Topography of a vacuolar-type H⁺-translocating ATPase: chromaffin-granule membrane ATPase I

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Proteins exposed on the cytoplasmic face of isolated chromaffin granules were labelled by lactoperoxidase-catalysed radioiodination and by non-enzymic biotinylation. Granule membranes were then prepared, and the H⁺-translocating ATPase isolated by fractionation with Triton X-114. The labelling of individual ATPase subunits was assessed by polyacrylamide-gel electrophoresis, followed by autoradiography or by blotting and decoration with ¹²⁵I-labelled streptavidin. Subunits of 72, 57 and 40 kDa were strongly labelled, and could be removed from the membrane at pH 11: they are therefore extrinsic proteins. The 120 kDa subunit was also labelled, but it was not solubilized at pH 11. Photolabelling with a hydrophobic probe indicated that this subunit penetrates the bilayer, and enzymic degradation studies showed the presence of *N*-linked oligosaccharides; this subunit therefore spans the chromaffin-granule membrane. Labelling of the 17 kDa subunit occurred predominantly on the extracytoplasmic (matrix) face of the granule membrane. These results are consistent with this V-type ATPase having a structure that is generally similar to that of mitochondrial (F-type) ATPases, although the attachment of the 120 kDa subunit may be asymmetrical.

INTRODUCTION

The secretory granules of the adrenal medulla maintain a low internal pH through the action of an ATP-dependent proton pump of the vacuolar type (Al-Awqati, 1986). This enzyme is termed ATPase I, to distinguish it from ATPase II (Apps *et al.*, 1983), a vanadate-sensitive ATPase, apparently of the P type, but which is of unknown function (Moriyama & Nelson, 1988). ATPase I is a multi-subunit complex of molecular mass about 500 kDa; it has been reported to contain five polypeptides of about 115, 70–72, 57, 39–41 and 16–17 kDa (Percy *et al.*, 1985; Cidon & Nelson, 1986), although their stoichiometry has not yet been determined. These polypeptides are referred to as subunits I–V throughout this paper, according to Moriyama & Nelson (1987). Another polypeptide, of 33 kDa, is found in the partially purified ATPase (Percy *et al.*, 1985), but can be removed without loss of ATPase and H⁺-pumping activity (Moriyama & Nelson, 1987).

Subunit II (about 72 kDa) contains the catalytic site (Percy & Apps, 1986; Moriyama & Nelson, 1987), being uniquely labelled by low concentrations of *N*-ethylmaleimide and protected by ATP. The 17 kDa subunit V is the site of reaction of the inhibitor *N,N'*-dicyclohexylcarbodi-imide (DCCD) (Sutton & Apps, 1981) and is homologous with the DCCD-reactive subunits of F-type ATPases (Mandel *et al.*, 1988), and so is presumed to form a transmembrane H⁺-conducting channel.

The functions of the other subunits have not yet been defined, and it is not even certain that all are genuine components of the ATPase. Reports of immunological cross-reactivity (Manolson *et al.*, 1986) and, more recently, of sequence similarity between H⁺-translocating

ATPases of the V and F type (Bowman *et al.*, 1988*a,b*; Zimniak *et al.*, 1988) suggest that subunits II and III (72 kDa and 57 kDa) are related to the α - and β -subunits of F-type ATPases, and therefore that the overall structures of V- and F-type ATPases may be similar. We now report the use of chemical labelling and enzymic degradation to map the membrane topography of the H⁺-ATPase subunits in intact chromaffin granules. In the chemical-labelling studies, we used lactoperoxidase-catalysed radioiodination and non-enzymic biotinylation to label proteins exposed on the cytoplasmic surface of intact chromaffin granules.

N-Hydroxysuccinimidylbiotin has been used to study the topography of proteins in the plasma membranes of leucocytes by Hurley *et al.* (1985), who concluded that the reagent did not penetrate and therefore only biotinylated those proteins exposed on the extracytoplasmic face of the membrane. We used the more hydrophilic sulphonated derivative (3-sulpho)-*N*-hydroxysuccinimidyl (SNHS)-biotin, which is even less likely to penetrate the bilayer (Ingalls *et al.*, 1986). Biotinylation has two major advantages: since lysine residues are modified, it is of more general application than the radioiodination of exposed tyrosines, and the detection of biotinylated proteins, with ¹²⁵I- or enzyme-conjugated streptavidin, is extremely sensitive. However, biotinylation alters the isoelectric points of proteins, so that two-dimensional gel electrophoresis is not a suitable means of analysis.

