Isolation of ATPase I, the proton pump of chromaffin-granule membranes

Judith M. PERCY, James G. PRYDE and David K. APPS

Department of Biochemistry, University of Edinburgh Medical School, George Square, Edinburgh EH8 9XD, U.K.

Chromaffin-granule membranes contain two ATPases, which can be separated by $(\text{NH}_4)_2\text{SO}_4$ fractionation after solubilization with detergents, or by phase segregation in Triton X-114. ATPase I (M_r 400 000) is inhibited by trialkyltin, quercetin and alkylating agents, and hydrolyses both ATP and ITP. It contains up to five types of subunit, including a low- M_r hydrophobic polypeptide that reacts with dicyclohexylcarbodi-imide; these subunits are unrelated to those of mitochondrial F_1F_0 -ATPase, as judged by size and reaction with antibodies. ATPase II (M_r 140 000) is inhibited by vanadate, and is specific for ATP; it has not been extensively purified. Proton translocation by resealed chromaffin-granule 'ghosts', measured by uptake of methylamine or by quenching of the fluorescence of 9-amino-6-chloro-2-methoxyacridine, is supported by the hydrolysis of ATP or ITP, and inhibited by quercetin or alkylating agents, but not by vanadate. ATPase I must therefore be the proton translocator involved in the uptake of catecholamines and possibly of other components of the chromaffin-granule matrix, whereas ATPase II does not translocate protons.

INTRODUCTION

The secretory granules of the adrenal medulla, known as chromaffin granules, store and secrete catecholamines, nucleotides and proteins. The mechanism by which the very large intragranular concentrations of catecholamines are accumulated and maintained is now well established (Njus *et al.*, 1981): an electrogenic H^+ -translocating ATPase acidifies the granule matrix, creating an ATP-dependent transmembrane pH gradient (ΔpH) and a membrane potential ($\Delta\psi$), both of which are used to drive catecholamine/proton exchange by a separate, electrogenic, amine carrier. A chemiosmotic mechanism may also apply to the uptake of nucleotides by the granules; this appears to be driven by $\Delta\psi$ alone (Weber & Winkler, 1981), although in experiments with resealed chromaffin-granule 'ghosts' it has not been possible to generate the large nucleotide concentration gradients that this model predicts (Grueninger *et al.*, 1983).

Considerable progress has already been made in the identification and isolation of the amine carrier (Gabizon *et al.*, 1982; Scherman & Henry, 1983), but the nucleotide carrier is yet to be characterized. The nature of the H^+ -translocating ATPase is controversial. Its inhibitor-sensitivity (Apps *et al.*, 1980c), catalysis of $\text{ATP}/[^{32}\text{P}]\text{P}_i$ isotope exchange (Roisin & Henry, 1982) and failure to catalyse $[^{14}\text{C}]\text{ADP}/\text{ATP}$ isotope exchange (Apps & Reid, 1977) suggest a mechanistic and possibly structural similarity to the F_1F_0 -ATPase of mitochondria, an idea supported by reconstitution studies (Buckland *et al.*, 1979; Giraudat *et al.*, 1980) and by electron microscopy (Schmidt *et al.*, 1982). An ATPase closely similar to mitochondrial ATPase was indeed found to be associated with chromaffin-granule membranes (Apps & Schatz, 1979), but it has since been shown (Cidon & Nelson, 1983; Cidon *et al.*, 1983) that chromaffin-granule membranes contain H^+ -translocating ATPase activity after removal of all polypeptides that react with antibodies to

mitochondrial F_1 -ATPase. Furthermore, a hydrophobic DCCD-reactive protein of low M_r was isolated from chromaffin-granule membranes and also shown to be structurally quite distinct from the mitochondrial DCCD-reactive proteolipid (Sutton & Apps, 1981); it appeared to be part of an ATPase complex (Apps *et al.*, 1982).

More recently it has been shown that purified chromaffin-granule membrane preparations contain, apart from small amounts of mitochondrial F_1 -ATPase, two ATPases with quite different properties (Apps *et al.*, 1983). After solubilization with non-ionic detergent one of these enzymes (termed ATPase I) behaves on gel filtration in the presence of detergent as a complex of apparent M_r 400 000, is inhibited by DCCD and trialkyltin, and contains the DCCD-reactive subunit (M_r 7000). The other (ATPase II) has an apparent M_r of 140 000, and is strongly inhibited by vanadate. By using the differences in inhibitor-sensitivity of the two ATPases, we have developed procedures for separating them, which yield ATPase I in a fairly pure state; we now report a partial structural characterization of this enzyme, and experiments with resealed chromaffin-granule 'ghosts' that correlate the inhibitor-sensitivity and substrate-specificity of nucleoside triphosphate-dependent amine uptake with those of ATPase I.

