Adenosine Triphosphatase and Adenosine Diphosphate/Adenosine Triphosphate Isotope-Exchange Activities of the Chromaffin-Granule Membrane

By DAVID K. APPS and GRAEME A. REID

Department of Biochemistry, University of Edinburgh Medical School,
Teviot Place, Edinburgh EH8 9AG, Scotland, U.K.

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The association of adenosine triphosphatase and ADP/ATP isotope-exchange activities with chromaffin-granule membranes was shown by sucrose-density-gradient centrifugation. The two activities were solubilized, and separated by differential sedimentation.

The membrane of the catecholamine-storage granule (chromaffin granule) of the bovine adrenal medulla has been reported to possess several enzymes that act on ATP: an Mg^{2+} -dependent ATPase* (EC 3.6.1.3; Hillarp, 1958), phosphatidylinositol kinase (EC 2.7.1.67; Phillips, 1973) and ATP-dependent catecholamine translocase (Phillips, 1974). ATP hydrolysis has been implicated in the creation of transmembrane pH gradients (Casey *et al.*, 1977) and ionic potentials (Pollard *et al.*, 1976) and in the enhancement of 8-anilino-naphthalene-1-sulphonic acid fluorescence by the granules (Bashford *et al.*, 1976). Recently, the association of ATPase with the granules has been claimed to be an artifact (Laduron *et al.*, 1976). The present paper reports the co-purification of ATPase activity with the granule membrane, and its solubilization and separation from ADP/ATP isotope-exchange activity, which is also associated with the granule membrane.

Methods

Enzyme assays

ATPase and [^{14}C]ADP/ATP exchange were assayed as described by Apps & Nairn (1977); the substrate concentrations for ATPase were 1 mM- $[\gamma\text{-}^{32}P]$ ATP (1 Ci/mol) and 10 mM- $MgCl_2$, and those for ADP/ATP exchange were 0.5 mM- $[8\text{-}^{14}C]$ ADP (5 Ci/mol), 5 mM-ATP, 10 mM- $MgCl_2$, and the buffer in each case was 0.1 M-triethanolamine chloride, pH 7.0. P_i /ATP exchange was assayed in 1 mM-ATP/10 mM- $MgCl_2$ /0.1–5.0 mM- $[\text{32}P]P_i$ (1 Ci/mol), the products being separated by chromatography on ion-exchange paper. Dopamine β -hydroxylase (EC 1.14.17.1) was assayed as described by Phillips (1973), with a tyramine concentration of 220 μ M. Monoamine oxidase (EC 1.4.3.4) was assayed by the method of Wurtman & Axelrod (1963), succinate dehydrogenase

* Abbreviations: ATPase, adenosine triphosphatase; dopamine, 3,4-dihydroxyphenethylamine; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

(EC 1.3.99.1) by the method of Porteous & Clark (1965), β -glucuronidase (EC 3.2.1.31) by the method of Gianetto & de Duve (1955) and protein as described by Bradford (1976). Enzyme assays were performed at 30°C, and where necessary ATPase and protein determinations were corrected for interference by detergent.

Fractionation methods

Crude chromaffin granules were prepared from fresh bovine adrenal medullae by differential centrifugation in 0.3 M-sucrose buffered with 10 mM-Hepes/NaOH, pH 7.0 (Phillips, 1974). Further purification was achieved by either centrifugation through 1.6 M-sucrose (Schneider, 1972) or isopycnic centrifugation on 60 ml linear gradients of 0.8–2.5 M-sucrose (3 h at 420 000 g, 4°C).

Chromaffin-granule membranes were prepared by hypo-osmotic lysis of the granules, and collected by centrifugation (1 h at 160 000 g, 4°C). They were washed in 10 mM-Hepes buffer, then in 0.25 M-KCl/10 mM-Hepes, and finally resuspended in 10 mM-Hepes at a concentration of 2–5 mg of protein/ml.

Sucrose-density-gradient analysis of membranes (Schneider, 1972) was performed by layering a 4.5 ml linear gradient of 0.3–1.4 M-sucrose over 0.5 ml of membrane suspension made 2 M in sucrose. The gradients were centrifuged (3 h at 270 000 g, 4°C) and fractionated.

