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Rat Liver Low M_r Phosphotyrosine Protein Phosphatase Isoenzymes: Purification and Amino Acid Sequences

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Two low M_r phosphotyrosine protein phosphatases have been isolated from rat liver. The enzymes were previously known as low M_r acid phosphatases, but several recent studies have demonstrated that this family of enzymes possesses specific phosphotyrosine protein phosphatase activity. We determined the complete amino acid sequences of the two isoenzymes and named them AcP1 and AcP2. Both consist of 157 amino acid residues, are acetylated at the NH₂-terminus, and have His as the COOH-terminus. The molecular weights calculated from the sequences are 18,062 for AcP1 and 17,848 for AcP2. They are homologous except in the 40–73 zone, where about 50% of residues are different. This fact suggests that the two isoenzymes are produced by an alternative splicing mechanism. There is no homology between these two isoenzymes and the receptor-like phosphotyrosine protein phosphatases LAR, CD45, human placenta PTPase 1B, and rat brain PTPase-1. AcP1 and AcP2 are also distinct from rat liver PTPase-1 and PTPase-2, since these last enzymes have higher molecular weights. AcP1 differs from AcP2 with respect to (1) substrate affinity and (2) its sensitivity to activators and inhibitors, thus suggesting a their different physiological function.

KEY WORDS: Phosphotyrosine protein phosphatases; acid phosphatase; phosphatase isoenzymes.

1. INTRODUCTION⁴

Protein phosphorylation on tyrosine residues is associated with receptor activity and cellular growth modifications. Alterations of the tyrosine phosphorylation pattern can lead to uncontrolled cell growth and

transformation (Yarden and Ulrich, 1988; Gould and Nurse, 1989; Hunter and Cooper, 1985, 1986). The level of protein tyrosine phosphorylation is the result of a balance between Tyr-protein kinase and phosphotyrosine protein phosphatase (PTPase) activities. Two families of PTPases have been described. The first is a receptor-like family with an extracellular domain, transmembrane spanning region, and cytoplasmic phosphatase domain(s), while the second consists of nonreceptorlike phosphatases with high specificity for tyrosine phosphate.

Various low M_r cytosolic acid phosphatases (EC 3.1.3.2) have intrinsic PTPase activity. Chernoff and Lee (1985) first demonstrated that a specific phosphotyrosine protein phosphatase activity, present in the heart, was associated with a low M_r acid phosphatase activity. Various authors demonstrated that this class of enzymes shows PTPase activity. Boivin and Galand (1986), Waheed *et al.* (1988), Ramponi

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⁴ Abbreviations used: PTPase, phosphotyrosine protein phosphatase; AcP, rat liver low M_r phosphotyrosine protein phosphatase; AP, acid phosphatase; SDS, sodium dodecylsulfate; PAGE, polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; LC, endoproteinase Lys-C peptides; AN, endoproteinase Asp-N peptides; SP, *Staphylococcus aureus* SPV8 protease peptides; TH, thermolysin peptides; TMA, trimethylamine; PMSF, phenylmethanesulfonylfluoride; DTT, dithiothreitol; FAB, fast atom bombardment; Ac, acetyl; Cm, carboxymethyl.

et al. (1989), and Zhang and Van Etten (1990) have all demonstrated that pure or highly purified preparations of low *M_r* cytosolic enzymes show dephosphorylating activity on Tyr-phosphorylated proteins. These enzymes are not able to dephosphorylate Ser/Thr phosphorylated proteins.

Low *M_r* cytosolic acid phosphatases have been isolated from many different sources (Waheed *et al.*, 1988; Zhang and Van Etten, 1990; Lawrence and Van Etten, 1981; Taga and Van Etten, 1982; Saeed *et al.*, 1990); recently, the amino acid sequence of the bovine liver enzyme has been reported (Camici *et al.*, 1989). It shows no homology with other known acid phosphatases or phosphotyrosine protein phosphatases related to PTPase 1B from human placenta, LAR, or CD45 (Tonks *et al.*, 1989). This enzyme possesses a peculiar substrate specificity; in fact, it catalyzes the hydrolysis of aryl phosphates (Taga and Van Etten, 1982; Henrikson, 1969) efficiently, while, on the other hand, aliphatic phosphate monoesters are poor substrates with the unique exception of flavin mononucleotide (Taga and Van Etten, 1982; Henrikson, 1969).

