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Three Types of Membranous ATPase on Rat Liver Lysosomes

Hidetoshi HAYASHI,[1a] Kunizo ARAI, Osamu SATO,[1b] Akiyoshi SHIMAYA,[1c] Yoshimichi SAI, and Shoji OHKUMA*

Department of Biochemistry, Faculty of Pharmaceutical Sciences, Kanazawa University, Takara-machi 13-1, Kanazawa, Ishikawa 920, Japan.
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At least three types of vanadate-insensitive membranous ATPase were identified on rat liver lysosomes: bafilomycin A_1-sensitive Mg^{2+}-ATPase (H^+-ATPase), *N*-ethylmaleimide (NEM)-sensitive but bafilomycin A_1-insensitive Mg^{2+}-ATPase (ATPase I), and NEM-insensitive Ca^{2+}/Mg^{2+}-ATPase (ATPase II). They showed different sensitivity to chemicals and ions with apparent molecular masses of 700—800, 500—650, and 360 kDa, respectively. Of these membranous ATPases, H^+-ATPase seemed to constitute only one tenth of the ATPase activity on rat liver lysosomes and to be the only ATPase that exposed its active site to the cytoplasmic side of the lysosomal membranes.

Keywords bafilomycin A_1; H^+-ATPase; NEM-sensitive ATPase; Ca^{2+}/Mg^{2+}-ATPase; rat liver lysosomes

Introduction

Lysosomal pH is maintained by a MgATP-dependent proton pump which transports protons into lysosomes.[2,3] The lysosomal proton pump is unique both in its sensitivity to *N*-ethylmaleimide (NEM), 7-chloro-4-nitrobenzo-2-oxo-1,3-diazole (NBD-Cl), quercetin, adenosine 5′-diphosphate (ADP) and *N,N′*-dicyclohexylcarbodiimide (DCCD) and insensitivity to oligomycin, vanadate and ouabain.[3,4] This type of proton pump is ubiquitous in the vacuolar system.[5] We showed that vanadate-insensitive alkaline Mg^{2+}-ATPase activity is also associated with membrane ghosts derived from rat liver lysosomes.[4] It had some characteristics that were similar to those of the proton pump. However, there were differences between the proton pump and ATPase activities in sensitivity to chemicals; namely, in contrast to the proton pump, the ATPase activity was only marginally sensitive to SH-reagents (NEM, *p*-chloromercuribenzoic acid), and resistant to NBD-Cl and quercetin at concentrations sufficient to inhibit proton pump activity. Furthermore, ATPase activity was expressed even in the presence of Ca^{2+}, contrary to the proton pump activity.[4] Although "uncoupling" is a possible explanation for this disparity, the existence of other type(s) of ATPase on lysosomal membranes should be considered.

In this paper, we show that there are at least two other types of ATPase on lysosomal membranes besides H^+-ATPase, and that H^+-ATPase constitutes only part of the lysosomal ATPase activity.

Materials and Methods

Materials Triton WR-1339 was purchased from the Ruger Chemical Co. (Irvington, NJ). Most of the other chemicals used in this study were obtained from Sigma (St. Louis, MO). Bafilomycin A_1 was a kind gift from Prof. K. Altendorf (University of Osnabrück, Germany).

Preparation of Rat Liver Lysosomes Triton-filled lysosomes (tritosomes) were prepared from rats (Wistar/ST, male) by means of a flotation gradient as previously described.[4] Dextran-filled lysosomes (dextranosomes) were prepared as described previously.[6]

Solubilization of Tritosomal Membranes The tritosomal membranes were solubilized with polyoxyethylene 9-lauryl ether ($C_{12}E_9$): the lysosomal membrane ghosts (final protein concentration, 1 mg/ml) were adjusted to 0.02% (w/v) $C_{12}E_9$ in solubilization buffer [20 mM 3-[*N*-morpholino]propanesulfonic acid (Mops)–tetramethylammonium hydroxide (TMAH) (pH 7.0), 10 mM dithiothreitol (DTT), 20% (v/v) glycerol, 1 mM EDTA, 5 μg/ml protease inhibitors (pepstatin, antipain, chymostatin, leupeptin), and 0.2 mg/ml asolectin]. Octylthioglucoside was occasionally used as a detergent for solubilization at a concentration of 1.0% (w/v). The mixture was immediately vortexed for 15 s, allowed to stand on ice for 10 min, then sedimented at 106000 × *g* for 1 h. The resulting supernatant was used as the solubilized fraction.

