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Metal Ion-Activated Acid Adenosine Triphosphatase from Chicken Liver Lysosomes—Purification and Enzyme Properties

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A metal ion-activated acid adenosine triphosphatase (ATPase) of chicken liver lysosomes was purified from the 100000 × *g* supernatant of lysosomal extract, by fractionation with $(NH_4)_2SO_4$, column chromatographies on Phenyl-Sepharose CL-4B, Sephacryl S-300 and Affi-Gel 501, isoelectric focusing, and gel filtration on Sepharose 6B, with a recovery of 0.38% and a 19-fold increase in specific activity.

Although the purified enzyme had a molecular weight ranging from 700000 to 800000 according to Sephacryl S-300 gel filtration, the enzyme was dissociated into several subunits having molecular weights ranging from 26000 to 93000 on sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis. By isoelectric focusing, the pI of the enzyme was found to be 4.12. The percentage activity ratios of acid phosphatase and phosphodiesterase to ATPase in the enzyme preparations became lower with the progress of purification, those in the finally purified preparation being much lower than reported previously.[2]

The enzyme preparation mainly hydrolyzed nucleoside triphosphates, but also acted weakly on adenosine diphosphate (ADP), adenosine monophosphate (AMP), *p*-nitrophenylphosphate or bis(*p*-nitophenyl) phosphate. In terms of K_m values, adenosine triphosphate (ATP) was the most accessible to the enzyme of all the nucleoside triphosphates tested. At 0.1 mM, nucleoside triphosphates were hydrolyzed in the absence of metal ions in the following order: ATP > GTP > CTP, UTP > dTTP. In the presence of metal ions, the order of nucleotide preference was only slightly affected.

The ATPase activity was markedly inhibited by $HgCl_2$, but other reagents including several SH-blockers and ATPase inhibitors were without effect.

Keywords——metal ion-activated acid ATPase; chicken liver lysosome; enzyme purification; enzyme property

Lysosomes contain many phosphohydrolases such as acid phosphatase, acid adenosine triphosphatase (ATPase) and phosphodiesterase. In the foregoing studies, we first found an acid ATPase having phosphodiesterase activity in chicken liver lysosomes,[3] then carried out partial purification and characterization of the enzyme.[4] Although this enzyme proved to be associated with the lysosomal membrane, its exact role was not established. Furthermore, we discovered a new acid ATPase which was activated by divalent metal ions such as Zn^{2+}, Mg^{2+} and Ca^{2+}.[2] This enzyme must be of significance in relation to the lysosomal Mg^{2+}-adenosine triphosphate (ATP)-driven H^+-pump, which may be involved in the maintenance of acidic pH at the interior of lysosomes.[5]

In the present study, we improved the procedures of enzyme purification and characterized this ATPase.

Experimental

Materials——The substrates such as nucleoside mono-, di-, and triphosphates were purchased from Boehringer Mannheim and bis(*p*-nitrophenyl) phosphate was from Seikagaku Kogyo Co., Ltd. These substrates and other

chemicals were of analytical grade, if available.

Enzyme Assays——Acid ATPase activity was determined according to the method described by Nakabayashi and Ikezawa[4] with a slight modification. In a final volume of 1 ml, the standard reaction mixture contained 0.1 M acetate buffer (pH 5.4), 1 mM ATP, 1 mM $ZnCl_2$ and enzyme solution. The enzyme reaction was terminated by the addition of 10% perchloric acid, then the reaction mixture was analyzed for inorganic phosphate by the method of Eibl and Lands[6] as modified by Nakabayashi and Ikezawa.[7]

Phosphodiesterase activity was measured according to the method of Brightwell and Tappel[8] except that the pH of the assay medium was 5.0 (0.1 M acetate buffer).

Acid phosphatase activity was measured with *p*-nitrophenylphosphate as a substrate according to the method of Brightwell and Tappel[8] with a slight modification. The pH of the assay medium was 5.4 (0.1 M acetate buffer).