In addition, this isoenzyme efficiently catalyzes the hydrolysis of acyl phosphate such as carbamoyl phosphate, acetyl phosphate as well as synthetic acyl phosphates, such as benzoyl phosphate (Taga and Van Etten, 1982; Ramponi *et al.*, 1980). Furthermore, it has a specific PTPase activity (Ramponi *et al.*, 1989).

The existence of multiple molecular forms of low *M_r* cytosolic acid phosphatase was demonstrated in some tissues [e.g., avian pectoral muscle (Baxter and Suelter, 1985), human erythrocytes (Dissing and Svensmark, 1990; Dissing *et al.*, 1991), and rat liver (Fujimoto *et al.*, 1988)].

This paper reports (1) an original purification procedure of two low *M_r* rat liver isoenzymes (both having phosphotyrosine protein phosphatase activity), and (2) the determination of their complete amino acid sequences. Our results demonstrate that both isoenzymes, which consist of 157 amino acid residues, are acetylated at the NH₂-terminus and show histidine as the COOH-terminal residue. Nevertheless, they are different both structurally and in their properties.

2. EXPERIMENTAL PROCEDURES

Rat liver was frozen at -20°C and then brought to 4-5°C before use. Endoproteinase Lys-C (sequencing grade) and endoproteinase Asp-N (sequencing grade) were obtained from Boehringer. *S. aureus* V8

protease was obtained from Miles. Pierce supplied BNPS-skatole. Sequencer solvents and reagents were provided by Milligen. All other reagents were of analytical grade or the best commercially available one. HPLC columns: Aquapore RP300 (4.6 × 250 mm, 7 μm) was obtained from Brownlee; Vydac protein and peptide C₁₈ column was obtained from Vydac.

2.1. Purification of AcP1 and AcP2

Step 1. Rat liver (1 kg) was minced and then homogenized with 3 vol of 0.3 M acetate buffer, pH 5.0, containing 1 mM EDTA, 2 mM 2-mercaptoethanol, and 0.1 mM PMSF. The homogenate was centrifuged at 2800g for 30 min and the supernatant was collected for the next step.

Step 2. Solid ammonium sulfate to 29% saturation was slowly added. After standing for 30 min, the precipitate was collected by centrifugation at 2800g for 50 min, and discarded.

The supernatant was brought to 60% ammonium sulfate saturation and the precipitate was collected by centrifugation at 2800g for 60 min. The precipitate was then dissolved in 500 ml of 0.01 M acetate buffer, pH 5.1, containing 1 mM EDTA, 2 mM 2-mercaptoethanol, and 0.1 mM PMSF, and the solution was dialysed overnight against the same buffer.

Step 3. The dialyzate was clarified by centrifugation at 10,000g for 30 min and the supernatant was applied to a SP-Sephadex C50 column (8 × 40 cm) equilibrated with 0.01 M acetate buffer, pH 5.1, containing 1 mM EDTA, 2 mM 2-mercaptoethanol, and 0.1 mM PMSF. The column was washed with the same buffer until the effluent optical density at 280 nm was less than 0.1; the enzyme was then eluted with 0.3 M Pi adjusted to pH 5.5, containing 1 mM EDTA, 2 mM 2-mercaptoethanol, and 0.1 mM PMSF. During column washing acid phosphatase activity (lysosomal) was eluted. Only active fractions eluted by Pi were pooled for the next step. The enzyme was precipitated by adding solid ammonium sulfate to 70% saturation and collected by centrifugation at 16,000g for 30 min. The precipitate was dissolved in 10 ml of 0.01 M acetate buffer, pH 4.9, containing 1 mM EDTA and 1 mM DTT.

