ISOLATION AND CHARACTERIZATION OF MAGNESIUM ADENOSINETRIPHOSPHATASE FROM THE CHROMAFFIN GRANULE MEMBRANE

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1. Introduction

Catecholamine storage vesicles (chromaffin granules) of the bovine adrenal medulla have long been thought to contain a Mg–ATPase (EC 3.6.1.3.) [1,2]. Although the association of this activity with chromaffin granules has recently been questioned [3], its co-purification with the granule membrane has been reported [4]. There is considerable evidence that ATP hydrolysis by chromaffin granules and resealed 'ghosts' results in acidification of the interior of the vesicle [5–7] and the uptake of catecholamines in response to transmembrane pH-gradients has been demonstrated in resealed chromaffin granule 'ghosts' (Phillips, J. H., personal communication). In the present study we report the isolation of a soluble form of Mg–ATPase from chromaffin granule membranes and an investigation of its structure and properties. Electrophoretic analysis suggests the presence of at least 3 types of subunit, which have similar electrophoretic mobility to the major subunits of mitochondrial ATPase. However, the enzymes from mitochondria and chromaffin granules differ in their sensitivity to some inhibitors.

Abbreviations: ATPase, adenosine triphosphatase; bicine, *NN'*-bis (2-hydroxyethyl) glycine; Hepes, *N*-2-hydroxyethylpiperazine *N'* 2-ethane sulphonic acid

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2. Materials and methods

2.1. *Assays*

ATPase was assayed as described [8], the standard assay medium (0.3 ml) containing 1 mM [γ-^{32}P]ATP (approx. 1 Ci/mol), 2 mM $MgCl_2$ and 0.1 M bicine–NaOH buffer, pH 8.3. Protein was determined by the method [9] using crystalline bovine serum albumin as standard. Succinate dehydrogenase (EC 1.3.99.1) was assayed at 30°C, according to [10].

2.2. *Inhibitors*

Efrapeptin (A23871) was the generous gift of R. L. Hamill, Lilly Research Laboratories, Indianapolis. Oligomycin was from Boehringer and Söhne, quercetin from Sigma and trimethyltin chloride from BDH. All were added as ethanolic solutions, the concentration of ethanol in assay media never exceeding 3%, v/v.

2.3. *Fractionation methods*

Chromaffin granules were prepared from fresh bovine adrenal medullae by differential centrifugation of the homogenate in 0.3 M sucrose [11] and further purified by centrifugation through 1.8 M sucrose [12]. Chromaffin granule membranes were prepared, and washed in buffered 0.25 M KCl, as described [4]. Alternatively both mitochondria and chromaffin granules were prepared by isopycnic centrifugation of the post-nuclear supernatant to equilibrium (2 h at 25 000 rev/min, 4°C, in a Beckman SW25.2 rotor) on 60 ml linear gradients of 0.8–2.5 M sucrose: the two fractions were collected separately, and washed membranes prepared from each. All

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solutions were buffered with 10 mM Hepes–NaOH, pH 7.0.

2.4. *Isolation of chromaffin granule membrane ATPase*

After solubilisation by extraction of purified chromaffin granule membranes with dichloromethane [4], the enzyme was concentrated approx. 5-fold in an Amicon ultrafilter with a PM30 membrane. Portions, 0.5 ml, were applied to 12.5 ml linear gradients of 8–35% w/v glycerol in 10 mM Hepes, pH 7.0, 0.2 mM EDTA, 1 mM dithiothreitol, 3 mM ATP and centrifuged for 15 h at 39 000 rev/min in a Beckman SW41 rotor at 4°C. The gradients were divided into 16 fractions, and assayed for protein and ATPase activity; the 6th and 7th fractions from the bottom of the tube reproducibly contained pure ATPase.

2.5. *Electrophoresis*

Disc gel electrophoresis of native protein was performed on 6 cm cylindrical gels of 6% acrylamide in 25 mM Tris, 0.1 M glycine, pH 8.6. Gels were stained for protein with Coomassie blue, or for activity by immersion for 1 h at 30°C in 3 mM ATP, 5 mM $Mg(CH_3CO_2)_2$, 1 mM $Pb(NO_3)_2$, 0.1 M Tris-acetate, pH 7.5. Electrophoresis in the presence of sodium dodecyl sulphate was on slab gels of 8% acrylamide, essentially as described [13], or on similar gels with acrylamide concentration increasing exponentially from 10–15%. Molecular weights were estimated from electrophoretic mobilities, using bovine serum albumin, ovalbumin and human γ-globulin as standards. Gels were scanned at 570 nm in a Gilford gel scanner.

