Biochimica et Biophysica Acta, 946 (1988) 33–39
Elsevier

BBA 74216

An immunoreactive 8-azido ATP-labeled protein common to the lysosomal and chromaffin granule membrane

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(Received 9 March 1988)

Key words: Chromaffin granule; Lysosome; Immunoreactivity; 8-Azido-ATP; (Rat liver)

The H^+-ATPases of eukaryotic cell organelles, including the rat liver lysosomal (tritosomal) H^+-ATPase and the bovine chromaffin granule ATPase, exhibit similarities in function, substrate requirements, and inhibitor responses. We have explored the possibility that these pumps also exhibit immunological similarities, and that common determinants may be present on polypeptides important to function, such as ATP binding. Toward this end, antibodies were produced in rabbits against a highly purified, detergent-solubilized and fractionated chromaffin granule proton pump preparation. This antibody reacted with a 70–80 kDa protein of the lysosomal membrane on Western blots. We have previously shown that photolysis with 8-azido-ATP inhibits lysosomal *N*-ethylmaleimide-sensitive, vanadate-, ouabain- and oligomycin-insensitive ATP hydrolysis and H^+ transport, with concomitant labeling of a 70–90 kDa membrane protein, amongst others. Here, we report that the photolysis with 8-azido-ATP also leads to inhibition of chromaffin granule H^+ pump function and pump-related ATP hydrolysis, with concomitant *N*-ethylmaleimide-sensitive, ATP-protectable, 8-azido-[α-^{32}P]ATP labeling. The anti-chromaffin granule antibody reacts with an approx. 70 kDa protein of the chromaffin granule and the lysosome. This raises the possibility that the 70 kDa 8-azido-ATP-reactive, immunologically similar proteins may play a similar role in pump function such as ATP binding and/or hydrolysis in these organelles.

Introduction

The lysosome is a member of the class of intracellular organelles including the chromaffin granule and others, which have been shown to generate and maintain pH gradients of approx. 2 pH units through the action of an electrogenic [1,2] uniport proton pump [3]. The functional role of acidification of the lysosome [4,5] and chromaffin granule [6,7] is well understood. Similarities in the functional attributes of the pumps from various organelles have been noted, as well as some differences. All are insensitive to inhibition by vanadate and low concentrations of oligomycin and ouabain, and are sensitive to *N*-ethylmaleimide. It will be essential to identify the polypeptides which are responsible for proton pump function to further understand the mechanism of regulation of acidification of these organelles. Proton pump preparations derived from the chromaffin granule [8,9] and the kidney vacuolar mem-

Abbreviation: Pipes, 1,4-piperazinediethanesulfonic acid.

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branes [10] are amongst the best characterized. The chromaffin granule pump has been reconstituted from detergent extracts [11]. The polypeptide profile of this preparation is similar to that of detergent-extracted and immunopurified [10] kidney vacuolar preparations. Between five and nine major polypeptides were present in each of these preparations, and most of these may have some role in proton pump function [10]. Less is known of the lysosomal H^+ pump. We have previously shown that photolysis with 8-azido-ATP inhibited lysosomal *N*-ethylmaleimide-sensitive-, oligomycin- and vanadate-insensitive ATP hydrolysis and lysosomal proton pump function [2]. We report here that a 70 kDa 8-azido-ATP-reactive polypeptide of the lysosomal membrane is labeled in the presence of inhibitors of proteolysis and inhibitors of other ion pumps.

We have also compared and contrasted the effect of photolysis with 8-azido-ATP upon ATP hydrolysis and proton pump function in the lysosomal and chromaffin granule preparations. In this paper we show the labeling pattern when these membranes were photolyzed with 8-azido-[α-^{32}P]ATP, and we show that a similar polypeptide is labeled in the chromaffin granule membrane. The polypeptide from detergent-solubilized lysosomal membrane preparations was immunoreactive with antibody raised in rabbits by injection of the chromaffin granule preparation. These immunologically related, 8-azido-ATP-reactive proteins may play a similar role in lysosomal and chromaffin granule pump function.

Methods

Chromaffin granule membranes

Bovine chromaffin granule membranes were prepared as described [8,9].