¹²⁵I-labelled 3-(trifluoromethyl)-3-(*m*-[¹²⁵I]iodophenyl)-diazirine (TID) was introduced as a hydrophobic probe for labelling the regions of membrane proteins in contact with the bilayer by Brunner & Semenza (1982). We have used this reagent to label the membrane-embedded polypeptides of ATPase I and have used enzymic

Abbreviations used: SNHS-biotin, (3-sulpho)-*N*-hydroxysuccinimidylbiotin; [¹²⁵I]TID, 3-(trifluoromethyl)-3-(*m*-[¹²⁵I]iodophenyl)diazirine; DCCD, *N,N'*-dicyclohexylcarbodi-imide.

degradation of oligosaccharide chains to investigate which subunits are glycosylated, since glycosylation also has implications for the arrangement of polypeptides in the chromaffin-granule membrane.

MATERIALS AND METHODS

Crude chromaffin granules were prepared by differential centrifugation of fresh homogenates of bovine adrenal medullae in iso-osmotic (0.3 M) sucrose (Phillips, 1974). Labelling, either with $^{125}\text{I}^-$ and lactoperoxidase or with SNHS-biotin (described below), was performed on these crude granules, suspended in slightly hyper-osmotic medium, to minimize granule lysis (Hiram *et al.*, 1982). The labelled granules were then further purified by layering 1.0 ml aliquots over 10 ml of 1.7 M-sucrose, 10 mM-Hepes/NaOH, pH 7.0, and centrifugation for 60 min at 50000 rev./min in a Beckman Ti50 rotor (170000 g) at 2 °C. The pelleted granules were lysed by resuspension in 10 ml of 10 mM-Hepes/NaOH, pH 7.0 (0 °C), and the membranes recovered by re-centrifugation.

Radioiodination was performed in a medium of the following composition: crude chromaffin granules, approx. 1 mg of protein/ml; 0.4 M-sucrose; 20 mM-Hepes/NaOH, pH 7.0; 3.7 MBq of Na^{125}I /ml; 1.1 unit of glucose oxidase/ml (EC 1.1.3.4; Sigma type IV); 5 mM-glucose; 1.1 units of lactoperoxidase/ml (EC 1.11.1.7; Boehringer); 2 μg of butylated hydroxytoluene/ml; 1 μM -*p*-trifluoromethoxyphenylhydrazine. After incubation for 30 min at approx. 25 °C, the mixture was chilled and approx. 10 mg of chromaffin granules/ml added. The granules were then further purified as described above, and the membranes isolated; during this isolation stage, all solutions contained 1 mM-NaI.

Biotinylation was performed in a medium of the following composition: crude chromaffin granules, 5 mg/ml; 0.35 M-sucrose; 50 mM-Bicine/NaOH, pH 8.3; 0.1 mM-SNHS-biotin (Pierce, Rockford, IL, U.S.A.). After incubation for 30 min at 30 °C, the reaction was stopped by addition of 2 mM-ethanolamine; the granules were then further purified and their membranes isolated; all solutions contained 0.2 mM-ethanolamine.

Trypsin treatment was performed in a medium of the following composition: crude chromaffin granules, 5 mg/ml; 0.35 M-sucrose; 20 mM-Hepes/NaOH, pH 7.0; 10 μg of trypsin/ml (EC 3.4.21.4; Sigma type III). After 60 min at 30 °C, proteolysis was stopped by adding soybean trypsin inhibitor (0.25 mg/ml). The granules were then pelleted, resuspended, biotinylated and purified as described above, and membranes prepared.

To remove extrinsic proteins, chromaffin-granule membranes were suspended in 0.1 M- Na_2CO_3 /NaOH/10 mM-EDTA, pH 11.0 (Fujiki *et al.*, 1982). After 30 min at 0 °C, the membranes were collected by centrifugation. The washings were dialysed against 1 mM-EDTA, pH 7.0, and freeze-dried.

