Characterization of Cationic Acid Phosphatase Isozyme from Rat Liver Mitochondria

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Acid phosphatase isozyme was highly purified from rat liver mitochondrial fraction. The enzyme showed an isoelectric point value of above 9.5 on isoelectric focusing, and the apparent molecular weight was estimated to be 32000 by Sephadex G-100 gel filtration or 16000 by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The enzyme catalyzed the hydrolysis of adenosine 5′-triphosphate, adenosine 5′-diphosphate, thiamine pyrophosphate, inorganic pyrophosphate, and phosphoprotein such as casein and phosvitin, but not of several phosphomonoesters, except for *p*-nitrophenyl phosphate and *o*-phosphotyrosine. The enzyme was not inhibited by L-(+)-tartrate, and was significantly activated by Fe^{2+} and reducing agents such as ascorbic acid, L-cysteine, and dithiothreitol. The enzyme was found to be distributed in various rat tissues including liver, spleen, kidney, small intestine, lung, stomach, brain and heart, but not in skeletal muscle.

Keywords acid phosphatase; protein phosphatase; rat liver; mitochondria

Acid phosphatase (APase, EC 3.1.3.2) is widely distributed in nature, and often occurs in multiple forms.[1] Several mammalian tissues have been shown to contain three types of APase, based on difference in molecular size: high-molecular-weight (HMW, molecular weight, $M_r \geqq 100000$) APases,[2] intermediate-molecular-weight (IMW, $M_r \fallingdotseq 40000$) APases,[3] and low-molecular-weight (LMW, $M_r \leqq 20000$) APases.[4] These types are clearly distinguishable from each other in localization of the cells, substrate specificity, and susceptibility to selected inhibitors.

We recently purified an APase isozyme from rat liver mitochondrial fraction and characterized it as IMW APase[3b]; it has a molecular weight of approximately 40000. It catalyzes the hydrolysis of a wide variety of natural phosphomonoesters, except for phosphoproteins, but not of adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP) and inorganic pyrophosphate (PPi). It is not inhibited by tartrate, a potent inhibitor of HMW APase.

During the studies on IMW APase, we found that additional APase isozyme with intermediate molecular weight exists in rat liver mitochondrial fraction, and that the enzyme is clearly different from so-called IMW APase, appearing similar in some properties to APases isolated from bovine,[5] rat[6] and human[7] spleen, rat[8] and human[9] bone, and rat epidermis.[10] Although an APase similar to this one with regard to substrate specificity and molecular weight has been reported from rat liver as mitochondrial protein phosphatase,[11] details of its properties have not been identified.

In the present study, highly purified APase isozyme from rat liver mitochondrial fraction was characterized as to its molecular weight, isoelectric point (pI), substrate specificity, and susceptibility to inhibitors and activators.

Experimental

Materials α-Casein and phosvitin were obtained from Sigma Chemical Co., carboxymenthyl (CM)-cellulose (CM-23) from Whatman Inc., SP-Sephadex C-50, Blue-Sepharose, Mono S column, and ampholyte were from Pharmacia Fine Chemicals. Molecular weight standard proteins used in electrophoresis were from Daiichi Pure Chemicals Co. The sources of other materials were as described previously.[3b]

Animal Male Wistar rats (weighing 200—250 g) which had fasted for 16 h were used in all experiments.