MATERIALS AND METHODS

Chemicals

Octa(ethylene glycol) dodecyl ether (C_{12}E_8) was obtained from the Kouyoh Trading Co. (Tokyo, Japan). Triton X-114 was obtained from Fluka, and purified by the method of Bordier (1981). Tributyltin and sodium orthovanadate were supplied by BDH Chemicals. Efrapreptin was from Eli Lilly Co. Quercetin, 4-chloro-7-nitrobenzofurazan, ATP (vanadate-free) and ITP

Abbreviations used: ACMA, 9-amino-6-chloro-2-methoxyacridine; C_{12}E_8 , octa(ethylene glycol) dodecyl ether; DCCD, *NN'*-dicyclohexylcarbodi-imide.

were from Sigma Chemical Co. Phosphoenolpyruvate, pyruvate kinase and lactate dehydrogenase were from Boehringer. *NN'*-Dicyclohexyl[^{14}C]carbodi-imide ([^{14}C]DCCD) was from C.E.A., Gif-sur-Yvette, France. Cholic acid (BDH Chemicals) was recrystallized from aq. 70% (v/v) ethanol. 9-Amino-6-chloro-2-methoxyacridine (ACMA) was a gift from Dr. R. Kraayenhof (Vrije Universiteit, Amsterdam, The Netherlands), and duramycin was a gift from Dr. O. Shotwell (Midwest Area Northern Regional Research Center, Peoria, IL, U.S.A.).

Assays

Hydrolysis of ATP or ITP was measured at 37 °C by spectrophotometric measurement of NADH oxidation in a medium of the following composition: 2 mM-ATP (or 2 mM-ITP), 10 mM-MgSO₄, 1 mM-phosphoenolpyruvate, 0.2 mM-NADH, 50 mM-KCl, 50 mM-Hepes/KOH buffer, pH 7.0, lactate dehydrogenase (3.6 units/ml) and pyruvate kinase (3.0 units/ml). ATP-dependent uptake of 5-hydroxy[^{14}C]tryptamine or of [^{14}C]methylamine by resealed chromaffin-granule 'ghosts' was measured as described previously (Apps *et al.*, 1980c). Quenching of ACMA fluorescence was measured at 25 °C in a Perkin-Elmer 3000 fluorimeter, with excitation and emission wavelengths of 400 nm and 525 nm respectively. The assay medium contained 3 mM-ATP (or 3 mM-ITP), 3 mM-MgSO₄, 40 mM-KI, 0.3 M-sucrose, 10 mM-Hepes/NaOH buffer, pH 7.6, 0.5 μM -ACMA and a 'ghost' concentration of 100 μg of protein/ml. Protein was determined by the modified Folin-Lowry procedure (Hartree, 1972), with bovine serum albumin as standard.

Electrophoresis

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed on slab gels containing exponential gradients of 8–15% acrylamide, with the buffer systems of Laemmli (1970). Before electrophoresis, lipid and detergent were removed by precipitation of proteins with 50 vol. of acetone/ethanol (1:1, v/v) at –15 °C; the samples were redissolved in buffer containing 5% (w/v) sodium dodecyl sulphate and 5% (v/v) 2-mercaptoethanol. Electrophoretic transfer to cellulose nitrate sheets and detection of mitochondrial F_1 -ATPase were as described previously (Apps *et al.*, 1983). Scanning of Coomassie Blue-stained gels was on a Joyce-Loebl Chromoscan 3 densitometer. Radiolabelling of ATPase I was with 20 μM -[^{14}C]DCCD (specific radioactivity 1.85 TBq/mol) in the presence of 10 mM-ATP, 1 mM-EDTA and 10 mM-Hepes/NaOH buffer, pH 7.0, for 16 h at 4 °C. After electrophoresis, gels were fixed in methanol/acetic acid/water (2:1:7, by vol.), washed in water, soaked in 1 M-sodium salicylate for 1 h, dried on to paper and autoradiographed at –70 °C for 14 days.

Fractionation methods

Chromaffin-granule membranes and resealed 'ghosts' were prepared as previously described (Apps *et al.*, 1980c). Fractionation of membrane proteins with (NH₄)₂SO₄ was by a modification of the procedure described previously (Apps *et al.*, 1980b). Membranes were suspended at a concentration of 4 mg of protein/ml in 20 mM-Hepes/NaOH buffer, pH 7.0, containing 1 mM-dithiothreitol and 0.1 mM-EDTA; C₁₂E₈ [10% (w/v) solution] was added to a final concentration of 10 mg/ml, and sodium cholate [10% (w/v) solution, pH 7.0] to 5 mg/ml. One-quarter volume of a saturated

(NH₄)₂SO₄ solution, pH 7.0 was then added, and the solution was centrifuged in 12 ml tubes at 40 000 rev./min for 30 min in a Beckman SW41 rotor (1.96 $\times 10^5 g_{av}$). The white floating cake was removed from the solution, resuspended in 0.8 M-(NH₄)₂SO₄ and re-centrifuged, and then finally resuspended in one-tenth of the original volume of 20 mM-Hepes/NaOH buffer, pH 7.0, containing 1 mM-dithiothreitol and 0.1 mM-EDTA. The insoluble and soluble fractions were assayed for ATPase activity.