Isoelectric Focusing——The enzyme solution was fractionated by isoelectric focusing with 0.5% Pharmalyte (pH 2.5—5) in a sucrose gradient (0—50%), using a 110 ml column (LKB-Produkter, Stockholm, Sweden) at 4 °C for 24 h at 900 V, according to the method of Vesterberg and Svensson.[9] Fractions of 2 ml were collected and subjected to measurement of pH. Then each fraction was diluted with 0.5 ml of 1 M Tris–HCl buffer containing 0.5 mM $ZnCl_2$ (pH 7.5) and assayed for enzyme activities and protein content.

Assay of Protein——Protein was determined by the measurement of A_{280} or by the method of Lowry *et al.*[10] using bovine serum albumin as a standard. In the case of electrofocused samples, protein content was measured by the method of Bensadoun and Weinstein.[11]

Sodium Dodecyl Sulfate (SDS)–Polyacrylamide Gel Electrophoresis——SDS–gel electrophoresis was performed by the method of Laemmli[12] in the medium containing 0.1% SDS on 12% polyacrylamide slab gels. Electrophoresis was carried out at 45 V for 18 h. Gels were removed and silver-stained.[13] The molecular weight of ATPase was estimated from a calibration curve of the relative mobilities of standard proteins plotted against the logarithm of enzyme molecular weight. The standard proteins used were phosphorylase B (M.W. 92500), bovine serum albumin (66200), ovalbumin (45000), carbonic anhydrase (31000), soybean trypsin inhibitor (25000), and lysozyme (14400).

Results

Purification of Metal Ion-Activated Acid ATPase from Chicken Liver Lysosomes

Preparation of Lysosomal Extract——Male Arbor Acres-New Fuji chickens (10 weeks old), weighing 2.5—2.6 kg, were killed by decapitation. The livers were excised and minced. The resulting paste was suspended in ice-cold 0.25 M sucrose–10 mM Tris–HCl buffer (pH 7.5) and homogenized in a Waring blender for 1 min. Lysosomal fractions were then prepared by the method of de Duve *et al.*[14] All procedures were carried out at 4 °C. Then, the lysosomal pellet was suspended in 10 mM Tris–HCl buffer (pH 7.5) containing 30 mM 1-*O*-*n*-octyl-β-D-glucopyranoside (octylglucoside). The suspensions were subjected to freezing (−20 °C) and thawing (4 °C) 3 times, and then centrifuged at $100000 \times g$ for 60 min. The supernatant obtained was used as the starting extract.

$(NH_4)_2SO_4$ Fractionation——To the $100000 \times g$ supernatant, 20% saturated solid ammonium sulfate was added, and the resulting precipitate was removed by centrifugation at $27000 \times g$ for 15 min.

Phenyl-Sepharose CL-4B Column Chromatography——After centrifugation at $27000 \times g$ for 15 min, the resulting supernatant was applied to a column of Phenyl-Sepharose CL-4B (5 × 24 cm). The elution of the column was carried out according to the method of Nakabayashi *et al.*[2] In the first elution using double linear gradients of 20—0% saturated $(NH_4)_2SO_4$ and 0—20% ethylene glycol in 10 mM Tris–HCl buffer (pH 7.5), acid ATPase activity without metal-ion activation was obtained (the peak corresponding to fraction A in the foregoing study).[2] The column was washed with 20% ethylene glycol in 10 mM Tris–HCl buffer (pH 7.5), then with a linear Triton X-100 gradient from 0 to 0.2% in 10 mM Tris–HCl buffer (pH 7.5) containing 0.1 mM $ZnCl_2$ as a stabilizing agent. Finally, metal ion-activated acid ATPase was obtained as a single peak by elution with 0.2% Triton X-100 in 10 mM Tris–HCl buffer (pH 7.5) containing 0.1 mM $ZnCl_2$. Acid phosphatase and phosphodiesterase activities were still contained in this fraction. The active fraction corresponding to fraction D in the foregoing study,[2] was pooled and concentrated by the use of a PT 10000 NMWL filter (Millipore).

