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Porcine Liver Low M_r Phosphotyrosine Protein Phosphatase: The Amino Acid Sequence

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Porcine low M_r phosphotyrosine protein phosphatase has been purified and the complete amino acid sequence has been determined. Both enzymic and chemical cleavages are used to obtain protein fragments. FAB mass spectrometry and enzymic subdigestion followed by Edman degradation have been used to determine the structure of the NH_2-terminal acylated tryptic peptide. The enzyme consists of 157 amino acid residues, is acetylated at the NH_2-terminus, and has arginine as COOH-terminal residue. It shows kinetic parameters very similar to other known low M_r PTPases. This PTPase is strongly inhibited by pyridoxal 5'-phosphate ($K_i = 21\ \mu M$) like the low M_r PTPases from bovine liver, rat liver (AcP2 isoenzyme), and human erythrocyte (Bslow isoenzyme). The comparison of the 40–73 sequence with the corresponding sequence of other low M_r PTPases from different sources demonstrates that this isoform is highly homologous to the isoforms mentioned above, and shows a lower homology degree with respect to rat AcP1 and human Bfast isoforms. A classification of low M_r PTPase isoforms based on the type-specific sequence and on the sensitivity to pyridoxal 5°-phosphate inhibition has been proposed.

KEY WORDS: Phosphotyrosine protein phosphatase; acid phosphatase; phosphatase sequence.

1. INTRODUCTION[3]

Low M_r phosphotyrosine protein phosphatase (EC 3.1.3.48) was previously known as low M_r acid phosphatase (EC 3.1.3.2), since it displays an optimum *p*H in the acidic range when low M_r substrates (such as *p*-nitrophenyl phosphate and L-phosphotyrosine) were used. Starting from 1985, various authors demonstrated that the enzyme possesses phosphatase activity vs. Tyr-phosphorylated protein substrates (Chernoff and Lee, 1985; Boivin and Galand, 1986; Waheed *et al.*, 1988; Ramponi *et al.*, 1989; Shekels *et al.*, 1992), whereas it shows no activity on Ser/Thr-phosphorylated proteins (Ramponi *et al.*, 1989). Recent studies on the reaction mechanism of the enzyme demonstrated that the enzyme forms a transient phosphorylated covalent complex during the catalytic process and that the hydrolysis of this complex is the rate-limiting step of the process (Zhang and Van Etten, 1991, 1992). Phosphoenzyme covalent intermediate trapping experiments enable the Van Etten group to demonstrate that a thiol-phosphate linkage is present in the phosphoenzyme intermediate (Wo *et al.*, 1992). Using phosphoenzyme trapping and site-directed mutagenesis, we were able to demonstrate that Cys12 is the residue that performs the nucleophilic

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[3] Abbreviations used: PTPase, phosphotyrosine protein phosphatase; TFA, trifluoroacetic acid; SDS, sodium dodecylsulfate; T, tryptic peptides; SP, endoproteinase Glu-C peptides; FAB, fast atom bombardment; Ac, acetyl; HPLC, high-performance liquid chromatography; OPA, *o*-phtaldialdehyde; PMSF, phenylmethylsulfonyl fluoride; CD45, leukocyte common-antigen PTPase; LAR, leukocyte-antigen-related PTPase; PTP 1B, human placental PTPase.