Fractionation with Triton X-114 was by a modification of the procedure of Bordier (1981). To chromaffin-granule membranes (4 mg of protein/ml in 150 mM-NaCl/10 mM-Tris/HCl buffer, pH 7.6), Triton X-114 [10% (w/v) stock] was added to give a final concentration of 2% (w/v). After a few minutes at 0 °C, the solution was centrifuged at 25 000 rev./min for 30 min at 4 °C in a Beckman SW50.1 rotor (5.8 $\times 10^4 g_{av}$). The supernatant was decanted and kept at 0 °C. The white pellet (P1) was resuspended in 150 mM-NaCl/10 mM-Tris buffer containing 2% Triton X-114, and re-centrifuged. This washing procedure was repeated in the absence of detergent, and the pellet was finally resuspended in 150 mM-NaCl/10 mM-Tris/HCl buffer, pH 7.6.

The supernatant (S1) was divided into 1.6 ml portions, and each was layered over 1.4 ml of 0.25 M-sucrose in 0.15 M-NaCl/10 mM-Tris/HCl buffer, pH 7.6, containing 0.06% Triton X-114; the tubes were warmed at 30 °C for 3 min, then centrifuged for 5 min at 4000 rev./min in a bench centrifuge (2 $\times 10^3 g_{av}$). The aqueous layer was removed and kept at 0 °C, and the sucrose 'cushion' was decanted from the red pellet (P2), which was resuspended in 0.15 M-NaCl/10 mM-Tris/HCl buffer, pH 7.6, and again centrifuged through a sucrose 'cushion'; finally it was resuspended in 0.15 M-NaCl/10 mM-Tris/HCl buffer, pH 7.6.

Triton X-114 was added to the S2 fraction to give a final concentration of 0.5% at 0 °C, and then the solution layered on to sucrose 'cushions', warmed to 30 °C and centrifuged as before. The aqueous supernatant was again removed and made 2% in Triton X-114, warmed at 30 °C over sucrose 'cushions' and centrifuged again. The final aqueous supernatant (S2) was removed from the sucrose 'cushion' and retained.

The P1, P2 and S2 fractions were assayed for ATPase activity.

RESULTS

Fractionation of detergent-solubilized membranes

We previously (Apps *et al.*, 1983) reported separation of two distinct ATPases from chromaffin-granule membranes, by solubilization with taurodeoxycholate or with the non-ionic detergent C₁₂E₈, followed by exclusion chromatography on Sephacryl S-300, or centrifugation through glycerol density gradients, in the presence of C₁₂E₈. We have now developed two fractionation procedures that can be performed more rapidly, and on a larger scale. These are described in detail in the Materials and methods section.

The first method is derived from the procedure used to purify cytochrome *b*-561 from chromaffin-granule membranes (Apps *et al.*, 1980b); after solubilization with C₁₂E₈ and addition of cholate, (NH₄)₂SO₄ (20% saturation) precipitates a white lipid-rich fraction, which floats to the surface on centrifugation. This contains the DCCD-

Table 1. ATPase and ITPase activities of chromaffin-granule membrane protein fractions produced by $(\text{NH}_4)_2\text{SO}_4$ fractionation, or by fractionation with Triton X-114

'Recovery' refers to the total ATPase activity in each fraction. —, Not tested. For experimental details see the text.

Fraction	Recovery (%)	Specific activity					
		ATPase (nmol/min per mg)			ITPase (nmol/min per mg)		
		Control	+ 2 μM -Tributyltin	+ 2 μM -Vanadate	Control	+ 2 μM -Tributyltin	+ 2 μM -Vanadate
Membranes	100	220	40	170	60	20	60
$(\text{NH}_4)_2\text{SO}_4$ -insoluble	15	470	30	490	100	40	90
$(\text{NH}_4)_2\text{SO}_4$ -soluble	76	240	220	100	100	160	90
Triton X-114 P1	19	550	10	530	100	30	90
Triton X-114 P2	18	200	190	70	50	90	50
Triton X-114 S2	1	10	—	—	—	—	—

sensitive ATPase activity (ATPase I), whereas the vanadate-sensitive ATPase activity (ATPase II) remains in solution. A detergent/protein ratio of 2.5:1 was routinely used; this produced the highest ATPase activity in the precipitated fraction, at the cost of some contamination with unwanted proteins (such as cytochrome *b*-561) compared with fractionation with a detergent/protein ratio of 5:1.

The second procedure is based on the temperature-dependent phase separation of Triton X-114 (Bordier, 1981), which segregates membrane proteins according to their hydrophobicity. After solubilization of chromaffin-granule membranes with this detergent at 0 °C, a precipitate forms, which contains ATPase I; the supernatant contains ATPase II, which, however, separates in the detergent-rich phase on warming at 30 °C.

In each case, ATPase I appears in the most hydrophobic fraction of detergent-solubilized proteins, the specific activity being increased 2–3-fold relative to chromaffin-granule membranes (Table 1); this is not an accurate reflection of the purification of ATPase I, since the activities of the two major ATPases in these membranes may be differently affected by solubilization. As shown in Table 1, they can be distinguished by their characteristic sensitivities to tributyltin and vanadate.

Polypeptide content of separate ATPase fractions

The polypeptide composition of each fraction was determined by one-dimensional polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate, after removal of lipid and detergent by precipitation with acetone/ethanol (Fig. 1).

