Acta Biochimica Polonica

Vol. 50 No. 2/2003

555–566

QUARTERLY

The 35 kDa acid metallophosphatase of the frog *Rana esculenta* liver: studies on its cellular localization and protein phosphatase activity

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Received: 18 December, 2002; revised: 27 March, 2003; accepted: 18 May, 2003

Key words: acid phosphatase, frog liver, metallophosphatase, protein phosphatase, tyrosine protein phosphatase

The cellular localization of the 35 kDa, low molecular mass acid metallophosphatase (LMW AcPase) from the frog (*Rana esculenta*) liver and its activity towards P-Ser and P-Tyr phosphorylated peptides were studied. This enzyme was localized to the cytoplasm of hepatocytes but did not appear in other cells of liver tissue (endothelium, macrophages, blood cells). This LMW AcPase does not display activity towards ^{32}P-phosphorylase *a* under conditions standard for the enzymes of PPP family. Proteins containing P-Ser: rabbit ^{32}P-phosphorylase *a* and phosvitin are hydrolysed only at acidic pH and are poor substrates for this enzyme. The frog AcPase is not inhibited by okadaic acid and F^- ions, the Ser/Thr protein phosphatase inhibitors. Moreover, the frog enzyme does not cross-react with specific antisera directed against N-terminal fragment of human PP2A and C-terminal conserved fragment of the eukaryotic PP2A catalytic subunits. These results exclude LMW AcPase from belonging to Ser/Thr protein phosphatases: PP1c or PP2Ac. In addition to P-Tyr, this enzyme hydrolyses efficiently at acidic pH P-Tyr phosphorylated peptides (hirudin and

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Abbreviations: AcPase, acid phosphatase; BCA, bicinchoninic acid; BSA, bovine serum albumin; CIAP, calf intestine alkaline phosphatase; Con A, concanavalin A; DAB, diaminobenzidine tetrachloride; DTT, dithiothreitol; HRP, horseradish peroxidase; LMW AcPase, lower molecular mass acid phosphatase; PAP, purple acid phosphatase; PBS, phosphate-buffered saline ; PP1c, catalytic subunit of type-1 protein phosphatase; PP2Ac, catalytic subunit of type-2 protein phosphatase class A; PP2B, type-2 protein phosphatase class B (calcineurin); PPP, gene family of Ser/Thr protein phosphatases comprising PP1, PP2A and PP2B; PMSF, phenylmethylsulfonyl fluoride; pNPP, *p*-nitrophenylphosphate; P-Ser, phosphoserine; P-Thr, phosphothreonine; PTPase, protein tyrosine phosphatase; P-Tyr, phosphotyrosine; TRAP, tartrate-resistant acid phosphatase; TPBS, PBS containing 0.05% Tween 20.

gastrin fragments). K_m value for the hirudin fragment ($7.55 \pm 1.59 \times 10^{-6}$ M) is 2–3 orders of magnitude lower in comparison with other substrates tested. The enzyme is inhibited competitively by typical inhibitors of protein tyrosine phosphatases (PTPases): sodium orthovanadate, molybdate and tungstate. These results may suggest that the LMW AcPase of frog liver can act as PTPase *in vivo*. A different cellular localization and different response to inhibition by tetrahedral oxyanions (molybdate, vanadate and tungstate) provide further evidence that LMW AcPase of frog liver is distinct from the mammalian tartrate-resistant acid phosphatases.

Acid phosphatases (non-specific phosphomonoesterases, EC 3.1.3.2) are a group of ubiquitous enzymes of diverse structures and biochemical properties. They hydrolyze a broad spectrum of substrates *in vitro* but their natural substrates and physiological function is still obscure. It is suggested that many of them function as protein phosphatases.

The low molecular mass acid phosphatase (LMW AcPase, *M* 35 kDa) from the frog liver described by our group has been shown to be a novel metallophosphatase activated by reducing agents and displaying tartrate-resistance (Jańska *et al.*, 1989: Szalewicz *et al.*, 1999a). It differs from the mammalian, tartrate-resistant purple acid phosphatases by the involvement of different metal ions in the catalytic process and in the way of iron coordination. There is also no immunological relationship between the protein moieties of these enzymes (Szalewicz *et al.,* 1999a). We previously showed that the frog LMW AcPase is able to hydrolyze phosphotyrosine at acidic pH and its optimum for this substrate is shifted towards neutral pH in the presence of some divalent metal cations (Szalewicz *et al.*, 1999b). The enzyme does not display activity towards other phosphorylated amino acids present in phosphoproteins, i.e., P-Ser and P-Thr. This suggests a posssible function of this enzyme as protein tyrosine phosphatase although the catalytic mechanism differs from that of other PTPases which require Cys residue in phosphate hydrolysis (Fauman & Saper, 1996). On the other hand, some features of the frog enzyme, i.e., the molecular mass and the presence of binuclear metal center, show similarity to the catalytic subunits of eukaryotic protein Ser/Thr phosphatases of the PPP family, i.e., PP1c and PP2Ac (Wera & Hemmings, 1995; Barford, 1996).