Step 4. The enzyme solution from step 3 was applied to a Sephadex G 75 column (5 × 100 cm) equilibrated with 0.1 M acetate buffer, pH 5.5, containing 1 mM EDTA and 1 mM DTT. The column was eluted with the same buffer at a flow rate of 50 ml/hr and 12 ml fractions were collected. Figure 1A reports the elution profile. Active fractions were pooled, and the

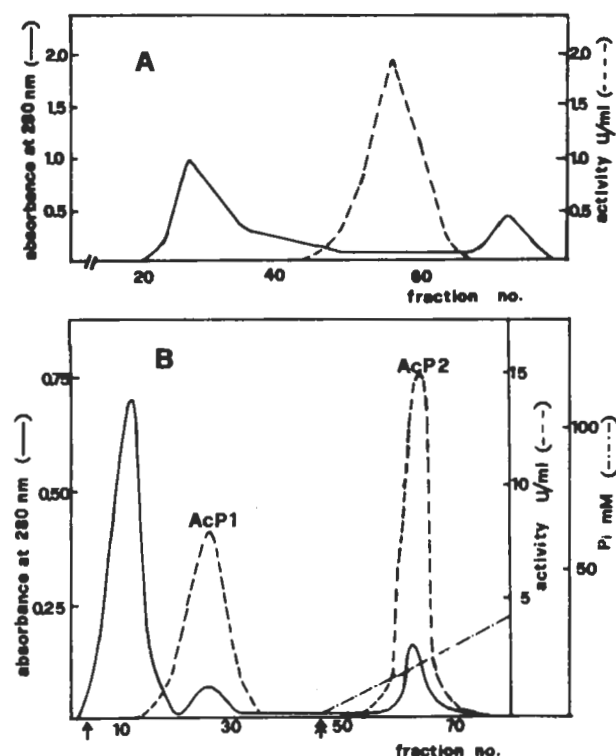


Fig. 1. (A) Elution profile of Sephadex G75 gel filtration. The column (5 × 100 cm) was eluted at a flow rate of 50 ml/hr and 12 ml fractions were collected. (B) Elution profile of *p*-aminobenzyl phosphonic acid-agarose affinity chromatography. The column (1 × 6 cm) was eluted at a flow rate of 15 ml/hr and 2 ml fractions were collected. Arrow indicates the start of the Pi gradient.

enzyme was precipitated by adding solid ammonium sulfate to 70% saturation. The precipitate was collected by centrifugation at 23,000g for 45 min, dissolved in 6 ml of 0.1 M sodium citrate buffer, pH 6.5, containing 1 mM EDTA, 1 mM DTT and 0.02% Triton X-100. The enzyme solution was then dialyzed overnight against the same buffer.

Step 5. The dialysate was filtered to clarify the solution and then applied to an affinity chromatography column (1 × 6 cm) of *p*-aminobenzylphosphonic

acid-agarose equilibrated with the dialysis buffer. The column was washed at a flow rate of 15 ml/hr and 2 ml fractions were collected. During the washing phase, foreign proteins were eluted first and then a well-separated acid phosphatase peak was eluted (AcP1). AcP2 was successively eluted by applying a linear gradient from 0 to 0.1 M Pi (100 ml of 0.1 M citrate buffer, pH 6.5, containing 1 mM EDTA, 1 mM DTT and 0.02% Triton X-100 and 100 ml of Pi solution adjusted to pH 6.5). Figure 1B shows the elution profile. Table I shows the data relative to the purification procedure. It can be seen that AcP1 was purified 1290-fold and AcP2 1029-fold with respect to the liver extract.