Purification of APase Isozyme (P-II-1) The liver perfused with cold 0.9% NaCl was homogenized with a Teflon-glass homogenizer in 9 volumes of 3 mM Tris–HCl buffer (pH 7.4) containing 0.25 M sucrose. The homogenate was centrifuged at 5000 × *g* (10 min) to obtain the precipitate. After being washed with the sucrose solution, the precipitate was resuspended in 10 mM phosphate buffer (pH 7.0) containing 0.5 M NaCl (buffer A). The suspension was combined with the same volume of 10% Triton X-100 containing 0.1 mM phenylmethylsulfonylfluoride and 20 μg each of antipain, chymostatin, leupeptin and pepstatin in buffer A. The suspension was stirred for 1 h at 4 °C, and then centrifuged at 105000 × *g* (60 min). The supernatant (crude extract) obtained was loaded onto a Sephadex G-75 column (5.6 × 115 cm) equilibrated and eluted with buffer A. Three peaks were obtained, corresponding to HMW, IMW, and LMW APases, which were designated P-I(I′), P-II, and P-III, respectively, in Fig. 1 of our previous paper.[3b] The pooled P-II fraction was dialyzed against 10 mM phosphate buffer (pH 7.0), and then applied to diethylaminoethyl (DEAE)-Sephadex column (4 × 32 cm) equilibrated with the same buffer. After washing of the column with the same buffer, adsorbed enzyme was eluted with a linear 0—0.5 M NaCl gradient at pH 7.0. Two enzyme activity peaks, designated as P-II-1 and 2 in order of their elution, were obtained (Fig. 1). P-II-2 fraction has already been purified and characterized as IMW APase.[3b] In the present experiment, another APase fraction (P-II-1), not adsorbed on DEAE-Sephadex resin at pH 7.0, was subjected to further purification. The pooled P-II-1 fraction was added to 0.5 M NaCl, and then applied to Blue-Sepharose column (2.4 × 21 cm) equilibrated with buffer A. After extensive washing of the column with the same buffer, the enzyme was eluted with 10 mM phosphate buffer (pH 7.0) containing 2 M NaCl. Pooled, active fraction was dialyzed with 10 mM phosphate buffer

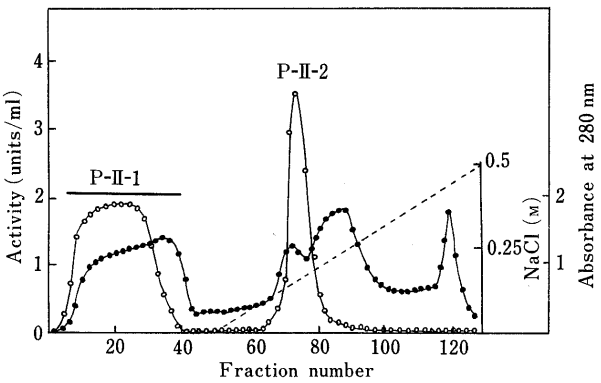

Fig. 1. DEAE-Sephadex A-50 Column Chromatography of P-II Fraction from the Sephadex G-75 Column

The P-II fraction obtained from Sephadex G-75 column was applied to DEAE–Sephadex A-50 column equilibrated with 10 mM phosphate buffer (pH 7.0). Elution was carried out initially with the equilibration buffer, followed by a linear gradient from 0 to 0.3 M NaCl (– – –) in the buffer. Fractions, 10 ml each, were collected and analyzed for absorbance at 280 nm (—●—) and APase activity (—○—). Fractions with bold line at the top of the figure were pooled and subjected to further purification.

(pH 7.0) containing 0.1 M NaCl, and then applied to CM-cellulose column (2.3 × 24 cm) equilibrated with the same buffer. The enzyme was eluted with linear gradient of 0.1—0.6 M NaCl at pH 7.0. The active fractions were pooled, dialyzed against 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 6.8), and applied to Mono S column (0.5 × 5 cm) equilibrated with the same buffer. The enzyme was eluted with 0—0.6 M NaCl gradient (Fig. 2). The active fractions were pooled and dialyzed with 50 mM MES buffer (pH 6.8). The dialyzed solution was applied to the above Mono S column and the enzyme was eluted with a linear gradient of 0—0.5 M NaCl. The enzyme preparation thus obtained was used as P-II-1 in further experiments.