Sedimentation analysis of solubilized membrane proteins was performed on 12.5 ml linear gradients of 8–35% (w/v) glycerol in 10 mM-Hepes, centrifuged for 15 h at 185 000 g at 4°C.

Solubilization of ATPase and ADP/ATP isotope-exchange activities

(a) *Detergent solubilization.* Membrane suspensions were made 2.0% in Nonidet P.42 (Shell Chemical Co., Shell Centre, London S.E.1, U.K.) by addition of a 25% (w/v) deionized solution of the detergent.

Insoluble material was removed by centrifugation (1 h at 160 000g, 4°C).

(b) *Solubilization by lipid extraction.* Membrane suspensions (2 ml) were made 1 mM in EDTA and 3 mM in ATP; 1 ml of dichloromethane was added, the mixture brought to room temperature (22°C), vortexed for 30 s and centrifuged in Corex glass tubes (20 min at 26 000g, 4°C). The upper (aqueous) layer was decanted from dichloromethane and precipitated protein, which were discarded.

Results and Discussion

Enzyme activities of the chromaffin-granule membrane

Fig. 1 shows the distribution of marker enzymes in a sucrose density gradient after centrifugation of membranes to equilibrium. Despite previous centrifugation of the granules through 1.6 M-sucrose, some contamination with mitochondrial markers is apparent; nonetheless, ATPase activity is associated with dopamine β -hydroxylase, used as a marker for the chromaffin-granule membrane. Fig. 2 shows the results of centrifuging membranes obtained by hypotonic lysis of granules previously purified on continuous sucrose gradients. In this case, lysosomal and inner- and outer-mitochondrial-membrane markers could not be detected, and ATPase, ADP/ATP exchange and dopamine β -hydroxylase equilibrated in parallel. These results were obtained in three separate experiments; qualitatively similar results were obtained from gradients containing 0.5 M-KCl or 1.0 M-urea. In all cases ATPase activity co-equilibrated with dopamine β -hydroxylase, less than 15% being found in other parts of the gradient.

Chromaffin-granule-membrane ATPase was not inhibited by oligomycin (20 μ g/ml) or ouabain (2 mM); but it was inhibited 50% by quercetin (Sigma

Chemical Co., Kingston-upon-Thames, Surrey, U.K.) (40 μ g/ml) and 28% by efrapectin (a gift from Dr. R. L. Hamill, Lilly Research Laboratories, Indianapolis, IN, U.S.A.) (30 μ g/ml). Taken with the above results, this suggests that ATPase activity is not due to a contaminant, but is a function of the chromaffin-granule membrane.

Solubilization of enzyme activities

Trifaro & Warner (1972) reported solubilization of chromaffin-granule ATPase by the poly(ethylene oxide)-based non-ionic detergent Lubrol PX, but we found that, of the related detergents Lubrol PX, Lubrol WX, Triton X-100, Tween-80 and Nonidet P.42, only Nonidet produced total solubilization. Chloroform extraction of membrane lipids solubilizes yeast mitochondrial ATPase (Takeshige *et al.*, 1976); trying a similar approach, we found that dichloromethane extraction produced higher yields (consistently 40–60% in ten experiments) of soluble chromaffin-granule-membrane ATPase than did 1,2-dichloroethane, diethyl ether, chloroform, carbon tetrachloride, pentan-1-ol or octan-1-ol. The activity of both detergent- and solvent-solubilized ATPase declined rapidly on storage at –20°C, 4°C or room temperature, but addition of glycerol (25%, w/v) and 1 mM-dithiothreitol permitted storage at –20°C with no loss of activity in 2 months, although dithiothreitol produced inactivation of dopamine β -hydroxylase. Specific enzyme activities of the various preparations are shown in Table 1.