The 17 kDa DCCD-reactive subunit of the ATPase was extracted from purified chromaffin-granule membranes as described by Sutton & Apps (1981). The chloroform/methanol extract was concentrated by rotary evaporation, and analysed without further purification.

Fractionation of chromaffin-granule membranes with Triton X-114 was performed as described by Pryde & Phillips (1986), giving a detergent-insoluble fraction (P-1), a detergent-rich fraction (P-2) and an aqueous fraction

(S-2). ATPase I was solubilized and further purified from the P-1 fraction, and reconstituted using endogenous lipids (J. R. Perez-Castineira, unpublished work). One- and two-dimensional electrophoretic separation of proteins was performed as described elsewhere (Gavine *et al.*, 1984). After electrophoretic transfer to nitrocellulose sheets (0.45 μm pore size, from Schleicher & Schüll, Dassel, Germany), biotinylated proteins were detected by washing the sheets with the following solutions, all in 150 mM-NaCl/10 mM-Tris/HCl, pH 7.4, at room temperature with gentle shaking: (i) 0.25% (w/v) Tween 20, for 60 min; (ii) ^{125}I -labelled streptavidin (10 Bq/ μl ; approx. 25 MBq/mg) for 60 min; (iii) 0.05% (w/v) Tween 20 (three changes, 15 min each). For 14 cm $\times$ 16 cm sheets, 50 ml of each solution was used. Sheets were air-dried, then autoradiographed on Hyperfilm MP (Amersham) for 4–24 h at 70 °C, using Kodak intensifying screens. Autoradiographs were scanned with a Joyce-Loebl Chromoscan 3. Streptavidin was obtained from Sigma, and radioiodinated by the Iodogen method (Pierce, Rockford, IL, U.S.A.). Free $^{125}\text{I}^-$ was removed by passage of the radioiodinated protein (0.2 ml) through a Bio-Gel P6-DG column (1.0 ml) equilibrated with 0.15 M-NaCl, 0.05 M-sodium phosphate; bovine serum albumin (5 mg/ml) was added and the ^{125}I -labelled streptavidin stored at –10 °C.

Peanut lectin (Sigma) was dissolved at 1 mg/ml in 0.1 M-NaCl/0.05 M-Bicine/NaOH, pH 8.3, and biotinylated by reaction with SNHS-biotin (0.1 mM) for 30 min at 30 °C. After passage through a column of Bio-Gel P6-DG equilibrated with 0.15 M-NaCl, 0.01 M-Tris/HCl, pH 7.4, the lectin was used to decorate blots at a concentration of 10 μg /ml in this buffer. The blot was then washed and decorated with ^{125}I -labelled streptavidin, as described above.

For degradation of oligosaccharide chains with neuraminidase, chromaffin-granule membranes (1 mg/ml) or the P-1 fraction from Triton X-114 fractionation (0.25 mg/ml) were incubated for 3 h at 30 °C in 0.2 M-Mes/NaOH buffer, pH 5.5, containing 1.6 mM-benzamidine, 6 μg of pepstatin/ml, 6 μg of leupeptin/ml, 0.4 mM-phenylmethanesulphonyl fluoride, 0.05 mM-*N*- α -*p*-tosyl-L-lysylchloromethane, 0.5 mM-*N*-tosyl-L-phenylalanylchloromethane, and 1 unit of neuraminidase/ml (EC 3.2.1.18, Sigma type VI). For treatment with glycopeptidase F, the conditions were 4 h at 30 °C in 20 mM-Hepes/NaOH, pH 7.4, containing 9 mM-EDTA, 1% (v/v) 2-mercaptoethanol, 6 μg of pepstatin/ml, 6 μg of leupeptin/ml, 1.6 mM-benzamidine and 33 units of glycopeptidase F/ml (EC 3.2.2.18; Boehringer). In some experiments octyl β -glucoside [1% (w/v) final concentration] or SDS (0.1%, w/v) was included.