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attack at the phosphorus atom of the substrate to form the phosphoenzyme covalent intermediate (Cirri *et al.*, 1993*a*). In addition, we demonstrated that both Cys17 and Arg18 participate to the active site of the enzyme, since they are involved in the binding of the phosphate moiety of substrates and phosphorylated inhibitors during the formation of the enzyme-substrate and enzyme-inhibitor complexes (Cirri *et al.*, 1993*a*, *b*). The above results enable us to identify a continuous sequence involved in the catalysis located in the 12–18 polypeptide stretch. This includes the active site residues Cys12, Cys17, and Arg18. A similar active site motif (CXXXXXR) was found in all PTPase family members (Cirri *et al.*, 1993*a*); also, in the main family, the Cys and Arg residues are essential for activity. No general homology between this enzyme and the members of the general PTPase family was observed. This suggests that this particular enzyme should be considered as a distantly related member of this continuously growing family, which contains both receptor-like (CD45, LAR) and nonreceptor-like (PTP 1B, cdc25 gene products) PTPases. PTPase are thought to antagonize the action of phosphotyrosine protein kinases in the cell. These last enzymes are involved in growth signal transduction, since they are cytoplasmic domains of growth factor receptors. Altered forms of phosphotyrosine protein kinases are products of several oncogenes, both related (such as v-erbB) and nonrelated (such as src) to growth factors.

The present paper describes the purification procedure and reports the amino acid sequence determination of the porcine liver low M_r PTPase.

2. EXPERIMENTAL PROCEDURES

Porcine liver was obtained from the local slaughterhouse. The reagents used for Edman degradation and derivatized polyvinylidene difluoride membranes (Sequelon-AA and Sequelon-DITC) were purchased from Milligen. *p*-Nitrophenyl phosphate was from SIGMA. BNPS-skatole and *p*-aminobenzylphosphonate-agarose were purchased from Pierce. All other reagents were of analytical grade or the best commercially available.

2.1. Activity Determination

Enzyme activity was determined at *p*H 5.5 and 37°C using *p*-nitrophenyl phosphate as substrate. The substrate was dissolved in 0.1 M acetate buffer, *p*H 5.5, containing 1 mM EDTA. The final volume of the test was 1.0 ml. The assay was started by adding aliquots of enzyme solution (5–100 μl) and blocked by adding 4 ml of 0.1 M KOH at the chosen incubation time. Similar tests without the enzyme were used as controls for spontaneous substrate hydrolysis. The released *p*-nitrophenolate ion was measured spectrophotometrically, using the extinction coefficient $\varepsilon_{400} = 18{,}000\ M^{-1}\,cm^{-1}$. The unit is defined as the amount of enzyme that hydrolyzes 1 μmol of *p*-nitrophenyl phosphate per min. Specific activity is expressed as units per mg of protein.

2.2. Protein Determination

In tissue and ammonium precipitate extracts, protein concentration was assayed by the biuret method (Beiseherz *et al.*, 1953). Bovine serum albumin was used as standard protein. In column effluents, protein concentration was estimated spectrophotometrically at 280 nm.

2.3. Enzyme purification

Step 1. Porcine liver (3 kg) was minced and then homogenized (using a Waring blender) with 3 volumes of 0.3 M acetate buffer, *p*H 5.0, containing 1 mM EDTA, 2 mM 2-mercaptoethanol, and 0.1 mM PMSF. The enzyme was extracted for 1 hr with stirring and then the homogenate was centrifuged at 1600 *g* for 1 hr. The precipitate was discarded, and the supernatant was collected for the next step.

Step 2. Ammonium sulfate (29% saturation) was slowly added. After 30 min from the complete dissolution of the salt, the mixture was centrifuged at 1600 *g* for 1 hr and the precipitate was discarded. The supernatant was brought to 60% saturation with ammonium sulfate. The protein precipitate was collected by centrifugation at 1600 *g* for 1 hr, dissolved in 1.3 l of 0.01 M acetate buffer, *p*H 5.0, containing 1 mM EDTA, 2 mM 2-mercaptoethanol, and 0.1 mM PMSF. This solution was dialyzed against 15 volumes of 0.01 M acetate buffer, *p*H 5.1, containing 0.1 M ammonium sulfate, 1 mM EDTA, 2 mM 2-mercaptoethanol, and 0.1 mM PMSF.