ATP-Dependent H^+-Pump Activity and ATPase Activity Lysosomes containing fluorescein-isothiocyanate dextran were prepared and ATP-driven acidification of lysosomes was measured as described before.[3] ATPase activity was determined by measuring the rate of liberation of inorganic phosphate from ATP. Briefly, samples were incubated at 30 °C for 20 to 40 min in an assay buffer containing 40 mM *N*-[3-Hydroxyethyl]piperazine-*N′*-[2-ethanesulfonic acid] (Hepes)–TMAH (pH 7.5), 0.1 M KCl, 0.5 mM $MgCl_2$ and 0.5 mM ATP-Na_2. Inorganic phosphate was determined colorimetrically according to the method of Chan *et al.*[7]

ATPase Activity Staining Gels were stained for ATPase activity, after undenatured polyacrylamide gel electrophoresis, by incubation at 37 °C overnight in a substrate solution containing 1 mM ATP, 20 mM Tris–maleate (pH 8.5), 5 mM $MgCl_2$ and 1 mM $Pb(NO_3)_2$.[8] The gels were rinsed in H_2O and incubated in 0.5% Na_2S to develop dark brown PbS precipitates.

DEAE-Sephadex A-25 Column Chromatography The $C_{12}E_9$ solubilized fraction was applied to DEAE-Sephadex A-25 pre-equilibrated with buffer A [50 mM Tris–HCl (pH 6.5), 0.5 mM DTT, 0.5 mM EGTA, 0.02% $C_{12}E_9$ and 8 μg/ml asolectin] then eluted with a linear gradient (0 to 1.0 M) of NaCl in buffer A.

Results

H^+-ATPase Constitutes Only Part of the Membranous ATPases of Rat Liver Lysosomes As shown in Fig. 1, ATP dependent proton uptake into lysosomes was totally inhibited by 1 mM or higher concentrations of NEM ($IC_{50} \fallingdotseq 100\ \mu M$).[3,4] Proton pump activity was also completely inhibited by bafilomycin A_1, a potent selective inhibitor against the vacuolar H^+-ATPase recently isolated from *Streptomyces* sp.,[9] at concentrations of 50 nM or higher ($IC_{50} \fallingdotseq 1$ nM). On the other hand, the Mg^{2+}-ATPase activity was little (10 to 20%) affected by either NEM or by bafilomycin A_1 ($\leq 10\%$), although it was insensitive to vanadate (Fig. 1). This suggests that most of the lysosomal ATPases are not related to proton pumping and that the H^+-ATPase constitutes only a portion of the lysosomal ATPase activity. Furthermore, the inhibitory effect on Mg^{2+}-ATPase of NEM was biphasic ($IC_{50} \fallingdotseq 100\ \mu M$ and 1 mM), whereas that of bafilomycin A_1 was monophasic ($IC_{50} \fallingdotseq 1$ nM), suggesting that there are two enzymes with different sensitivities to NEM. That with the higher sensitivity to NEM is probably also sensitive to bafilomycin A_1.