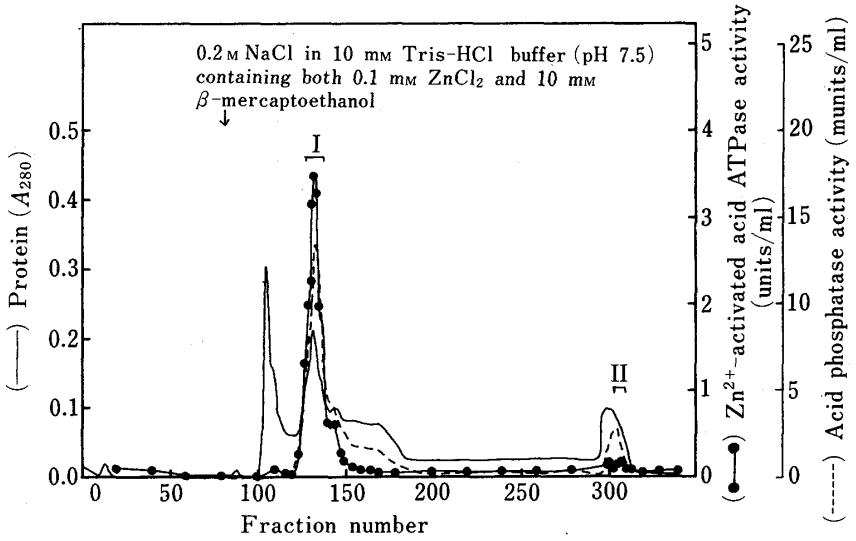

Fig. 1. Column Chromatography on Affi-Gel 501

The enzyme solution was applied to an Affi-Gel 501 column (4 × 18.5 cm) equilibrated with 0.2 M NaCl in 10 mM Tris–HCl buffer (pH 7.5) containing 0.1 mM $ZnCl_2$. In the elution, 15 ml fractions were collected. Other eluting conditions are described in the text. ——, absorbance at 280 nm (due to protein); ----, acid phosphatase activity; ●—●, Zn^{2+}-activated ATPase activity.

Sephacryl S-300 Gel Filtration——After addition of 0.5 M NaCl, the concentrated enzyme solution was applied to a Sephacryl S-300 column (2.6 × 100 cm) equilibrated with 0.5 M NaCl in 10 mM Tris–HCl buffer (pH 7.5) containing 0.1 mM $ZnCl_2$ as a stabilizing agent. The active fractions eluted were pooled and concentrated by ultrafiltration with a Centriflo CF-25 filter (Amicon).

Affinity Chromatography with Affi-Gel 501——The concentrated, active fractions were applied to an Affi-Gel 501 column (Bio-Rad, Organomercurial Agarose, 4 × 18.5 cm) equilibrated with 0.2 M NaCl in 10 mM Tris–HCl buffer (pH 7.5) containing 0.1 mM $ZnCl_2$ (Fig. 1). First, the proteins which were not adsorbed on this affinity gel were eluted with the same buffer as the breakthrough fraction. Then the column was eluted with the 0.2 M NaCl in 10 mM Tris–HCl buffer (pH 7.5) containing both 0.1 mM $ZnCl_2$ and 10 mM β-mercaptoethanol. Two activity peaks of metal ion-activated acid ATPase were obtained (fractions I and II). Fraction I, the major fraction of ATPase, contained a very minor quantity of acid phosphatase activity corresponding to 0.76% of that of Zn^{2+}-activated ATPase. This fraction was pooled and concentrated by ultrafiltration with a Centriflo CF-25 filter (Amicon).

Isoelectric Focusing——The concentrated enzyme solution was dialyzed against distilled water for 1 h. The dialyzed enzyme solution was subjected to isoelectric focusing in the pH range of 2.5 to 5, as described in Experimental. Metal ion-activated acid ATPase, acid phosphatase and phosphodiesterase appeared in the same fraction as a single peak with a pI value of 4.12.

Sepharose 6B Gel Filtration——For the removal of Pharmalyte, the active fractions after isoelectric focusing were applied to a Sepharose 6B column (2.6 × 100 cm) equilibrated with 0.5 M NaCl in 10 mM Tris–HCl buffer (pH 7.5) containing 0.1 mM $ZnCl_2$. The active fractions eluted were pooled and concentrated by ultrafiltration with a Centriflo CF-25 filter (Amicon). After concentration, the enzyme solution was used for further experiments as the partially purified preparation.

