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SEPARATION OF RAT LIVER LYSOSOME MEMBRANE ADENOSINE TRIPHOSPHATASE ACTIVITIES BY POLYACRYLAMIDE GEL ELECTROPHORESIS

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Solubilization of rat liver lysosome membranes with octyl glucoside or lauryl sarcosinate and analysis of ATPase activities in sections of polyacrylamide gels after electrophoresis revealed one major peak at pH 8 and two peaks at pH 5. The pH 8 ATPase peak was not localized in the same peak with pH 5 ATPase activity, suggesting that these were catalyzed by different proteins. Ca^{2+}- and Mg^{2+}-ATPase activities at pH 8 were present in the same major peak, with Ca^{2+} activity predominating. The pH 8 Ca^{2+}-ATPase was also not present in the same area of the gels as Ca^{2+}-ADPase.

Introduction

Sufficient evidence now exists for the presence of an ATP-energized proton or acid pump in lysosome membranes [1–8]. These membranes contain ATPase activity [9,10], which is obligatory for such a pump. However, this ATPase has a broad pH optimum ranging from about 4 to 9. Furthermore, the activity at pH 8 is dependent on Ca^{2+} or Mg^{2+} but considerable activity is present in the absence of cations at pH 5 [10]. This suggests at least two ATPases, one active at pH 5 in the absence of cations and one with a pH 8 optimum that requires divalent cations for activity.

Collins and Wells [11] have reported the presence of a Ca^{2+}-dependent protein kinase in the lysosome membrane that phosphorylates a low molecular weight peptide. More recently these workers have identified a phosphatidylinositol kinase in these membranes [12]. This enzyme is Mg^{2+}-dependent and active at neutral pH. The lysosomal proton pump activity described by Ohkuma et al. [8] exhibits a strict requirement for Mg^{2+}, and Ca^{2+} will not replace Mg^{2+}. The stimulatory effects of ATP on intralysosomal proteolysis at pH 8, which has been interpreted in terms of proton-pump activity [7,13,14], exhibits a requirement for either Ca^{2+}, Mg^{2+} or Mn^{2+} [14]. Thus, it is of interest to determine whether more than one ATPase activity is present in the lysosome membrane or whether the various observations described above may be attributed to the same enzyme.

Materials and Methods

Lysosome membrane preparation. Adult, random-bred albino rats (Charles River Breeding Laboratories, Wilmington, MA) were injected with Triton WR-1339 and lysosomes were isolated from livers according to the procedure of Leighton et al. [15] as described by Dean [16] with some minor modifications. These changes consisted of an additional removal of the microsome fraction ('pink fluffy layer') from initial centrifugation pellets and an increase in the high-speed centrifugation time from 2 h to 3.5 h at 110000 × *g* (Beckman Model L2-65B ultracentrifuge, SW-27 rotor). The increased centrifugation time reduced contamination of purified lysosomes by mitochondria.

Purified Triton WR-1339-filled lysosomes (tritosomes) were broken by suspension in distilled water and dialysis for 4 h against 1 liter ice-cold distilled water. The preparations were then centrifuged at 40000 × *g* for 20 min to sediment membranes. Tritosome membranes were washed twice by suspension in 2.5 ml 0.5 M KCl and centrifugation and then with 2.5 ml H_2O to remove residual KCl. Pellets were stored at −30 °C until used. Yields were about 0.4 mg membrane protein per 300 g rat.

Assays. ATPase was assayed in 0.5 ml reaction mixtures consisting of 4 mM [γ-^{32}P]ATP ((0.4–2) · 10^5 cpm/μmol), 5 mM $CaCl_2$ and 0.1 M Tris-acetate buffer. Liberated [^{32}P]phosphate was extracted with isobutanol/benzene/acetone [17] after addition of 5 ml 1.2% (w/v) ammonium molybdate in 1 M HCl [18]. Radioactivity was measured in a liquid scintillation spectrometer with automatic sampler changer. ADPase activity was determined by phosphate assay according to Fiske and Subbarow [19] or by the method of Chen et al. [20]. Protein was assayed according to Lowry et al. [21] after solubilization with 10% (w/v) deoxycholate, pH 11. Crystalline bovine serum albumin standards prepared in the same manner were run with each assay. Specific activities of enzyme activities were calculated as μmoles phosphate/min per mg protein.