Sedimentation analysis of enzyme activities

Fig. 3 shows the distribution of soluble enzyme activities in glycerol density gradients; continued centrifugation resulted in complete sedimentation of these enzymes. Marker proteins of known sedimentation coefficient were added to some gradients,

Fig. 1. Distribution of enzyme activities in sucrose gradients after isopycnic centrifugation of chromaffin-granule membranes

For details, see the text. $\circ$, 5 $\times$ Dopamine β -hydroxylase activity; $\bullet$, ATPase; $\triangle$, 100 $\times$ monoamine oxidase activity; $\blacktriangle$, 5 $\times$ succinate dehydrogenase activity; ∇ , 2 $\times$ β -glucuronidase activity.

Fig. 2. Distribution of enzyme activities in sucrose gradients after isopycnic centrifugation of highly purified chromaffin-granule membranes

For details, see the text. $\bullet$, ATPase; $\circ$, 2 $\times$ dopamine β -hydroxylase activity; $\triangle$, ADP/ATP exchange.

Table 1. *Specific enzyme activities of membrane preparations*

The results are expressed as nmol/min per mg of protein and are means $\pm$ S.D. for the numbers of experiments given in parentheses.

Preparation	ATPase	ADP/ATP isotope exchange	Dopamine β -hydroxylase
Purified membranes	170 $\pm$ 30 (6)	175 $\pm$ 30 (4)	17 $\pm$ 5 (6)
Nonidet P.42 supernatant	200 $\pm$ 40 (6)	260 $\pm$ 30 (4)	22 $\pm$ 6 (6)
Dichloromethane extract	60 $\pm$ 20 (4)	200 $\pm$ 40 (4)	0

Fig. 3. *Separation of solubilized enzyme activities by centrifugation through glycerol gradients*

For experimental details, see the text. $\circ$, $\bullet$, ADP/ATP isotope exchange; $\triangle$, $\blacktriangle$, ATPase. Open symbols, solvent solubilization; filled symbols, detergent solubilization.

Fig. 4. *Determination of sedimentation coefficients of solubilized enzymes by glycerol-density-gradient centrifugation*

permitting determination of the sedimentation coefficients of the enzymes under study (Fig. 4), since under the conditions used migration was directly proportional to $s_{20,w}$ (Martin & Ames, 1961). ATPase and ADP/ATP isotope-exchange activities were well separated, and had sedimentation coefficients 13.5S and 6.8S respectively, whereas detergent-solubilized dopamine β -hydroxylase had $s_{20,w} = 11.2$ S (three

experiments). Detergent solubilization produced broader peaks than did dichloromethane extraction of the membrane, presumably because the detergent-solubilized enzymes were still associated with some lipid, and showed micro-heterogeneity. This heterogeneity was more pronounced when the membranes had been frozen and thawed before detergent treatment.

Functions of the ATPase and ADP/ATP-exchange enzyme

The mechanism of catecholamine accumulation by chromaffin granules is poorly understood; kinetic evidence suggests that the ATP-dependent catecholamine translocase may be an entity separate from the ATPase (Phillips, 1974), but there is also substantial evidence for the involvement of the ATPase in catecholamine uptake. The present study reveals only one major solubilizable ATPase activity, but it should be noted that ATP hydrolysis in resealed chromaffin-granule 'ghosts' proceeds at 100–200 times the rate of catecholamine accumulation (Phillips, 1974), that dichloromethane extraction solubilizes only 40–60% of the total activity, and that Nonidet P.42, although it apparently solubilizes all of the ATPase activity, yields a rather heterogeneous product.

The function of the enzyme that catalyses isotope exchange between ATP and ADP is unknown; its activity greatly exceeds those of phosphatidylinositol kinase and the catecholamine translocase (Phillips, 1973, 1974), although this does not exclude such an identity. Adenylate kinase (EC 2.7.4.3), which catalyses isotope exchange between AMP, ADP and ATP, was simultaneously assayed by the same chromatographic procedure as was used to monitor ADP/ATP exchange (Apps & Nairn, 1977) and accounted for less than 2% of the observed isotope exchange in all experiments. The exchange is presumably the result of reversible phosphorylation of the enzyme, a second substrate then accepting or causing release of the phosphoryl residue; P_i /ATP-exchange activity was not detected under any conditions (upper limit 0.01 nmol/min per mg of protein; four experiments), suggesting that total reversal of ATP hydrolysis is not the mechanism of isotope exchange.

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