Volume 282, number 1, 69–72 FEBS 09623 April 1991

ADONIS 0014579391003304

The vacuolar H^+-translocating ATPase of renal tubules contains a 115-kDa glycosylated subunit

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Received 14 November 1990; revised version received 19 February 1991

Kidney microsomes were fractionated with Triton X-114, to give a fraction enriched in the renal tubule H^+-translocating ATPase, as judged by the sensitivity of its ATPase activity to bafilomycin A_1, and its content of two polypeptides recognized by antibodies directed against subunits of plant tonoplast ATPases. This fraction contained a polypeptide of apparent molecular mass of 115 kDa, that was recognized by an antibody to the largest (120 kDa) subunit of chromaffin-granule membrane H^+-ATPase, and, like this subunit, was reduced in molecular weight on treatment with glycopeptidase F. We conclude that, like other mammalian vacuolar H^+-ATPases, the kidney H^+-ATPase contains a large, glycosylated subunit.

Kidney; Proton translocation; ATPase; Glycoprotein

1. INTRODUCTION

Three classes of H^+-translocating ATPase have been recognized [1]: F-type (the ATP-synthases of energy-transducing membranes), P-type (proton pumps, located in the plasma membranes of plant, fungal and some specialized animal cells), and V-type (endomembrane proton pumps). The V-type ATPases have a wide distribution, occurring in the membranes of most (probably all) types of acidic intracellular compartments in eukaryotic cells [2]. In renal tubular cells, ATP-driven proton transport is carried out by V-type ATPases that are inserted into the apical plasma membrane by fusion of exocytotic vesicles, in response to changes in acid/base status [3,4].

V-type ATPases are of high molecular weight (400–600 kDa) and complex subunit composition. There is now a consensus that they have a minimum of 3 subunit types: (i) 66–73 kDa; (ii) 55–62 kDa; and (iii) 13–17 kDa, as judged by SDS-polyacrylamide gel electrophoresis. These subunits have been implicated in ATP hydrolysis, regulatory nucleotide-binding and H^+-translocation, respectively [5–8]. Subunits of intermediate size (20–40 kDa) are also present in most preparations. In some cases up to 5 polypeptides in this range have been reported, but none has yet been characterized functionally.

An interesting controversy surrounds the largest (100–120 kDa) subunit. This was originally found in just two types of mammalian V-ATPase [9–11], but a polypeptide of comparable size has now been reported to occur in V-ATPases from beet and yeast vacuoles [12,13], although most groups working with plant or fungal V-type ATPases do not find it in their preparations. A subunit of this size was not found in immunoaffinity-purified ATPase of bovine renal cortex and medulla [14], and it was not detected by immune blotting of liver lysosomal membranes and kidney microsomes [14]. We now report the immunochemical detection of a 115 kDa *N*-glycosylated component in partially-purified kidney microsomal V-ATPase and in the ATPase of chromaffin granules isolated from human phaeochromocytoma.

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2. MATERIALS AND METHODS

Kidney microsomes were prepared by an adaptation of a published procedure [16]. All solutions contained 10 mM Tris-HCl, 1 mM $NaHCO_3$, 1 mM EDTA, 1 mM DTT (pH 8.0, 0°C), plus sucrose at the required concentration. Bovine renal medullary tissue was minced and homogenized in 0.25 M sucrose, and centrifuged for 10 min at g_{av} = 1600. After centrifugation of the supernatant for 60 min at g_{av} = 41 000, crude microsomes were removed from the surface of the pellet by swirling, collected by centrifugation, resuspended in 0.25 M sucrose, overlaid onto step gradients of 0.7, 1.0 and 1.5 M sucrose, and centrifuged for 5 h at 35 000 rpm in a swingout rotor (Beckman SW41). The microsomal fraction was collected from the 0.7 M/1.0 M sucrose interface, diluted, centrifuged and finally resuspended in 0.25 M buffered sucrose containing benzamidine (2 mM), pepstatin (5 μg/ml), leupeptin (5 μg/ml).