Polyacrylamide gel electrophoresis. Lysosome membranes were solubilized in 2% (w/v) octyl glycoside or lauryl sarcosinate in 50 mM Tris-acetate, pH 9. Preparations were stirred in an ice-water bath for 30 min, sonicated for 30 s in a bath sonicator, and centrifuged at 40000 × *g* for 20 min. Polyacrylamide gels were prepared according to Williams and Reisfeld [22] in their pH 8 system in 110 × 4 mm (internal diameter) glass tubes. Stacking and resolving gels were prepared with 1% octyl glucose or lauryl sarcosinate and the electrophoresis buffer (Tris-diethylbarbiturate, pH 7), containing 0.1% detergent. About 400 μg solubilized membrane protein were applied and electrophoresis was performed at 3 mA per tube at 4 °C for 4–6 h. Gels were then removed and one was stained with 1% Coomassie blue (R-250) in 10% acetic acid/40% methanol. Destaining was performed by diffusion in the same solution. Remaining gels (usually one or two) were cut into 4-mm sections, or they were first split lengthwise and then cut into sections. The gel sections were incubated in appropriate media for 12–24 h at 37 °C in 0.5 ml reaction mixtures. Controls without gels were incubated with each set. Inorganic phosphate produced in these controls was subtracted from that produced in mixtures containing gel sections. This phosphate never exceeded 5% of the total hydrolyzable material.

Chemicals. ATP, ADP, sucrose, octyl glucoside, lauryl sarcosinate and buffer materials were obtained from Sigma Chemical Co., St. Louis, MO, or from Calbiochem (San Diego, CA). Triton WR-1339 was purchased from Ruger Chemical Co., Irvington, NJ. [γ-^{32}P]ATP was purchased from ICN, Irvine, CA. This ATP was purified by adsorption on charcoal and elution with 40% ethanol in 1% ammonium hydroxide. Ethanol and ammonia were removed by evaporation at 37 °C under reduced pressure or by lyophilization. Either procedure produced satisfactory results with less than 1% isobutanol/benzene/acetone-extractable counts after treatment with molybdate reagent as described in the preceding section.

Results

When the ATPase of tritosome membranes is assayed by chemical phosphate determination, the activity exhibits a broad pH spectrum from 4 to 9 [10]. ATPase assay by liberation of [^{32}P]phosphate from [γ-^{32}P]ATP also showed the broad pH spectrum, but in most experiments the activities at pH 6–7 were reduced with respect to the activities at pH 5 and pH 8, suggesting two peaks (Fig. 1).

Solubilization of tritosome membranes with octyl glucoside or lauryl sarcosinate resulted in a reduction of activity at pH 7–9 similar to that noted with Triton X-100 [10]. Dialysis of the octyl glucoside- or sarcosinate-solubilized membranes did not restore the pH 8 activity, suggesting irreversible damage (unpublished data).

Electrophoresis of octyl glucoside-solubilized membranes revealed two peaks of ATPase activity when gel sections were incubated in media containing [γ-[^{32}P]ATPase pH 5 (Fig. 2). The same two peaks, one more rapidly moving sharp band followed by a broad band, were obtained by electrophoresis of membranes solubilized with lauryl