The ATPase I fraction from the $\text{C}_{12}\text{E}_8/(\text{NH}_4)_2\text{SO}_4$ procedure (track 5) contains six major bands, of apparent M_r 140 000, 70 000, 57 000, 41 000, 33 000 and 16 000: these are designated bands 1–6 respectively. There are traces of other components, but these are eliminated if a higher concentration of detergent is employed during solubilization of the membranes, with a slight loss of ATPase activity. The soluble fraction (track 6), with ATPase II activity, contains the remainder of the chromaffin-granule membrane proteins.

The first precipitate (P1) in the Triton X-114 procedure, which has ATPase I activity, also contains bands 1–6, together with some others (track 2); this

Fig. 1. Gel electrophoresis of chromaffin-granule fractions

Track 1, purified membranes; track 7, matrix proteins. Tracks 2, 3 and 4; Triton X-114 fractionation of membranes: P1, P2 and S2 fractions. Tracks 5 and 6, $(\text{NH}_4)_2\text{SO}_4$ fractionation of detergent-solubilized membrane proteins: insoluble and soluble fractions. Outer tracks, M_r markers. Numbers to the left of the Figure refer to ATPase I polypeptides (tracks 2 and 5); the positions of dopamine β -hydroxylase (DBH, M_r 75 000, tracks 3 and 6), chromogranin A (CGA, M_r 70 000, tracks 4 and 7) and cytochrome *b*-561 (*b*-561, M_r 27 000, tracks 3 and 6) are also indicated.

precipitation is thus less selective than that obtained with C_{12}E_8 . ATPase II occurs in the detergent-rich fraction P2 (track 3), which is, however, dominated by dopamine β -hydroxylase (M_r 75 000) and cytochrome *b*-561 (M_r 27 000). The soluble phase S2 (track 4) contains the most hydrophilic proteins, including the matrix protein

Fig. 2. Autoradiograph of fractions obtained by Triton X-114 fractionation of membranes labelled with [^{14}C]DCCD

Track 1, membranes; track 2, P1; track 3, P2; track 4, S2. Left-hand track, ^{14}C -labelled M_r markers. The position of the major chromaffin-granule DCCD-reactive protein (ATPase I, subunit 5) is indicated by a horizontal arrow.

chromogranin A (M_r 70000) and several components identified as glycoproteins by lectin blotting (Gavine *et al.*, 1984).

The smallest component of the ATPase I fractions, band 6, was identified as the DCCD-reactive protein previously characterized (Sutton & Apps, 1981) by labelling chromaffin-granule membranes with [^{14}C]DCCD before fractionation, and gel electrophoresis of the separate fractions, followed by autoradiography (Fig. 2). The minor labelled band, running just above the chromaffin-granule DCCD-reactive protein, is the mitochondrial protein, which has a higher M_r (Sutton & Apps, 1981) and is present here as a contaminant.

The distribution of mitochondrial F_1 -ATPase was assayed by immune blotting (Towbin *et al.*, 1979), with an antiserum raised against bovine heart mitochondrial F_1 , which reacts with the α , β and γ subunits (Apps & Schatz, 1979). As shown in Fig. 3, only minute traces of this appear in the ATPase I fractions (tracks 2 and 5), most remaining in solution when ATPase I is precipitated.

Use of inhibitors to distinguish ATPase I from ATPase II

In order to distinguish the two enzymes, we investigated the relative effects of single concentrations of several inhibitors on the various fractions (Table 2).

ATPase I is strongly inhibited by tributyltin, by quercetin and by preincubation with the alkylating agents *N*-ethylmaleimide and 4-chloro-7-nitrobenzofurazan; vanadate is not inhibitory. In contrast, ATPase II is very

Fig. 3. Distribution of mitochondrial F_1 -ATPase during fractionation of chromaffin-granule membranes

Proteins were separated by electrophoresis, transferred to a nitrocellulose sheet, decorated with anti- F_1 -ATPase serum and ^{125}I -protein A and autoradiographed. Track 1, membranes; tracks 2, 3 and 4, Triton X-114 fractionation of membranes: P1, P2 and S2 fractions. Tracks 5 and 6, $(\text{NH}_4)_2\text{SO}_4$ fractionation of detergent-solubilized membranes: insoluble and soluble fractions. Outer tracks, ^{14}C -labelled M_r markers.

sensitive to vanadate, but only slightly affected by tributyltin, quercetin or the alkylating agents. Efrapentin, a rather specific inhibitor of mitochondrial F_1 -ATPase activity, does not inhibit ATPase I, although it has a slight inhibitory effect on the ATPase activity of the membranes, indicating some contamination by F_1 -ATPase.

These results are in agreement with those obtained earlier with ATPases separated by exclusion chromatography (Apps *et al.*, 1983), apart from the effects of quercetin, which was previously found to inhibit the vanadate-sensitive ATPase. This question has now been investigated in more detail (see below).

