Proton-translocating adenosine triphosphatase of chromaffin-granule membranes

The active site is in the largest (70 kDa) subunit

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The proton-translocating adenosine triphosphatase (ATPase) of bovine chromaffin granules contains up to five different polypeptides. Its activity is inhibited by *N*-ethylmaleimide, and ATP protects the enzyme from inhibition. After treatment of membranes with *N*-[2-³H]ethylmaleimide, only one polypeptide is strongly radiolabelled: this is the largest (70 kDa) subunit of the proton-translocating ATPase. This subunit therefore contains the ATP-hydrolysing site. Two-dimensional electrophoresis reveals heterogeneity in this polypeptide.

INTRODUCTION

The secretory granules of the adrenal medulla, called chromaffin granules, store and secrete catecholamines, nucleotides and proteins, their very high internal catecholamine concentrations being achieved by chemiosmotic coupling between an inwardly-directed, electrogenic, H⁺-translocating ATPase, and a separate H⁺/catecholamine exchanger (Njus *et al.*, 1981). In fact, like the secretory vesicles of the pituitary (Russell, 1984), isolated bovine chromaffin-granule membranes contain two ATPases, of different *M_r* and inhibitor sensitivity (Apps *et al.*, 1983).

The smaller of these (*M_r* 140 000), ATPase II, has not yet been purified. The larger (*M_r* 400 000), ATPase I, has been obtained in a soluble, highly-purified form, and its inhibitor sensitivity and substrate specificity shown to be consistent with its being the proton pump involved in acidification of the intragranular matrix, and hence in amine uptake (Percy *et al.*, 1985). This enzyme is probably the same as that solubilized and reconstituted into liposomes, the H⁺-translocating activity of which was then directly demonstrable (Cidon & Nelson, 1983). The purified enzyme contains five types of polypeptide, the smallest being the very hydrophobic, DCCD-reactive protein already characterized (Sutton & Apps, 1981), but the functions of the other subunits are unknown – indeed, it is not even certain that they are all components of the ATPase.

ATP hydrolysis and H⁺-translocation by chromaffin granule membranes are inhibited by alkylating agents such as NEM and NbfCl (Hasselbach & Taugner, 1970; Apps *et al.*, 1980; Flatmark *et al.*, 1982), which, although potentially nonspecific in their reaction with proteins, can be used to label specifically particularly sensitive sites, if low concentrations are used. We now report the use of chemical labelling by [³H]NEM to identify the largest (70 kDa) subunit of chromaffin granule membrane ATPase I as the site of ATP hydrolysis.

MATERIALS AND METHODS

Chromaffin granule membranes were purified from fresh bovine adrenal medullae (Apps *et al.*, 1980) and fractionated with Triton X-114 as described elsewhere (Bordier, 1981; Percy *et al.*, 1985; Pryde & Phillips, 1986).

In protein labelling experiments, *N*-[ethyl-2-³H]ethylmaleimide, specific radioactivity 1.95 × 10¹⁵ Bq/mol (New England Nuclear) was added to membranes as an ethanolic solution, the final ethanol concentration not exceeding 5%. For identification of labelled proteins, membranes (3 mg/ml in 10 mM-Hepes/NaOH, pH 7.0, with 1 mM-EDTA) were treated with 1 μM-[³H]NEM for 20 min at 20 °C. The reaction was stopped by adding 50 vol. of cold acetone/ethanol (1:1, v/v), the membranes were collected by centrifugation, and proteins separated by one- or two-dimensional electrophoresis. Gels were soaked in 1 M-sodium salicylate (Chamberlain, 1979), dried and autoradiographed on Agfa–Gevaert RP-1 film (–70 °C, 4–14 days, Kodak X-Omatic intensifying screen). Alternatively, membranes were labelled under the same conditions, washed with 10 mM-Hepes buffer, and fractionated with Triton X-114 (Percy *et al.*, 1985). Membrane fractions were then extracted with acetone/ethanol before electrophoresis. Densitometric scanning of autoradiographs was in a Joyce-Loebl Chromoscan 3.

Electrofocusing of membrane proteins was performed in rod gels, using Bio-Lyte 3–10 ampholyte (Bio-Rad); samples were lyophilized, dissolved in sample buffer containing 8 M-urea and 2% Triton X-100, and applied at the basic (cathodic) end of the gel. Second-dimension slab gel electrophoresis was as described previously (Gavine *et al.*, 1984).

