[Phosphotyrosine]protein phosphatase in rat brain

A major [phosphotyrosine]protein phosphatase is a 23 kDa protein distinct from acid phosphatase

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A [phosphotyrosine]protein phosphatase (PTPPase) was purified almost to homogeneity from rat brain, with [³²P]p130^{gag-fps}, an oncogene product of Fujinami sarcoma virus, as substrate. The characteristics of the purified preparation of PTPPase were as follows: (1) the enzyme was a monomer with a molecular mass of 23 kDa; (2) its optimum pH was 5.0–5.5; (3) its activity was not dependent on bivalent cations; (4) its activity was strongly inhibited by sodium vanadate, but was not inhibited by ZnCl₂, L(+)-tartrate or NaF; (5) it catalysed the dephosphorylation of [³²P]p130^{gag-fps}, [³²P]Tyr]casein, *p*-nitrophenyl phosphate and L-phosphotyrosine, but did not hydrolyse [³²P]Ser]tubulin, L-phosphoserine, DL-phosphothreonine, 5'-AMP, 2'-AMP or β -glycerophosphate significantly. During the purification, most of the PTPPase activity was recovered in distinct fractions from those of conventional low-molecular-mass acid phosphatase (APase), which was reported to be a major PTPPase [Chernoff & Li (1985) *Arch. Biochem. Biophys.* **240**, 135–145], from DE-52 DEAE-cellulose column chromatography, and those two enzymes could be completely separated by Sephadex G-75 column chromatography. APase also showed PTPPase activity with [³²P]p130^{gag-fps}, but the specific activity was lower than that of PTPPase with molecular mass of 23 kDa, and it was not sensitive to sodium vanadate. These findings suggested that PTPPase (23 kDa) was the major and specific PTPPase in the cell.

INTRODUCTION

Much attention is now focused on tyrosine-specific protein kinase, since it may play a triggering role in cellular responses to some growth factors and RNA viruses (Hunter & Cooper, 1985). On the other hand, little is known about whether [phosphotyrosine]protein phosphatase (PTPPase) acts as a counter-regulator of these cellular responses. Several types of PTPPase have been identified in a number of animal cells and tissues, for example rat muscle and liver (Foulkes *et al.*, 1981), A431 cells (Swarup *et al.*, 1984), rabbit kidney (Shriner & Brautigan, 1984), chicken brain (Foulkes *et al.*, 1983) and bovine heart (Chernoff & Li, 1983), but they have not hitherto been thoroughly purified, and their characteristics and classification have not been fully elucidated. Some investigators (Leis & Kaplan, 1982; Swarup *et al.*, 1982; Li *et al.*, 1984) reported that acid phosphatase and alkaline phosphatase acted as PTPPase. Chernoff & Li (1985) have demonstrated that a major PTPPase was closely associated with conventional low-molecular-mass acid phosphatase (APase) (Lawrence & Van Etten, 1981; Taga & Van Etten, 1982). However, which enzyme is the most active and specific PTPPase in the cell has remained unknown.

We found that continuous infusion of insulin into rat brain resulted in complete loss of the circadian rhythm of feeding behaviour (Nagai *et al.*, 1982). From this and the suggestion that the tyrosine kinase activity of insulin receptor might have some role in the action of insulin (Kasuga *et al.*, 1983), we speculated that a phosphoryl-

ation–dephosphorylation cycle might be involved in the regulation of the temporal signal of the circadian clock in brain. Brugge *et al.* (1985) reported that post-mitotic neuronal cells expressed high concentrations of the activated forms of pp60^{c-src}, the cellular homologue of the oncogene product of Rous sarcoma virus. This indicated that tyrosine kinase activity might serve some specific neuronal function. Thus PTPPase, as a counter-regulator, may also take part in the regulation of a neuronal function.