Enzyme Assay The assay mixture contained, in a final volume of 1.0 ml, 100 μmol of sodium acetate buffer (pH 5.5), 2.5 μmol of *p*-nitrophenyl phosphate and the enzyme, unless otherwise noted. After incubation for an appropriate period at 37 °C, the reaction was stopped by the addition of 3 ml of 0.25 M NaOH, and the absorbance was measured at 410 nm. One unit of the enzyme activity was defined as an increase in the absorbance of 1.0 per minute under the above conditions. The enzyme activity toward a number of other phosphorylated compounds except for phosphoproteins was assayed in 1.0 ml of the same reaction mixture containing 2.5 μmol of substrate. The reaction was stopped by adding 3.0 ml of 0.6 N H_2SO_4, and the liberated orthophosphate (Pi) was determined by the method of Chen *et al.*[12] For the unstable substrates, ATP, ADP, thiamine pyrophosphate (TPP) and PPi, 1.0 M acetate buffer (pH 4.0) containing 20 mM $CuSO_4$ was used to stop the reaction, and the liberated Pi was determined by the method of Delsal and Manhouri.[13] The enzyme activity toward phosphoprotein substrates was also assayed in the same reaction mixture as described above, except that phosphorylated substrate was replaced by 0.3% phosphoprotein. The reaction was stopped by the addition of 2.0 ml of 15% trichloroacetic acid, and the liberated Pi was determined in the clear supernatant by the method of Chen *et al.*[12]

Analysis of the P-II-1 Fraction in Various Rat Tissues Tissues perfused with cold 0.9% NaCl were removed, chilled and chopped. The 5000 × *g* precipitate from each tissue and the 105000 × *g* supernatant from the precipitate were prepared by the methods described in the above purification section of P-II-1. Each 105000 × *g* supernatant was applied onto Sephadex G-100 column equilibrated with buffer A. Active fractions corresponding to P-II were pooled, dialyzed against 10 mM phosphate buffer (pH 8.0), and then applied to SP-Sephadex A-50 column equilibrated with the same buffer. After extensive washing of the column with the same buffer, the enzyme corresponding to P-II-1 was eluted with 10 mM phosphate buffer (pH 8.0) containing 0.5 M NaCl. Under the above conditions, HMW and LMW APases were not adsorbed on the SP-Sephadex column.

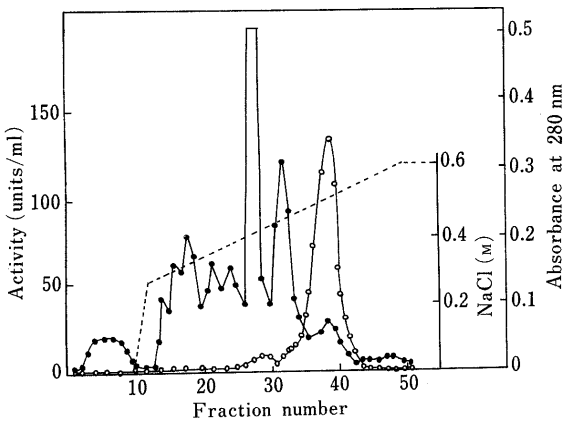

Fig. 2. Mono S Column Chromatography of P-II-1 Fraction from CM-Cellulose Column

P-II-1 fraction obtained from CM-cellulose column was applied to Mono S column equilibrated with 50 mM MES buffer (pH 6.8). Elution was carried out initially with a linear gradient from 0 to 0.3 M NaCl, followed with that from 0.3 to 0.6 M NaCl (---). Fractions, 1 ml each, were collected and analyzed from absorbance at 280 nm (——) and APase activity (—○—).

Molecular Weight Determination Molecular weight data was obtained by gel permeation chromatography,[14] using a Sephadex G-100 column (2 × 67 cm) equilibrated with 10 mM phosphate buffer (pH 6.5) containing 2 M NaCl and bovine serum albumin, ovalbumin, chymotrypsinogen, and horse heart cytochrome c as standard proteins, and also by sodium dodecyl sulfate–polyacrylamide disc gel electrophoresis (SDS-PAGE)[15] in 10—20% acrylamide gel.

Isoelectric Focusing The P-II-1 fraction was applied to isoelectric focusing[16] in 0.8% ampholyte with a gradient of sucrose (0—48%) in the pH range 3.5—10 using 110 ml-volume column. Electrophoresis was performed at 800 V for 48 h at 4 °C.