Tritosomes. Rat liver lysosomes were prepared by the method of Trouet et al. [12], with the inclusion of 1 mM EDTA/0.5 mM phenylmethylsulfonyl fluoride/2 μg/ml pepstatin/5 μg/ml leupeptin [11].

Lysosomal membranes. These were prepared by the method of Moriyama et al. [13].

Reconstituted lysosomal membrane proteins. These were obtained using the procedure of Moriyama and Nelson [11]. Care was taken in all preparations and procedures to include inhibitors of proteolysis and to work rapidly at low temperatures.

Antibody to a 70 kDa protein of the chromaffin granule

Antisera to the chromaffin granule membrane were raised in rabbits by repeated intradermal injection of the purified pump preparation [8,9]. Immunodetection was carried out on nitrocellulose replicates of the sodium dodecyl sulfate separated polypeptides using ^{125}I-protein A [14]. Molecular weights were determined from internal standards cut from a strip of the nitrocellulose replicate prior to blocking and visualized by staining with amido black.

Photoaffinity inactivation and labeling with 8-azido-ATP

Photoaffinity inactivation of the chromaffin granule membrane ATP hydrolysis activity proteins was carried out using 8-azido-ATP [16] essentially as described earlier for the photoaffinity inactivation of the lysosomal ATPase [2], including pepstatin and leupeptin to inhibit proteolysis without inhibition of the proton pump function [11]. 8-Azido-[α-^{32}P]ATP-labeling was carried out using 4 μM 8-azido-[α-^{32}P]ATP, 2.4 μCi/assay. *N*-Ethylmaleimide pretreatment, where indicated, was carried out in the absence of nucleotide at 0°C with 0.5 mM *N*-ethylmaleimide for 5 min. 0.33 mM 5'AMP or 0.165 mM 5'AMP plus 0.165 mM ATP was added to all reaction mixtures, as indicated, to block nonspecific binding of 8-azido-ATP and to serve as an internal quench control when ATP was included [16]. Reaction mixtures for *N*-ethylmaleimide pretreatment and labeling contained (in 0.1 ml), 57 μM NH_4 vanadate/57 μM EGTA/57 μM EDTA/1.14 μg/ml pepstatin/1.14 μg/ml leupeptin/5.7 μg/ml oligomycin/280 μM ouabain/28 μM Pipes-Tris (pH 7.4). Where indicated, $MgCl_2$ was added. 8-azido-ATP was added in the dark, and photolysis carried out by irradiation for 30 s through the top of an open 1.5 ml Eppendorf microfuge tube at 0°C using an ultraviolet light source from a 150 W Nikon mercury lamp in a Diaphot mounting bracket with quartz lens focused at the center of the sample at a distance of 5.5 cm. The pho-

tolysis reaction was terminated by the addition of 0.05 ml standard Laemmli sample solution [17].

Measurement of proton transport and ATP hydrolysis

Rates and extents of proton transport in lysosomal fractions and chromaffin granule membranes were carried out using the fluorescence quench of acridine orange, as described in Ref. 2. Rates of ATP hydrolysis were measured in a radioactive assay as described in Ref. 18.

Miscellaneous

Protein was estimated using the Lowry method [19]. 1.5 mm SDS gel electrophoresis, with 3.5% stacking gel and 10% running gel, was carried out as described by Laemmli [17]. Molecular weight standards were included as internal standards on all gels. Autoradiograms were obtained from stained, dried gels at −80°C using Cronex 4 (Dupont) film and intensifying screens as described in Ref. 15. [γ-^{32}P]ATP was prepared as described in Ref. 18. WR1339 (Taloxypol), oligomycin, ouabain, nigericin, and 8-azido-ATP were obtained from Sigma. 8-Azido-[α-^{32}P]ATP was obtained from ICN. Molecular weight standards were obtained from Bio-Rad. All other reagents were of the highest grade available. Radioactive inorganic [^{32}P]phosphate was obtained from Amersham. Enzyme grade sucrose was a gift from Accurate Chemical Company.