From these findings, we have been interested in the regulation mechanism of PTPPase in brain, and, as a first step, we attempted to clarify the problem of which PTPPase is the most active and specific for natural [phosphotyrosine]protein substrates. For identification of a specific PTPPase, we thought that a naturally occurring substrate was most suitable, and so used [³²P]p130^{gag-fps}, an autophosphorylated oncogene product of Fujinami sarcoma virus, as substrate during purification. The present paper deals with the purification and some enzymic properties of a PTPPase from rat brain cytosolic fraction. The major PTPPase is distinct from conventional low-molecular-mass APase.

MATERIALS AND METHODS

Animals

Adult male Wistar strain rats were killed by decapitation. The whole rat brains were removed quickly and stored at –70 °C until use.

Abbreviations used: PTPPase, [phosphotyrosine]protein phosphatase; APase, acid phosphatase; pNPPase, *p*-nitrophenyl-phosphatase; [³²P]Tyr]casein, α -casein phosphorylated at tyrosine residues; [³²P]Ser]tubulin, tubulin phosphorylated at serine residues; pNPP, *p*-nitrophenyl phosphate.

Chemicals

[γ - 32 P]ATP was purchased from Amersham Japan (Tokyo, Japan). α -Casein, L-phosphotyrosine, L-phosphoserine, DL-phosphothreonine, 5'-AMP, 2'-AMP, ATP, dithiothreitol, Mes, phenylmethanesulphonyl fluoride, deoxycholic acid, di-isopropyl phosphorofluoridate, leupeptin and Tris were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). EDTA, EGTA, trichloroacetic acid, sodium orthovanadate, pNPP, SDS, ammonium molybdate, sodium tartrate, 2,5-diphenyloxazole ('DPO') and 1,4-bis-(5-phenyloxazol-2-yl)benzene ('POPOP') were from Wako Chemicals (Osaka, Japan). Triton X-100, toluene, sucrose and $(\text{NH}_4)_2\text{SO}_4$ were from Katayama Chemicals (Osaka, Japan). Bovine serum albumin was from Nakarai Chemicals (Kyoto, Japan). Sephadex G-75 (superfine grade), Sephadex G-25 (medium grade) and Protein A-Sepharose CL-4B were from Pharmacia (Uppsala, Sweden). Bio-Gel HT and molecular-mass marker proteins were from Bio-Rad Laboratories (Richmond, CA, U.S.A.). DE-52 DEAE-cellulose was from Whatman (Maidstone, Kent, U.K.). X-ray film was from Eastman Kodak (Rochester, NY, U.S.A.). Silver staining kit was from Daiichi Chemicals (Tokyo, Japan).

Preparation of [32 P]p130^{gag-fps}

[32 P]p130^{gag-fps} was prepared by the method of Feldman *et al.* (1980) with some modifications. A 500 μ l portion of lysate of Fujinami-sarcoma-virus-transformed 3Y1 cells was incubated with 10 μ l of the antiserum against p19 protein for 60 min on ice. A 40 μ l volume of Protein A-Sepharose CL-4B was added and mixed for 30 min on ice. Then the Sepharose pellet was washed five times with RIPA buffer (50 mM-Tris/HCl buffer, pH 8.2, containing 0.15 M-NaCl, 10 mM-EDTA, 20 mM-NaF, 1% Triton X-100, 1% deoxycholic acid, 0.1% SDS, 0.1 mM-di-isopropyl phosphorofluoridate, 50 μ g of phenylmethanesulphonyl fluoride/ml, 5 μ g of leupeptin/ml and 100 kallikrein-inhibitory units of Trasylol/ml), and once with kinase buffer (20 mM-Tris/HCl buffer, pH 7.4, containing 10 mM-MnCl₂). To the packed pellet of immune complex-Protein A-Sepharose, 45 μ l of kinase buffer and 30 μ Ci of [γ - 32 P]ATP (3000 Ci/mmol) were added. The tube was incubated for 20 min at 30 °C. The reaction was stopped by washing three times with RIPA buffer and once with dialysis buffer (25 mM-Tris/HCl buffer, pH 7.4, containing 0.1 mM-dithiothreitol and 0.1 mg of bovine serum albumin/ml). To the packed pellet, 500 μ l of elution buffer (50 mM-Tris/HCl buffer, pH 7.5, containing 0.1 mM-dithiothreitol, 0.1% Triton X-100, 0.1 mg of bovine serum albumin/ml and 3 M-KSCN) was added, and the tube was shaken vigorously overnight at 4 °C. After centrifugation at 10000 g for 1 min, the supernatant was dialysed overnight at 4 °C. The dialysis residue, immune complex of [32 P]p130^{gag-fps} and IgG, was used as substrate.