Three Types of ATPase on Lysosomal Membranes Solubilization of the tritosomal membranes with $C_{12}E_9$,

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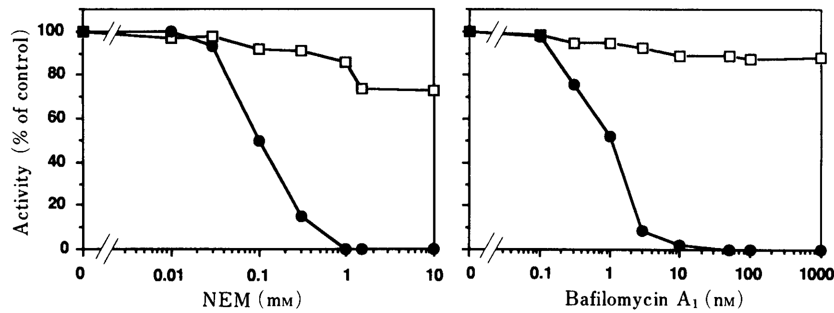

Fig. 1. Effects of NEM and Bafilomycin A_1 on the Lysosomal H^+-Pump and ATPase Activities

The H^+-pump activity was measured by ATP-driven fluorescence quenching of fluorescein isothiocyanate-dextran incorporated within lysosomes.[3,13] The ATPase activity was measured as described in Materials and Methods. Activity is expressed as a percentage of control values (in the absence of inhibitors). —□—, Mg^{2+}-ATPase activity; —●—, proton pump activity.

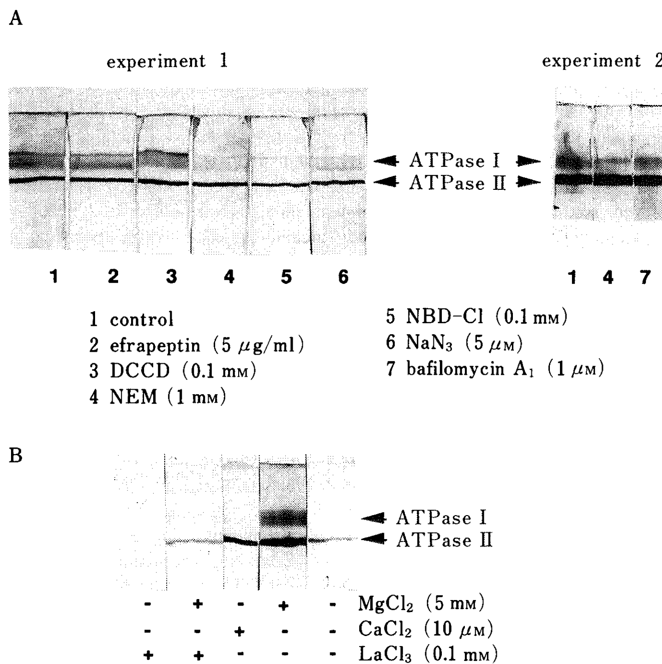

Fig. 2. ATPase Activity Staining of Solubilized Lysosomal Membranes

Activity staining of Mg^{2+}-ATPase was performed as described in Materials and Methods after application of $C_{12}E_9$ extracts of tritosomal membranes. A: Effect of inhibitors. B: Effect of divalent cations. The upper arrow indicates NEM-sensitive Mg^{2+}-ATPase (ATPase I) and the lower arrow indicates Ca^{2+}/Mg^{2+}-ATPase (ATPase II).

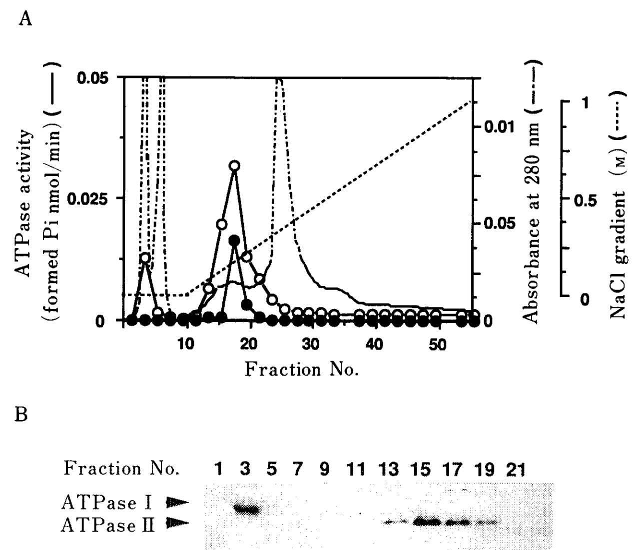

Fig. 3. Elution Profile of Tritosomal Membrane Extract on DEAE-Sephadex A-25 and ATPase Activity Staining of the Fractions