The above observations prompted us to search for further evidence that the LMW AcPase can act as a protein phosphatase. We have therefore investigated both the protein tyrosine and protein Ser/Thr phosphatase activities of this enzyme towards specific phosphorylated peptide or protein substrates. For comparative purpose we have also examined the immunological relationship between the frog LMW AcPase and eukaryotic PP2Ac. In addition we studied the cellular localization of the enzyme within the frog liver tissue.

MATERIALS AND METHODS

Materials

The enzyme was isolated from the frog (*Rana esculenta*) livers removed immediately after animal death, rinsed with isotonic solution (0.65% NaCl) and frozen at –20°C.

Chemicals

BCA, 3,3′-diaminobenzidine, DTT, pNPP, Malachite Green were purchased from Sigma (U.S.A); Bio-Gel P-200 from Bio-Rad (U.S.A.); BSA from Roth (Germany); CIAP from MBI Fermentas (Lithuania); *O*-phospho-L-tyrosine, phosvitin from egg yolk, PMSF, Tween 20, ammonium molybdate, magnesium, manganese and calcium chlorides from Fluka (Switzerland); Con A-Sepharose 4B and Mono S HR 5/5 column from Pharmacia (Sweden).

A nonradioactive tyrosine phosphatase assay kit, okadaic acid, peroxidase-labeled goat

anti-rabbit-IgG from Boehringer Mannheim-Roche Molecular Biochemicals (Germany); DAKO LSAB$^{(R)2}$ kit HRP from DAKO Corporation (Denmark); rabbit ^{32}P-phosphorylase *a* and rabbit antisera specific towards human PP2Ac were kindly supplied by the late Professor Stanisław Żołnierowicz of Intercollegiate Faculty of Biotechnology UG-MUG, Gdańsk (Poland).

Other chemicals were from POCh (Poland). All reagents used were of analytical grade.

Enzyme purification

The LMW acid phosphatase from frog (*Rana esculenta*) livers was extracted with 0.1 M Na/acetate buffer, pH 5.0, containing 0.1 mM PMSF and 1 mM DTT, and purified subsequently by ammonium sulfate fractionation, Con A-affinity chromatography, Bio-Gel P-200 filtration and ion exchange chromatography on Mono S column as described earlier (Jańska *et al.*, 1989; Szalewicz *et al.*, 1999a).

Analytical methods

Immunohistochemical localization of the LMW AcPase in frog liver cells. The reaction was carried out according to the modified ABC method (Hsu *et al.*, 1981). Frog livers obtained after cervical dislocation were sliced, fixed in Bouin fluid and embedded in paraffin. Paraffin sections were cut, dewaxed, hydrated through graded alcohol to PBS (pH 7.4) and treated with 1% hydrogen peroxide in methanol to abolish endogenous peroxidase activity. The sections were then probed with primary antibodies, i.e., polyclonal rabbit IgG directed against the LMW AcPase, prepared as described earlier (Szalewicz *et al.*, 1992). In parallel experiments two kinds of antibodies, raised against the separated main forms of the enzyme differing in pI , i.e.: AcPase III (pI 5.9) and AcPase IV (pI 6.2), were used. Incubation with specific antibodies was carried out overnight at 4°C. The secondary antibodies (biotinylated goat IgG anti-rabbit IgG) was applied for 45 min at room temperature, followed by 1 h incubation with streptavidin-biotin-HRP complex. Between the incubations, sections were washed 3 times with PBS. Except for the primary antibodies, the components of DAKO LSAB$^{(R)2}$ kit HRP were used. Finally the sections were stained with diaminobenzidine solution (0.05% DAB, 0.01% H_2O_2 in 50 mM Tris/HCl buffer, pH 7.6) for 7 min and the reaction was stopped by rinsing with distilled water.

Sections in which treatment with primary antibodies was omitted served as negative controls.

Protein concentration was determined by the BCA protein assay (Smith *et al.*, 1985) using crystalline BSA as a standard or spectrophotometrically, based on absorbance measurements at 235 and 280 nm (Whitaker & Granum, 1980).

Acid phosphatase activity towards pNPP was measured by following the release of *p*-nitrophenol from 2.5 mM sodium *p*-nitrophenyl phosphate in 0.1 M Na/acetate buffer, pH 5.0, at 30°C. One unit of the enzyme activity corresponds to the hydrolysis of 1 μmole of *p*-nitrophenyl phosphate per min under the assay conditions.