Preparation of [32 P]Tyr]casein

To prepare [32 P]Tyr]casein, 100 μ g of heat-inactivated α -casein was added to the p130^{gag-fps} kinase reaction mixture. After incubation, the supernatant was applied to a Sephadex G-25 (medium grade) column (1.5 cm $\times$ 30 cm) equilibrated with 50 mM-Tris/HCl buffer, pH 7.5, and eluted with the same buffer to remove salt and [32 P]ATP. [32 P]Tyr]casein with a specific radioactivity of 250000 c.p.m./ μ g was obtained.

Preparation of [32 P]Ser]tubulin

Tubulin was purified from rat brain by the method of Shelansky *et al.* (1973). This preparation was co-purified with Mn²⁺-stimulated serine-specific protein kinase. We utilized the protein kinase to prepare [32 P]Ser]tubulin. The reaction mixture (500 μ l) contained 50 mM-Tris/HCl buffer, pH 7.4, containing 10 mM-MnCl₂, 30 μ Ci of [γ - 32 P]ATP (3000 Ci/mmol) and 100 μ l of tubulin solution. After incubation at 30 °C for 30 min, the reaction mixture was applied to a Sephadex G-25 (medium grade) column (1.5 cm $\times$ 30 cm) equilibrated with 50 mM-Tris/HCl buffer, pH 7.5, and eluted with the same buffer. [32 P]Ser]tubulin with a specific radioactivity of 140000 c.p.m./ μ g was obtained.

PTPPase assay

The standard assay mixture (60 μ l) contained 50 mM-sodium acetate buffer, pH 5.0, 0.1 mM-dithiothreitol, 1 mM-EDTA, 100 μ g of bovine serum albumin, 10 μ l of substrate (100000 c.p.m.) and 10 μ l of enzyme. After incubation at 37 °C for 10 min, the reaction was stopped by adding 10 μ l of 100% (w/v) trichloroacetic acid. The mixture was cooled for 5 min on ice and centrifuged at 10000 g for 3 min, and the radioactivity in the supernatant was counted in a scintillation counter. One unit of enzyme activity was defined as the amount releasing 1 pmol of [32 P]P_i/10 min.

p-Nitrophenyl-phosphatase (pNPPase) activity and the phosphatase activities towards phospho amino acids, 5'-AMP, 2'-AMP and β -glycerophosphate were determined in 0.3 ml of assay mixture that contained 50 mM-sodium acetate buffer, pH 5.0, 0.1 mM-dithiothreitol, 1 mM-EDTA and 10 mM-pNPP (2 mM for other substrates). pNPPase activity was determined by the method of Knowles *et al.* (1981), whereas other phosphatase activities were assayed by measuring P_i liberation by the method of Itaye & Ui (1966). One unit of enzyme activity was defined as the amount releasing 1 μ mol of P_i/10 min.

Other methods

Phospho amino acids were analysed by electrophoresis on cellulose plates at pH 3.5 for 90 min at 500 V by the method of Hunter & Sefton (1980). The labelled phospho amino acids were detected by autoradiography.

SDS/polyacrylamide-gel electrophoresis was carried out by the method of Laemmli (1970). Protein was detected by silver staining (Merrill *et al.*, 1981).

Protein concentration was determined by the method of Bradford (1976), with bovine serum albumin as a standard.