Results

Photolysis with 8-azido-ATP inhibits pump and ATP hydrolysis function

Lysosomal proton pump and pump-related ATP hydrolysis activities were sensitive to inhibition by photolysis with 250 μM 8-azido-ATP [2], as is Na^+/K^+-ATPase [16,20]. We have investigated the effect of photolysis with 8-azido-ATP on the highly purified chromaffin granule proton pump [8,9]. Frozen chromaffin granule membranes, stored at −80°C, were thawed in 100 vol. of 300 mM sucrose, washed by centrifugation, frozen, thawed, and the wash procedure repeated to remove exogenous ATP [8]. The chromaffin granule membranes were then photolyzed with 8-azido-ATP in the presence of ouabain and EGTA to prevent 8-azido-ATP hydrolysis by Na^+/K^+-ATPase and Ca^{2+}-ATPase, respectively. Vanadate was also added to prevent labelling of the metal ion pumps, and oligomycin to inhibit the activity of the mitochondrial proton pump. As a final precaution against ATP or 8-azido-ATP hydrolysis during photolysis, EDTA was included in all reactions [2]. As shown in Fig. 1, photolysis with 250 μM 8-azido-ATP leads to over 75% inhibition of chromaffin granule pump-related ATP hydrolysis. The rate of inhibition is slowed by the inclusion of a 10-fold excess of ATP, and irradiation alone, or treatment with 8-azido-ATP is without effect. These results are similar to those observed previously with the lysosomal proton pump [2]. With both pumps, Mg^{2+} is not required for inhibition by photolysis. As shown in Fig. 1b, proton transport is also inactivated by photolysis with 250 μM 8-azido-ATP.

Labeling of pump polypeptide

The inactivation of pump-related ATP hydrolysis and proton pump function by photolysis with 8-azido-ATP suggested that it would be possible to covalently tag the ATP-binding proteins of the lysosomal and chromaffin granule membrane using radioactive 8-azido-ATP. In Fig. 2a, lysosomal membranes, prepared by osmotic lysis in the presence of NaCl [13] were reacted with 8-azido-ATP as described in Methods. Label incorporation at a broad band of 70–80 kDa was observed. Label incorporation was prevented by the inclusion of ATP. Mg^{2+} inclusion did not significantly alter the labeling pattern, other than to increase background labeling, in contrast to the results obtained with clathrin-coated vesicles with inclusion of Mg^{2+} during ultraviolet irradiation with ATP [22]. In Fig. 2b, the 8-azido-[α-^{32}P]ATP-labeling pattern of osmotically lysed lysosomal membranes was compared with the labeling pattern of the chromaffin granule preparation in the same experiment. Two 8-azido-ATP-labeled proteins of the lysosomal membrane were resolved on these gels. However, only the lower band shows *N*-ethylmaleimide-sensitive labeling, expected for the lysosomal proton pump from ATP protection of *N*-ethylmaleimide inhibition of pump fraction [2]. A broad band of label incorporation was obtained in the 70–80 kDA range of the chromaffin granule