A: Elution profile of Mg^{2+}-ATPase on DEAE-Sephadex A-25 from extracts of tritosomal membranes. The $C_{12}E_9$ extract of tritosomal membranes was applied to DEAE-Sephadex A-25 and 2 ml fractions were collected at a flow rate of 0.5 ml/min. —○—, total Mg^{2+}-ATPase activity; —●—, bafilomycin A_1 (10 μM)-sensitive Mg^{2+}-ATPase activity. ---, absorption at 280 nm; —·—, linear NaCl gradient (0—1.0 M). B: ATPase activity staining of fractions (No. 1—19) from DEAE-Sephadex A-25. The arrows indicate the position of the NEM-sensitive Mg^{2+}-ATPase (ATPase I) and the NEM-insensitive Ca^{2+}/Mg^{2+}-ATPase (ATPase II), respectively.

which has also been used to extract H^+-ATPase from chromaffin granules[10] and coated vesicles,[11] yielded an extract with maximum recovery of the total lysosomal ATPases (recovery: 53%) including NEM-sensitive ATPase activity (90%) and bafilomycin A_1-sensitive Mg^{2+}-ATPase (85%), which were minimally contaminated by F_0F_1-ATPase. When the $C_{12}E_9$-solubilized membranes were stained for ATPase after undenatured polyacrylamide gel electrophoresis, two efrapeptin-insensitive ATPase bands appeared, with apparent molecular masses of 500—650 (upper band, ATPase I) and 360 kDa (lower band, ATPase II)), respectively (Fig. 2A). The membrane ghosts from highly purified lysosomes (dextranosomes) prepared by the different principle from rat liver[6] yielded essentially the same ATPase bands, suggesting that the two ATPases are not derived from contamination of other organella but actually reside on the lysosomal membranes.

The upper band was sensitive to NEM and not expressed when Mg^{2+} was replaced with a low concentration (10 μM) of Ca^{2+} (Fig. 2B). The lower band was resistant to NEM and appeared even in the absence of Mg^{2+} or when Mg^{2+} was replaced with low concentrations of Ca^{2+}, but was inhibited by La^{3+} (Fig. 2B). Both ATPases expressed activities at a broad pH range around neutrality. However, neither of these two ATPases were sensitive either to bafilomycin A_1 or DCCD (Fig. 2A).

These two ATPases recognized by activity staining were separated by DEAE-Sephadex A-25 (Fig. 3). The NEM-sensitive Mg^{2+}-ATPase was not adsorbed to DEAE-Sephadex A-25, while the Ca^{2+}/Mg^{2+}-ATPase was adsorbed to and eluted from it by a linear gradient (0 to 1.0 M) of NaCl. However, the DEAE-Sephadex A-25 unbound fraction containing NEM-sensitive ATPase, was insensitive to bafilomycin A_1. On the contrary, bafilomycin A_1-sensitive ATPase was bound to DEAE-Sephadex A-25 and eluted from it, as was Ca^{2+}/Mg^{2+}-ATPase, by a

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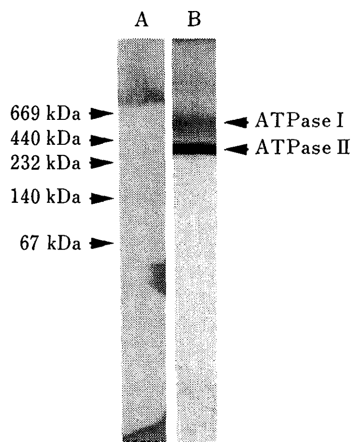

Fig. 4. Immunoblotting with Anti-vacuolar H^+-ATPase Antiserum and ATPase Activity Staining of Solubilized Tritosomal Membranes

An octylthioglucoside extract of tritosomal membranes was separated by undenatured polyacrylamide gel electrophoresis (4—20% linear gradient). A: Immunoblotting with antiserum raised against a 16 kDa peptide of rat vacuolar H^+-ATPase.[12] B: Activity staining of Mg^{2+}-ATPase. The arrows indicate the position of the NEM-sensitive Mg^{2+}-ATPase (ATPase I) and the NEM-insensitive Ca^{2+}/Mg^{2+}-ATPase (ATPase II), respectively.