2.2. Enzyme Assay, Kinetic Parameters, and Inhibition

Acid phosphatase activity was assayed using *p*-nitrophenyl phosphate as a substrate at pH 5.5, as previously described (Ramponi *et al.*, 1989). The unit of activity is defined as the amount of enzyme that catalyzes the hydrolysis of 1 μmol of *p*-nitrophenyl phosphate per min at pH 5.5 and 37°C. L-phosphotyrosine phosphatase activity was determined according to Apostol *et al.* (1985). K_m and V_{max} were determined measuring the initial rates at pH 5.5 and 37°C, using several different substrate concentrations; the results were plotted according to Lineweaver and Burk (1934). Pyridoxal 5'-phosphate studies were carried out in 0.1 M acetate buffer, pH 5.5, containing 1 mM EDTA, at 37°C. The initial rates were determined using three different pyridoxal 5'-phosphate concentrations and several different *p*-nitrophenyl phosphate concentrations. K_i were calculated by plotting the apparent K_m determined at the various inhibitor concentrations vs. inhibitor concentrations.

2.3. Protein Determination

Protein concentration was determined by the biuret method (Beisenherz *et al.*, 1953). In chromato-

Table I. Purification of AcP1 and AcP2 Isoenzymes from Rat Liver (1 kg)

Step	Volume (ml)	Activity (U)	Protein (mg)	Specific activity (U/mg)	Yield (%)
1. Tissue extract	2800	4648	67,720	0.07	100
2. Ammonium sulfate (29–60%)	680	2339	21,760	0.11	50.3
3. SP-Sephadex C50	1750	411	248.5	1.65	8.8
4. Sephadex G75	325	344	24.7	13.9	7.4
5. Affinity chromat. AcP1	40.5	177	2.0	88.5	3.8
AcP2	15	173	2.4	72.1	3.7

graphic effluents, the relative protein concentration was estimated by measuring the absorbance at 280 nm.

2.4. Polyacrylamide Gel Electrophoresis

SDS-PAGE was performed using the method of Laemmli (1970).

2.5. Amino Acid Analysis

Amino acid analysis was carried out as previously described (Manao *et al.*, 1985). Cysteine was determined as carboxymethylcysteine. Tryptophan was determined by the Edelhoch method (Edelhoch, 1967) or by that of Penke *et al.* (1974). Values for serine and threonine were corrected for loss during hydrolysis. Alternatively, the amino acid analysis of the peptides (10–500 pmol) was performed by analyzing the naphthylcarbamoyl derivatives of amino acids by HPLC on a Waters Pico-Tag column (3.9 × 150 mm, 4 μ m), according to Neidle *et al.* (1989), with minor modifications.

2.6. Carboxymethylation

AcP1 or AcP2 (10 nmol) were dissolved in 0.2 ml of 0.1 M Tris-HCl buffer, pH 8.5, containing 6 M guanidinium chloride and treated with 10 μ l of 2-mercaptoethanol overnight at 40°C in a nitrogen atmosphere. Then 0.2 ml of a solution of iodoacetic acid (freshly recrystallized, 120 mg/ml) in 3 M guanidinium chloride, adjusted to pH 8.7 with Tris base, was added. After 15 min at room temperature, the Cm-proteins were separated from reagents by HPLC on Aquapore RP300, using a TFA/acetonitrile gradient.

2.7. Enzymatic digestions

Before protease addition, the protein solutions were incubated for 3 min in a boiling water bath and then chilled in ice. *S. aureus* V8 protease digestion was performed as previously described (Liguri *et al.*, 1986). Endoproteinase Asp-N digestion was performed as follows: 10 nmol of Cm-AcP1 or Cm-AcP2 were dissolved in 50 μ l of 50 mM sodium phosphate buffer, pH 8.0, and then 50 μ l of a solution containing 2 μ g of endoproteinase was added. The mixture was incubated at 37°C for 18 hr. Endoproteinase Lys-C digestion was carried out as follows: 10 nmol of Cm-AcP1 or Cm-AcP2 in 50 μ l of 25 mM Tris-HCl buffer, pH 8.5, containing 1 mM EDTA, were mixed with

5 μ g of endoproteinase Lys-C dissolved in 50 μ l of 50 mM tricin buffer, pH 8.0, containing 10 mM EDTA; the mixture was incubated at 37°C for 20 hr. Carboxypeptidase Y digestion was performed by incubating Cm-AcP1 (5 nmol), Cm-AcP2 (5 nmol), and LC1-TH1 peptides (2.2 nmol) with CPY (at an enzyme/protein (peptide) molar ratio of 1/25) in 25 μ l of 0.1 M pyridine-acetate buffer, pH 5.6, at 30°C. Aliquots were withdrawn at different interval times and analyzed for released amino acids.