Triton X-114 (Fluka) was precondensed as described [17]. It was used to fractionate kidney microsomes essentially as described for chromaffin-granule membranes [18,19], except that the final concentration of detergent was 17.5 mg/ml. The Triton-insoluble fraction was used for immune blotting as described below. Chromaffin granule membranes were prepared from bovine adrenal medulla and human phaeochromocytoma as described [20] and fractionated with Triton X-114 [19]. Antiserum to chromaffin granule H^+-ATPase was

raised by injecting reconstituted holo-ATPase [18] into rabbits. Rabbit antisera to individual subunits of the tonoplast H^+-ATPase of *Kalanchoe daigremontiana* were raised by injection of proteins electroeluted from gels. Immune blots were developed using the ECL system (Amersham). Deglycosylation of proteins in the Triton-insoluble fraction was carried out by incubation (8 h, 20°C) of 100 μg protein with 1 unit endoglycosidase F (Boehringer) in 50 mM HEPES-NaOH, pH 7.0, containing 2 mM benzamidine, 5 μg/ml pepstatin, 5 μg/ml leupeptin, 0.2 mM PMSF, 0.5% deoxycholate, 1.0% Triton X-100, 20 mM EDTA and 10 mM dithiothreitol (total volume 105 μl).

3. RESULTS

The purified kidney microsomes had a specific ATPase activity of 0.37 μmol/min/mg protein, and this could be inhibited 15% by 2 μM bafilomycin A_1, a specific inhibitor of V-type ATPases [21]. We established that the optimal concentration of Triton X-114 for fractionating kidney microsomes was 1.75% (w/v); the Triton-insoluble fraction had only a slightly higher specific ATPase activity than did the membranes (0.48 μmol/min/mg protein), but it was inhibited about 80% by bafilomycin, indicating an enrichment in vacuolar ATPase.

This enrichment is confirmed by the immune blots shown in Fig. 1. Rabbit antisera directed against the 57 kDa and 67 kDa subunits of the tonoplast H^+-ATPase of *Kalanchoe daigremontiana* recognize polypeptides of similar molecular weight in bovine chromaffin granule membranes and kidney microsomes, and in each case the immunoreactive polypeptides appear in the Triton-insoluble fraction. The anti-57 kDa serum recognizes a doublet in the kidney fraction (Fig. 1b, track 4); a similar result was obtained with antiserum directed against the 57 kDa subunit of beet tonoplast H^+-ATPase (not shown).

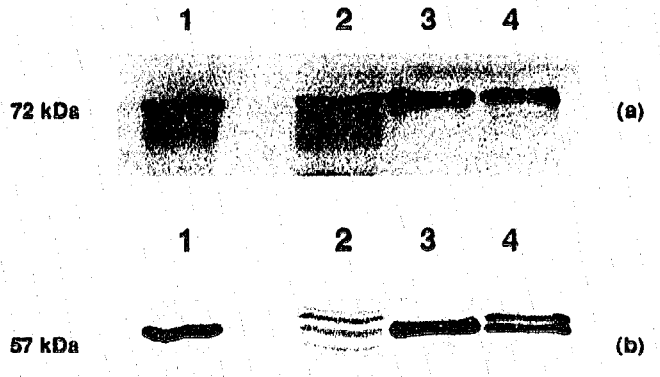

Fig. 1. Immunoblots showing the enrichment in vacuolar ATPase obtained by fractionating membranes with Triton X-114 (Track 1) Chromaffin granule membranes (60 μg protein). (Track 2) Kidney microsomes (60 μg). (Track 3) Triton-insoluble fraction from chromaffin granule membranes (10 μg). (Track 4) Triton-insoluble fraction from kidney microsomes (30 μg). Antibodies were against the 67 kDa (a) and 57 kDa (b) subunits of a plant tonoplast ATPase; note that in animals these recognize proteins of 72 and 57 kDa, respectively. In each case only the relevant part of the blot is shown, but there is no significant immunoreactivity elsewhere.