Table I. ATPase Activity of Intact and Disrupted Lysosomes

Types of ATPase and conditions	ATPase activity			
	nmol/min/mg protein	% control	% maximum[a]	% total[b]
(1) bafilomycin A_1-sensitive Mg^{2+}-ATPase				
Intact (control)	1.055	100.0	100.0	76.3
+ freeze/thaw[c]	0.464	44.0	44.0	3.78
(2) NEM-sensitive/bafilomycin A_1-insensitive Mg^{2+}-ATPase				
Intact (control)	0.093	100.0	10.4	6.70
+ freeze/thaw[c]	0.895	962	100.0	7.30
(3) NEM-insensitive Ca^{2+}/Mg^{2+}-ATPase				
Intact (control)	0.235	100.0	2.16	17.0
+ freeze/thaw[c]	10.90	4640	100.0	88.9

Mg^{2+}-ATPase activities of dextranosomes were assayed in the presence of 2.5 μM FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) to express the full activity of H^+-ATPase (bafilomycin A_1-sensitive Mg^{2+}-ATPase). Activation by 2.5 μM FCCP was about 277% from 0.381 nmol/min/mg protein. Intactness of lysosomes before and after incubation was 96.7% and 94.5%, respectively, as assessed by the release of fluorescein isothiocyanate-dextran incorporated into lysosomes.[13] Corrections were not made for the possible inactivation of the ATPase activities by freeze/thaw disruption. *a*) Percent maximum indicates the values relative to the maximum (intact or freeze-thawed) value for each enzyme activity. *b*) Total ATPase activity of intact and freeze-thawed samples were 1.383 and 12.26 nmol/min/mg protein, respectively. c) Corrections were made for the small ATP hydrolyzing activity of the lysosomal matrix (15.2%, 5.01% and 8.54% of the total activity of freeze-thawed dextranosomes, respectively, for each ((1) to (3)) of the corresponding activities).

linear gradient of NaCl. Furthermore, when the lysosomal membranes were immunoblotted with antibody against 16 kDa subunit of rat vacuolar H^+-ATPases,[12] after undenatured polyacrylamide gel electrophoresis, a reactive band appeared at 700—800 kDa that was at a slightly higher molecular mass than that of the NEM-sensitive Mg^{2+}-ATPase (Fig. 4). These results show that there are at least three ATPases on rat liver lysosomes; two NEM-sensitive ATPases (one is a bafilomycin A_1-sensitive H^+-ATPase and the other is a bafilomycin A_1-insensitive Mg^{2+}-ATPase, ATPase I) and one NEM-insensitive ATPase (CA^{2+}/Mg^{2+}-ATPase, ATPase II).

Position of ATPases on Lysosomal Membranes The ATPase activities of the NEM-insensitive (ATPase II) and the NEM-sensitive/bafilomycin A_1-insensitive (ATPase I)

Table II. Three Types of ATPase on Rat Liver Lysosomes

Types of ATPase	Apparent molecular mass (kDa)	Cation requirement	Selected inhibitors
(1) H^+-ATPase	700—800	Mg^{2+}	NEM, NBD-Cl, bafilomycin A_1
(2) NEM-sensitive/bafilomycin A_1-insensitive Mg^{2+}-ATPase (ATPase I)	500—650	Mg^{2+}	NEM, NBD-Cl
(3) NEM-insensitive Ca^{2+}/Mg^{2+}-ATPase (ATPase II)	360	Mg^{2+}, Ca^{2+}	

enzymes were enhanced 10 to 46 folds in lysosomal membranes disrupted by freeze-thawing (Table I). A similar enhancement of these activities was attained after detergent treatment (data not shown). As the latency values of these activities become almost complete when corrected for the disruption of lysosomes as assessed by the release of incorporated fluorescein isothiocyanate-dextran,[13] their active sites must be exposed to the lysosomal interior. The behaviors of these two ATPases are contrary to that of bafilomycin A_1-sensitive Mg^{2+}-ATPase (H^+-ATPase), whose active site should be on the exterior of lysosomal membranes facing the cytoplasm because its activity was not enhanced, but rather decreased, by freeze-thawing (Table I).