Step 3. The dialysate was centrifuged at 7000 *g* for 40 min to eliminate the precipitate formed during the dialysis. The supernatant was applied to

a column (8 × 40 cm) of SP-Sephadex C50 equilibrated with the dialysis buffer. The column was washed with the same buffer at a flow rate of 350 ml/hr until the absorbance at 280 nm was less than 0.1. During washing, about 60% of the *p*-nitrophenyl phosphatase activity present in the dialysate from step 2 was eluted. Most of this phosphatase activity is strongly inhibited by tartrate; this suggests that this *p*-nitrophenyl-phosphatase activity is due to high M_r acid phosphatases such as that present in the lysosomes. The low M_r enzyme was then eluted at a flow rate of 120 ml/hr with a 0.3 M Pi solution adjusted to *p*H 5.1 and containing 1 mM EDTA, 2 mM 2-mercaptoethanol, and 0.1 mM PMSF. Fractions of 20 ml were collected (see Fig. 1A). The enzyme-containing fractions were pooled, and the phosphatase was precipitated by salting-out (70% saturation of ammonium sulfate). After standing 12 hr at 4°C, the precipitate was collected by centrifugation at 24,000 *g* for 50 min and then dissolved in 20 ml of 0.1 M sodium citrate buffer, *p*H 6.5, containing 1 mM EDTA and 1 mM dithiothreitol, and dialyzed against the same buffer.

Step 4. The dialysate was applied to a small affinity chromatography column (1 × 12 cm) containing *p*-aminobenzylphosphonate-agarose. The foreign proteins were washed out with the same buffer and the enzyme was then eluted with a linear Pi gradient (100 ml of 0.1 M citrate buffer containing 1 mM EDTA and 1 mM dithiothreitol and 100 ml of 0.1 M Pi in the same buffer). Fractions of 4 ml were collected at a flow rate of 18 ml/hr. Figure 1B shows the chromatogram.

2.4. Carboxymethylation of the Enzyme

The enzyme was carboxymethylated using the method previously described (Manao *et al.*, 1992).

2.5. Amino Acid Analysis

Amino acid analysis was performed on 6 M HCl hydrolysate using a Carlo Erba 3A29 Amino Acid Analyzer equipped with a computing integrator. The hydrolysis was carried out in vacuum-sealed vials at 110°C for 20 and 48 hr for protein samples, and 20 hr for peptide samples. Tryptophan was determined according to Edelhoch (1967) or to Penke *et al.* (1974). Cysteine was determined as carboxymethyl-cysteine.

2.6. Enzymic Hydrolysis and Chemical Cleavages

Endoproteinase Glu-C and trypsin hydrolyses were performed as previously described (Cappugi *et al.*, 1980), except that the protein digestion with the endoproteinase Glu-C was carried out in the presence of 1 M guanidinium chloride. CNBr cleavage was carried out as described by Pazzagli *et al.* (1993). BNPS-Skatole cleavage was performed as described by Manao *et al.* (1992). Carboxypeptidase B digestion was carried out as previously described (Camici *et al.*, 1989).

2.7. Sequence Determination

The sequence of peptides was determined using both manual and automatic Edman degradation. The Milligen Protein Sequencer, which operates solid-phase Edman degradation on peptides covalently linked to special membranes (Sequelon-DITC or Sequelon-AA), was used. In some cases, both membrane types were applied contemporaneously to the sequencer reactor.

2.8. FAB Mass Spectrometry

FAB mass spectra were obtained using a VG Analytical 11-250J instrument.

3. RESULTS AND DISCUSSION

Table I summarizes the results of the purification procedure. The enzyme was purified 2632-fold from the extract. It shows a specific activity of 100 units per mg of protein. The SDS-polyacrylamide gel electrophoresis analysis showed a main band with a molecular weight of 18 kD and an additional band at about 14 kD that account for about 10% of total protein. The 14 kD band was separated from the 18 kD enzyme using reversed-phase HPLC. Figure 1C shows the chromatogram. Sequence analysis trials performed both before and after CNBr treatment demonstrated that this 14 kD band is a fragment of the enzyme that lost about 30 residues at the N-terminus. This was probably originated through protoelysis during the purification of the enzyme. Thus, the sequence determination presented in this paper refers to the 18 kD band which corresponds to the entire enzyme. The main kinetic parameters of the porcine liver low M_r PTPase are reported in Table II. The found values of K_m and V_{max} relative