RESULTS

Purification of PTPase (23 kDa) and low-molecular-mass APase

Preparation of crude extract and $(\text{NH}_4)_2\text{SO}_4$ fractionation. All operations were carried out at 0–4 °C. About 200 g of whole rat brain was homogenized in 4 vol. of 0.32 M-sucrose containing 0.1 mM-dithiothreitol and 1 mM-phenylmethanesulphonyl fluoride with a Teflon-pestle homogenizer and centrifuged at 96000 g for 90 min. The supernatant was fractionated by 40–60% saturation with $(\text{NH}_4)_2\text{SO}_4$. The precipitate was dissolved in and dialysed against 50 mM-Tris/HCl buffer, pH 8.0, containing 0.1 mM-EGTA and 0.1 mM-dithiothreitol (buffer A).

Fig. 1. Elution profiles of PTPase (●) and pNPPase (○) activities on a DE-52 DEAE-cellulose column (a), on a Sephadex G-75 (superfine grade) column (b) and on a Bio-Gel HT column (c)

The procedures for these column chromatographies are as described in the text. -----, Protein; ----, KCl gradient (a) or $(\text{NH}_4)_2\text{SO}_4$ gradient (c). On the Sephadex G-75 (superfine grade) column (b) the molecular mass was estimated by comparison of the elution position with those of marker proteins: lysozyme (14.4 kDa), chymotrypsinogen A (26 kDa), ovalbumin (45 kDa) and bovine serum albumin (68 kDa).

Table 1. Purification of PTPase (23 kDa) from rat brain

Crude extract is the supernatant of brain homogenate obtained by centrifugation at 96000 *g* for 90 min. For further details of the purification procedure see the text. The net PTPase activities were determined by subtracting the activities in the presence of 0.1 mM-sodium vanadate from those in the presence of 1 mM-EDTA to suppress non-specific phosphatase activities. The incubations were carried out at 37 °C for 10 min in the presence of [³²P]p130^{gag-fps} (100000 c.p.m.).

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purity (fold)	Yield (%)
Crude extract	4314	810	0.19	1	100
(NH ₄) ₂ SO ₄ fractionation	1241	130	0.11	0.6	16
DE-52 DEAE-cellulose chromatography	35	66.5	1.9	10	8.2
Sephadex G-75 (superfine grade) chromatography	0.57	14.5	25.5	134	1.8
Bio-Gel HT chromatography	0.035	5.0	142.0	747	0.62

DE-52 DEAE-cellulose column chromatography. The dialysis residue was applied to a DE-52 DEAE-cellulose column (3 cm × 21 cm) equilibrated with buffer A. The column was washed with the same buffer and the material was eluted with 10 column volumes of a linear gradient of 0–0.15 M-KCl in buffer A. The enzyme activity was assayed with [³²P]p130^{gag-fps} (PTPase) and *p*NPP (*p*NPPase) as substrates. The elution profile is shown in Fig. 1(a). Two peaks of *p*NPPase activity were recovered. The major peak seemed to be due to a conventional low-molecular-mass APase. Most of the PTPase activity was eluted after the APase peak, and the minor peak of *p*NPPase activity was coincident with the major PTPase activity. These results indicate that the major PTPase activity is not associated with APase and is due to another enzyme molecule.