Discussion

We showed that the lysosomal membranes of rat liver possess at least two other types of ATPase besides H^+-ATPase: an NEM-sensitive but bafilomycin A_1-insensitive Mg^{2+}-ATPase (ATPase I) and an NEM-insensitive Ca^{2+}/Mg^{2+}-ATPase (ATPase II) (Table II). Rat liver lysosomal membranes contain AMPase (5′-nucleotidase)[14] and FADase (flavin adenine dinucleotidase)[15] activities with an optimum at pH 6—8. However, they do not hydrolyze ATP suggesting that they are unrelated to any of these ATPase activities. Furthermore, both NEM-sensitive Mg^{2+}-ATPase and Ca^{2+}/Mg^{2+}-ATPase did not hydrolyze *p*-nitrophenylphosphate (data not shown). The presence of multiple forms of lysosomal ATPase has been suggested also by Schneider[16] and Mego,[17] although the evidence is fragmentary. The profile of multiple forms of ATPase is however, not universal to all lysosomes, because NEM-insensitive Ca^{2+}/Mg^{2+}-ATPase (ATPase II) has not been detected on rat kidney lysosomes.[18]

Recently, we isolated lysosomal H^+-ATPase from the other two ATPases by Mono Q column chromatography as a bafilomycin A_1-sensitive ATPase having cross-reactivity with antibody against vacuolar H^+-ATPases.[12,19] The full characterization of the isolated lysosomal H^+-ATPase shows that the difference in the effect of chemicals and divalent cations that we observed previously between the proton pump and ATPase activities[4] can be explained by the existence on lysosomal membranes of these major ATPase activities that are different from H^+-ATPase. It also suggests to us that the H^+-ATPase was hard to detect on activity staining (Figs. 2, 4) because of its sensitivity to Pb^{2+}.[19]

The characteristics of the NEM-insensitive ATPase are

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quite similar to that of ecto-ATPase.[20] Furthermore, the proposed sidedness (facing the lysosomal matrix, Table I), tissue distribution (not detected on rat kidney lysosomes[18]) and glycoprotein nature (adsorbed to Concanavalin A-column, data not shown) of the NEM-insensitive ATPase, are all consistent with the notion that it was derived from internalization of the plasma membrane ecto-ATPase. However, there are significant differences between these two ATPases. (1) The ecto-ATPase seems to be confined to the plasma membranes, as determined by immunocytochemical analysis using anti-ecto ATPase antibody.[21] (2) The N-terminal of the lysosomal NEM-insensitive Ca^{2+}/Mg^{2+}-ATPase purified recently is different from that of ecto-ATPase.[22] The relationship between these two activities remains to be solved.

Also, the location of the NEM-sensitive Mg^{2+}-ATPase suggests that it has nothing to do with kinesin-like motor ATPases for organellar movement[23] or any fusion factors such as NSF (NEM-sensitive factor for vesicular transport of Golgi system) which possesses ATPase activity (M. Tagaya, personal communication).

The functions of both the NEM-sensitive Mg^{2+}-ATPase and the Ca^{2+}/Mg^{2+}-ATPase remain unclear. The possible roles of these ATPases in lysosomal physiology remain to be elucidated.

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1) Present address: *a) Department of Hygienic Chemistry, Faculty of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabedori, Mizuho-ku, Nagoya 467, Japan; b) Research and Development, Mochida Pharmaceutical Company, Ltd., Yotsuya 1-7, Shinjuku-ku, Tokyo 160, Japan; c) Tsukuba Research Institute, Yamanouchi Pharmaceutical Company, Ltd., Miyukigaoka 21, Tsukuba, Ibaragi 305, Japan.*
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