Protein Ser/Thr phosphatase activity towards rabbit ^{32}P-phosphorylase a. Enzymatic tests were carried out at both acidic and neutral pH (50 mM Na/acetate buffer, pH 5.3, and 50 mM Tris/HCl buffer, pH 7.4, respectively) at 37°C. The reaction mixtures contained 10 μM ^{32}P-phosphorylase *a*, 0.03% β-mercaptoehtanol, 0.03 mM EDTA and 0.1% BSA. The reaction was terminated by the addition of ice-cold trichloroacetic acid to a final concentration of 15% (Yang *et al.*, 1980). After centrifugation of the precipitate, the released $^{32}P_i$ was determined by measuring the supernatant radioactivity. One unit of the enzyme activity corresponds to 1 μmole of $^{32}P_i$ released from the substrate per min under the assay conditions.

Protein Ser/Thr phosphatase activity towards phosvitin from egg yolk was measured

by following the release of inorganic phosphate, estimated by the Malachite Green method. The enzyme activity was tested in 50 mM Na/acetate buffer, pH 5.1, and in 50 mM Tris/HCl buffer, pH 7.4, at 37°C. Enzyme reactions were carried out in a volume of 0.1 ml, then terminated by addition of 1 ml of the Malachite Green-ammonium molybdate reagent (Harder *et al.*, 1994). After 20 min incubation at room temperature the absorbance at 620 nm was read and the amount of inorganic phosphate was determined from the calibration curve (2–20 nmoles of KH_2PO_4). One unit of the enzyme activity corresponds to the release of 1 μmole of P_i per min under the assay conditions.

Protein tyrosine phosphatase (PTPase) activity was determined towards two phosphorylated synthetic oligopeptides corresponding to the hirudin 53–65 C-terminal fragment, phosphorylated on Tyr 63 (PPS1), and to the human gastrin 1-17 N-terminal fragment, phosphorylated on Tyr 12 (PPS2), using a nonradioactive tyrosine phosphatase assay kit (Boehringer Mannheim-Roche Molecular Biochemicals, Germany). The enzymatic reaction was carried out on biotinylated substrates bound to streptavidin-coated microtiter plates (6 pmoles and 1 pmole, respectively). The amount of nonhydrolyzed substrate was measured with the use of HRP-labeled murine monoclonal antibodies (clone 3-365-10) directed against phosphotyrosine, according to the manufacturer's instruction. The activity of LMW AcPase was determined both at acidic (0.1 M Na/acetate buffer, pH 5.0) and neutral pH (20 mM Tris/HCl buffer, pH 7.2, and 50 mM diethylbarbiturate/acetate buffer, pH 7.4, containing different concentrations of $MnCl_2$) at 37°C. As a reference, unspecific alkaline phosphatase from calf intenstine (CIAP) in 10 mM Tris/HCl buffer, pH 7.5, containing 10 mM $MgCl_2$ was used with these substrates. In each series of experiments a separate control sample (substrate treated with an appropriate buffer without the enzyme) was included.

Estimation of K_m constants for peptidic substrates

The K_m constant for phosvitin was determined on the basis of the initial reaction velocity measured at a 5.89×10^{-7} M to 5.89×10^{-6} M phosphoprotein that corresponded to 6.45×10^{-5} M – 6.45×10^{-4} M of phosphate residues (all in the form of P-Ser, 110 phosphate residues per protein molecule (Shainkin & Perlman, 1971; Byrne *et al.*, 1984; and supplier information)). The measurements were carried out in 0.1 M Na/acetate buffer, pH 5.0, at 37°C. The amount of inorganic phosphate released was determined by the Malachite Green method as described above.

The K_m constant for hirudin 53–65 C-terminal fragment was estimated on the basis of the initial reaction velocity measured at the substrate concentration range 0.5–50 10^{-6} M in 0.1 M Na/acetate buffer, pH 5.0, using a general procedure of the nonradioactive tyrosine phosphatase assay kit (Boehringer Mannheim-Roche Molecular Biochemicals, Germany). The reaction was carried out in a total volume of 10 μl and stopped by addition of 10 μl of 10 mM phosphate. After an appriopriate dilution with PBS, 30 μl of each sample corresponding to 4 pmoles of the substrate, was transferred to the wells of the microtiter plate and the reaction was processed according to the manufacturer's instructions. The amount of non-dephosphorylated substrate was determined on the basis of the standard curve set up in 0.1–6 pmoles range of the phosphopeptide.

All kinetic assays were performed in triplicate. For both substrates K_m and V_{max} were estimated by the Michaelis–Menten formula using the Slide Write Plus 2.0 computer program (Advanced Graphics Software Inc., U.S.A.).