Sephadex G-75 (superfine grade) column chromatography. The materials in the fractions with PTPase activity were concentrated by 70% saturation with (NH₄)₂SO₄. The precipitate was dissolved in a minimum volume of 20 mM-Tris/HCl buffer, pH 8.0, containing 0.1 mM-EGTA, 0.1 mM-dithiothreitol and 5% (v/v) glycerol (buffer B) and applied to a Sephadex G-75 (superfine grade) column (2 cm × 86 cm) equilibrated with buffer B. The elution profile is shown in Fig. 1(b). Two major peaks of PTPase activity could be separated by this chromatography. The peak with higher PTPase activity was termed peak I, and the rest was peak II, as indicated in Fig. 1(b). Both peaks were accompanied with *p*NPPase activity, but the ratios of *p*NPPase activity to PTPase activity were substantially different and that of peak II was at least 15-fold higher than that of peak I. The molecular masses of peak I and peak II material were estimated to be 25 kDa and 15 kDa respectively from this chromatography. By this step, peak II material was purified almost to homogeneity. As shown in Fig. 2(b), SDS/polyacrylamide-gel electrophoresis of peak II material gave a single protein band, and its molecular mass was estimated to be 18 kDa by comparison with the migrations of marker proteins. The peak II enzyme showed a strict substrate specificity and, besides [³²P]p130^{gag-fps}, hydrolysed only *p*NPP and L-phosphotyrosine significantly. Furthermore, it was not sensitive to L(+)-tartrate and NaF, potent inhibitors of high-molecular-mass APase. These findings suggest that

peak II is due to low-molecular-mass APase, which is a contaminant in DE-52 DEAE-cellulose fractions of PTPase activity. The peak II material was used as highly purified APase to compare the enzymic properties with those of PTPase.

Bio-Gel HT column chromatography. The fractions of peak I were combined and applied to a Bio-Gel HT column (2 ml) equilibrated with buffer B. The column was washed with the same buffer and the material was eluted with a linear gradient of 0–0.1 M-(NH₄)₂SO₄ in buffer B (100 ml). As shown in Fig. 1(c), a peak of PTPase activity was recovered, and it was almost coincident with *p*NPPase activity, which seemed to be an intrinsic activity of PTPase.

By this step, the PTPase was purified almost to homogeneity and could be separated from APase completely. The purification procedure is summarized in Table 1. PTPase of rat brain was purified about 750-fold to a specific activity of 142 units/mg of protein in a yield of 0.6%. The enzyme was extremely unstable, thus the purification procedures resulted in a substantial loss of activity. The activity of the highly purified preparation was completely lost in only a week, when stored at 0 °C.

Purity and molecular mass

The highly purified preparation was analysed by SDS/polyacrylamide-gel electrophoresis, and the electrophoretogram gave a single protein band detected by silver staining. The molecular mass was estimated to be 23 kDa by comparison with the migrations of marker proteins (Fig. 2a). All fractions with PTPase activity eluted from columns of Sephadex G-75 (superfine grade) and Bio-Gel HT were subjected to SDS/polyacrylamide-gel electrophoresis and their protein bands were detected by silver staining. The electrophoretograms showed that the stained intensity of the protein band with the molecular mass of 23 kDa was proportional to original PTPase activity of the fraction applied, indicating that the enzyme activity resided in the 23 kDa protein (results not shown). The agreement between the size estimates from gel filtration and SDS/polyacrylamide-gel electrophoresis indicates that the PTPase is a monomer protein with a molecular mass of 23 kDa. In accordance with this we termed the enzyme PTPase (23 kDa).

Fig. 2. SDS/polyacrylamide-gel electrophoretogram of the purified PTPase (a) and APase (b) from rat brain

The enzyme was subjected to SDS/polyacrylamide-gel electrophoresis in a 14% polyacrylamide gel by the method of Laemmli (1970). The molecular mass was estimated by comparison of the relative mobility with those of marker proteins: phosphorylase *b* (92.5 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa).

Substrate specificity

PTPase (23 kDa) hydrolysed [³²P]p130^{gag-fps} with phosphorylated tyrosine residues (Figs. 3a and 3b). The specific activities of highly purified preparation of PTPase (23 kDa) and APase from rat brain towards phosphoprotein and pNPP are shown in Table 2. The specific activity of PTPase (23 kDa) towards [³²P]p130^{gag-fps} was 142 units/mg. It also dephosphorylated [[³²P]Tyr]casein with a specific activity of 66 units/mg. However, it did not hydrolyse [[³²P]Ser]tubulin. APase also possessed PTPase activity and dephosphorylated [³²P]p130^{gag-fps} and [[³²P]Tyr]casein with specific activities of 89 and 72 units/mg respectively, and also did not hydrolyse [[³²P]Ser]tubulin. PTPase (23 kDa) showed higher specific activity towards [³²]p130^{gag-fps} than towards [[³²P]Tyr]casein, whereas APase showed similar specific activity towards both substrates. Furthermore, the specific activity of PTPase (23 kDa) towards [³²P]p130^{gag-fps} was at least 1.6-fold higher than that of APase. These results suggest that, since PTPase (23 kDa) showed higher specific activity towards the