ATPase activity was measured at 30 °C by a coupled spectrophotometric assay (Percy *et al.*, 1985). Inhibition by NEM was studied by preincubation at 20 °C of chromaffin granule membranes (4 mg/ml) or partially-purified ATPase I (1–2 mg/ml) with various concentra-

Abbreviations used: ATPase, adenosine triphosphatase; DCCD, *NN'*-dicyclohexylcarbodi-imide; NbfCl, 4-chloro-7-nitrobenzofurazan; NEM, *N*-ethylmaleimide.

Fig. 1. Inhibition of ATPase activity by NEM

(a) Effect of preincubation (15 min) with various concentrations of NEM, on chromaffin granule membranes (○) and partially-purified ATPase I (Δ). (b) Time course of inhibition by 10 μM-NEM of partially-purified ATPase I, in the absence of ATP (▲), or in the presence of 2 mM-ATP (●). Initial specific activities were 162 nmol/min per mg (○) or 1190 nmol/min per mg (●, Δ, ▲).

tions of the inhibitor in 20 mM-Hepes/NaOH, pH 7.0, samples being taken after 15 min and diluted 40-fold into the assay cuvette. The time-course of inhibition was determined with 10 μM-NEM and ATPase I (1.8 mg/ml), samples being taken for assay at various times.

RESULTS

Inhibition of ATPase activity by NEM

The hydrolysis of ATP by chromaffin granule membranes is maximally inhibited about 50% by NEM (Fig. 1a). Fractionation of these membranes with Triton X-114 completely separates the two ATPases (Percy *et al.*, 1985), giving an ATPase I-containing fraction which is inhibited more than 90% by NEM (Fig. 1a), and in which the ATPase activity is protected from NEM-inhibition by the substrate, ATP (Fig. 1b), 2 mM-ATP reducing the pseudo-first-order rate constant for inhibition by 10 μM-NEM from $1.8 \times 10^{-3} \text{ s}^{-1}$ to $3.0 \times 10^{-5} \text{ s}^{-1}$. In contrast, ATPase II is totally insensitive to alkylating agents (Percy *et al.*, 1985); this explains the large NEM-insensitive component of ATPase activity noted by us and others (Flatmark *et al.*, 1982).

Identification of the NEM-reactive subunit

One-dimensional SDS/polyacrylamide-gel electrophoresis of [³H]NEM-labelled membranes, followed by autoradiography, revealed that the majority of radiolabel was incorporated into a polypeptide of apparent M_r 70000 (Fig. 2b, track 1). Densitometric scanning of autoradiographs showed that $73 \pm 3\%$ of the total label incorporated was in this band, no other band containing

more than 13%. Labelling and subsequent fractionation of the membranes with Triton X-114 (Fig. 2a, tracks 2–4) gave three fractions (Percy *et al.*, 1985; Pryde & Phillips, 1986): an insoluble fraction, containing ATPase I and a few other proteins (track 2), a detergent-rich fraction, containing many membrane proteins, including dopamine β-hydroxylase, cytochrome b_{561} and ATPase II (track 3), and an aqueous fraction, containing mainly soluble proteins such as chromogranins (track 4). The 70-kDa [³H]NEM-labelled protein occurred only in the first fraction (Fig. 2b, track 2) and can be confidently identified as subunit 1 of ATPase I. No other subunit of the enzyme was labelled under these conditions; however, if membranes were first fractionated, then the Triton X-114-insoluble fraction labelled, specificity was lost, and radioactivity was found in subunits 1, 2, 3 and 4, as well as in other proteins (results not shown).

Two-dimensional gel electrophoresis

All of the putative ATPase I subunits are major components of chromaffin granule membranes (Percy *et al.*, 1985), but one-dimensional SDS/polyacrylamide-gel electrophoresis fails to separate subunit 1 from several other membrane proteins of similar M_r . Two-dimensional electrophoresis of [³H]NEM-labelled membranes shows that the only label detectable by autoradiography is in a compact cluster of small spots, of apparent M_r 70000 and pI 5.0. The appearance of this area is shown in Fig. 3(d), with, for comparison, an enlargement (Fig. 3b) of the same area of a Coomassie-stained gel (Fig. 3a). Coomassie staining shows the same cluster of spots with, in addition, another of slightly lower M_r and pI. This

Fig. 2. Fractionation of chromaffin granule membranes labelled with [^3H]NEM

(a) Coomassie-Blue-stained gel of fractions obtained with Triton X-114 (Percy *et al.*, 1985). Track 1, unfractionated membranes; 2, insoluble fraction; 3, detergent-rich fraction; 4, aqueous fraction. (b) Autoradiograph of the same fractions. The positions of the five ATPase subunits are indicated by arrows on the left of the Figure; these subunits are identifiable as the corresponding major Coomassie-stained bands in track 2 of (a).

latter spot is not present in the Triton X-114-insoluble fraction (Fig. 3c) and is therefore not a component of ATPase I.