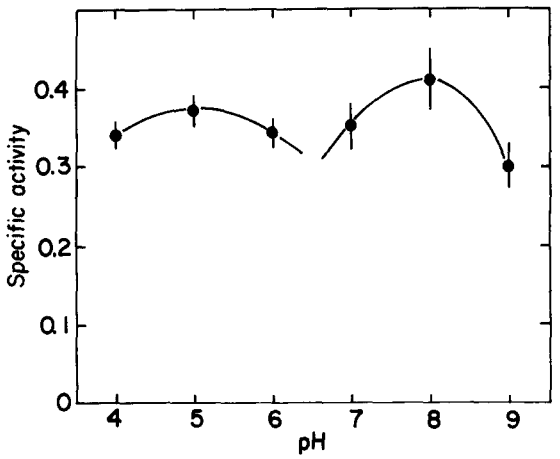

Fig. 1. pH profile of ATPase activities in KCl-washed lysosome membranes. Reaction mixtures (1 ml) contained 4 mM [γ-^{32}P]ATP (142000 cpm/μmol), 5 mM $CaCl_2$ and 0.1 M Tris-acetate buffers. Specific activity: μmol phosphate/min per mg protein. Vertical lines represent standard errors of the means of five experiments.

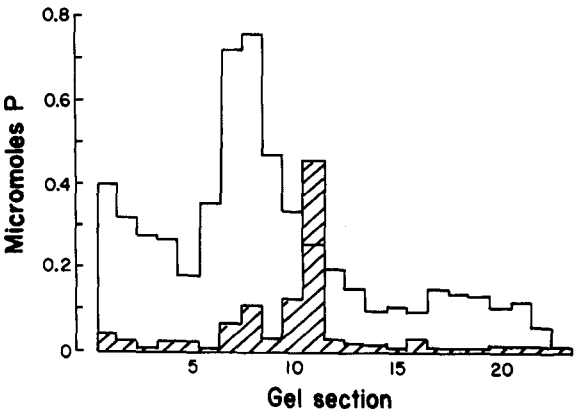

Fig. 3. ATPase assay at pH 8 (shaded areas) and pH 5 (unshaded) in 4-mm polyacrylamide gel sections containing octyl glucoside-solubilized lysosome membrane proteins. Electrophoresis time was 4 h. Gel sections were incubated for 15 h (pH 5) and 21 h (pH 8) in 0.5 ml reaction mixtures as described in the legend to Fig. 1.

sarcosinate (not shown). Analysis of ATPase activities at pH 5 and pH 8 in the same gel sections revealed one peak of pH 8 activity that did not coincide with a pH 5 peak (Fig. 3). Fig. 3 also shows the second pH 5 ATPase peak beginning to enter the resolving gel.

Fig. 4 shows that the pH 8 activity was higher in the presence of Ca^{2+} than Mg^{2+}, but both activities were present in the same peak. ATPase activity is negligible at pH 8 in the absence of divalent cations either in intact [10] or in solubilized preparations (unpublished data). Thus, assays of pH 8 ATPase was not performed in gel

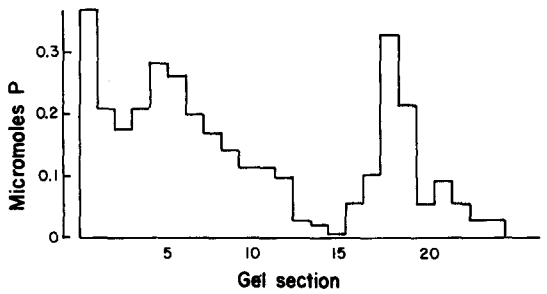

Fig. 2. ATPase activity at pH 5 in 4-mm polyacrylamide gel sections after 6 h electrophoresis of octyl glucoside-solubilized rat liver lysosome membranes. Gel sections were incubated for 15 h in 0.5 ml reaction mixtures containing 4 mM [γ-^{32}P]ATP, 5 mM $CaCl_2$ and 0.1 M Tris-acetate, pH 5.