The results of the purification procedure are summarized in Table I. The specific activity of the Zn^{2+}-activated ATPase preparation was 30.3 units/mg. Although not more than 19-fold purification over the original 100000 × *g* supernatant had been accomplished (Table

TABLE I. Summary of Purification of Zn^{2+}-Activated Acid ATPase from Chicken Liver Lysosomes

(A)

Fraction	Total protein (mg)	Zn^{2+}-ATPase		
		Total activity (units)	Specific activity (units/mg)	Recovery (%)
100000 × *g* Supernatant	4437	7180	1.62	100
20% $(NH_4)_2SO_4$ Supernatant	3496	6267	1.79	87.3
Phenyl-Sepharose CL-4B	295	4687	15.9	65.3 (100)[a]
Sephacryl S-300	66.8	1960	29.3	27.3 (41.8)
Affi-Gel 501	18.4	555	30.2	7.7 (11.8)
Isoelectric focusing	4.89	148	30.3	2.1 (3.2)
Sepharose 6B	0.902	27.3	30.3	0.38 (0.6)

a) The numbers in parentheses represent the percentage recovery when the total activity of the metal ion-activated acid ATPase fraction from Phenyl-Sepharose CL-4B column chromatography is taken as 100.

(B)

Zn^{2+}-ATPase	ATPase	Acid phosphatase	Phosphodiesterase
1.62 (100)[a]	0.311 (19.1)[a]	0.140 (8.6)[a]	0.007 (0.40)[a]
1.79 (100)	0.322 (18.0)	0.140 (7.8)	0.006 (0.30)
15.9 (100)	2.01 (12.7)	0.300 (1.9)	0.014 (0.09)
29.3 (100)	3.07 (10.5)	0.400 (1.4)	0.011 (0.038)
30.2 (100)	2.74 (9.1)	0.210 (0.76)	0.005 (0.017)
30.3 (100)	2.70 (8.9)	0.199 (0.66)	0.005 (0.017)
30.3 (100)	2.70 (8.9)	0.111 (0.37)	0.004 (0.013)

a) The numbers in parentheses represent the percentage specific activities when the activity against ATP in the presence of 1 mM $ZnCl_2$ is taken as 100.

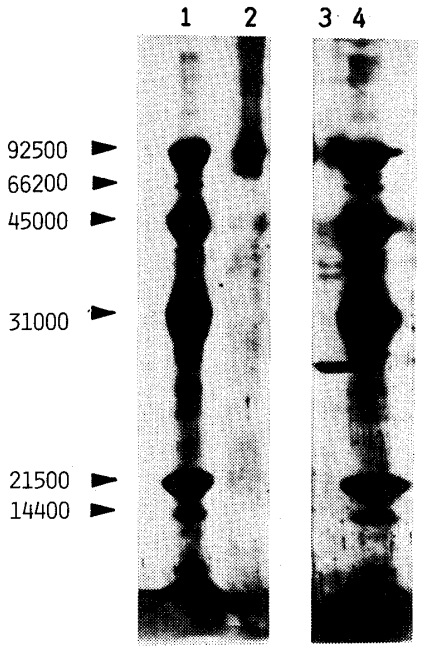

Fig. 2. SDS–Gel Electrophoresis of the Metal Ion-Activated Acid ATPase

Lane 2: The sample applied was 15 μg of ATPase (M.W.: 700000—800000), which was obtained as the eluate from Sepharose 6B column chromatography in the final step of purification. This sample was purified according to the foregoing method,[2] in which Affi-Gel 501 chromatography was not included. Lane 3: 16 μg of the ATPase purified by the present improved method, in which Affi-Gel 501 was used for the enzyme purification.

Lanes 1, 4: Marker proteins; phosphorylase B (92500), bovine serum albumin (66200), ovalbumin (45000), carbonic anhydrase (31000), soybean trypsin inhibitor (25000), and lysozyme (14400).

I(A)), the percentage activity ratios of acid phosphatase and phosphodiesterase to Zn^{2+}-ATPase in the enzyme preparations became lower with the progress of purification (Table I(B)). Although the specific activity of ATPase was not increased in the steps of Affi-Gel 501, isoelectric focusing and Sepharose 6B, the relative activities of these phosphohydrolases to ATPase became much lower than those reported previously.[2)]

SDS-Polyacrylamide Gel Electrophoresis

Figure 2 shows the results of SDS–polyacrylamide gel electrophoresis of the purified metal ion-activated acid ATPase. As compared with the ATPase purified by the previous method[2)] lacking the step of Affi-Gel 501 chromatography, higher purity of the enzyme was obtained by the present method of purification, in that several contaminating proteins were removed. In the foregoing study, the molecular weight of this ATPase was estimated to be 640000 daltons (Da) by gel filtration on Sephacryl S-300. In the present study, we estimated the molecular weight to be 700000—800000 by the same method. Therefore, this enzyme seems to be composed of several subunits having molecular weights ranging from 26000 to 93000 Da, judging from SDS–polyacrylamide gel electrophoresis (Fig. 2).