Results

Purification and Purity of P-II-1 As outlined in Table I, P-II-1 was purified about 19000-fold with 1.5% recovery from the crude extract obtained from the 5000 × *g* precipitate of rat liver homogenate. The first Mono S column chromatography gave a peak of acid phosphatase activity coinciding with that of the protein (Fig. 2), and in the second Mono S column chromatography, the peak of acid phosphatase activity as well as protein concentration was symmetrical (not shown in figure). When the purified P-II-1 preparation was passed through a Sephadex G-100 column equilibrated with 10 mM phosphate buffer (pH 6.5) containing 2 M NaCl, the activity peak of acid phosphatase coinciding with the protein peak was eluted at the position corresponding to the molecular weight of approximately 32000. Specific activity of the active fractions was similar to that of the P-II-1 preparation finally obtained from the 2nd Mono S column. The active fractions were collected, dialyzed against deionized water, and lyophilized. SDS-PAGE of the lyophilized sample gave one major protein band corresponding to an apparent molecular weight of 16000 (Fig. 3). These findings suggest that the finally obtained enzyme preparation is highly purified.

Properties of P-II-1 A. Isoelectric Point To determine the isoelectric point, P-II-1 fraction was applied to isoelectric focusing. Distribution of phosphatase activity obtained after electrophoresis is shown in Fig. 4. Phosphatase activity was found to be located near the bottom of the column to

TABLE I. Purification of APase (P-II-1) from Rat Liver

Step	Total protein[b] (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Recovery (%)
Crude extract[a]	8.64×10^4	4.67×10^4	0.54	1.0	100
Sephadex G-75	5.05×10^3	4.76×10^3	0.94	1.7	10.2
DEAE-Sephadex A-50	1.99×10^3	2.51×10^3	1.3	2.4	5.4
Blue-Sepharose	33.3	2.39×10^3	72	1.33×10^2	5.1
CM-cellulose	1.5	2.22×10^3	1.48×10^3	2.74×10^3	4.8
1st Mono S	0.39	7.84×10^2	2.01×10^3	3.72×10^3	1.7
2nd Mono S	6.8×10^{-2}	7.06×10^2	1.04×10^4	1.93×10^4	1.5

a) Starting from 510 g of rat liver. The enzyme was extracted with Triton X-100 (5%) from 5000 × *g* precipitate of rat liver homogenate. *b*) Protein concentration was determined from the absorbance at 280 nm, assuming that the extinction coefficient, $E_{1\%}^{1cm}$, at 280 nm was 10.0.

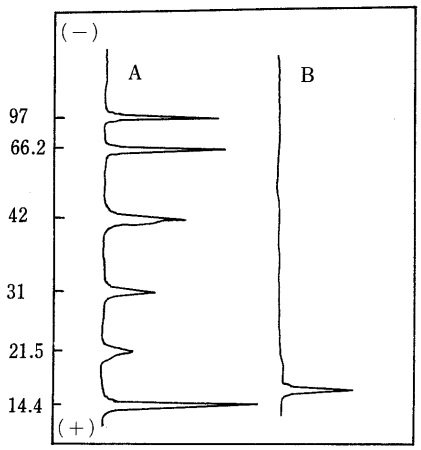

Fig. 3. SDS-PAGE of Purified P-II-1

The enzyme and standard proteins were subjected to SDS-PAGE. The gel stained by silver staining was scanned at 385 nm with a Shimadzu Dual-wavelength flying-spot scanner CS-9000. The scale at left is molecular weight $\times 10^{-3}$. Protein samples: A, molecular weight standards (from top to bottom, phospholylase B, bovine serum albumin, aldolase, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme); B, P-II-1.

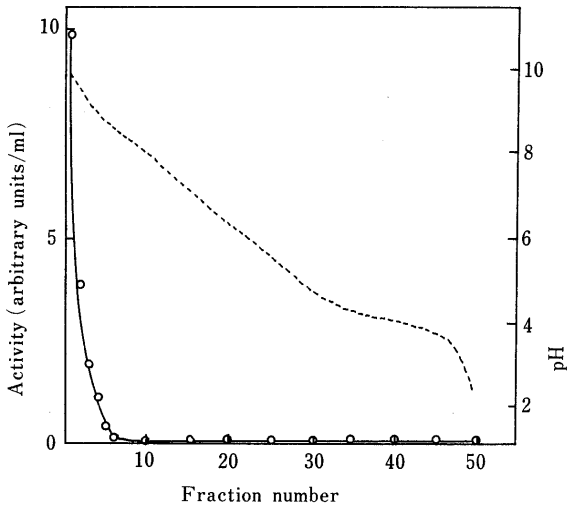

Fig. 4. Determination of the Isoelectric Point of P-II-1

P-II-1 fraction obtained from the final purification step was subjected to column electrophoresis with carrier ampholyte, pH gradient between 3.5 and 10. The fractions, 2 ml each, were collected after isoelectrofocussing for 48 h, and analyzed for APase activity (—○—) and pH (– – –).

which the cathodic electrode was attached. The results showed that the pI of P-II-1 might be above 9.5.