Inhibition studies

The effect of okadaic acid (1 μM) on the LMW AcPase activity towards rabbit ^{32}P-phosphorylase *a* was determined in 50

mM Na/acetate buffer, pH 5.3, as described above.

The type of inhibition and the K_i values of the inhibitors of pNPP hydrolysis were estimated using the Dixon method. The initial velocity of pNPP hydrolysis was measured in the presence of the following inhibitors: inorganic phosphate (1–15 × 10^{-4} M), molybdate (1–16 10^{-7}M), vanadate (2–20 × 10^{-6} M) tungstate (1–16 × 10^{-8} M) and P-Tyr (5–50 × 10^{-4} M), minimum at six different inhibitor concentrations. In each experiment, the initial velocity of pNPP hydrolysis was measured at three different concentrations of the substrate: 1 mM, 2 mM and 4 mM in the case of inorganic phosphate and P-Tyr and 1 mM, 1.5 mM and 2 mM in the case of molybdate, vanadate and tungstate. The type of inhibition and the K_i values were estimated using Dixon plots: $v/v_i = f([I])$ and $1/v_i = f([I])$, respectively.

The inhibitory effect of pNPP and sodium orthovanadate on PTPase activity was examined by the standard PTPase assay, as described above.

Antibodies against PP2Ac and immunoblotting

Polyclonal rabbit antibodies directed against peptides corresponding to the C-terminal 9 aa fragment of eukaryotic PP2Ac (^{301}TRRTPDY(PO_4)FL^{309}) and against the N-terminal 20 aa fragment of human PP2Acα (^{1}MDEKVFTKELDQWIEQLNEC^{20}) were obtained by immunization with synthetic peptides conjugated to hemocyanin from horseshoe crab (*Limulus polyphemus*) hemolymph (Świątek *et al.*, 2000). They served as primary antibodies and were applied in the form of appropriate sera at 1 : 20 and 1 : 100 dilution in TPBS, respectively.

The frog enzyme was immobilized on NC membranes by dot blotting (Gershoni, 1988) or transferred from the gel after PAGE (Davis, 1964; Kinzkofer-Peresch *et al.*, 1988) or SDS/PAGE (Laemmli, 1970; Towbin *et al.*, 1979). After blocking with 3% BSA in PBS, the NC sheets were incubated with the primary antibodies as described above. HRP-labeled goat anti-rabbit-IgG diluted 1 : 400 with TPBS were used as the secondary antibody. The enzyme conjugated probes were detected with 0.06 % 4-chloro-1-naphthol and 0.01% H_2O_2 in 50 mM Tris/HCl, pH 7.5.

RESULTS

Immunohistochemical localization of the LMW AcPase

Cells expressing LMW AcPase were identified with the use of specific polyclonal antibodies. In two series of experiments identical results were obtained with the two kinds of antibodies directed towards the separated main forms of the frog enzyme, i.e. AcPase III (pI 5.9) and Acpase IV (pI 6.2). A positive reaction indicating the presence of the LMW AcPase was detected only in hepatocytes, the most abundant liver cells (Fig. 1A and B). The immunochemical complexes are localized exclusively to the cytoplasm of hepatocytes, with the nuclei remaining negative (Fig. 1C). Other cells of liver tissue, i.e., endothelium, macrophages and blood cells do not react with the antibodies.

No reaction was found in control sections, incubated without the primary antibodies, confirming the specificity of the reaction (Fig. 1D).

Protein Ser/Thr phosphatase activity of LMW AcPase

The activity of LMW AcPase was assayed towards Ser-phosphorylated proteins: the rabbit ^{32}P-phosphorylase *a* (P-Ser^{14}) – the substrate specific for PP1 and PP2A and towards phosvitin – the egg yolk protein rich in P-Ser residues. It has been found (Table 1) that LMW AcPase was unable to hydrolyze rabbit ^{32}P-phosphorylase *a* at standard conditions for Ser/Thr protein phosphatases, i.e., at pH

7.4 but hydrolysis took place at acidic pH. However, specific activity of the enzyme was about 5 orders of magnitude lower than that for the synthetic substrate, pNPP (Tables 1 and 2). Moreover, this activity was unaffected by okadaic acid even at 1 μM concentration which is inhibitory for Ser/Thr protein phosphatases PP1 and PP2A (Table 3). This inhibitor is used for classification of phosphatases due to its specific action (Bialojan & Takai, 1988; Cohen *et al.*, 1990).

Phosvitin was processed by the enzyme also only at acidic pH. In this case specific activity was much higher than that displayed towards ^{32}P-phosphorylase *a* but still much lower (by 2 orders of magnitude) than the unspecific activity towards pNPP under the same conditions (Table 2).