Substrate Specificity

Enzyme activities towards substrates such as ATP, adenosine diphosphate (ADP), adenosine monophosphate (AMP), *p*-nitrophenylphosphate and bis(*p*-nitrophenyl) phosphate are shown in Table II. The relative activities (%) of the hydrolysis of ADP, AMP, *p*-nitrophenylphosphate and bis(*p*-nitrophenyl) phosphate to that of ATP became lower than those reported previously.[2)] Thus, apyrase activity is not likely to exist in this enzyme preparation.

Effects of Divalent Metal Ions on the Hydrolysis of Nucleoside Triphosphates at 1 mM

The results of enzymatic hydrolysis of 1 mM nucleoside triphosphates in the presence or absence of 1 mM divalent metal ions are shown in Table III. The nucleoside triphosphates were hydrolyzed by the enzyme in the absence of metal ions in the following order: UTP > CTP > ATP, GTP > dTTP. However, such differences in the preference for substrates were almost lost in the presence of divalent metal ions such as Zn^{2+}, Mg^{2+}, Ca^{2+} and Mn^{2+}. As shown previously with ATP as a sole substrate,[2)] the stimulation of enzyme activity by Mg^{2+} was almost the same as that by Zn^{2+}. However, Mg^{2+} ion stimulated the hydrolysis of other nucleoside triphosphates more markedly than Zn^{2+} ion did.

Kinetic Parameters

The values of K_m and V_{max} for nucleoside triphosphates in the presence and the absence of divalent metal ions were calculated by means of Lineweaver–Burk plots.[15)] As shown in Table IV, there was no significant difference in K_m values between ATP and GTP in the absence of metal ions. The K_m values for CTP and UTP were ten times greater than that for ATP, whereas K_m for dTTP was intermediate between that for ATP or GTP and that for CTP

TABLE II. Substrate Specificity at pH 5.4

Substrate	Final concentration (mM)	Relative activity (%)
ATP	1	100
ADP	1	8.1
AMP	1	0.4
PNPP[a)]	2.5	2.9
BIS[a, b)]	2.5	0.14

a) PNPP, *p*-nitrophenylphosphate; BIS, bis(*p*-nitrophenyl) phosphate. *b*) Acetate buffer (pH 5.0).

TABLE III. Effects of Divalent Metal Ions on the Hydrolysis of Nucleoside Triphosphates at 1 mM

1 mM substrate	Specific activity (μmol/min·mg)				
	None	Zn^{2+}	Mg^{2+}	Ca^{2+}	Mn^{2+}
ATP	0.413 (100)[a]	3.69 (100)[a]	3.71 (100)[a]	1.69 (100)[a]	1.36 (100)[a]
GTP	0.419 (101)	3.20 (87)	3.81 (103)	1.94 (115)	1.11 (82)
CTP	0.749 (181)	3.77 (102)	4.52 (122)	2.04 (121)	1.73 (127)
UTP	0.936 (227)	3.71 (101)	4.52 (122)	2.32 (137)	2.36 (174)
dTTP	0.274 (66)	2.20 (60)	3.31 (89)	1.79 (106)	1.73 (127)

a) The numbers in parentheses represent the percentage specific activities when the activity towards ATP is taken as 100.