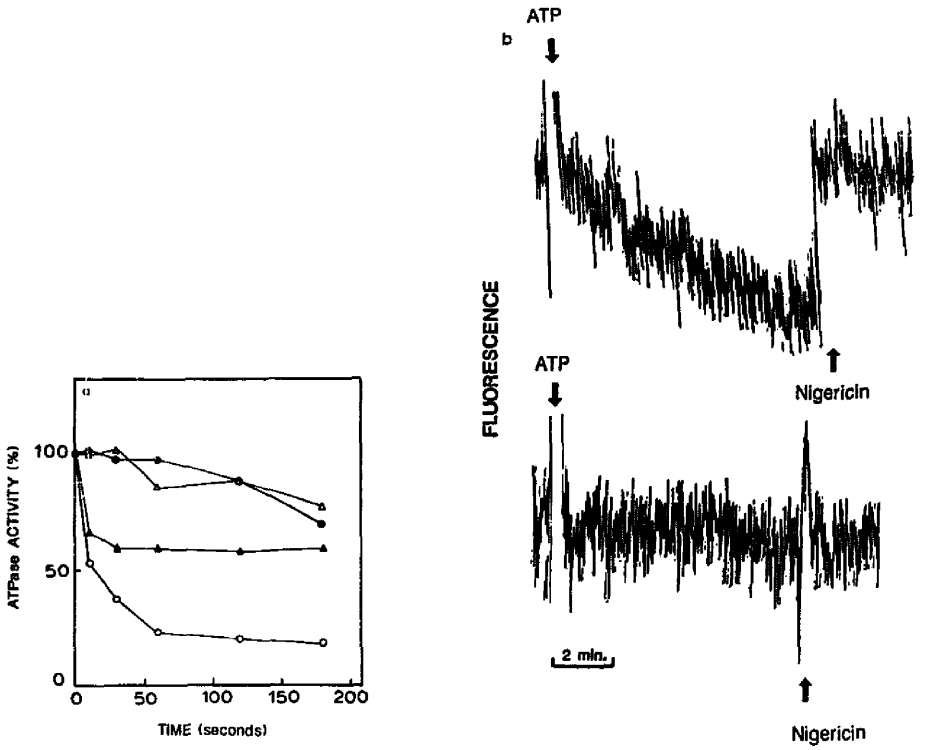

Fig. 1. (a) Photoinactivation of chromaffin granule Mg^{2+}-ATPase with 8-azido-ATP. Chromaffin granule membranes were incubated at 0 °C in the dark for the indicated times in the presence of 250 μM 8-azido-ATP (△△) or irradiated as described in Methods with 250 μM 8-azido-ATP (○○), with 250 μM 8-azido-ATP plus 2.5 mM ATP (▲▲), or without additions (●●). Following irradiation or incubation, the membranes were diluted 100-fold for assay using the radioactive ATPase assay as described. (b) Photoinactivation of chromaffin granule H^+ transport with 8-azido-ATP. In the upper control curve, chromaffin granule membranes were assayed for H^+ transport following a 30-s irradiation without 8-azido-ATP, followed by 30-s incubation with 250 μM 8-azido-ATP in the dark, prior to assay of proton transport. In the lower curve, 250 μM 8-azido-ATP was present during irradiation. ATP-Mg (1 mM) and nigericin (10 μM) were added as indicated.

preparation, which was not further resolved. The chromaffin granule preparation does not appear to contain a major 8-azido-ATP-labeling protein of 120 kDa suggested to be a subunit of the clathrin-coated vesicle ATPase [23], and Mg^{2+} inclusion does not result in label incorporation of high-molecular-weight proteins of the chromaffin granule, in agreement with the findings with the lysosomal membrane, but in contrast to the findings with the clathrin-coated vesicle [22].

The similar 8-azido-ATP-labeling patterns of the lysosome and the 70–80 kDa chromaffin granule membrane proteins suggested that the labeled polypeptides from the two sources might be sufficiently related to possess similar antigenic determinants. To test this hypothesis, antibody to the chromaffin granule preparation was raised in rabbits by successive intradermal injections [21]. The antibody reacts with polypeptides of the chromaffin granule membrane including a 70 kDa polypeptide (not shown). The antibody also reacts strongly with a 70–80 kDa polypeptide present in detergent extracts [9] of lysosomal membranes (Fig. 3).

Discussion

ATP-dependent proton pump activity of the lysosome and chromaffin granule were inhibited upon photolysis with 8-azido-ATP. A 70 kDa polypeptide is labeled with 8-azido-[α-^{32}P]ATP under these conditions. An antibody raised against purified bovine chromaffin granule pump reacts