B. Molecular Weight The molecular weight of P-II-1 was estimated to be approximately 32000 by gel filtration (data not shown) and approximately 16000 by SDS-PAGE (Fig.3). These results suggest that P-II-1 consists of two identical subunits with an apparent molecular weight of 16000.

C. Substrate Specificity The relative hydrolytic rates on the different phosphate esters are shown in Table II. P-II-1 efficiently catalyzed the hydrolysis of *p*-nitrophenyl phosphate and *o*-phosphotyrosine among the phosphomonoesters tested, and of ATP, ADP, TPP, and PPi. The enzyme was also active on phosphoproteins such as casein and phosvitin. The other phosphomonoesters including nucleoside monophosphates, sugar phosphates, *o*-phosphoserine, pyridoxal phosphate, nicotinamide adenine dinucleotide phosphate ($NADP^+$), flavin mononucleotide (FMN), and phosphodiesters including nicotinamide adenine dinucleotide (NAD^+) and diphenylphosphate, were not hydrolyzed to any measurable extent. P-II-1 exhibited the optimum pH at around 5.5 for the substrates including *p*-nitrophenyl phosphate, ATP, and casein.

TABLE II. Substrate Specificity

Substrate	Relative activity (%)[a]
p-Nitrophenyl phosphate	100
o-Phosphotyrosine	50
ATP	85
ADP	70
TPP	76
PPi	40
α-Casein (0.3%)	11
Phosvitin (0.3%)	8

a) The value represents the rate with 2.5 mM substrate relative to that with 2.5 mM *p*-nitrophenyl phosphate, unless otherwise specified. The following compounds were not hydrolyzed by P-II-1 at rates greater than 1% of the rate with *p*-nitrophenyl phosphate: adenosine 2′-monophosphate (2′-AMP), 3′-AMP, 5′-AMP, α-glycerophosphate, β-glycerophosphate, glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-diphosphate, ribose-5-phosphate, thiamine monophosphate, pyridoxal 5′-phosphate, FMN, 3-phosphoglycerate, *o*-phosphoserine, diphenyl phosphate, NAD^+ and $NADP^+$.

TABLE III. Effect of Various Substances on the Activity of P-II-1

Substance added	Conc. (M)	Relative activity (%)[a]
None		100
L-(+)-Tartrate	10^{-2}	100
KF	10^{-3}	36
N-Ethylmaleimide	10^{-2}	92
Iodoacetamide	10^{-2}	97
EDTA	10^{-2}	100
o-Phenanthroline	10^{-3}	91
$FeCl_3$	10^{-3}	3
$FeCl_3$	10^{-4}	17
$FeCl_3$	10^{-5}	71
$FeCl_3$	10^{-6}	95
$FeSO_4$	10^{-3}	43
$FeSO_4$	10^{-4}	123
Ascorbic acid	10^{-2}	150
L-Cysteine	10^{-2}	120
$FeSO_4$[b] + ascorbic acid[c]		213
$FeSO_4$[b] + L-cysteine[c]		192
Dithiothreitol	10^{-2}	200
Dithionite	5×10^{-2}	0
Dithionite	10^{-2}	10
Dithionite	10^{-3}	53
$(NH_4)_6Mo_7O_{24}$	10^{-6}	37
KH_2PO_4	10^{-3}	73
Na_2HAsO_4	10^{-3}	55

a) The activity was determined by incubation of the enzyme at 37 °C in the presence of 2.5 mM *p*-nitrophenyl phosphate and indicated additions in a total volume of 1.0 ml of 0.1 M acetate buffer (pH 5.5). The activity was expressed as percent of the no-addition run. *b*) 10^{-4} M. *c*) 10^{-2} M.