Immunological relationship between LMW AcPase and PP2Ac

The cross-reactivity tests between the frog LMW AcPase and the specific antisera directed against peptidic fragments of the PP2A catalytic subunits: N-terminal fragment of human PP2Acα and C-terminal conserved fragment of the eukaryotic PP2Ac were carried out using dot-blot and immunoblot techniques. Both experiments showed no reaction between the frog enzyme and the specific antisera, even when micrograms of the homogeneous frog protein were applied. On the other hand, nanograms of the control antigen (rabbit PP2Ac) were readily detected at the same serum dilution. It was thus evident that the antibodies directed against N-terminal frag-

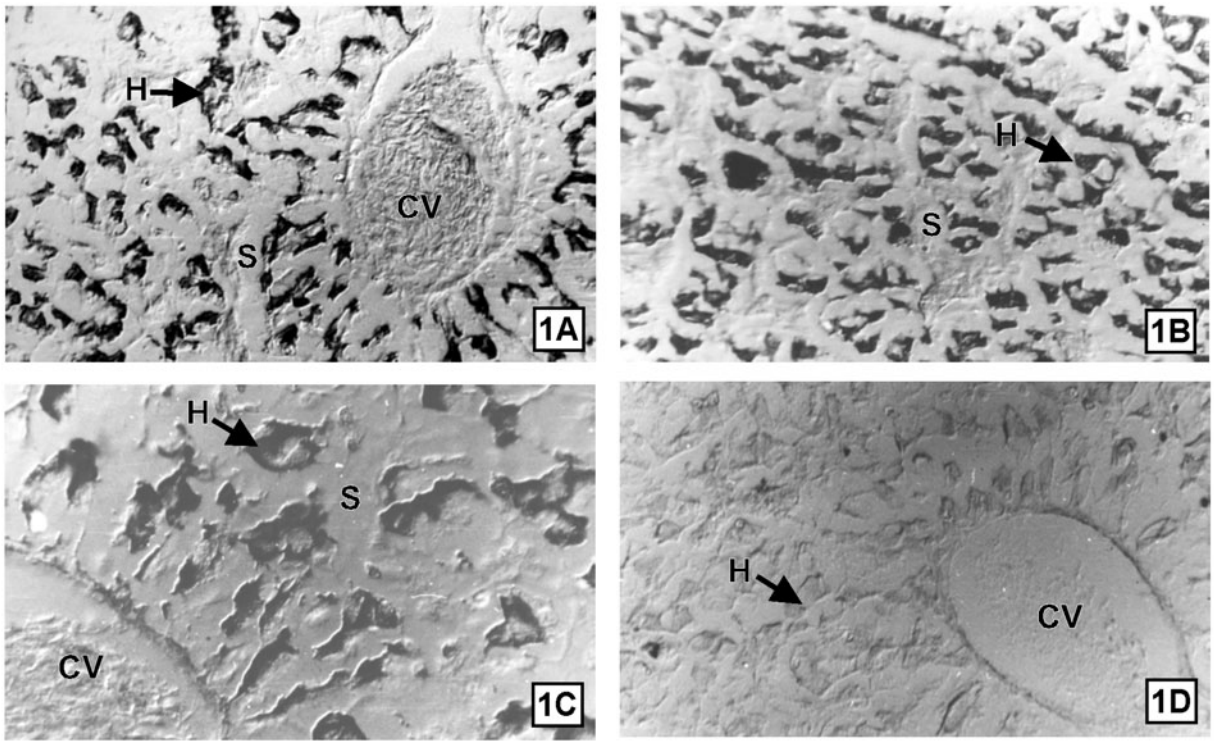

Figure 1. Immunochemical localization of the LMW AcPase in frog liver sections.

1A, 1B and 1C: a positive reaction; 1D: a negative (control) reaction. The reaction was carried out with polyclonal rabbit IgG against LMW AcPase as the primary antibody, and biotinylated goat IgG anti-rabbit IgG as the secondary antibody followed by streptavidin-biotin-HRP complex. For peroxidase reaction DAB was used as a substrate. In the control reaction incubation with primary antibodies was omitted. Details in Materials and Methods. H, hepatocytes; CV, central vein; S, sinusoids. Light microscope Olympus BH2 was used with magnification: 1A, 1B, 1D: ×250 ; 1C: ×500.

Table 1. The Ser/Thr protein phosphatase activity of LMW AcPase of frog liver*

Substrate	Specific activity
Rabbit ^{32}P-phosphorylase *a* (pH 7.4)	none
Rabbit ^{32}P-phosphorylase *a* (pH 5.3)	8.7×10^{-5} U/mg
Phosvitin (pH 7.4)	none
Phosvitin (pH 5.3)	0.22 U/mg

*Acid phosphatase activity towards pNPP, pH 5.0: 20 U/mg.

ment of human PP2Ac and C-terminal conserved fragment of eukaryotic PP2Ac do not cross-react with LMW AcPase, indicating a lack of homologous fragments in the frog enzyme (not shown).