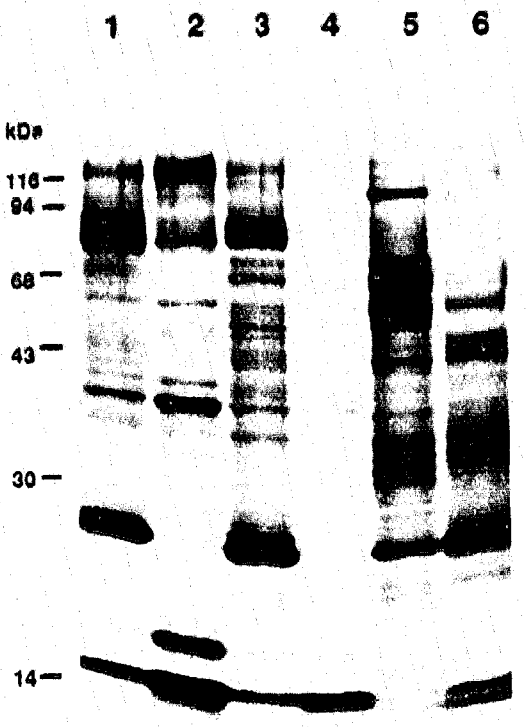

Fig. 2. Silver-stained gel showing unfractionated membranes (tracks 1, 3 and 5) and Triton-insoluble fractions (tracks 2, 4 and 6). (Tracks 1 and 2) Bovine chromaffin granules; (tracks 3 and 4) human phaeochromocytoma granules; (tracks 5 and 6) bovine kidney microsomes. Each track contained 3 μg protein. The positions of marker proteins are shown at the side; major bands in tracks 2 and 4 are at 120, 72, 57, 40, 19 and 16 kDa.

Although fractionation of chromaffin granule membranes with Triton X-114 purifies the H^+-ATPase to near-homogeneity [18], the Triton X-114-insoluble fraction from kidney microsomes contains many polypeptides besides those identified as H^+-ATPase subunits (Fig. 2).

The antiserum produced by immunization of rabbits with reconstituted chromaffin-granule H^+-ATPase vesicles [18] reacted with the 120 kDa subunit of this ATPase, and (more weakly) with dopamine β-monooxygenase (Fig. 3a, track 1). In unfractionated kidney microsomes, immunoreactivity was barely detectable (track 2), but the Triton X-114-insoluble fraction contained a protein of about 115 kDa that was clearly recognized by this serum (track 4).

Digestion of the Triton X-114-insoluble fraction with endoglycosidase F converted the diffuse immunoreactive band at 115 kDa into a sharper one at about 100 kDa (Fig. 3b, tracks 5 and 6). This is similar to the effect of deglycosylation on the 120 kDa H^+-ATPase subunit in both bovine and human chromaffin granules (Fig. 3b, and [10]).