D. Effect of Various Compounds Table III summarizes the effect of various compounds on the activity of P-II-1. The enzyme was not inhibited by high concentration of tartrate (10 mM), though it was significantly inhibited by KF (1 mM), both of which were strong HMW APase inhibitors.[2] It was little affected by sulfhydryl blocking agents such as *N*-ethylmaleimide and iodoacetate, which were strong LMW APase inhibitors.[4] Ascorbic acid, cysteine, and dithiothreitol significantly stimulated the

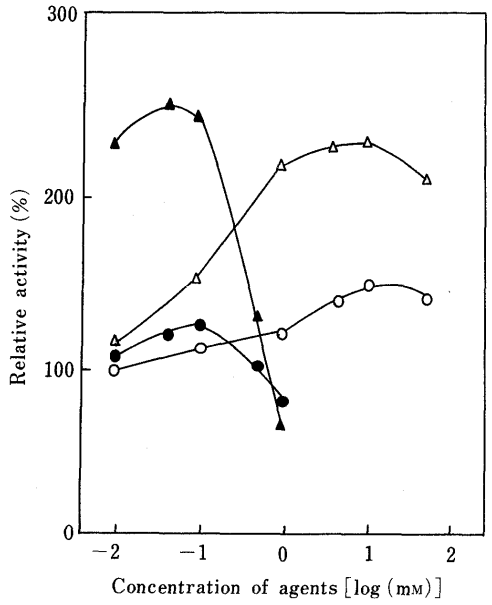

Fig. 5. Dose–Response Curves of the Effect of Ascorbic Acid and Ferrous Ions on the Activity of P-II-1

The activity was determined by incubation of the enzyme at 37 °C in the presence of 2.5 mM *p*-nitrophenyl phosphate (○, ●) or 0.3% casein (△, ▲), as a substrate, and ascorbic acid (△, ○) or Fe^{2+} (▲, ●), as a effector, at the indicated concentrations, in a total volume of 1.0 ml of 0.1 M acetate buffer (pH 5.5). Activity was expressed as percent of the no-addition run.

hydrolysis of *p*-nitrophenyl phosphate by P-II-1; Fe^{2+} also slightly stimulated the enzyme activity, though Fe^{3+} did not. The combined effect of Fe^{2+} and ascorbic acid, or cysteine was also observed, whereas a very strong reducing agent, dithionite, significantly inhibited the activity of the enzyme. *o*-Phenanthroline (1 mM) only minimally inhibited the enzyme, while ethylenediaminetetraacetic acid (EDTA) did not. General phosphatase inhibitors such as Pi, arsenate, and molybdate were found to significantly inhibit the enzyme activity.

P-II-1 was activated by Fe^{2+} and ascorbic acid even with casein as a substrate. Dose response curves for the effect of Fe^{2+} and ascorbic acid on the hydrolysis of the substrates, *p*-nitrophenyl phosphate and casein by P-II-1 are represented in Fig. 5. Although with both these substrates the optimum concentrations of ascorbic acid and Fe^{2+} for stimulation of the enzyme activity were about 10 and 0.1 mM, respectively, a greater stimulating effect was observed with casein than with *p*-nitrophenyl phosphate substrate.

E. Distribution of P-II-1 in Various Tissues of Rat To know the distribution of the reductant-activatable APase like P-II-1 in liver, we analyzed the existence of the enzyme in various rat tissues by the procedure described in Experimental. Under the chromatographic conditions used in isolating the enzyme, P-II-1 fraction could be separated completely from HMW, IMW and LMW APases. None of the P-II-1 fractions thus obtained from various tissues showed hydrolytic activity toward *p*-nitrophenyl phosphate as a substrate in alkaline pH range, indicating the absence of alkaline phosphatase in the enzyme fractions. All of the P-II-1 fractions were also found to be significantly activated by dithiothreitol (10 mM). Table IV lists the contents of P-II-1 fraction in rat various tissues. P-II-1 was found in various tissues including liver, kidney, lung, spleen, stomach, small intestine, and brain, but not in skeletal muscle. The activity of P-II-1 per wet weight of tissue was highest in the spleen among the tissues tested.