Hydrolysis of ITP by ATPase I

Chromaffin-granule membranes hydrolyse not only ATP, but also other nucleoside triphosphates; of these ITP is the best substrate (Kirshner, 1962). A comparison of the effects of inhibitors on the ITPase activity of chromaffin-granule membranes, and of the two ATPase fractions, shows that this activity is strongly inhibited by tributyltin in membranes and in the ATPase I fraction, whether this is prepared by $(\text{NH}_4)_2\text{SO}_4$ precipitation or by Triton X-114 fractionation (Table 1). Vanadate does not significantly affect the ITPase activity of any fraction. This suggests that ATPase I hydrolyses ITP, but that ATPase II does not; the ITPase activity of the ATPase II fraction is ascribed to contamination by vanadate-insensitive ATPases such as mitochondrial F_1 -ATPase. Surprisingly, tributyltin stimulates the $(\text{NH}_4)_2\text{SO}_4$ -soluble ITPase activity, though not ATPase; the reason for this is not known.

Table 2. Effects of inhibitors on various ATPase fractions

For experimental details see the text. Activities are expressed relative to controls assayed without inhibitors. For the alkylating agents 4-chloro-7-nitrobenzofurazan (NbfCl) and *N*-ethylmaleimide, assays were performed after preincubation of the enzyme (approx. 1 mg/ml, in 20 mM-Hepes/NaOH buffer, pH 7.0) with the inhibitor for 5 min at 20 °C.

Inhibitor	Concentration	Membranes	Relative activities			
			(NH ₄) ₂ SO ₄ -insoluble	(NH ₄) ₂ SO ₄ -soluble	Triton X-114	
					P1	P2
Tributyltin	2 μ M	18	2	91	2	87
Vanadate	2 μ M	78	91	40	98	34
NbfCl	25 μ M	16	16	96	—	—
<i>N</i> -Ethylmaleimide	25 μ M	29	24	94	—	—
Quercetin	30 μ M	62	36	82	20	78
Efrapeptin	2 μ g/ml	91	100	93	—	—

Fig. 4. Effects of quercetin on ATPase and transport activities of chromaffin-granule membranes

(a) ATPase activity of membranes (○), (NH₄)₂SO₄-insoluble fraction (ATPase I, △) and soluble fraction (ATPase II, ▲). (b) Resealed membrane 'ghosts': initial rate of uptake of 5-hydroxy[¹⁴C]tryptamine (●) and steady-state Δ pH (▼).

Inhibition by quercetin of H⁺ translocation and amine uptake in resealed chromaffin-granule 'ghosts'

As shown in Table 2, quercetin is a relatively specific inhibitor of ATPase I activity, and is therefore useful for investigating the role of ATPase I in amine transport. Fig. 4(a) shows the effects of increasing concentrations of quercetin on the ATPase activity of chromaffin-granule membranes, and on separated ATPase I and II fractions, and Fig. 4(b) shows its effect on active transport in resealed chromaffin-granule 'ghosts', namely uptake of 5-hydroxy[¹⁴C]tryptamine (which occurs via the catecholamine translocator) and generation of a transmembrane Δ pH (measured by the steady-state accumulation of [¹⁴C]methylamine). Direct comparison of Figs. 4(a) and (b) is difficult because of the low protein concentrations used in ATPase assays (0.02–0.04 mg/ml) compared with those in transport experiments (0.5 mg/ml). Furthermore measurements of Δ pH were performed in the presence of 40 mM-KI to minimize $\Delta\psi$ and maximize Δ pH; this was necessary because methylamine distribution cannot accurately measure Δ pH values less than 1.0. The data

in Fig. 4(b) establish that quercetin inhibits both ATP-dependent uptake of 5-hydroxytryptamine and H⁺ translocation, but cannot be used to assess the variation of transport rates with Δ pH.

The rate of uptake of 5-hydroxytryptamine in pH-jump experiments (Apps *et al.*, 1980a) was unaffected by quercetin up to 100 μ M (results not shown); this indicates that quercetin does not inhibit the catecholamine translocator, nor does it act as an uncoupler, collapsing the transmembrane Δ pH.

H⁺ translocation driven by hydrolysis of ATP

Hydrolysis of ITP supports only very low rates of uptake of 5-hydroxytryptamine (results not shown); this is consistent with the rather low ITP-induced steady-state Δ pH of 0.9, measured by the methylamine distribution technique. H⁺ translocation into 'ghosts' can be investigated qualitatively by measuring quenching of the fluorescence of ACMA, a permeant weak base. As shown in Fig. 5(a), ATP produces a rapid quenching of ACMA fluorescence, which is reversed by the uncoupler carbonyl

Fig. 5. Quenching of ACMA fluorescence by resealed chromaffin-granule 'ghosts', induced by ATP (a) or ITP (b)

'Ghosts' were preincubated with *N*-ethylmaleimide at various concentrations: 0 (traces A and F), 2.5 μ M (trace B), 5 μ M (trace G), 10 μ M (traces C and H), 15 μ M (trace D) or 20 μ M (trace E). Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was added to a final concentration of 5 μ M (traces A and F). Duramycin (5 μ g/ml) or vanadate (2 μ M) had no effect on the ATP-dependent or ITP-dependent quenching, traces in presence of these inhibitors being superimposable on traces A and F.

cyanide *p*-trifluoromethoxyphenylhydrazone; preincubation of the 'ghosts' with *N*-ethylmaleimide inhibits fluorescence quenching, but vanadate is without effect. ITP produces a slower and less extensive quenching of ACMA fluorescence (Fig. 5b); like ATP-dependent quenching, this is inhibited by *N*-ethylmaleimide but not by vanadate. Duramycin, an inhibitor of proton pumping in clathrin-coated vesicles (Stone *et al.*, 1984), had no effect on acidification of 'ghosts' (as judged by the quenching of ACMA fluorescence) at a concentration of 5 μ g/ml.