^{125}I -labelled TID, of specific activity > 370 TBq/mol, was obtained from Amersham. Chromaffin-granule membranes (0.25 mg) or 0.2 mg of purified reconstituted ATPase I, were incubated with 5 MBq of ^{125}I -labelled TID (total vol. 0.5 ml) for 15 min in the dark at room temperature, then illuminated for 20 min at 330 nm, in a 5 mm $\times$ 5 mm quartz cuvette in the sample holder of a Perkin-Elmer fluorimeter. Labelled proteins were analysed by electrophoresis and autoradiography.

Silver staining of gels was performed according to Wray (1981). Apparent molecular masses of protein bands were calculated from the mobilities of the following standard proteins (kDa): β -galactosidase (116),

phosphorylase *b* (97), serum albumin (66.25), ovalbumin (45), carbonic anhydrase (30), trypsin inhibitor (20.1) and lysozyme (14.3).

RESULTS

Fractionation of chromaffin-granule membranes by phase-separation in Triton X-114 (Pryde & Phillips, 1986) is a rapid and convenient procedure that yields three fractions, termed P-1, P-2 and S-2. In P-1, ATPase I is purified at least 10-fold, having a specific activity of about $2 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ when resolubilized (J. R. Perez-Castineira, unpublished work); its subunits can be recognized in SDS/polyacrylamide gels by comparison with the purified enzyme (Percy *et al.*, 1985) and the only major contaminants are proteins of apparent molecular mass 33 and 36 kDa. Fraction P-2 contains the membrane proteins dopamine β -hydroxylase, cytochrome b_{561} and ATPase II, and S-2 contains extrinsic proteins, notably chromogranins and the soluble form of dopamine β -hydroxylase. Triton X-114 fractionation, followed by one- or two-dimensional polyacrylamide-gel electrophoresis, provided a satisfactory method for the rapid analysis of ATPase I subunits, after labelling of intact chromaffin granules.

Labelling of lactoperoxidase-catalysed radioiodination

Crude, intact chromaffin granules were subjected to radioiodination, then re-purified to remove contaminating organelles and membrane fragments; membranes were then isolated from these granules, fractionated and analysed electrophoretically. A one-dimensional electrophoretogram is shown in Fig. 1. In the Triton-insoluble fraction P-1 (track 2), ATPase I subunits II (72 kDa), III (57 kDa) and IV (40 kDa) are strongly labelled, and subunit I (122 kDa) is weakly labelled. Subunit V (17 kDa) is unlabelled; the smallest labelled polypeptide, of apparent molecular mass 15 kDa, is not a component

Fig. 1. Triton X-114 fractionation of membranes from radiolabelled chromaffin granules: autoradiograph

Track 1, chromaffin-granule membranes (100 μg); track 2, P-1 fraction (60 μg); track 3, P-2 fraction (60 μg); track 4, S-2 fraction (50 μg).

Fig. 2. Two-dimensional electrophoretic separation of radioiodinated chromaffin-granule membrane proteins: autoradiographs

(a) Membranes from radioiodinated granules (150 μg); (b) membranes radioiodinated after isolation (150 μg); (c) proteins solubilized at pH 11, after radioiodination of intact granules (50 μg). Abbreviations used; A2, ATPase I subunit II; A3, ATPase I subunit III; B, cytochrome b_{561} ; C, chromogranin A; D, dopamine β -hydroxylase; G, glycoprotein III; H, glycoprotein H; J, glycoprotein J; K, glycoprotein K.

of ATPase I. The integrity of the granules is confirmed by the labelling patterns shown in fractions P-2 (track 3) and S-2 (track 4), which provide a control for this experiment. Cytochrome b_{561} is a 30-kDa transmembrane protein (Duong & Fleming, 1984; Perin *et al.*, 1988), although its electrophoretic mobility suggests a lower molecular mass. It is strongly iodinated (track 3), whereas dopamine β -hydroxylase and the chromogranins, located in the granule matrix (70–75 kDa), are not iodinated (tracks 3 and 4). No labelling was detected in the soluble proteins released on lysis of the granules (not shown).