AcPase activity towards P-Tyr phosphorylated peptides

The PTPase activity of frog LMW AcPase was tested towards two oligopeptides prebound to the solid phase: the hirudin 53–65 C-terminal fragment (PPS1) and the human gastrin 1–17 N-terminal fragment (PPS2), both containing one P-Tyr residue per molecule. In this experiment equal amounts of the substrate bound to the wells of the streptavidin coated microtiter plate were treated with different amounts of the frog enzyme (Fig. 2). The LMW AcPase hydrolyses both substrates at pH 5.0 with comparable efficiency: 1 mU of the enzyme is required to process about 50% of the substrate in 1 h. In order to compare this activity with that of an unspecific phosphatase, the activity of calf intestine alkaline phosphatase has been checked in a parallel test. This enzyme hydrolysed also both substrates, but its activity towards phosphorylated peptides was about 30 times lower compared to that of the frog LMW AcPase. Although the reaction procedure does not allow us to estimate the specific activity of the enzyme towards both tested peptides, one can assume that LMW AcPase is able to recognize specifically the P-Tyr phosphorylated peptides.

Very low activity was observed at neutral pH. Contrary to the activity of the frog LMW AcPase towards free P-Tyr which increased in the presence of the Mn^{2+} ions, the activity towards P-Tyr phosphorylated peptides remained unaffected (not shown).

Kinetic parameters of the LMW AcPase for peptidic substrates

In order to compare the protein Ser/Thr phosphatase and PTPase activities of the LMW AcPase, the K_m and V_{max} values for

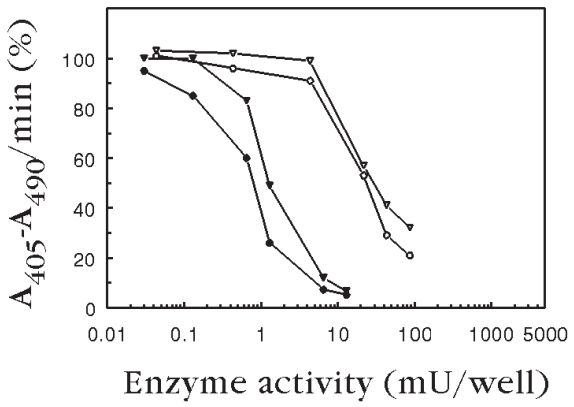

Figure 2. The PTPase activity of the frog LMW AcPase with phosphorylated oligopeptides.

The enzymatic reaction was carried out on biotinylated substrates bound to the streptavidin-coated microtiter plates. The amount of nonhydrolysed substrate was measured and expressed as percents of absorbance of the control sample (untreated with the enzyme). As the reference, unspecific alkaline phosphatase (CIAP) activity towards these substrates was included. Details in Materials and Methods. Hydrolysis of hirudin fragment (PPS1): (●), LMW AcPase; (○), CIAP; Hydrolysis of human gastrin (PPS2): (▼), LMW AcPase; (▽), CIAP.

phosvitin and P-Tyr phosphorylated hirudin fragment were determined (Table 2). All parameters were estimated at pH 5.0, which was found to be optimal also for the hydrolysis of the phosphorylated peptidic substrates. The K_m values for natural peptidic substrates were lower than those for pNPP compounds were estimated using the Dixon method. Vanadate, molybdate and tungstate are strong competitive inhibitors of the LMW AcPase, with K_i: 9.55×10^{-6} M, 5.5×10^{-7} M and 3.6×10^{-8} M, respectively. They are significantly more efficient inhibitors, with K_i 2–4 orders of magnitude lower than inorganic

Table 2. K_m and V_{max} values of the LMW AcPase for pNPP, P-Tyr and natural peptidic substrates, measured in 0.1 M Na/acetate buffer, pH 5.0, at 37°C.

Substrate	K_m (M)	V_{max} (IU/mg)	V_{max}/K_m
Hirudin 53–65 C-terminal fragment (containing P-Tyr 63)	$7.55 \pm 1.59 \times 10^{-6}$	0.42 ± 0.13	55.63×10^3
pNPP*	$1.39 \pm 0.07 \times 10^{-3}$	27.26 ± 0.80	19.61×10^3
P-Tyr*	$1.38 \pm 0.15 \times 10^{-3}$	11.09 ± 0.66	8.04×10^3
Phosvitin (per number of P-Ser residues)	$2.73 \pm 0.60 \times 10^{-4}$	0.77 ± 0.06	2.82×10^3

*According to Szalewicz *et al.*, (1999b).

or P-Tyr: 2.73×10^{-4} M for phosvitin (calculated per P-Ser residue) and 7.55×10^{-6} M for the hirudin fragment, latter being the lowest K_m value of all other substrates found so far. This indicates that the enzyme displays higher affinity for peptidic substrates, especially for the P-Tyr phosphorylated hirudin fragment, than for small phosphate esters. The P-Tyr phosphorylated hirudin fragment is also hydrolysed with the highest specificity, expressed by the highest V_{max}/K_m ratio value.