It is noticeable that ATP causes a small, non-energy-linked, quenching of ACMA fluorescence; this is seen in Fig. 5, which shows that addition of uncoupler does not produce a return of the fluorescence to exactly its starting value.

DISCUSSION

The fractionation procedures described here separate the two major ATPases of the chromaffin-granule membrane; they can be carried out rapidly, and are easily applied on a fairly large scale. In each case ATPase I appears in the most hydrophobic fraction and is associated with a relatively small number of polypeptides, whereas the more soluble ATPase II is purified much less.

The first fraction in the $C_{12}E_8$ /cholate/ $(NH_4)_2SO_4$ procedure, which is discarded during the purification of cytochrome *b*-561 (Apps *et al.*, 1980b), contains ATPase I in the purest state yet achieved. Of the six bands apparent after one-dimensional electrophoresis (Fig. 1), the largest (band 1, approx. M_r 140000) is an aggregate, since it has no equivalent in unfractionated membranes and is not seen if the sample is heated at 100 °C before electrophoresis (results not shown). The remaining five bands are all major components of the chromaffin-granule membrane, although band 2 (M_r 70000) is obscured in one-dimensional electrophoretograms by dopamine β -hydroxylase and chromogranin A, and is only resolved from these by two-dimensional electrophoresis (results not shown). Band 6 is the DCCD-reactive protein described previously (Sutton & Apps, 1981); its apparent

M_r on polyacrylamide gels is 14000, but amino acid analysis and comparison with the mobility of the mitochondrial DCCD-reactive protein suggest an M_r of 7000 (Apps *et al.*, 1982). If the chromaffin-granule membranes are contaminated with mitochondria, the mitochondrial DCCD-reactive protein, identifiable by its lower electrophoretic mobility, appears in the P2 fraction (J. G. Pryde, unpublished work).

It is not known which of these polypeptides (apart from band 6) are part of ATPase I. The same components are found in ATPase I purified by Triton X-114 fractionation (Fig. 1), exclusion chromatography (Apps *et al.*, 1983) or hydrophobic column chromatography (J. M. Percy, J. G. Pryde & D. K. Apps, unpublished work). The apparent M_r of ATPase I is 400000 (Apps *et al.*, 1983), suggesting that the enzyme is a complex, but elucidation of its subunit stoichiometry must await further purification. Gel scanning indicated that ATPase I purified by different procedures contained different relative amounts of bands 1–6, suggesting either that some of these band proteins may not be part of the ATPase I complex, or that the extraction procedures cause some loss of subunits, thus accounting for the rather low recovery of activity. When one-dimensional polyacrylamide gels of chromaffin-granule membranes were scanned, only bands 3, 4 and 5 could be resolved from other membrane proteins; these are present in approximately equal amounts, equivalent to 200–250 copies per granule. A similar number of copies was calculated from band 6, by radiolabelling it with [^{14}C]DCCD (Apps *et al.*, 1983). The reliability of the scanning procedure was checked by scanning the cytochrome *b*-561 band; this was found to constitute about 20% of the membrane protein, in good agreement with the estimate of 19% obtained immunologically (Apps *et al.*, 1984).

We previously showed (Apps *et al.*, 1980c) that ATP hydrolysis, H^+ translocation and amine uptake by resealed chromaffin-granule 'ghosts' were inhibited by 4-chloro-7-nitrobenzofurazan, although this inhibitor had no effect on the catecholamine translocase itself, since amine uptake driven by an imposed pH gradient was not inhibited. Flatmark *et al.* (1982) found that another

alkylating agent, *N*-ethylmaleimide, completely blocked H^+ translocation at concentrations that only partially inhibited ATPase activity: even at high concentrations of *N*-ethylmaleimide, only 50% inhibition of ATPase activity occurred. Rather similar results have now been obtained with quercetin; this is a potent inhibitor of ATPase I, but has rather little effect on ATPase II; indeed, it is possible that the slight inhibition of this fraction by quercetin is due to contamination with ATPase I. Quercetin inhibits, not only ATPase activity, but also ATP-dependent uptake of 5-hydroxy[^{14}C]tryptamine (Fig. 4), yet it has no effect on the rate or extent of uptake of 5-hydroxytryptamine when this is driven by a rapid pH jump, which imposes a pH of about 2.1 (Apps *et al.*, 1980a). The relationship between ATP hydrolysis, pH generation and amine uptake is complex, since the steady-state concentration of catecholamines within the 'ghosts' depends on the square of the proton concentration ratio, or $2\Delta pH$ (Njus *et al.*, 1981), and ΔpH depends upon the rate of active H^+ translocation, passive H^+ leakage, internal buffering capacity and the concentration of permeant anions in the external medium. A relatively small change in ΔpH (Fig. 4b) may therefore have profound effects on the rate of uptake of 5-hydroxytryptamine.