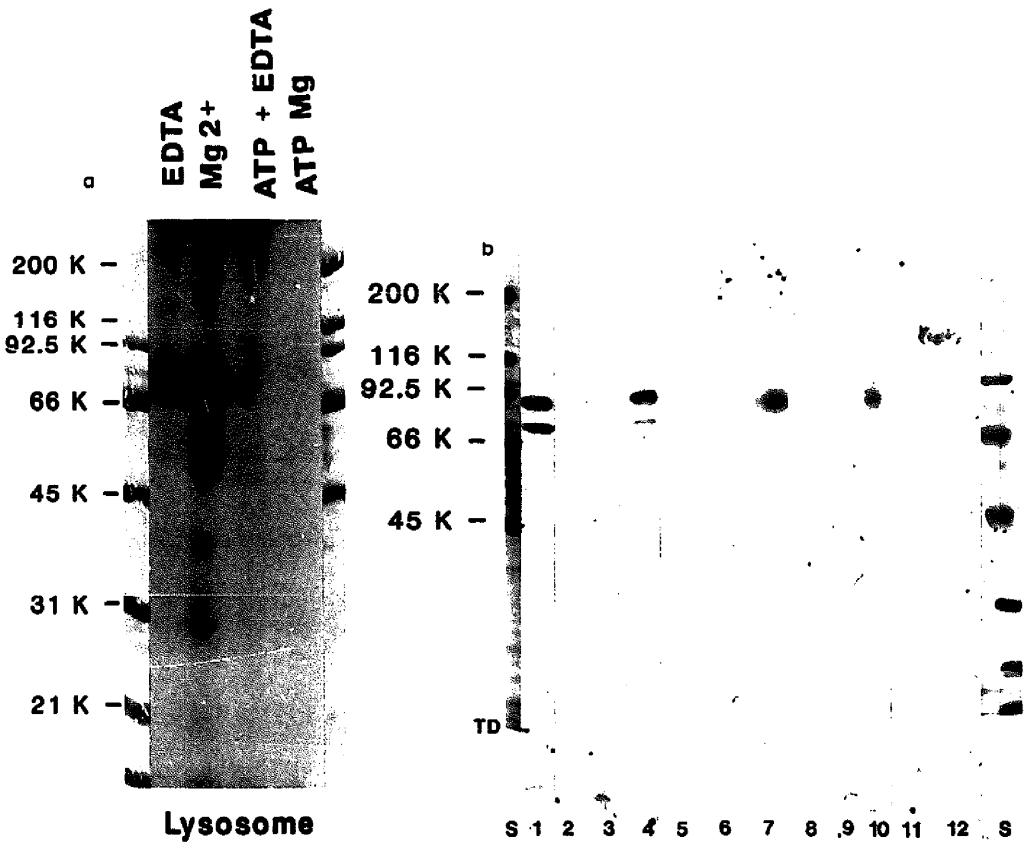

Fig. 2. (a) Photoaffinity labeling of lysosomal membrane proteins: Effect of Mg^{2+}. 100 μg of lysosomal membranes prepared by osmotic lysis were incubated with 8-azido-[^{32}P]ATP, irradiated, electrophoresed, and autoradiographed as described in Methods. In lane 1, the incubation mixture contained 1 mM EDTA, and the major label incorporation occurred in a band in the 70–80 kDa range. In lane 2, the incubation mixture contained in addition excess $MgCl_2$. No major shift in label intensity was observed upon addition of $MgCl_2$. Label incorporation was largely abolished by the addition of ATP. Also shown are the Coomassie blue-stained standards from the stained and dried gel. Molcular weights were determined from internal standards on the stained, dried gels. (b) Photoaffinity labeling of lysosomal and chromaffin granule membrane proteins. 50 μg lysosomal membranes were prepared by osmotic lysis, photoaffinity labeled with 8-azido-[α-^{32}P]ATP, separated on SDS gels, and autoradiographed as described in Methods. In lane 1, two ^{32}P-labeled bands are evident in the range of 70–80 kDa on the autoradiogram. Label incorporation is prevented by inclusion of 0.165 mM ATP (lane 2) or 0.165 mM Mg^{2+}-ATP (lane 3). In lanes 3–6, membranes were preincubated with 0.5 mM *N*-ethylmaleimide for 5 min prior to photolysis with 8-azido-[α-^{32}P]ATP. Label incorporation in the lower band was reduced by *N*-ethylmaleimide pretreatment. Similar results were obtained when chromaffin granule membranes were examined in the same experiment (lanes 7–12). In lane 7, 50 μg of chromaffin granule membranes were photolysed with 8-azido-[α-^{32}P]ATP, and a single broad band of labeling was observed at 70–80 kDa ATP (lane 8) and Mg^{2+}-ATP (lane 9) prevented labeling. In lanes 10–12, the chromaffin granule membranes were first pre-incubated with *N*-ethylmaleimide. Label incorporation was reduced by prior *N*-ethylmaleimide treatment. Also shown are the Coomassie blue-stained standards from the same gels. Molecular weights were determined from high- and low-molecular-weight internal standards on the stained, dried gels.