2.8. Separation of Peptides

Peptides were purified by reverse-phase HPLC on Aquapore RP300 or Vydac columns using TFA/acetonitrile or ammonium bicarbonate/acetonitrile gradients. The peaks containing more than one peptide were rechromatographed by using different solvent systems or different elution gradients.

2.9. NH₂-Terminal Analysis and Sequence Determination

Edman degradation was carried out using the Milligen Protein Sequencer mod. 6600 or the Tarr manual method (Tarr, 1977). The Milligen Sequencer operates solid-phase Edman degradation on peptides or proteins covalently immobilized on polyvinylidene difluoride membranes derivatized with 1,4-phenylene diisothiocyanate (Sequelon DITC) or arylamine groups (Sequelon AA). (Both membranes were purchased from Milligen.) FAB mass spectra were determined by a VG Analytical 70-70 EQ instrument as described (Camici *et al.*, 1983).

2.10. BNPS-Skatole and CNBr-Cleavages

Cm-AcP1 (2 nmol) was dissolved in 10 μ l of 0.25% (w/v) phenol and then treated with 20 μ l of a 7.2 mg/ml BNPS-skatole solution in acetic acid. The mixture was incubated for 21 hr at 40°C, diluted with 0.1 ml of water, and then extracted twice with 0.4 ml of diethyl ether and twice with 0.4 ml of ethyl acetate. The water phase was dried by a Savant vacuum evaporator. The residue was dissolved in a solution of TMA/water/acetonitrile (1/1/1) and dried again; the residue was dissolved in 40 μ l of 2% N-methylmorpholine containing 0.1% SDS and the peptide fragments were bound, as always, to a Sequelon-DITC membrane. Finally, the membrane was washed three times with 1 ml of acetonitrile and then applied to the

reaction chamber of the Milligen Protein Sequencer. After the programmed number of steps, the membrane was removed from the Sequencer and treated with *o*-phthalaldehyde/2-mercaptoethanol reagent (0.08% OPA, 0.2% 2-mercaptoethanol in 0.4 M potassium borate buffer, pH 10.4) to block all the free primary amino groups. Then, the membrane was washed with methanol, dried, and treated with a CNBr solution (5 mg/ml) in 70% formic acid. After incubation for 16 hr at room temperature, the membrane was removed from the CNBr solution, washed three times with 2 ml of 70% formic acid, twice with 50% ethanol, and twice with methanol. The membrane was again applied to the Sequencer reactor and the Edman degradation was continued on the postmethionine sequence.

AcP2 (2 nmol) was covalently linked to a Sequelon DITC membrane as described. The membrane was washed with 50% ethanol, methanol, and dried. It was then treated with CNBr, as described above.

3. RESULTS AND DISCUSSION

3.1. Isolation and Characterization of AcP1 and AcP2

With the procedure described in Materials and Methods, about 2 mg of pure AcP1 and AcP2 were obtained from 1 kg of rat liver. Their purity was checked by both SDS-PAGE and HPLC on Aquapore RP300 column. Amino acid analyses reported in Table II show that the two isoenzymes have a different amino acid composition, thus indicating structural differences. Both AcP1 and AcP2 have blocked NH₂-terminal residues and so give no results using Edman degradation. Several differences were found with regard to their kinetic parameters. More specifically, AcP1 shows a K_m for L-phosphotyrosine which is one order of magnitude lower than that of AcP2 and a V_{max} on the same substrate which is higher than that of AcP2. Furthermore, the K_m on *p*-nitrophenyl phosphate for the two isoenzymes is quite similar, while the V_{max} on the same substrate is higher for AcP1 than for AcP2. In addition, AcP1 shows a K_i for pyridoxal 5'-phosphate which is two orders of magnitude higher than that of AcP2 (Table III). Both isoenzymes have quite a similar pH optimum using *p*-nitrophenyl phosphate as a substrate. Moreover, AcP2 was activated by purine compounds as previously reported by Fujimoto *et al.* (1988), whereas AcP1 was insensitive to these compounds. We underline the very different