3. Results and discussion

Following the purification procedure detailed in table 1, the enzyme appeared to be homogeneous (fig.1), a single active band appearing on 6% polyacrylamide gel electrophoresis with the maximum loading used (25 μg protein). The sedimentation constant of 13.5 S [4] suggests an approx. mol. wt 400 000 as was obtained with a detergent-solubilised preparation [14]. This is close to that of the mitochondrial H^+-translocating ATPase, and electrophoresis in the presence of sodium dodecyl sulphate suggests a similar subunit composition (fig.2). The major subunits have app. mol. wt 50 000, 51 000 and

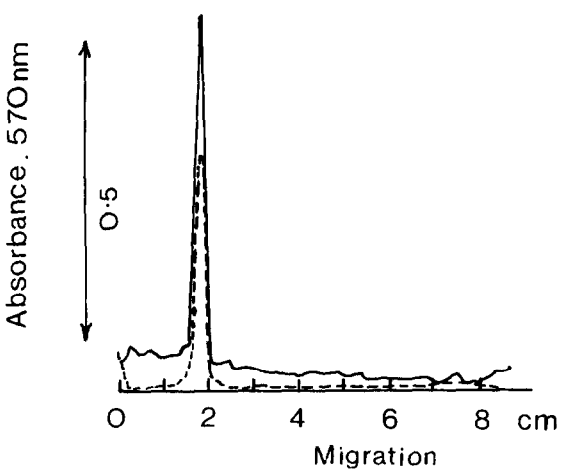

Fig.1. Disc electrophoresis of purified chromaffin granule membrane ATPase (10 μg protein), on 6% polyacrylamide gels, under non-denaturing conditions. (——) Stained for protein, with Coomassie blue. (– – – –) Stained for activity (see text).

Table 1
Purification of chromaffin granule membrane ATPase

Step	Total act. (units)	Protein (mg)	Spec. act. (units/mg)	Recovery (%)
Purified membranes	4530	35	129	100
Dichloromethane extract	3770	3.4	1108	83
Concentrated dichloromethane extract	3030	3.1	977	67
Peak fractions from glycerol gradients	1260	0.82	1537	28

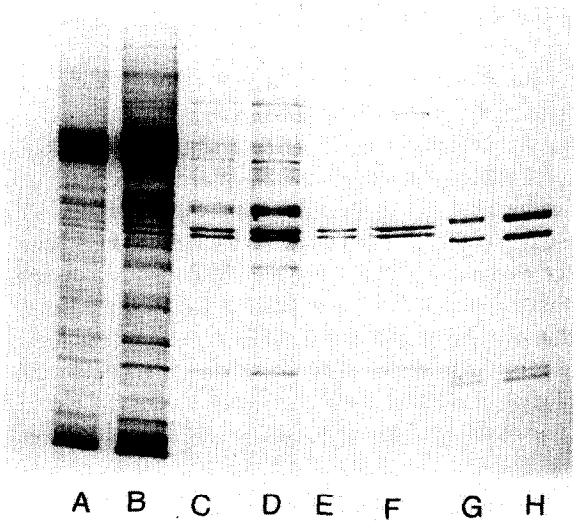

Fig.2. Slab electrophoresis of chromaffin granule membrane ATPase at various stages of purification, on 10–15% (exponential gradient) polyacrylamide gels, in presence of 0.1%, w/v, sodium dodecyl sulphate. Tracks A and B, chromaffin granule membranes (15 μg and 30 μg protein, respectively). Tracks C and D, dichloromethane extract of chromaffin granule membranes (9 μg and 18 μg). Tracks E and F, purified chromaffin granule membrane ATPase (2 μg and 4 μg). Tracks G and M, partially purified yeast mitochondrial F_1 ATPase (3 μg and 6 μg).

28 000; in addition, an apparently sub-stoichiometric amount of a polypeptide of mol. wt 89 000 is present. We cannot accurately assess the stoichiometry, as the relative intensity of staining of the bands is unknown, but from fig.2, the approximate apparent molar ratio of these polypeptides, in decreasing order of molecular weight, is 1:5:4:2. The bands of largest and smallest molecular weight could therefore be impurities, although excision of the single band obtained under non-denaturing conditions (fig.1) and re-electrophoresis in the presence of sodium dodecyl sulphate, produced a pattern similar to fig.2.

Figure 3 compares the effects of some inhibitors on membrane-bound ATPases from adrenal mitochondria and chromaffin granules. The chromaffin