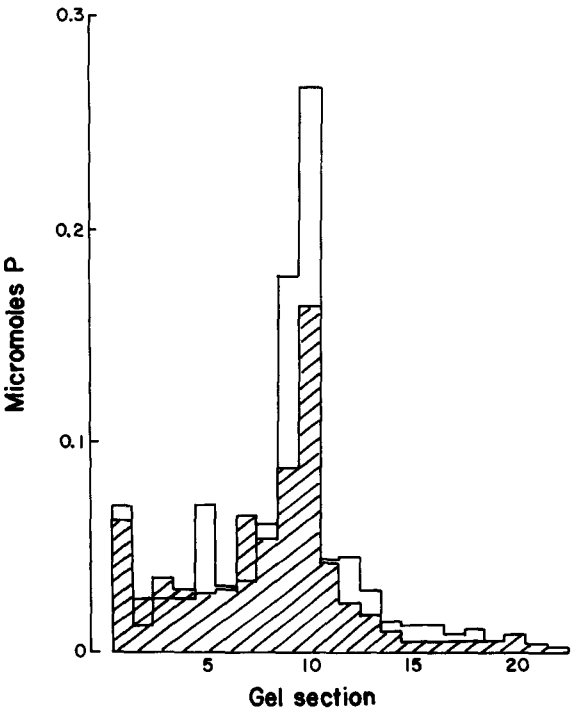

Fig. 4. Effects of Mg^{2+} (shaded) and Ca^{2+} on ATPase activities at pH 8 in polyacrylamide gel sections containing octyl-glycoside solubilized lysosome membranes. Electrophoresis was carried out for 4 h at 3 mA per tube. Gel sections were incubated for 22 h at 37 °C in 0.5 ml reaction mixture as described in the legend to Fig. 1.

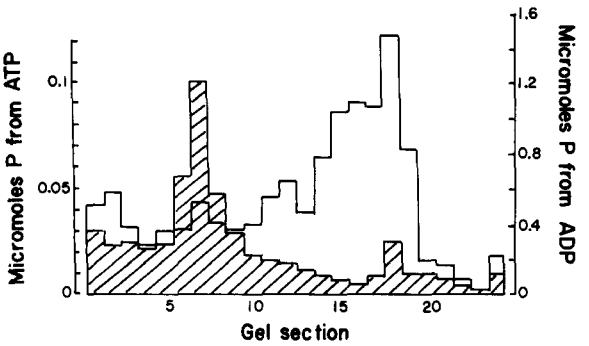

Fig. 5. ADPase (unshaded) and ATPase (shaded) activities in polyacrylamide gel sections containing octyl-glucoside solubilized lysosome membranes after 3 h electrophoresis. ADP was was assayed after 16 h incubation of gel sections in 0.5 ml reaction mixtures containing 4 mM ADP, 5 mM $CaCl_2$ and 0.1 M Tris-acetate, pH 6, by the Fiske and SubbaRow [17] procedure. ATPase was assayed after 22 h incubation of gel sections in 0.5 ml reaction mixtures as described in the legend to Fig. 1.

sections in the absence of divalent cations.

Analysis of ADPase activities in polyacrylamide gel sections revealed that the peak of this activity did not appear in the same sections as pH 8 ATPase (Fig. 5). ADPase activity was much higher in the gels than the pH 8 ATPase although ATPase is greater in intact or solubilized membrane preparations [10]. This was probably due to the apparently greater lability of the pH 8 ATPase as well as to the procedure used to assay ADPase. Chemical assay of inorganic phosphate in these preparations included that released by the action of a very active 5′-nucleotidase (AMPase) which has a pH optimum of 6–7 [10] as well as the phosphate released by ADPase.

Since pH 8 and pH 5 ATPase activities appeared to be catalyzed by different proteins, some studies were undertaken to compare kinetic parameters at pH 5 and pH 8. For example, Fig. 6 shows effects of substrate concentrations on ATPase activities at pH 5 and pH 8. The curves were similar although the activity of the pH 5 enzyme was greater at higher substrate concentrations. Apparent K_m values of the two enzymes were similar, 0.8 mM at pH 8 and 1.0 mM at pH 5.