Table I. Purification of Low M_r PTPase from Porcine Liver (3 kg)

Step	Volume (ml)	Protein (mg)	Activity (U)	Specific activity (U/mg)	Yield (%)
1. Tissue extract	7350	191,100	7350	0.036	100
2. Ammonium sulfate	2050	69,085	5119	0.070	69.6
3. SP-Sephadex C50	600	240	762	3.20	10.3
4. Affinity chromatography	24.5	4.8	480	100	6.5

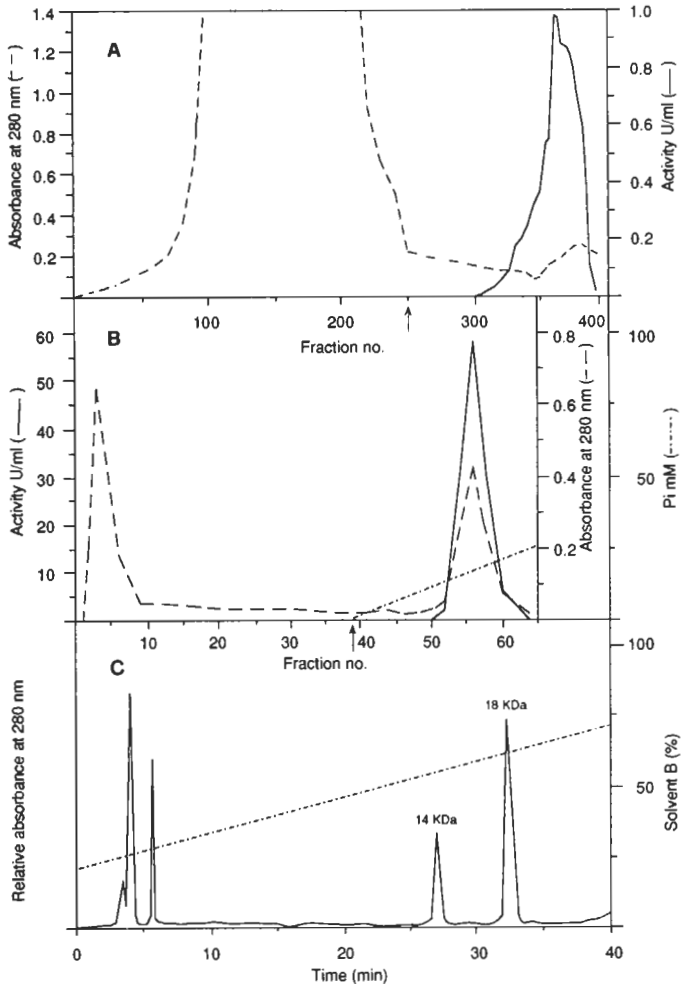

Fig. 1. (A) SP-Sephadex C50 chromatography. The column (8 × 40 cm) was eluted at a flow rate of 120 ml/hr and 20 ml fractions were collected. The arrow indicates the starting of elution with 0.3 M Pi. The acid phosphatase activity eluted during the washing is not reported. (B) Affinity chromatography on *p*-aminobenzylphosphonate-agarose. The column (1 × 12 cm) was washed and eluted at a flow rate of 18 ml/hr. The arrow indicates the starting of the Pi-gradient elution. (C) HPLC separation of the enzyme from the 14 kD impurity (see text). Column: Vydac Protein and Peptide C18, 4.6 × 250 mm, 5 μm. Solvent A: 10 mM TFA in water; solvent B: 10 mM TFA in acetonitrile. Flow rate, 0.8 ml/min (——), absorbance at 280 nm; (- - - - - -), elution gradient.