TABLE IV. P-II-1 Content in Various Rat Tissues

Tissue	P-II-1 activity[a] (units/g wet weight)
Spleen	23.4
Kidney	10.6
Liver	2.94
Small intestine	1.05
Lung	0.55
Stomach	0.52
Brain	0.23
Heart	0.11
Skeletal muscle	—[b]

Experimental conditions for preparation of P-II-1 fraction from various tissues were as described under Experimental. *a*) The values are expressed as the activity obtained from the SP-Sephadex column per gram wet weight of tissue. *b*) No activity peak corresponding to P-II by Sephadex G-100 gel filtration of the crude extract was detected.

Discussion

In the present study, we highly purified an APase isozyme, P-II-1, from rat liver mitochondrial fraction and characterized it for some properties. It was a cationic glycoprotein[17] (pI, above 9.5) with intermediate molecular weight ($M_r \fallingdotseq 35000$). It catalyzed the hydrolysis of ATP, ADP, TPP, and PPi, but not of several phosphomonoesters except for *p*-nitrophenyl phosphate and *o*-phosphotyrosine. It also showed an activity on phosphoproteins such as phosvitin and casein. It was significantly activated by Fe^{2+} and mild reducing agents such as ascorbic acid, cysteine, and dithiothreitol, whereas largely inhibited by dithionite, a strong reducing agent. Its activity was not inhibited by tartrate.

Another APase, P-II-2, was previously isolated from rat liver mitochondrial fraction,[3b] and has a molecular weight and pI of approximately 35000 and 7, respectively. It catalyzes the hydrolysis of a wide variety of phosphomonoesters, but not of ATP, ADP, PPi, or phosphoproteins. It is not inhibited by tartrate, and not activated by Fe^{2+} or the above reducing agents.[18] Therefore, P-II-1 is clearly different from P-II-2 with respect to substrate specificity, susceptibility to reductant, and pI, though both are tartrate-resistant APases with intermediate molecular weight.

Tartrate-resistant APases with intermediate molecular weight also have been isolated from bovine,[5] rat[6] and human[7] spleen, rat[8] and human[9] bone, and rat epidermis.[10] Some of them have been called Fe^{2+}-activated APase,[10] Type 5 APase,[7] or purple APase.[5,6,8b,c] They have been generally found to have the following common properties: They are cationic glycoproteins with molecular weight between 30000 and 40000. They catalyze the hydrolysis of ATP, ADP, PPi, and certain phosphoproteins. They can be activated several times by Fe^{2+} and reducing agents such as ascorbic acid and cysteine, whereas they are strongly inhibited by dithionite.[6,8c,9] These properties of spleen, bone, and epidermis APases parallel those of P-II-1, though the activation of P-II-1 by Fe^{2+} and reductant is much less than that of the former enzymes.

The tartrate-resistant, reductant-activatable APases can

be extracted from spleen,[6,7] bone,[8] and epidermis[9] by a high concentration of salt or by detergent such as Triton X-100, or by a combination of salt and detergent. This suggests that the enzymes may be bound to a particle fraction(s) of the cells, but not in soluble fraction. We previously demonstrated that APase fraction (P-II) with intermediate molecular weight ($M_r \fallingdotseq 40000$) is extracted efficiently by a solution containing Triton X-100 from particle fraction of liver cells, and that the P-II fraction comes from mitochondria.[3b] The present P-II-1 was isolated from the P-II fraction. These facts suggest that P-II-1 is also bound to particle fraction, probably mitochondria, of the cells.

In conclusion, the present study appears to indicate that at least two types of APases exist in rat liver mitochondrial fraction, tartrate-resistant, reductant-activatable APase (P-II-1) and so-called IMW APase (P-II-2), and that P-II-1 detected in liver may also exist in various tissues of rat including spleen, kidney, lung, small intestine, brain, stomach and heart.

Spleen[5,6] and bone[8b,c] APases are characteristically purple proteins and appear to contain iron. We were unable to confirm whether the P-II-1 was a purple colored protein containing iron or not, as it was difficult to obtain sufficient amounts of purified P-II-1 preparation because of the small amount present in the liver cells.

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18) S. Fujimoto and A. Ohara, unpublished data.