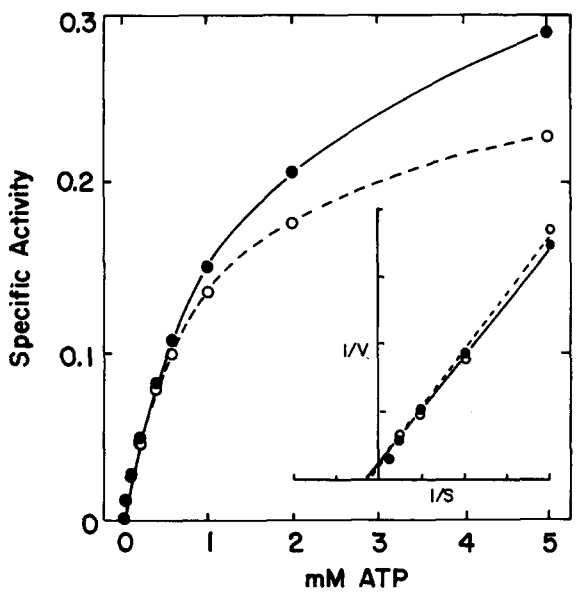

Fig. 6. Effects of substrate concentrations on ATPase reaction rates at pH 5 (●———●) and pH 8 (O— — —O) in intact (unsolubilized) lysosome membranes. Reactions were carried out as described in the legend to Fig. 1. A double reciprocal plot of the data is also shown.

Discussion

Rat liver lysosome membranes contain ADPase and AMPase (5′-nucleotidase) activities with optima at pH 6–7 [10]. Assays of ATPase in these membranes by release of inorganic phosphate therefore includes some phosphate from the hydrolysis of ADP and possibly AMP in this pH range. This should increase the apparent ATPase at pH 6–7, thus obscuring the two peaks shown in Fig. 1 of the present study. A requirement for divalent cations at pH 7–9 and not at pH 5, greater capacity to hydrolyze α,β-methylene ATP at pH 5 than at pH 7–9, and loss of ATPase activity at pH 7–9 after solubilization with detergents [10] suggest that at least two different enzymes with different pH optima are present. Evidence presented here shows that lysosomal membranes do indeed contain at least three ATPase activities, two active at pH 5 and one at pH 8.

Schneider [9] reported that rat liver lysosome membrane pH 8 ATPase activity is stimulated by either Ca^{2+} or Mg^{2+} and that the effects of these cations are not additive, suggesting only one enzyme. The results presented in the present studies and illustrated in Fig. 4 showed that the Ca^{2+}- and Mg^{2+}-ATPase activities migrated as one peak

in polyacrylamide gels. In our original studies on the stimulatory effects of ATP on intralysosomal proteolysis at alkaline pH, which were interpreted in terms of a proton pump [7], we reported that Mg^{2+} or Mn^{2+} were required and that Ca^{2+} inhibited the effect. We later reported that Ca^{2+} and Mg^{2+} were equally effective [14]. The former results are consistent with the observations of Ohkuma et al. [8] using a more clearly defined system in which these workers reported an inhibition of lysosomal proton pump activity by Ca^{2+}. The single Mg^{2+}-Ca^{2+} pH 8 ATPase peak shown in Fig. 4 in the present studies therefore must represent two different proteins. Another possibility is that divalent cations exert a variable, perhaps regulatory, function on lysosomal proton pump activity.

Tritosome membrane ATPase activity at pH 8 was partially inactivated immediately upon solubilization by detergents and a further loss of both pH 8 and pH 5 activities occurred during polyacrylamide gel electrophoresis. Loss of pH 8 ATPase activity was generally more extensive than loss of activity at pH 5. Intact membrane (non-solubilized) pH 8 ATPase activity was usually somewhat greater than the activity at pH 5 (Fig. 1). After solubilization and extensive electrophoresis, the pH 8 activity was greatly reduced with respect to the pH 5 ATPase (Fig. 3). Pontremoli et al. [23] have reported the presence of two proteinases in lysosome membranes. It is possible that the inactivation of ATPase activities observed in the present studies was due to these proteinases. Indeed, Schneider has described a procedure for reducing proteolysis of intact lysosome membranes during purification [2]. However, we do not observe a reduction of ATPase activities in lysosomal membranes even after prolonged storage in the frozen state or after 10 min incubation at 37 °C (unpublished data). Activity loss is apparent only immediately after solubilization with detergents.

Acknowledgement

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