Table II. Some Kinetic Parameters of Porcine Liver Low M_r PTPase

	K_m (mM)	V_{max} (units/mg)	[a]K_i (mM)
p-Nitrophenyl phosphate	0.24	105	
Pyridoxal 5′-phosphate			0.021
Inorganic phosphate			1.4

[a] Competitive.

Table III. Amino Acid Composition of the Carboxymethylated Low M_r PTPase from Porcine Liver

Amino acid		Sequence data
Cm-cysteine	8.4	8
Aspartic acid	21.9	23
Threonine	5.7	6
Serine	10.6	11
Glutamic acid	16.5	16
Proline	4.8	5
Glycine	6.0	6
Alanine	10.4	10
Valine	13.5	14
Methionine	0.8	1
Isoleucine	9.4	9
Leucine	10.8	11
Tyrosine	4.7	5
Phenylalanine	5.9	6
Lysine	9.1	9
Histidine	3.2	3
Arginine	12.0	12
Trpyptophan	1.8	2
NH_2-terminus (Edman)	None	
COOH-terminus (carboxypepdidase B)	Arginine	

The results are expressed as residues per molecule of enzyme. Values for Thr and Ser were determined by hydrolysis at 110°C for 20 and 70 hr in duplicate and extrapolated to zero time. Tryptophan was determined according to Edelhoch (1967). The value for Ile was that calculated in the 70 hr hydrolysate.

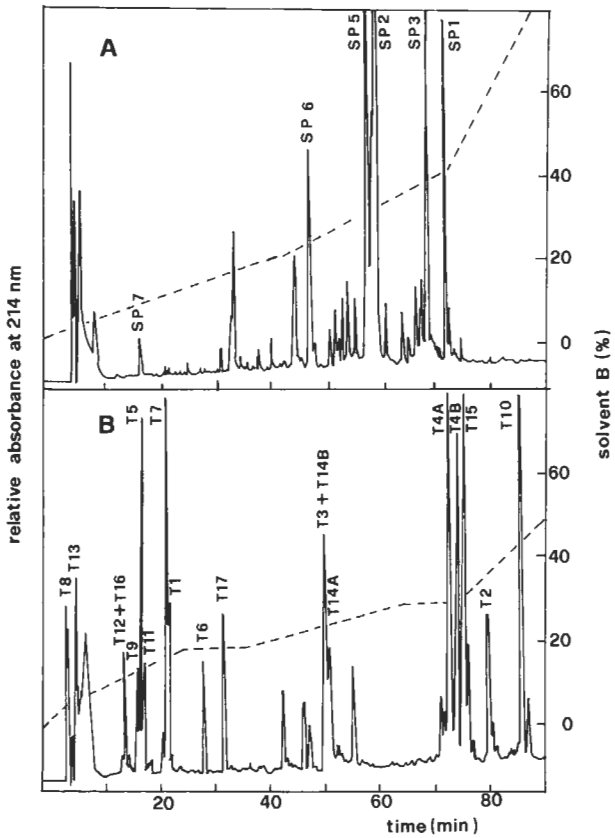

Fig. 2. The HPLC separations of endoproteinase Glu-C (A) and tryptic (B) peptides. Column: Vydac Protein and Peptide C18, 5 μm, 4.6 × 250 mm. Solvent A: 10 mM TFA in water; solvent B: 10 mM TFA in acetonitrile. Flow rate, 0.8 ml/min. (——), absorbance at 214 nm; (- - - - - -), elution gradient.