Although ITP is a relatively poor substrate for the chromaffin-granule ATPase, ITP hydrolysis by 'ghosts' generates a significant ΔpH , measurable by methylamine distribution or by quenching of ACMA fluorescence (Fig. 5). The inhibitor-sensitivity of ATPase activity (Table 1) suggests that ATPase I hydrolyses ITP but that ATPase II is more specific for ATP. Taken together, the substrate specificity of nucleoside triphosphate-driven H^+ translocation, and its sensitivity to quercetin, 4-chloro-7-nitrobenzofurazan and *N*-ethylmaleimide, show close parallels with the properties of ATPase I and demonstrate rather conclusively that ATPase I catalyses H^+ translocation. It seems unlikely that ATPase II translocates protons; quite apart from the redundancy of a second H^+ translocator in the same membrane, ΔpH generation and amine transport by 'ghosts' is completely insensitive to vanadate.

What structural and mechanistic similarities does ATPase I have to other H^+ -translocating ATPases? Its high M_r value suggests that it is a complex of several subunits, since the largest polypeptide in the ATPase I fraction has M_r 70000. Inhibition by DCCD apparently occurs by covalent modification of the smallest subunit of the complex, a very hydrophobic protein that can be extracted into chloroform/methanol (Sutton & Apps, 1981). This presumably forms a H^+ -conducting channel, since low concentrations of DCCD actually increase the ATP-generated ΔpH in 'ghosts' (Apps *et al.*, 1980c). The enzyme does not catalyse [^{14}C]ADP/ATP isotope exchange (Apps & Reid, 1977), suggesting that a phosphoryl-enzyme does not occur as an intermediate in the catalytic cycle; however, it does catalyse ATP/[^{32}P]P $_i$ isotope exchange, as would be expected for a reversible H^+ -translocating ATPase (Roisin & Henry, 1982).

These properties clearly distinguish ATPase I from the E_1E_2 type of H^+ -translocating ATPase, such as occurs in gastric mucosa and the fungal plasma membrane, and suggest some similarity to H^+ -translocating ATPases of the F_1F_0 type, found in mitochondria, chloroplasts and bacteria. However, these ATPases are not inhibited by low concentrations of *N*-ethylmaleimide; the DCCD-

reactive subunit of ATPase I shows significant differences from its mitochondrial counterpart; and, most significantly, antiserum raised against bovine mitochondrial F_1 does not react with any component of chromaffin-granule ATPase I (Fig. 3). It thus appears that, although ATPase I may be mechanistically similar to F_1F_0 -ATPase, it is structurally similar only in its large size and in the fact that multiple subunits are attached to a DCCD-sensitive H^+ -conducting channel. It is generally similar to the H^+ -translocating ATPases that have been partially characterized in lysosomes (Moriyama *et al.*, 1984), Golgi vesicles (Glickman *et al.*, 1983), clathrin-coated vesicles (Xie *et al.*, 1984; Forgac & Cantley, 1984), the vacuolar membranes of fungi (Bowman & Bowman, 1982; Uchida *et al.*, 1985) and plants (Churchill & Sze, 1983), and the secretory vesicles of platelets (Dean *et al.*, 1984) and the pituitary (Russell, 1984). It may be that all of these enzymes are of the same type, and that work on chromaffin-granule ATPase I will yield results applicable to exocytotic and endocytotic organelles. However, it is noteworthy that chromaffin-granule ATPase I, unlike the H^+ -translocating ATPase of coated vesicles, is not inhibited by duramycin.

It has been suggested that the knob-like protuberances seen on the cytoplasmic faces of chromaffin granules (Schmidt *et al.*, 1982) and synaptic vesicles (Stadler & Tsukita, 1984) may be the H^+ -translocating ATPase. This is consistent with the proposed structure of ATPase I, and the diameter of these particles (8–9 nm) would be consistent with its M_r value. However, at the measured frequency of 22 particles per granule, such particles would be only about 3% of the membrane protein, whereas bands 3–5 of the ATPase I fraction each comprise 2–3% of the membrane protein, or about 200 copies per granule. Although ATPase I may contain multiple copies of some subunits, these rough calculations suggest either that some of the bands in the ATPase I fraction are not part of the enzyme, or that the number of ATPase molecules per granule is underestimated by electron microscopy.

What is the significance of the F_1 -like ATPase present in chromaffin-granule membrane preparations? Despite the fact that it is present in higher relative concentrations than mitochondrial markers (Apps *et al.*, 1983), it appears to be a contaminant: it is probable that F_1 -ATPase particles become detached from mitochondria during cell fractionation, and associate with chromaffin granules to a greater extent than do intrinsic mitochondrial membrane proteins.

Little can be said of the structure or function of ATPase II. It has not been purified, and, since there is no specific assay for it, we cannot yet be certain that it is genuinely a component of chromaffin granules. After separation by exclusion chromatography (Apps *et al.*, 1983), ATPases I and II appear to have approximately equal activity, and to account for all of the ATPase activity of chromaffin-granule membranes; in the present study, the ATPase II activity recovered exceeds that of ATPase I, because ATPase I is partially inactivated by the fractionation procedure. The existence of two ATPases accounts for the partial inhibitions produced by several ATPase inhibitors when tested on intact membranes (Apps *et al.*, 1980c; Flatmark *et al.*, 1982). It is noteworthy that secretory vesicles from the pituitary also contain two ATPases, the properties of which are quite similar to those of the chromaffin-granule enzymes (Russell, 1984).

