by Lambeth & Lardy (1971), 84% of the particulate ATPase units was extracted, but about one-half of these units were lost during protamine sulphate fractionation, perhaps owing to the removal of ATPase attached to very small particles resistant to sedimentation by centrifugation. The final percentage yield of purified ATPase and the degree of purification of the chloroform-released liver enzyme is in good agreement with the data of Catterall & Pedersen (1971) and Lambeth & Lardy (1971), but differs from the complete recovery of liver ATPase units reported by Drahota & Houstek (1977).

The apparent loss of ATPase activity occurring when the enzyme is released from the membrane cannot be explained by changes in the affinity of the enzyme for ATP, since no marked differences in K_m value were observed between the particulate and soluble enzymes. The low yield could be due to a change in the mechanism of ATP hydrolysis indicated by the loss of oligomycin-sensitivity of the soluble enzyme. If this were so, it might have been expected that the restoration of oligomycin-sensitivity when the soluble ATPase recombined with the membrane would be associated with an increased ATPase activity, but no increase was observed. Since the percentage recovery of ATPase units in the aqueous layer after chloroform extraction is similar to the percentage recovery of particle protein in the same fraction, an alternative explanation for the low yield of ATPase is that only 40% of the submitochondrial particles extracted escape denaturation by chloroform.

Beechey *et al.* (1975) observed that the chloroform-released ATPase from ox heart submitochondrial particles was cold-stable, and suggested that the enzyme was released in combination with the ATPase-inhibitor protein described by Pullman & Monroy (1963), which confers cold-stability on the heart ATPase. In contrast, the chloroform-released liver ATPase is cold-labile and therefore appears not to contain the inhibitor protein. The latter protein has been isolated from rat liver mitochondria, but the inhibitor activity (2.1 units/mg of protein; Chan & Barbour, 1976) is about one-twenty-eighth of the activity of ox heart mitochondria (59 units/mg of protein; Horstman & Racker, 1970). The marked difference in the ATPase-inhibitor content of liver and heart mitochondria is probably responsible for differences between the behaviour of the liver and heart ATPase during extraction of the enzyme into soluble form.

Salt extraction of liver submitochondrial particles by the method of MacLennan *et al.* (1968) removed about 90% of the ATPase activity. ATPase could be restored by mixing the extracted particles with soluble ATPase, but cold-inactivated ATPase had no effect. Cold-inactivation is associated with the dissociation of the enzyme into individual subunits (Penefsky & Warner, 1965). Thus restoration of particulate ATPase was only possible by recombination between the membrane and the native ATPase and not between the membrane and the subunits, indicating the absence from extracted liver particles of inactive ATPase molecules partially depleted of subunits but capable of re-binding added subunits and regaining catalytic activity.

The liver ATPase appears to contain a small amount of carbohydrate material. It is obviously important to investigate this question in more detail by using specific analytical methods. The function of carbohydrate moieties in membrane-bound glycoproteins is poorly understood (Marshall, 1972).

Provided that a buffer containing ATP is used at a suitable temperature during chloroform treatment, the liver ATPase can be purified very easily and the amount of enzyme obtained is proportional to the amount of particulate enzyme in the starting material even at low particle protein concentrations. The present studies therefore support the conclusions of Beechey *et al.* (1975) that the chloroform method is superior to previous purification methods in simplicity and speed and in its application to small biological samples. Studies of the mechanism of action of chloroform should provide new information on the forces binding the ATPase to the mitochondrial membrane and improve our understanding of the process by which newly synthesized ATPase is incorporated into the membrane in order to fulfil its central role in cellular energy metabolism.

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