to *p*-nitrophenyl phosphate and the K_i value relative to Pi are very close to those determined for other known low M_r PTPases (Cirri *et al.*, 1993*b*). On the other hand, the K_i values relative to pyridoxal 5′-phosphate (21 μM) clearly indicate that the low M_r PTPase from porcine liver can be classified as type-2 isoenzyme, since these molecular forms have very high affinity vs. this compound (Cirri *et al.*, 1993*b*). Type-1 isoenzymes, like AcP1 from rat liver and Bfast from human erythrocytes, show k_i values for pyridoxal 5′-phosphate about two orders of magnitude higher than those of type-2 isoenzymes. We emphasize that type-1 and type-2 isoenzymes from the same species differ only in the 40–73 zone, where a type-specific sequence is present (Dissing *et al.*, 1991; Manao *et al.*, 1992). Table III reports the amino acid composition and the terminal residue analyses of the enzyme. The HPLC separations of tryptic and endoproteinase Glu-C peptides are reported in Fig. 2A and B, respectively. Tables IV and V report the amino acid composition of tryptic and endoproteinase Glu-C peptides, respectively. The amino acid sequence of the T1 peptide, which is N-acetylated, was obtained as follows: i) the amino acid composition was determined; ii) the peptide was cleaved with the endoproteinase Glu-C at the level of the second residue, and the post-Glu sequence was determined by the Edman degrada-

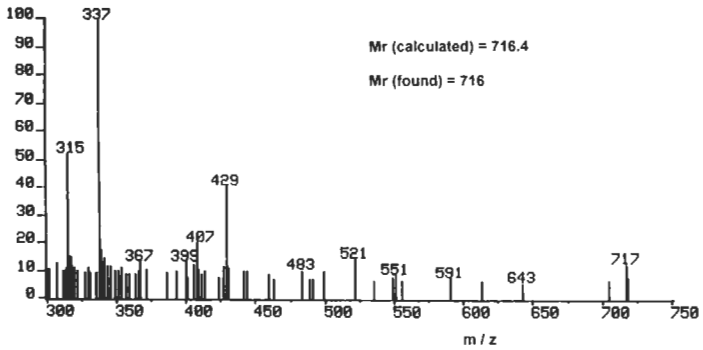

Fig. 3. Positive FAB mass spectrum of the T1 peptide.

tion; iii) the FAB-mass spectrum reported in Fig. 3 demonstrates that the acyl-blocking group is an acetyl, since an M_r of 716 was deduced from the observed protonated molecular ion ($M_r = 717$). All these data indicate the sequence Ac-Ala-Glu-Gln-Val-Thr-Lys. The sequence of the SP1 peptide (Fig. 4), which contains a Glu-Gln bond not cleaved during the protein digestion, was newly digested using a higher enzyme/substrate ratio with respect to that used in the first digestion. The second digestion enabled us to cleave that bond and therefore the post-Glu2 sequence was determined using the Protein Sequencer. The post-Met sequence was obtained by treating a sample of enzyme with CNBr; in this case the separation of fragments was not performed, since the enzyme contains a single Met residue and, in addition, is N-acylated. Thus, the CNBr-cleaved peptide mixture was bound to the Sequelon membranes and directly applied to the Sequencer reactor. A clear unique sequence was obtained (see Fig. 4). Also, in the case of BNPS-skatole cleavage, the fragments were not separated from each other. The mixture was covalently bound to Sequelon membranes (both AA- and DITC-); then five Edman degradation steps were performed. The membranes were then