It is not clear which of the two enzymes was separated by Cidon & Nelson (1983), but it may be ATPase II, since it sedimented at a significantly lower rate than did mitochondrial F_1 -ATPase; in their study, treatment with NaBr to remove F_1 -ATPase apparently inactivated one of the chromaffin-granule ATPases as well.

This work was supported by a grant from the Medical Research Council. We thank Dr. J. H. Phillips for discussion of the manuscript.

REFERENCES

- Apps, D. K. & Reid, G. A. (1977) *Biochem. J.* **167**, 297–300
- Apps, D. K. & Schatz, G. (1979) *Eur. J. Biochem.* **100**, 411–419
- Apps, D. K., Pryde, J. G. & Phillips, J. H. (1980a) *FEBS Lett.* **111**, 386–390
- Apps, D. K., Pryde, J. G. & Phillips, J. H. (1980b) *Neuroscience* **5**, 2279–2287
- Apps, D. K., Pryde, J. G., Sutton, R. & Phillips, J. H. (1980c) *Biochem. J.* **190**, 273–282
- Apps, D. K., Pryde, J. G. & Sutton, R. (1982) *Ann. N.Y. Acad. Sci.* **402**, 134–145
- Apps, D. K., Pryde, J. G. & Sutton, R. (1983) *Neuroscience* **9**, 687–700
- Apps, D. K., Boisclair, M. D., Gavine, F. S. & Pettigrew, G. W. (1984) *Biochim. Biophys. Acta* **764**, 8–16
- Bordier, C. (1981) *J. Biol. Chem.* **256**, 1604–1607
- Bowman, E. J. & Bowman, B. J. (1982) *J. Bacteriol.* **151**, 1326–1337
- Buckland, R. M., Radda, G. K. & Wakefield, L. M. (1979) *FEBS Lett.* **103**, 323–327
- Churchill, K. A. & Sze, H. (1983) *Plant Physiol.* **71**, 610–617
- Cidon, S. & Nelson, N. (1983) *J. Biol. Chem.* **258**, 2892–2898
- Cidon, S., Ben-David, H. & Nelson, N. (1983) *J. Biol. Chem.* **258**, 11684–11688
- Dean, G. E., Fishkes, H., Nelson, P. J. & Rudnick, G. (1984) *J. Biol. Chem.* **259**, 9569–9574
- Flatmark, T., Gronberg, M., Husebye, E. & Berge, S. V. (1982) *FEBS Lett.* **149**, 71–74
- Forgac, M. & Cantley, L. (1984) *J. Biol. Chem.* **259**, 8101–8105
- Gabizon, R., Yetinson, T. & Schuldiner, S. (1982) *J. Biol. Chem.* **257**, 15145–15150
- Gavine, F. S., Pryde, J. G. & Apps, D. K. (1984) *J. Neurochem.* **43**, 1243–1252
- Giraudat, J., Roisin, M.-P. & Henry, J.-P. (1980) *Biochemistry* **19**, 4499–4505
- Glickman, J., Croen, K., Kelly, S. & Al-Awqati, Q. (1983) *J. Cell Biol.* **97**, 1303–1308
- Grueninger, H. A., Apps, D. K. & Phillips, J. H. (1983) *Neuroscience* **9**, 917–924
- Hartree, E. F. (1972) *Anal. Biochem.* **48**, 422–427
- Kirshner, N. (1962) *J. Biol. Chem.* **237**, 2311–2317
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–688
- Moriyama, Y., Takano, T. & Ohkuma, S. (1984) *J. Biochem. (Tokyo)* **95**, 995–1007
- Njus, D., Knoth, J. & Zallakian, M. (1981) *Curr. Top. Bioenerg.* **11**, 107–147
- Roisin, M. P. & Henry, J.-P. (1982) *Biochim. Biophys. Acta* **681**, 292–299
- Russell, J. T. (1984) *J. Biol. Chem.* **259**, 9496–9507
- Scherman, D. & Henry, J.-P. (1983) *Biochemistry* **22**, 2805–2810
- Schmidt, W., Winkler, H. & Plattner, H. (1982) *Eur. J. Cell Biol.* **27**, 96–104
- Stadler, H. & Tsukita, S. (1984) *EMBO J.* **3**, 3333–3337
- Stone, D. K., Xie, X.-S. & Racker, E. (1984) *J. Biol. Chem.* **259**, 2701–2703
- Sutton, R. & Apps, D. K. (1981) *FEBS Lett.* **130**, 103–106
- Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354
- Uchida, E., Ohsumi, Y. & Anraku, Y. (1985) *J. Biol. Chem.* **260**, 1090–1095
- Weber, A. & Winkler, H. (1981) *Neuroscience* **6**, 2269–2276
- Xie, X.-S., Stone, D. K. & Racker, E. (1984) *J. Biol. Chem.* **259**, 11676–11678

Received 27 March 1985/24 June 1985; accepted 28 June 1985