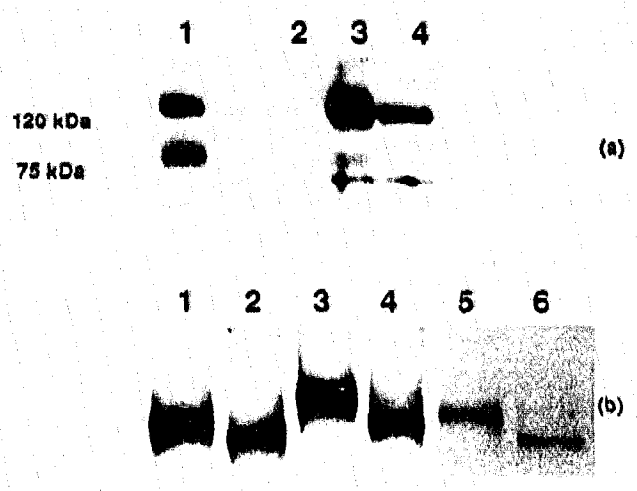

Fig. 3. Immunoblots showing that an antibody to the 120 kDa subunit of chromaffin granule membrane H^+-ATPase recognizes a similar component in kidney membranes. (a) Fractionation with Triton X-114. (Track 1) Chromaffin granule membranes (20 μg protein); (track 2) kidney microsomes (20 μg); (track 3) chromaffin granule membrane Triton-insoluble fraction (5 μg); (track 4) kidney microsome Triton-insoluble fraction (28 μg). The immunoreactive band at 75 kDa is dopamine β-monooxygenase. (b) Digestion with endoglycosidase F; Triton-insoluble fractions from: (tracks 1 and 2) human phaeochromocytoma granule (5 μg protein); (tracks 3 and 4) bovine chromaffin granule (5 μg); (tracks 5 and 6) bovine kidney microsomes (25 μg). Tracks 1, 3 and 5 are controls; tracks 2, 4 and 6 are endoglycosidase F-treated.

4. DISCUSSION

Fractionation with Triton X-114 [16,18] has proved useful in purifying V-type H^+-ATPases from chromaffin granule membranes [18] and plant tonoplasts [22], essentially in one step. When applied to kidney microsomes, it produces a Triton-insoluble fraction that is enriched about 5-fold in V-ATPase, as judged by its sensitivity to bafilomycin, but which contains many other polypeptides (Fig. 2). The activity of this preparation can be increased by the addition of phospholipids (cf. [18]) but the conditions have not yet been optimized.

Our attempts to raise antibodies to chromaffin granule membrane H^+-ATPase by immunization with individual subunits have proved unsuccessful; however, antibodies to the 57 kDa and 67 kDa subunits of plant tonoplast H^+-ATPases crossreact with the mammalian counterparts [23], and antisera raised against individual subunits of the tonoplast H^+-ATPase from the crassulacean plant *Kalanchoe daigremontiana* have been used to follow the kidney H^+-ATPase during Triton X-114 fractionation. Immunoblotting using these antisera confirms the results obtained, using bafilomycin-sensitive ATPase activity as an assay.

Immunization of rabbits with reconstituted chromaffin-granule H^+-ATPase produced an antiserum that reacted with the largest (120 kDa) subunit. Its slight reactivity with dopamine β-monooxygenase, a contaminant of the purified H^+-ATPase, was not a nuisance in these experiments, since this enzyme is absent from kidney; it could in any case be overcome by absorption of the serum with purified dopamine β-monooxygenase, without affecting the reactivity of the serum with the larger component. The antiserum recognizes a polypeptide in kidney microsomes that has an electrophoretic mobility close to that of the 120 kDa subunit of chromaffin-granule H^+-ATPase, and is glycosylated to a similar extent. In fact, the bovine kidney polypeptide is slightly smaller than that in bovine chromaffin granules, with an apparent molecular weight of about 115 kDa; this is reduced by about 15 kDa on treatment with endoglycosidase F (specific for N-linked oligosaccharide chains). It is established that the 120 kDa subunit is a component of the H^+-ATPase of chromaffin granules and clathrin-coated vesicles [9–11] and this result suggests that the kidney H^+-ATPase is similar, this polypeptide having previously been undetected by protein staining and immune blotting. Several of the properties of this subunit make it difficult to detect: it is glycosylated, so appears as a diffuse band in electrophoretograms; it stains poorly with Coomassie blue; it is extremely sensitive to proteolysis; and it aggregates when heated in SDS.

Interestingly, the antibody raised against bovine H^+-ATPase fails to recognize any antigen in plant tonoplasts [22], although the presence of a 100 kDa subunit in beet H^+-ATPase has been reported [13]. The question of whether a polypeptide of 100–120 kDa is a necessary component of all V-type ATPases, or is a species- or tissue-specific subunit, remains open.

Acknowledgements: This work was supported by grants from the Medical Research Council and the Science and Engineering Research Council. We thank Dr. J.H. Phillips for his comments on the manuscript.

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