Table IV. Amino Acid Composition of Tryptic Peptides

	T1	T2	T3	T4A	T4B	T5	T6	T7	T8	T9	T10	T11	T12	T13	T14A	T14B	T15	T16	T17
Cm-Cys		2.3					0.7				0.9			0.9			1.1	1.9	
Asp		1.1		6.7	6.7	1.0		1.1			4.3	2.0	1.0	1.0	1.0	1.0	4.4		
Thr	1.1			1.0	1.0			1.1		0.9	1.1								
Ser		1.0	0.9	3.9	3.8	0.9	1.2				1.0				1.1	1.2	1.2		
Glu	2.2		1.1	1.3	1.2					1.1	2.3		1.1		2.3	2.2	5.3		1.2
Pro			1.1			2.1													
Gly		0.9		1.1	1.0			1.0					1.1		0.9	1.1	1.0		
Ala	1.1		1.9	1.1	1.0		1.0	1.1	1.0		1.0				1.1		1.1		1.1
Val	1.1	1.8	0.9	4.6	4.8		0.8						0.9				2.1		
Met											0.7								
Ile		0.9	1.0	0.9	1.0			0.9		1.1	1.0				1.0	0.9	1.6		
Leu		2.0		1.1	1.0		1.3				2.3	1.1			1.8	2.0	1.0		1.0
Tyr											1.0				0.8	0.9	2.6		
Phe		0.8	0.9								1.9						0.8		0.9
Lys	1.0			1.0				1.0		1.0			1.0		1.7	1.0			1.0
His								2.9											
Arg		1.0	1.0	1.0	1.0	1.0	1.0		1.0		1.0	1.0		1.0			1.0	1.0	
Trp				1.9	1.8														
Position	1 ↓ 6	7 ↓ 18	19 ↓ 27	28 ↓ 53	29 ↓ 53	54 ↓ 58	59 ↓ 64	65 ↓ 73	74 ↓ 75	76 ↓ 79	80 ↓ 97	98 ↓ 101	103 ↓ 107	108 ↓ 110	111 ↓ 123	113 ↓ 123	124 ↓ 147	148 ↓ 150	151 ↓ 155
Yield (%)	80	46	38	37	19	81	37	63	66	62	56	88	42	57	42	37	63	51	81

[a] The digestion was performed on 10 nmol of carboxymethylated enzyme. The values are expressed as molar ratio. Values for Ser and Thr were corrected for 15% and 5% destruction, respectively. Yield was calculated as moles obtained from moles of digested protein.

Table V. Amino Acid Composition of Endoproteinase Glu-C Peptides

	SP1	SP2	SP3	SP5	SP6	SP7
Cm-Cys	2.1	1.2	1.2		3.2	
Asp	1.1	8.7	2.7	4.9		
Thr	1.0	3.0	0.8			
Ser	1.8	5.7		1.8		
Glu	3.3	3.2	1.0	3.7	3.1	
Pro	0.9	1.9		2.1		
Gly	1.1	2.2		1.8		
Ala	2.0	4.9	0.9		2.1	
Val	2.7	6.7			1.9	0.9
Met			0.7			
Ile	2.0	2.9	0.9	1.7		
Leu	2.0	2.0	1.0	3.0	1.1	
Tyr			0.8	2.8	0.9	
Phe	1.0	1.3	1.7	1.0	1.0	
Lys	1.2	2.9		1.0		1.0
His		3.0				
Arg	1.0	4.7			1.9	1.0
Trp		1.8				
Position	1 ↓ 23	24 ↓ 80	81 ↓ 93	115 ↓ 139	140 ↓ 154	155 ↓ 157
Yield (%)	52	20	81	67	74	63

[a] The digestion was performed on 15 nmol of carboxymethylated-enzyme. Details are the same as in Table IV.