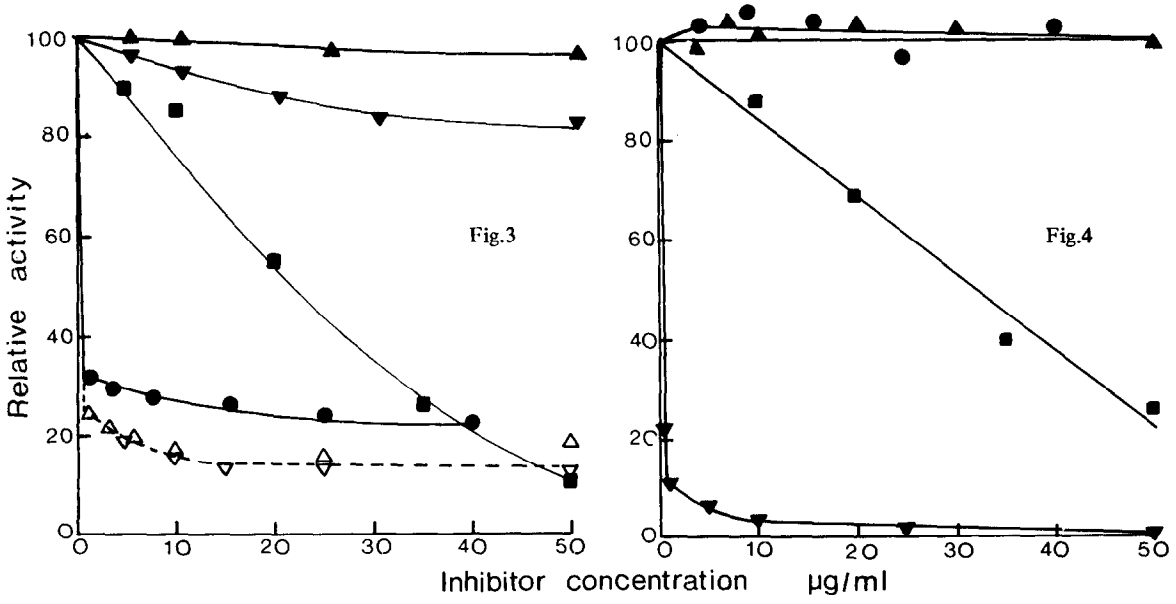

Fig.3. Effects of inhibitors on membrane ATPase activity. Purified mitochondrial membranes (open symbols, 100 μg protein/ml) or chromaffin granule membranes (solid symbols, 100 μg/ml) were assayed as described in the text, in presence of various inhibitors. (△,▲) Oligomycin; (▽,▼) efrapeptin; (■) quercetin; (●) trimethyltin chloride. Relative activities were corrected for the effects of ethanol, which were not greater than 5%. Fig.4. Effects of inhibitors on purified chromaffin granule ATPase (8 μg/ml). Symbols and conditions as in fig.3.

granule enzyme is insensitive to oligomycin and efrapeptin, but becomes sensitive to efrapeptin on solubilisation (fig.4).

These results raise the following two questions:

1. Could the enzyme we have isolated arise through mitochondrial contamination?

2. If it derives from the chromaffin granule membrane, is it the sole or even the major ATPase present?

Despite its similarity to the mitochondrial enzyme, we believe the ATPase cannot be of mitochondrial origin: the specific activity of the inner mitochondrial membrane marker succinate dehydrogenase in chromaffin granule membranes is less than 3% of its value in purified adrenal mitochondrial membranes (2.8 ± 0.6 and 108 ± 23 nmol/mg protein/min, respectively, mean ± SD, 4 experiments) whilst that of ATPase is similar (127 ± 54 and 125 ± 36 nmol/mg/min); furthermore only the mitochondrial ATPase is sensitive to oligomycin (fig.3). The enzyme appears to be the major ATPase of the chromaffin granule membrane, as about 80% of the activity detected in the granule is solubilised by dichloromethane. Of course, the specific activity might change on solubilisation, and indeed the pH optimum for activity rises from 7.0–8.3, the latter pH being used in all assays reported here. Gel electrophoresis of the crude dichloromethane extract, followed by staining for activity, revealed a single active band, with a mobility identical to that of the purified enzyme; furthermore the non-ionic detergent Nonidet P42 solubilised all of the ATPase activity of the membrane without altering the pH optimum, and this preparation had the same sedimentation constant as the dichloromethane-solubilised enzyme [4] and the same electrophoretic mobility.

We conclude that the chromaffin granule membrane possesses an ATPase of high molecular weight, containing at least two and perhaps four types of subunit. It bears considerable resemblance to mitochondrial ATPase solubilised in a similar way [15,16] and it is tempting to speculate that the two enzymes may possess some subunits in common: since oligomycin binds to a mitochondrially-synthesized protein in mitochondrial ATPase, whereas efrapeptin appears to bind to the active site [17], this might explain the difference in inhibitor sensitivity of chromaffin granule membrane ATPase.

It is noteworthy, however, that decyclohexyl carbodiimide, which has effects on mitochondrial ATPase similar to those of oligomycin, inhibits membrane-bound chromaffin granule ATPase; and aurovertin, an inhibitor of mitochondrial ATPase in its soluble and insoluble forms, fails to inhibit the chromaffin granule enzyme [18]. The two proton translocating ATPases therefore are similarly inhibited by decyclohexyl carbodiimide, and (in their soluble forms, at least) by efrapeptin; but they differ in the resistance of chromaffin granule ATPase to aurovertin and oligomycin. The molecular basis of these similarities and differences remains to be elucidated.

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