Inhibition of LMW AcPase

In order to characterize further the frog enzyme, the effect of the inhibitors specific for protein phosphatases was tested. As shown in Table 3, the enzyme was inhibited by the typical phosphotyrosine phosphatase inhibitors: sodium orthovanadate and molybdate, but unaffected by okadaic acid and F^- ions, the typical Ser/Thr phosphatase inhibitors. Besides molybdate and vanadate, also tungstate, another tetrahedral oxyanion, was found to exert an inhibitory effect. The type of inhibition and the inhibition constants for the tested phosphate ($K_i = 2.13 \times 10^{-4}$ M), another competitive inhibitor.

The effect of orthovanadate was also tested at pH 7.4 in the reaction with P-Tyr and at pH 5.0 with phosphorylated peptides in the PTPase test. In both cases this compound caused 60% and 20% inhibition, respectively, at 0.1 mM concentration.

In order to establish whether pNPP, P-Tyr and the P-Tyr phosphorylated peptides are hydrolyzed at the same active site, the inhibitory effect of P-Tyr on pNPP hydrolysis and the effect of pNPP on PTPase activity were studied. As shown in Table 3 P-Tyr inhibited hydrolysis of pNPP, acting as a competitive inhibitor with K_i = 1.54 mM. This value is very close to the K_m for pNPP as a substrate and also to the K_m value for P-Tyr, both estimated in 0.1 M Na/acetate buffer, pH 5.0 (Table 2). pNPP also exerts an inhibitory effect on the P-Tyr hydrolysis of the peptidic fragments; at the concentration of 5 mM pNPP reduced PTPase activity by half under the standard assay conditions (not shown). This indicates that all substrates tested, i.e., pNPP, P-Tyr and P-Tyr phosphorylated peptidic fragments act as competitive inhibi-

Table 3. Effect of different phosphatase inhibitors and phosphotyrosine, a substrate analogue, on the LMW AcPase activity

Inhibitor	Type of inhibition	K_i (M)
Inorganic phosphate[a]	competitive	$2.13 \pm 0.31 \times 10^{-4}$
Molybdate[a]	competitive	$5.52 \pm 0.83 \times 10^{-7}$
Tungstate[a]	competitive	$3.60 \pm 0.50 \times 10^{-8}$
Orthovanadate[a]	competitive	$9.55 \pm 2.39 \times 10^{-6}$
Okadaic acid[b]	none	–
F^- ions[a,c]	none	–
Phosphotyrosine[a]	competitive	$1.54 \pm 0.35 \times 10^{-3}$

[a] with pNPP, in 0.1 M Na/acetate buffer, pH 5.0, for details see Methods; [b] in the presence of 1 μM inhibitor, with ^{32}P-phosphorylase *a*, pH 5.3; [c] in the presence of 1 mM inhibitor.

tors to each other, and are processed within the same active center.

DISCUSSION

Immunocytochemical studies revealed that frog LMW AcPase is the enzyme of hepatocytic origin and was not found in macrophages in frog liver tissue. This is a further feature that differentiates the frog enzyme from the mammalian tartrate-resistant acid phosphatases (TRAPs). Althought this enzyme was detected in diverse human and animal tissues, it is expressed mainly in specialized cells: osteoclasts, macrophages and dendritic cells (Vincent *et al.*, 1990; Fleckenstein & Drexler, 1997; Hayman *et al.*, 2000; Oddie *et al.*, 2000; Hayman *et al.*, 2001). TRAP was either not detected in human and animal livers or, even when detected at considerably high level, it was never present in hepatocytes (Hayman *et al.*, 2000; Oddie *et al.*, 2000; Hayman *et al.*, 2001). However, significant number of TRAP containing cells of non macrophage origin were reported in human placenta: in leukocytes (Schindelmeiser & Muenstermann, 1991), decidual cells and syncytiotrophoblast (Janckila *et al.*, 1996).