removed from the sequencer and treated for 15 min at room temperature with 200 μl of 0.4 M potassium borate buffer, *p*H 10.4, containing 0.08% (w/v) *o*-phtaldialdehyde and 0.2% (v/v) 2-mercaptoethanol. Then the membranes were washed twice with 50% ethanol and methanol. This treatment caused the derivatization of all primary amino groups, whereas the secondary amino groups (Pro) remained available for Edman degradation. Successively, the membranes were newly applied to the Sequencer reactor and an unique sequence, starting from Pro55, was obtained (see Fig. 4). All sequence results are reported in Fig. 4, which shows the reconstructed amino acid sequence of the enzyme. This consists of 157 residues, like similar enzymes from different species previously sequenced (Manao *et al.*, 1992). All eight cysteines are conserved. We previously demonstrated that all are in the free thiol form. Also His66 and His72, which are indicated as active site residues, are conserved. Comparison of this sequence with those previously known (Fig. 5) clearly indicates a high conservation of the enzyme in the mammalian species. The figure presents a classification of the known low M_r PTPases. This classification arises from the presence of two isoforms of this particular enzyme in mammalian tissues; they have been recently isolated both from human erythrocytes and from rat liver and their complete amino acid sequence has been determined (Dissing *et al.*, 1991; *Manao et al.*, 1992). In both species, the isoforms, which are probably originated from a single gene through an alternative splicing mechanism, differ exclusively in the sequence 40–73 that is isoform-specific. In this alternative zone all the porcine liver, the rat liver AcP2, the human erythrocyte Bslow, and the bovine liver isoenzymes have the common 48–53 sequence motif DWNVGR. On the contrary the rat liver AcP1 and the human erythrocyte Bfast isoenzymes have the 48–53 sequence XYEXGN. Other amino acids, such as Arg40 and Met63, are present only in this latter isoform. Thus, AcP1 and Bfast isoenzymes are classified as type-1, while all other forms are classified as type-2. This classification is justified also by the high affinity of type-2 isoenzymes for pyridoxal 5'phosphate as shown above. To date, type-1 isoforms have not been isolated from bovine and porcine tissues, but their presence in these species seems to be indicated by electrophoretic zymograms (unpublished results). This suggests that the contemporaneous expression of both isoform types in mammalian tissues is a general rule. At present, the significance of the expression of both types in mammalian tissues is not understood; the different sensitivity to activity regulators, such as pyridoxal 5'-phosphate (inhibitor of type-2) and purine-derivatives (activator of type-2), suggests that the physiological function of the two types is different (Manao *et al.*, 1992).

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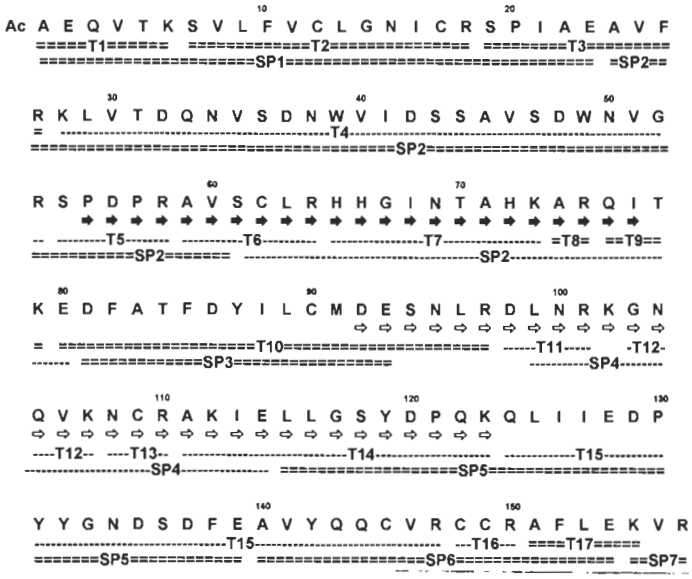

Fig. 4. The primary structure of the porcine liver low M_r PTPase. T, tryptic peptides; SP, endoproteinase Glu-C peptides; Ac, acetyl. →, This symbol indicates the Edman degradation results obtained on the BNPS-skatole cleaved protein. The cleavage products were submitted to five cycles of Edman degradation and then treated with the OPA-2-mercaptoethanol reagent to derivatize the primary amino groups. The symbol (⇨) indicates the sequence results obtained on the CNBr-cleaved protein. The symbol (=) was used to indicate the extent of the determined amino acid sequence on T and SP peptides. Dashed lines indicate sequence information which was inferred from the amino acid composition of the peptide and from data on sequence analysis of other peptides.

Fig. 5. Alignment of low M_r PTPases. Bfast and Bslow are from human erythrocytes [Dissing *et al.* (1991); Manao *et al.* (1992)]. AcP1 and AcP2 are from rat liver [Manao *et al.* (1992)]. Bovine PTPase [Camici *et al.* (1989)].

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