Two-dimensional electrophoresis was also used to separate radioiodinated membrane proteins. The position of subunit II (72 kDa) in these gels has previously been demonstrated by [^3H]N-ethylmaleimide labelling (Percy

& Apps, 1986); both it and subunit III (57 kDa) are iodinated (Fig. 2a), but no other subunits of ATPase I are visible on this autoradiograph, probably because they fail to enter the first-dimension (electrofocusing) gel. If chromaffin-granule membranes, as opposed to intact granules, are radiiodinated, many additional proteins are labelled (Fig. 2b); these include dopamine β -hydroxylase, glycoprotein III and the glycoproteins previously termed H, J and K (Gavine *et al.*, 1984; Wood *et al.*, 1985), two of which were subsequently identified as isoforms of the carboxypeptidase involved in neuropeptide processing (Laslop *et al.*, 1986). Fig. 2(c) shows the radioiodinated polypeptides that could be removed from the membranes by washing at high pH; these include subunits II and III of ATPase I.

Fig. 3. Biotinylation of chromaffin-granule membrane proteins: densitometric scans of autoradiographs

(a) P-1 fraction (60 μg); (b) P-2 fraction (50 μg); (c) S-2 fraction (76 μg); (d) unfractionated membranes (130 μg); (e) unfractionated membranes from trypsin-treated granules (100 μg); (f) proteins solubilized at pH 11 (50 μg). Full scale, absorbance = 2.0. Numbers above peaks are apparent molecular masses (kDa).

Labelling by biotinylation

Chromaffin granules were labelled with SNHS-biotin at pH 8.3 and 30 °C, in slightly hyper-osmotic sucrose. Granules were then re-purified, and membranes prepared and fractionated with Triton X-114. This technique was also applied to trypsin-treated granules. Fig. 3 presents densitometric scans of autoradiograms obtained from individual gel tracks; the densities are all to the same absorbance scale. Electrophoretic migration was from left to right and the apparent molecular masses of individual peaks are indicated. All of the subunits of ATPase I are biotinylated and appear in the P-1 fraction (Fig. 3a). As expected in intact granules, cytochrome b_{561} is labelled, and dopamine β -hydroxylase is not (Fig. 3b); there is very little labelling of proteins in the S-2 fraction (Fig. 3c), which consists mainly of soluble proteins from the granule matrix. As with lactoperoxidase-catalysed radioiodination, no labelling was detected in the soluble proteins released on lysis of the granules (not shown).

Subunits I, II, III and IV are major biotinylated components of unfractionated chromaffin-granule membranes (Fig. 3d); trypsin treatment of the granules degrades subunit II from 72 to 66 kDa, and subunit III from 57 to 50 kDa (Fig. 3e). The molecular masses of the degradation products of subunits II and III were established by Triton X-114 fractionation of membranes from trypsin-treated granules (not shown). Subunit I is more extensively proteolysed and cannot be recognized in Fig. 3(e), nor was it found after Triton X-114 fractionation. As reported elsewhere (Apps *et al.*, 1984), cytochrome b_{561} is clipped to an apparent molecular mass of 21 kDa, during proteolysis of chromaffin granules. Washing the membranes at pH 11 elutes subunits II, III and IV, but not I or V (Fig. 3f), nor cytochrome b_{561} , known to be an intrinsic membrane protein.

The results of these experiments can be summarized as follows: (1) all five subunits of the H^+ -ATPase are accessible to SNHS-biotin in intact granules and must therefore be exposed on the cytoplasmic face of the granule membrane; (2) washing at pH 11 solubilizes subunits II, III and IV, but not I or V, which must therefore be intrinsic membrane proteins; (3) trypsin treatment of intact granules leads to extensive degradation of subunit I, and partial degradation of subunits II and III, confirming that all three are exposed on the cytoplasmic face of the granule membrane.

Extraction of subunit V

Although subunit V is biotinylated in intact granules (Fig. 3a; 17 kDa), its labelling is weak in comparison with that of other polypeptides and in relation to the intensity of protein staining. This hydrophobic polypeptide is soluble in chloroform/methanol (Sutton & Apps, 1981) and was extracted from membranes prepared from biotinylated granules, and from membranes which had been biotinylated after isolation. Extracts from equal quantities of membranes were then run on a 10–15% (w/v) polyacrylamide gel and their biotinylation compared (Fig. 4). Labelling of the 17 kDa polypeptide was far more intense in membranes than in intact granules (note the difference in scales).