Studies on the LMW AcPase activity towards both P-Tyr and P-Ser containing peptide substrates shed some light on its possible function as a protein phosphatase and its relationship to other enzymes displaying such an activity. Despite the apparent similarities in the general features between the frog enzyme and the catalytic subunits of the Ser/Thr phosphatases of the PPP family, these proteins are not homologous. This can be concluded from: i: a lack of the activity towards ^{32}P-phosphorylase *a* in the standard reaction for the enzymes of PPP family, ii: resistance to okadaic acid and F^- ions, specific inhibitors of the PPP enzymes, PP1 and PP2A, and iii: a lack of immunological relationship between LMW AcPase and eukaryotic PP2Ac proved with two kinds of antibodies, especially with those directed against C-terminal conserved fragment of PP2Ac. Since C-terminal sequence of PP2Ac is conserved from yeast to man (Wera & Hemmings, 1995), the negative reaction with the antibodies recognizing this fragment excludes the frog enzyme from this class of proteins.

Although the range of substrates tested is insufficient for precise definition of the physiological role of the frog enzyme, nevertheless its considerable activity towards two different P-Tyr phosphorylated peptides, susceptibility to typical inhibitors of protein tyrosine phosphatase (vanadate, molybdate and tungstate) as well as very high affinity towards P-Tyr phosphorylated natural substrate hirudin (K_m 2–3 orders of magnitude lower than the K_m values for other substrates) and very high efficiency of its hydrolysis compared with other

substrates tested, support our earlier suggestion that this enzyme can function as a phosphotyrosine phosphatase *in vivo*. In this respect LMW AcPase of frog liver seems to resemble TRAPs since for most human and animal enzymes belonging to this class PTPase activity was demonstrated (Lau *et al.*, 1987; Vincent *et al.*, 1990; Janckila *et al.*, 1992; Nuthmann *et al.*, 1993; Fleckenstein & Drexler, 1997; Hallen *et al.*, 1998; Oddie *et al.*, 2000). It is noteworthy that although some of TRAPs are able to hydrolyse phosphopeptides also in neutral conditions, e.g., the enzyme from bovine cortical bone matrix (Lau *et al.*, 1987) or human hairy cells (Janckila *et al.*, 1992), thus differing from the requirements for the frog enzyme, nevertheless their pH optima are usually at pH 5.0–6.0 due to their lysosomal localization (Hallen *et al.*, 1998; Hayman *et al.*, 2000; Oddie *et al.*, 2000).

The LMW AcPase is also able to process phosvitin with a higher affinity than pNPP or P-Tyr (lower K_m value), but the efficiency of this reaction is the lowest among all substrates tested. The ability to dephosphorylate Ser and Thr residues in peptidic substrates seems to differ among the TRAP enzymes and probably depends also on the kind of substrate. The activity towards P-Ser or P-Thr residues in proteins was also reported for a few TRAP enzymes, especially those from rat and bovine bone and from human Gaucher spleen (Vincent *et al.*, 1990; Hallen *et al.*, 1998; Oddie *et al.*, 2000 and references therein).

Like other acid phosphatases, the frog liver LMW AcPase is inhibited by tetrahedral oxyanions. Inhibition studies revealed that all oxyanions tested (i.e., vanadate, molybdate and tungstate) inhibit pNPP hydrolysis in a competitive fashion, being far more effective inhibitors than P_i. Thus, one can conclude that there is a single oxyanion binding site in the frog enzyme. On the one hand, these results indicate that the mechanism of phosphate esters hydrolysis is common for LMW AcPase and TRAPs as tetrahedral oxyanions act as transition state analogues. On the other hand, the presence of a single tetrahedral oxyanion binding site differentiates the frog enzyme from the mammalian ones for which the noncompetitive or mixed character of inhibition suggest a presence of multiple binding sites for these inhibitors (Vincent *et al.*, 1991; Crans *et al.*, 1992; Vincent *et al.*, 1992).

In our earlier paper (Szalewicz *et al.*, 1999a) we have presented the data indicating that the frog liver LMW AcPase is a novel metallophosphatase different from those belonging to the class of mammalian TRAPs. The new data presented in this paper allow us to extend our comparison. We have found that the LMW AcPase shares with TRAPs the same reaction mechanism and similar activity towards phosphopeptides but being a potential PTPase it differs from them in cellular localization, and is therefore probably involved in other metabolic processes. LMW AcPase is a new example of the enzyme of lower vertebrate origin that may act as a PTPase *in vivo*.

The authors wish to express their gratitude to the late Professor Stanisław Żołnierowicz for his helpful advice and discussion as well as for kindly providing materials for the experiments on the Ser/Thr protein phosphatase and the immunological relationship between the frog LMW AcPase and eukaryotic PP2Ac. We are indebted to Professor Zofia Szyfter for her kind help in interpretation of the results on the immunochemical localization of the enzyme. We also thank the Department of Animal Physiology, Faculty of Veterinary Medicine, the Agricultural Academy of Wrocław, for kindly providing the material for the experiments.

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