Fig. 3. (a) Autoradiogram of PTPase (23 kDa) activity for [³²P]p130^{gag-fps} and (b) phospho amino acid analysis

(a) PTPase (23 kDa) was incubated with [³²P]p130^{gag-fps} (20000 c.p.m.) and the various substances; no enzyme (lane a), no addition (lane b), 1 mM-EDTA (lane c), 0.1 mM-sodium vanadate (lane d) and 0.1 mM-ZnCl₂ (lane e). The reaction was stopped by addition of SDS/polyacrylamide-gel-electrophoresis sample buffer, and electrophoresis was carried out in a 10% gel. The gel was then dried and exposed to X-ray film overnight at -70 °C. (b) [³²P]p130^{gag-fps} was eluted from the gel, partially hydrolysed with 6 M-HCl and separated in one dimension on a cellulose plate as described in the Materials and methods section. The positions of the internal phospho amino acid standards are indicated.

naturally occurring substrate, it might act as a specific and active PTPase in a cell rather than APase. PTPase (23 kDa) could also dephosphorylate pNPP, but its specific activity was about 7-fold lower than that of APase and the *K_m* values for pNPP of PTPase (23 kDa) and APase were calculated to be about 2 mM and 0.1 mM respectively. These findings further support the high specificity for [phosphotyrosine]protein of PTPase (23 kDa). Table 3 shows substrate specificities of PTPase (23 kDa) and APase from rat brain towards low-molecular-mass phosphomonoesters. PTPase (23 kDa) hydrolysed pNPP, L-phosphotyrosine, L-

Table 2. Substrate specificities of PTPase (23 kDa) and APase from rat brain towards phosphoprotein and pNPP

The enzyme activities were determined with various substrates as indicated. The incubations were carried out at 37 °C for 10 min.

Enzyme	Specific activity (units/mg)			
	[³² P]p130 ^{gag-fps} (*)	[[³² P]Tyr]casein (6.7 µg/ml)	[[³² P]Ser]tubulin (4.7 µg/ml)	pNPP (10 µM)
PTPase (23 kDa)	142	66	0.2	41.1
APase	89	72	0.4	292.2

* The protein concentration could not be determined accurately.

Table 3. Substrate specificities of PTPase (23 kDa) and APase from rat brain towards low-molecular-mass phosphomonoesters

The enzyme activities were determined with various substrates as indicated. The incubations were carried out at 37 °C for 10 min. The K_m values were calculated from double-reciprocal plots.

Substrate (2 mM)	PTPase (23 kDa)		APase	
	Relative activity (%)	K_m (mM)	Relative activity (%)	K_m (mM)
pNPP	100	2	100	0.1
L-Phosphotyrosine	55	2	16	—
L-Phosphoserine	9	—	0	—
DL-Phosphothreonine	10	—	1	—
5'-AMP	7	—	0	—
2'-AMP	8	—	1	—
β -Glycerophosphate	7	—	0	—

phosphoserine, DL-phosphothreonine, 5'-AMP, 2'-AMP and β -glycerophosphate, but the relative proportions of the enzyme activity with these substrates were 100:55:9:10:7:8:7. The K_m values for pNPP and L-phosphotyrosine were calculated to be about 2 mM. In contrast, APase showed strict substrate specificity and could hydrolyse only pNPP and L-phosphotyrosine significantly. The ratio of the activity with these two substrates was 100:16.

pH optima

Fig. 4 shows pH-activity profile of PTPase (23 kDa) with [32 P]p130^{gag-fps} and pNPP. The activity was maximal at pH 5.0–5.5 with both substrates. However, the activity with [32 P]p130^{gag-fps} was higher than that with the pNPP at pH 5.5–7.0, but lower at pH 3.8–4.8. APase also showed maximum activity at pH 5.0–5.5 (results not shown).