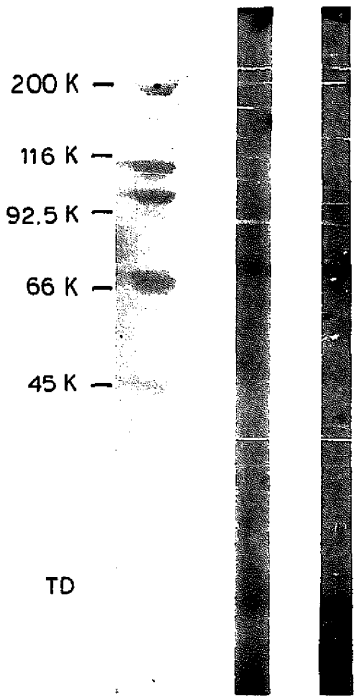

Fig. 3. Immunoreactivity of integral lysosomal membrane proteins to anti-chromaffin granule membrane antibody. 100 μg of membrane proteins prepared by osmotic lysis, detergent extraction, and reconstitution were separated, electroblotted and probed with antibody prepared against the chromaffin granule membrane using ^{125}I-protein A. Molecular weights, which were determined from internal standards on the same nitrocellulose replicate, were visualized by staining a strip of the replicate (prior to blocking), using amido black stain as described in Methods (lane 1). A membrane protein of the lysosomal membrane of approx. 70 kDa shares antigenic determinants in common with the chromaffin granule membrane proteins. Duplicate immunoblots are shown in lanes 2 and 3.

with a 70 kDa polypeptide of the lysosomal membrane on Western blots.

Previous attempts at labeling the lysosomal membranes with 8-azido-ATP showed that a 70–80 kDa protein was the major polypeptide labeled in the lysosomal membrane [2]. We have now resolved the labeled polypeptides in this region and have shown that one, at 70 kDa, shows *N*-ethylmaleimide-sensitive label incorporation. We have also used a combination of osmotic lysis at high ionic strength and detergent extraction in the presence of appropriate inhibitors of proteolysis [8]. These procedures result in simplification of the labeling pattern.

In addition, we have now shown for the first time that the chromaffin granule proton pump and pump-related ATP hydrolysis were inhibited by photolysis with 8-azido-ATP. A 70 kDa polypeptide of the chromaffin granule membrane is labeled by 8-azido-[α-^{32}P]ATP to a similar extent as lysosomal membrane protein of similar size. 8-azido-ATP-labeling is reduced by approx. 50% by prior treatment of the membranes with *N*-ethylmaleimide, and ATP reduces both the rate of inactivation and the extent of labeling.

It has recently been shown that the clathrin-coated vesicle proton pump is labeled upon photolysis with radioactive ATP [22]. Interestingly, Mg^{2+} changed the labeling pattern in that study. In the absence of Mg^{2+}, major label incorporation occurred at a 70 kDa band. In the presence of Mg^{2+}, an increase in label incorporation was observed at a 120 kDa band. From this and other studies [23], the clathrin-coated vesicle pump is thought to be a polypeptide of 120 kDa. In contrast, the purified chromaffin granule preparation employed here appears to lack a major 120 kDa polypeptide, and in this study, label incorporation in the 120 kDa region was not detectable. Mg^{2+} did not increase the label incorporation of high-molecular-weight polypeptides. Under control conditions, minor labeling of an approx. 120 kDa lysosomal polypeptide does occur, but this labeling is not sensitive to competition with ATP. Thus, the lysosomal and chromaffin granule proton pump appear to differ from the clathrin-coated vesicle pump in these regards, despite the similarity of function and similarity in inhibitor responses.

The availability of antibodies to the purified chromaffin granule membrane allowed determination of antigenic determinants shared by the lysosomal membrane and the chromaffin granule membrane. We found that 70–80 kDa polypeptide(s) of the lysosomal membrane, which were extracted by Triton X-100, were reactive on Western blots with the anti-chromaffin granule antibody. These studies thus provide strong evidence that the major 8-azido-ATP-reactive polypeptides of the lysosomal and chromaffin granule membranes are functionally and structurally related.

The inhibition data suggest that the 8-azido-ATP-reactive 70 kDa polypeptide is involved in proton pump function. Further studies are in progress to define the role that this protein plays in proton pump function.

Acknowledgements

We thank Lisa M. Cannon for expert technical assistance. Supported by NIH DK 38808 and VA Merit (J.C.), and AHA 87-1025 and ACS CD-362 (GED).

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