TABLE IV. Kinetic Parameters for Nucleoside Triphosphates in the Presence and the Absence of Divalent Metal Ions

Substrate	1 mM metal ion	K_m (mM)	V_{max} (μmol/min·mg)	V_{max}/K_m (μmol/min·mg·mM)
ATP	(−)	0.130	4.70	34.6
GTP	(−)	0.223	3.56	16.0
CTP	(−)	1.24	8.92	7.19
UTP	(−)	1.47	15.0	10.2
dTTP	(−)	0.611	3.68	6.02
ATP	Zn^{2+}	0.0769	29.6	384
ATP	Mg^{2+}	0.0725	25.8	353
ATP	Ca^{2+}	0.0439	14.2	323
ATP	Mn^{2+}	0.0489	16.5	337

or UTP. On the other hand, it should be noted that the values of V_{max} for CTP and UTP were greater than those for ATP and GTP. Thus, the ease of access of nucleoside triphosphates to the enzyme active site must be in the order ATP > GTP > UTP > CTP, according to the magnitude of V_{max}/K_m. Therefore, ATP is the best substrate among the nucleoside triphosphates tested.

The K_m values for ATP in the presence of metal ions became generally lower than that in the absence of metal ions. The K_m for ATP in the presence of Zn^{2+} was almost the same as that in the case of Mg^{2+}, and a similar relationship held between the K_m values in the presence of Ca^{2+} and Mn^{2+}. Judging from the values of V_{max}/K_m, the ease of access of ATP to the enzyme active site was almost the same in the presence of all the metal ions examined.

Effects of Divalent Metal Ions on the Hydrolysis of Nucleoside Triphosphates at 0.1 mM

The results of enzymatic hydrolysis of 0.1 mM nucleoside triphosphates in the presence or absence of 1 mM divalent metal ions are shown in Table V. The nucleoside triphosphates were hydrolyzed by the enzyme in the absence of metal ions in the following order: ATP > GTP > CTP, UTP > dTTP. Also, such differences in the preference of substrates were fairly well retained in the presence of divalent metal ions such as Zn^{2+}, Mg^{2+}, Ca^{2+} and Mn^{2+}. With ATP as a substrate, the stimulation of enzyme activity by Zn^{2+} was almost the

Table V. Effects of Divalent Metal Ions on the Hydrolysis of Nucleoside Triphosphates at 0.1 mM

0.1 mM substrate	Specific activity (μmol/min·mg)				
	None	Zn^{2+}	Mg^{2+}	Ca^{2+}	Mn^{2+}
ATP	0.210 (100)[a]	2.08 (100)[a]	2.02 (100)[a]	1.56 (100)[a]	1.59 (100)[a]
GTP	0.125 (60)	1.35 (65)	1.34 (66)	1.29 (83)	1.30 (82)
CTP	0.0825 (39)	1.10 (53)	1.07 (53)	1.07 (69)	0.903 (67)
UTP	0.0846 (40)	1.16 (56)	1.15 (57)	1.08 (69)	1.03 (65)
dTTP	0.0664 (32)	1.02 (52)	1.08 (53)	1.08 (69)	1.28 (81)

a) The numbers in parentheses represent the percentage specific activities when the activity towards ATP is taken as 100.

Table VI. Effects of Various Compounds on ATPase Activity

Compound	Final concentration (mM)	Relative activity (%)
None		100
$HgCl_2$	0.2	0
NEM[a]	4	113
Iodoacetamide	20	102
PCMB[a]	0.1	97
Oligomycin	1[b]	102
Tri-*n*-butyltin	0.1	113
NBD-Cl[a]	0.1	100
NaN_3	16	105
DCCD[a]	0.1	94
Ouabain	0.02	99
Quercetin	0.1	92

a) NEM, *N*-ethylmaleimide; PCMB, *p*-chloromercuribenzoic acid; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; DCCD, *N*,*N*′-dicyclohexylcarbodiimide. *b*) μg/ml.

same as that by Mg^{2+}, being 30% higher than that by Ca^{2+} or Mn^{2+}. With other nucleoside triphosphates as substrates, however, the stimulation of enzyme activity by Zn^{2+} was almost the same as that by other metal ions such as Mg^{2+}, Ca^{2+} and Mn^{2+}

Effects of Various Compounds on the Enzyme Activity

Various reagents were examined for their effects on the activity of ATPase, as shown in Table VI. The enzyme activity was markedly inhibited by $HgCl_2$, while other reagents including several SH-blockers and so-called ATPase inhibitors were without effect.