Effect of inhibitors

As shown in Fig. 3(a), PTPase (23 kDa) activity with [32 P]p130^{gag-fps} was strongly inhibited by sodium vanadate. The effect of sodium vanadate on it is further shown in Fig. 5. The activity was inhibited 50% by 250 nM-sodium vanadate. The PTPase (23 kDa) activity was not significantly inhibited by 0.1 mM-ZnCl₂, but was slightly enhanced by 1 mM-EDTA. Table 4 summarizes the effects of inhibitors on PTPase (23 kDa) and APase from rat brain. Sodium vanadate inhibited pNPPase activity of PTPase (23 kDa). Molybdate also inhibited PTPase and pNPPase activity of the enzyme. These results further support the view that both activities reside in the same enzyme molecule. However, APase activity with [32 P]p130^{gag-fps} and pNPP was not effectively inhibited by sodium vanadate and molybdate. L(+)-Tartrate, which is known to inhibit high-molecular-mass APase strongly, did not inhibit PTPase (23 kDa) significantly. NaF at 10 mM inhibited [phosphoserine]-protein phosphatase, but it inhibited PTPase (23 kDa) only slightly. P_i and PP_i were not inhibitory, but ATP caused 55% inhibition at a concentration of 1 mM. PTPase (23 kDa) did not require bivalent cations for activity, and in fact was inhibited by Mg²⁺ (5 mM). APase was also not inhibited by L(+)-tartrate and NaF, which is a typical property of conventional low-molecular mass APase.

Fig. 4. Effect of pH on PTPase (23 kDa) activity

PTPase was assayed with [32 P]p130^{gag-fps} (●) and pNPP (○) at various pH values obtained with sodium acetate buffer (pH 3.8–5.5), Mes/NaOH buffer (pH 5.5–7.4) and Tris/HCl buffer (pH 7.0–7.4). The activities are shown as percentages of the maximum activities.

DISCUSSION

A PTPase was purified almost to homogeneity from rat brain cytosolic fraction. It has an acidic pH optimum, but was a distinct enzyme molecule from a conventional low-molecular-mass APase. It efficiently hydrolysed natural [phosphotyrosine]protein ([32 P]-p130^{gag-fps}). The molecular mass of the enzyme was estimated to be 23 kDa from SDS/polyacrylamide-gel electrophoresis, and it was a monomer protein. The molecular masses of previously reported PTPases, except for APase (18 kDa), ranged from 34 kDa to 100 kDa, and thus PTPase (23 kDa) appears to be a new type of PTPase.

Chernoff & Li (1985) reported that acidic PTPase activity was higher than its neutral counterpart in the extract of several tissues, and proposed that acidic

Table 4. Effects of inhibitors of PTPase (23 kDa) and APase from rat brain

The enzymes were assayed with [32 P]p130^{gag-fps} (100 000 c.p.m.) and pNPP (10 mM) in the presence of various compounds. The incubations were carried out at 37 °C for 10 min.

Inhibitor	Relative activity (%)			
	PTPase (23 kDa)		APase	
	[32 P]p130 ^{gag-fps}	pNPP	[32 P]p130 ^{gag-fps}	pNPP
No addition	100	100	100	100
EDTA (1 mM)	124	82	102	112
Vanadate (0.01 mM)	3	8	44	104
Molybdate (0.01 mM)	0	0	57	99
L(+)-Tartrate (10 mM)	80	58	103	97
NaF (10 mM)	74	76	91	95
KP ₁ (1 mM)	79	88	85	82
NaPP ₁ (1 mM)	93	95	99	82
ZnCl ₂ (0.1 mM)	82	86	104	102
MgCl ₂ (5 mM)	47	81	92	105
5'-ATP (1 mM)	45	45	102	101

Fig. 5. Inhibition of PTPase (23 kDa) by sodium vanadate

PTPase was assayed in the presence of various concentrations of sodium vanadate instead of EDTA.