Enzymic deglycosylation

Purified chromaffin-granule membranes were treated

Fig. 4. Biotinylation of subunit V: densitometric scan of autoradiograph

Chloroform/methanol extracts of chromaffin-granule membranes (a) prepared from biotinylated granules (2.2 µg of protein; full scale, absorbance = 0.5) and (b) biotinylated after isolation (3.4 µg of protein; full scale, absorbance = 2.0).

with neuraminidase and with glycopeptidase F, at the appropriate pH values for these enzymes, and in the presence of proteinase inhibitors. Digested membrane proteins were separated on 6–15% (w/v) polyacrylamide gels which were silver-stained. Of the putative ATPase I polypeptides, only subunit I was affected. Neuraminidase treatment produces a slight reduction in its apparent molecular mass (Fig. 5a). As with several other proteins of the chromaffin-granule membrane (Gavine *et al.*, 1984), this polypeptide binds the galactose-specific peanut lectin after neuraminidase treatment, but not before (not shown). However, this lectin binds non-specifically to several other proteins in the P-1 fraction. More convincing evidence for glycosylation of this subunit is the significant reduction in molecular mass (from 120 to 110 kDa) that is produced by digestion with glycopeptidase F (Fig. 5b). No other subunit of ATPase I is affected. The faint band of apparent molecular mass about 71 kDa that appears on glycopeptidase F treatment (Fig. 5b, tracks 6–8) is the deglycosylated form of dopamine β -hydroxylase, present as a contaminant in the P-1 fraction; it is seen more clearly in tracks 3 and 4.

Labelling with [125 I]TID

In chromaffin-granule membranes, the hydrophobic photoactivated probe 125 I-labelled TID labels subunits I and V of the H^+ -ATPase; these are the most intensely labelled of all polypeptides and, as expected, cytochrome b_{561} is also labelled (Fig. 6a). (Note that in this scan, subunit V is not resolved from other components of low molecular mass, which are probably lipids.) With purified, reconstituted ATPase I, the results are even more clear-cut (Fig. 6b); subunits I and V are the only labelled polypeptides.

DISCUSSION

The results for individual ATPase I subunits are summarized in Table 1, which also includes data on the 33 kDa polypeptide that was originally postulated to be a subunit of ATPase I (Percy *et al.*, 1985), but which can

Fig. 5. Enzymic degradation of oligosaccharide chains: upper parts of silver-stained SDS/polyacrylamide gels

Tracks 1–4, chromaffin-granule membranes; tracks 5–8, P-1 fraction. (a) Neuraminidase digestion; (b) glycopeptidase-F digestion. Tracks 1 and 5, controls (no enzyme); 2 and 6, digested without detergent; 3 and 7, with 1% (w/v) octyl β -glucoside; 4 and 8, with 0.1% (w/v) SDS.

Fig. 6. Photolabelling with [125 I]TID: densitometric scan of autoradiograph

(a) Chromaffin-granule membranes; (b) purified ATPase I. Full scale, absorbance = 1.0.

be removed. A polypeptide of similar molecular mass is found in the ATPase of clathrin-coated vesicles (Arai *et al.*, 1987), but its function in the chromaffin granule is in doubt.

Subunit I is clearly exposed on the cytoplasmic face of the chromaffin-granule membrane and can be radioiodinated, biotinylated or proteolysed in intact granules. It is glycosylated, which suggests that it is a transmembrane protein, since the oligosaccharide chains of chromaffin-granule membrane glycoproteins, like those of other endomembrane glycoproteins, are located exclusively on the extracytoplasmic face of the granule (Huber *et al.*, 1979). In agreement with this, it is not solubilized at pH 11 and is labelled by [125 I]TID, though in comparison subunit V is more strongly labelled. The polypeptide chain of subunit I may only traverse the bilayer a small number of times or it may be screened by other subunits.

Subunits II, III and IV behave as extrinsic proteins on

Table 1. Summary of properties of ATPase I subunits

Abbreviations used: LPO, lactoperoxidase; Glyco-F, glycopeptidase F.