Discussion

Many workers have reported several properties and functions of ATPase in lysosomes.[5,16-20] In chicken liver lysosomes, we found a unique ATPase activated by divalent metal ions, in the foregoing study.[2] In the present study, we tried to decrease the contents of

other phosphohydrolases such as acid phosphodiesterase and acid phosphatase in the preparation of the purified ATPase, since these phosphohydrolases are usually major contaminating enzymes. As a result, the relative activities of these enzymes became very much lower than those reported previously,[2] although the specific activity of the metal ion-activated ATPase was not increased in the purification steps from Sephacryl S-300 column chromatography to gel filtration on Sepharose 6B. The reason for this may be that contaminating proteins acting as specific or non-specific activators of ATPase were removed.

Furthermore, the inclusion of Affi-Gel 501 column chromatography in the purification procedures significantly improved the purity of this ATPase, judging from SDS–gel electrophoresis (Fig. 2). The result of electrophoresis also suggests the presence of several distinct subunits having molecular weights ranging from 26000 to 93000 Da, since the ATPase activity appeared as a single protein peak having the molecular weight of 700000—800000 Da in the gel filtration on Sephacryl S-300.

The isoelectric point of this purified ATPase was estimated to be 4.12, being lower than that (4.8) reported previously.[2] This discrepancy might have resulted from the improvement of the purification procedures; for example, ATPase was solubilized by octylglucoside instead of Triton X-100 and acetone fractionation of the enzyme was omitted. The enzyme preparation obtained by the modified purification procedures seemed to contain some acidic, nonprotein components such as sialic acid and acidic phospholipid(s).

A number of compounds known as effectors for ATPase were examined for their effects on the activity of acid ATPase. Mitochondrial ATPase inhibitors such as oligomycin, DCCD, NBD-Cl and NaN_3 as well as ouabain (an inhibitor of plasma membrane Na^+, K^+-ATPase) were without effect. Therefore, this enzyme is considered not to be contaminated with mitochondrial or plasma membrane components. Tri-*n*-butyltin and quercetin were also without effect. Only Hg^{2+} was inhibitory for the ATPase activity of chicken liver lysosomes. Therefore, it is likely that the active site of the enzyme contains SH group(s). However, SH reagents such as *N*-ethylmaleimide (NEM), iodoacetamide and *p*-chloromercuribenzoic acid (PCMB) were without effect. Since this enzyme seemed to be associated with some nonprotein components such as phospholipid(s), these SH reagents might be unable to reach the active site of the enzyme due to some conformational restriction.

There were some interesting observations on substrate specificity and the effect of metal ions. At the substrate concentration of 1 mM, pyrimidine nucleoside triphosphates, UTP and CTP, were hydrolyzed more extensively than purine nucleoside triphosphates, ATP and GTP, in the absence of metal ions. On the other hand, the difference in the preferences for nucleoside triphosphates was significantly reduced in the presence of metal ions. Actually, both V_{max} and K_m for CTP and UTP were greater than those for ATP and GTP. Based on the values of K_m, 1 mM ATP or GTP was enough to saturate the enzyme, whereas 1 mM CTP or UTP was not enough, as shown in Table IV. Therefore, this difference in the preferences for nucleoside triphosphates between the absence and presence of metal ions seemed to be partly due to a difference in the stimulation of enzyme activity by metal ions between substrates at high saturation, such as purine nucleoside triphosphates, and substrates at low saturation, such as pyrimidine nucleoside triphosphates. Thus in order to find the physiological substrate(s) for the enzyme, we tried to examine the enzyme activity toward 0.1 mM nucleoside triphosphates. At this concentration, ATP was the best substrate of all the nucleoside triphosphates tested, both in the presence and absence of metal ions. Consequently, ATP seems to be the most preferred nucleotide for the enzyme under physiological conditions, where the concentrations of nucleoside triphosphates are *ca.* 0.1 mM. We also examined the effect of divalent metal ions, in order to look for physiologically required metal ions. With 1 mM ATP as a substrate, the stimulation of enzyme activity by Mg^{2+} was almost the same as that by Zn^{2+}, being much higher than that by Ca^{2+} or Mn^{2+}. This was also the case with

0.1 mM ATP. Therefore, Mg^{2+} or Zn^{2+} may be required, if this ATPase plays some role in membrane function. Further studies on the physiological functions of this ATPase are in progress.

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References and Notes

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