PTPase might play an important role in controlling dephosphorylation of [phosphotyrosine]protein in a cell. They showed that PTPase could be separated by DEAE-cellulose chromatography into three major peaks, termed Y1, Y2 and Y3. Y1 did not bind to DEAE-cellulose at neutral pH, and was the major acidic PTPase. They then purified the Y1 protein and suggested that the major active species in Y1 was closely associated with APase. As shown in Fig. 1(a), major acidic PTPase activity bound to DEAE-cellulose at our conditions (50 mM-Tris/HCl buffer, pH 8.0) and APase could be separated from the major PTPase activity. Furthermore, by Sephadex G-75 (superfine grade) column chromatography, PTPase (23 kDa) and APase could be completely separated, and thus the characteristics

of the highly purified preparations could be compared. PTPase (23 kDa) dephosphorylated [32 P]p130^{gag-fps}, a naturally occurring substrate, more efficiently than it did [32 P]Tyr]casein, an artificial substrate. APase could also dephosphorylate [32 P]p130^{gag-fps} and [32 P]Tyr]casein, but with similar efficiency. The specific activity of PTPase (23 kDa) for [32 P]p130^{gag-fps} was at least 1.6-fold higher than that of APase. APase hydrolysed pNPP effectively, and its specific activity was about 7-fold higher than that of PTPase (23 kDa). These results indicate that PTPase (23 kDa) is the most specific and active species of PTPase in the cell.

Leis & Kaplan (1982) reported that an APase in plasma membrane from a human astrocytoma showed marked specificity towards [phosphotyrosine]protein, and that it showed a shift of optimum pH towards a physiological pH when [phosphotyrosine]protein was used as substrate. This APase also did not hydrolyse 5'-AMP or β -glycerophosphate significantly, and was strongly inhibited by sodium vanadate. On the basis of these findings, they proposed that the enzyme acted as a specific PTPase in physiological conditions. The pH-activity profile of PTPase (23 kDa) also showed a slight shift towards neutral pH when [32 P]p130^{gag-fps} was used as substrate. Thus, with an adequate or inherent substrate, the brain enzyme may show marked activity at physiological pH. Therefore these two enzymes should be classified as the same type of PTPase. However, since the distribution of PTPase (23 kDa) in the cell has not been elucidated, the relationship between these two enzymes remains unknown.

PTPase (23 kDa) from rat brain was strongly inhibited by sodium vanadate, a concentration of 250 nM causing 50% inhibition. Leis & Kaplan (1982), Chernoff & Li (1983) and Swarup *et al.* (1984) also reported that PTPase was inhibited by sodium vanadate. Furthermore, these vanadate-sensitive PTPases do not require bivalent cations and are stimulated by EDTA. Thus inhibition by sodium vanadate and the absence of requirement for bivalent cations seem to be typical properties of PTPase. Dubyak & Kleinzeller (1980)

reported that sodium vanadate exhibited an insulin-mimetic effect in isolated rat adipocytes. Also, Klarlund (1985) demonstrated that the addition of sodium vanadate to NRK1 cells resulted in a maximal 40-fold increase in the phosphotyrosine content in cell protein, and induced transformation of the cells. These effects of sodium vanadate may depend on its inhibitory action on PTPase. Therefore vanadate-sensitive PTPase may have some regulatory role in the action of insulin and in the transformation of cells induced by tyrosine kinase activity. These facts also support the view that vanadate-sensitive PTPase (23 kDa) is the most important PTPase in a cell.

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Received 10 February 1986/21 April 1986; accepted 10 June 1986