Subunit	Molecular mass (kDa)	Labelling			Degradation		Solubilization at pH 11
		$^{125}\text{I}^-/\text{LPO}$	[^{125}I]-TID	SNHS-biotin	Trypsin	Glyco-F	
I	120	+	++	++	++	+	—
II	72	++	—	++	+	—	+
III	57	++	—	++	+	—	+
IV	41	++	—	+	—	—	+
V	17	—	++	(+)	—	—	—

the cytoplasmic face of the membrane, being accessible to non-penetrating reagents, solubilized at pH 11 and not glycosylated. They show no labelling by [125 I]TID, so are not embedded in the bilayer.

Subunit V can be slightly biotinylated in intact granules, but is much more strongly labelled in membrane fragments. Its amino acid sequence has recently been reported (Mandel *et al.*, 1988): its structure was predicted to consist of four transmembrane helices, connected by one cytoplasmic and two intragranular loops, with two lysines exposed on each face of the membrane. The *N*-terminus is blocked (Sutton & Apps, 1981). The strong labelling in membrane fragments suggests that labelling occurs mainly on the extracytoplasmic (matrix) face and that interactions with other subunits probably prevent labelling on the cytoplasmic face. The slight labelling of subunit V in intact granules may even be of damaged ATPase molecules, in which subunits II–IV have been lost, leaving subunit V accessible.

These results are consistent with a model in which subunit V (17 kDa) is intrinsic and subunits II–IV (72, 57 and 41 kDa) form an extrinsic complex on the cytoplasmic face of the granule membrane, protecting subunit V from labelling. This subunit arrangement is like that of F_1F_0 -ATPases, and this general similarity between ATPases of the V- and F-types has been demonstrated by immunological studies (Manolson *et al.*, 1987) and sequence determinations (Bowman *et al.*, 1988a,b; Mandel *et al.*, 1988). It is, however, difficult to reconcile the properties of subunit I (120 kDa) with an F_1F_0 -like structure. Although its polypeptide stoichiometry is unknown, the ATPase (molecular mass about 500 kDa) is unlikely to contain more than one copy of subunit I (120 kDa). This could imply that subunit I is associated asymmetrically with the complex. An alternative possibility, that it functions as a central core, seems unlikely, as no polypeptide larger than subunit II has been found in the V-type ATPases of plants and fungi (Bowman & Bowman, 1986), and the H^+ -ATPase of kidney cortex may also lack this subunit (Gluck & Caldwell, 1987, 1988). However, a component of similar molecular mass and properties occurs in the H^+ -ATPase of clathrin-coated vesicles (Xie & Stone, 1986; Arai *et al.*, 1987). It therefore seems that, if subunit I is really a component of some V-type ATPases, it does not have a fundamental role in catalysis, but may have a regulatory function.

The H^+ -translocating ATPases isolated from kidney microsomes and from clathrin-coated vesicles have reported subunit compositions that are more complex than that of the chromaffin-granule enzyme, but their morphology is similar. For the kidney enzyme, subunits of 70 and 56 kDa (presumably corresponding to subunits II and III) have been located on the cytoplasmic face of the membrane by immunological methods and electron microscopy suggests a 9.5 nm cytoplasmic domain that is morphologically similar to that of F-type ATPases (Brown *et al.*, 1987, 1988). Recent studies on the coated-vesicle ATPase (Arai *et al.*, 1988) have also resulted in a structural model that is in most respects similar to that proposed here for the chromaffin-granule enzyme. The major differences are that the coated-vesicle ATPase may have more subunit types, polypeptides of 38, 34, 33 and 19 kDa being present in addition to polypeptides apparently similar to the subunits I–V described here, and that subunit I (100 kDa in the coated-vesicle ATPase) is

not glycosylated. A subunit of 40 kDa was found to be integral to the membrane, but this may not be equivalent to subunit IV in the chromaffin-granule enzyme. The subunit stoichiometry of the coated-vesicle ATPase was elegantly measured by amino acid analysis (Arai *et al.*, 1988) and a more refined model of the structure of the chromaffin granule enzyme must await similar studies on this ATPase.

Note added in proof (received 2 August 1989)

Moriyama & Nelson (1989) have recently reported that subunits of 72, 57 and 34 kDa are released from chromaffin granules during cold-inactivation of ATPase I. Those results are entirely consistent with those obtained in the labelling studies reported here.

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