Inhibition of labelling by ATP

When chromaffin granule membranes were incubated with $10\ \mu\text{M}$ - ^3H]NEM, inclusion of $2\ \text{mM}$ -ATP led to a reduction in the labelling of the $70\ \text{kDa}$ band; densitometric scanning of autoradiographs showed that this inhibition was by 67% during the first 2 min of incubation, but only 30% after 8 min. This suggests that not all the NEM-reactive groups of subunit 1 are in the active site, and that other sites, not protected by ATP, may become alkylated during prolonged incubation with NEM. This being so, the maximum incorporation of ^3H , which is about $0.35\ \text{nmol/mg}$ of protein, cannot be used to estimate the concentration of subunit 1 in the membranes. Hasselbach & Taugner (1970) obtained an incorporation of about $1.5\ \text{nmol/mg}$ of protein, using ^{14}C]NEM at $10\ \mu\text{M}$.

DISCUSSION

Although the most highly-purified preparations of ATPase I contain five types of polypeptide (Percy *et al.*, 1985), it is not yet certain that they are all components of the enzyme, nor is the function of any of them known. The smallest, subunit 5, is a hydrophobic DCCD-reactive protein of M_r about 7000 (although its apparent M_r on gels is higher); it is different in size and amino acid content from its mitochondrial counterpart (Apps *et al.*, 1982) but may have a similar H^+ -conducting function, as DCCD decreases the proton-permeability of chromaffin granule membranes (Apps *et al.*, 1980).

We can now identify subunit 1 as the site of ATP hydrolysis, since NEM inhibits this activity and, at low concentrations at least, labels this subunit specifically; furthermore, ATP protects ATPase I from inactivation by NEM, and inhibits labelling of subunit 1. On two-dimensional gels subunit 1 shows charge heterogeneity; this is not an effect of alkylation, as it appears as a similar cluster of spots in untreated membranes. There is

Fig. 3. Two-dimensional polyacrylamide gel electrophoresis

(a) Chromaffin granule membranes. I-1, I-2, ATPase I, subunits 1 and 2; CGA, chromogranin A; CYT, cytochrome b_{561} ; DBH, dopamine β -hydroxylase; GP III, glycoprotein III. (b) Enlargement of the area containing subunit 1 of ATPase I. (c) The same area from a gel of the detergent-insoluble fraction from Triton X-114 fractionation (Percy *et al.*, 1985). (d) Autoradiograph of the same area from a gel of [3 H]NEM-labelled chromaffin granule membranes.

no evidence that this polypeptide is glycosylated (Gavine *et al.*, 1984).

It is noteworthy that the α and β subunits of the mitochondrial H^+ -ATPase complex also show extensive heterogeneity on two-dimensional electrophoresis, due to proteolysis at the *N*-termini (Walker *et al.*, 1985).

In its sensitivity to NEM and other inhibitors, ATPase I resembles the H^+ -ATPases of other secretory vesicles and also of endosomes, Golgi, coated vesicles, lysosomes and vacuoles of fungi and higher plants. Some of these enzymes have been purified (Mandala & Taiz, 1985; Manolson *et al.*, 1985; Uchida *et al.*, 1985; Randall & Sze, 1986; Xie & Stone, 1986), and have a structural resemblance to ATPase 1, since they contain a small, DCCD-reactive component and probably two other subunits (about 72 kDa and 60 kDa), the larger of which is specifically labelled by NEM and NbfCl (Bowman *et al.*, 1986). The fact that ATPase 1 preparations contain at least five polypeptides suggests that some of them may not be part of the enzyme, and it is now an urgent necessity to define the functions of subunits 2 (57 kDa), 3 (40 kDa) and 4 (33 kDa).

These 'vesicular' ATPases seem to have a structure that is simpler than that of mitochondrial F_1F_0 -ATPase, but more complex than that of the simple one-subunit H^+ -ATPases of fungal plasma membranes (Goffeau & Slayman, 1981).

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