Table II. Amino Acid Composition of AcP1 and AcP2^a

Amino acid	AcP1	AcP2
Cm-cysteine	7.9 (8)	8.3 (8)
Aspartic acid	21.3 (22)	24.1 (24)
Threonine	5.6 (6)	4.7 (5)
Serin	9.6 (10)	11.9 (12)
Glutamic acid	18.1 (18)	15.5 (15)
Proline	5.1 (5)	6.0 (6)
Glycine	7.4 (7)	5.6 (6)
Alanine	9.3 (9)	10.7 (11)
Valine	9.6 (10)	12.5 (12)
Methionine	2.9 (3)	0.9 (1)
Isoleucine	9.7 (10)	9.1 (9)
Leucine	11.3 (11)	12.4 (12)
Tyrosine	6.8 (7)	5.0 (5)
Phenylalanine	6.1 (6)	6.0 (6)
Lysine	11.3 (11)	9.9 (10)
Histidine	4.0 (4)	3.0 (3)
Arginine	9.1 (9)	10.0 (10)
Tryptophan	1.1 (1)	1.9 (2)

^a The results are expressed as residues per molecule of enzyme. Values for Thr and Ser were determined by hydrolyses at 110°C for 22 and 70 hr in duplicate and extrapolation to zero time. The value for Ile was that calculated in the 70 hr hydrolysate. Trp was determined by the method of Edelhoch (1967).

Table III. Effect of Some Purine Nucleotides on the Activities and Some Kinetic Properties of AcP1 and AcP2

Nucleotide added	Relative activity ^a	
	AcP1	AcP2
None	100	100
5'-GMP (1 mM)	107	147
cGMP (1 mM)	113	325
cGMP (0.1 mM)	102	150
cAMP (1 mM)	95	114
K_m (mM)		
(on <i>p</i> -nitrophenyl phosphate)	0.31	0.27
K_m (mM)		
(on L-phosphotyrosine)	0.6	6.0
V_{max}		
(on <i>p</i> -nitrophenyl phosphate)	98.9	69.3
V_{max}		
(on L-phosphotyrosine)	85.9	47.9
K_i (mM)		
(on pyridoxal 5'-phosphate, competitive)	0.4	0.004
pH-optimum	4.5–5.5	4.5–5.5

^a Relative activity was determined using *p*-nitrophenyl phosphate as a substrate at pH 5.5 and at 37°C.

activation ability of cGMP with respect to cAMP on AcP2. This could be of physiological significance, and we think that this aspect merits additional work.

3.2. Sequence Determination

The Cm-AcP1 isoenzyme was digested with Lys-C endoproteinase and the peptides were separated by HPLC (Fig. 2). Eight peptides were purified and their amino acid compositions are reported in Table IV. The sequence of each peptide was determined manually or automatically using the Milligen Sequencer. LC1 peptide gave no sequence results: this was as expected for the NH₂-terminal peptide since the protein is NH₂-acylated. The LC1 structure was determined as follows: (i) the FAB mass spectrum reported in Fig. 3 shows a protonated molecular ion at $m/z = 632$, this value corresponds to a M_r of 631; (ii) the digestion of LC1 with thermolysin generates a free α -NH₂ group by cleavage at the amino terminus of Val 3. The sequence 3–6 was determined by the Edman degradation on an aliquot of the digest without peptide separation since one of the two thermolytic peptides was NH₂-acylated. Another aliquot was applied to an AG-50W X4 column (0.25 \times 2.5 cm, H⁺ form, Bio-Rad) and the α -NH₂ acylated peptide was collected by washing with distilled water. The amino acid composition of this peptide (LC1-TH1) was Glu₁, Ala₁. LC1-TH1 digestion with carboxypeptidase Y

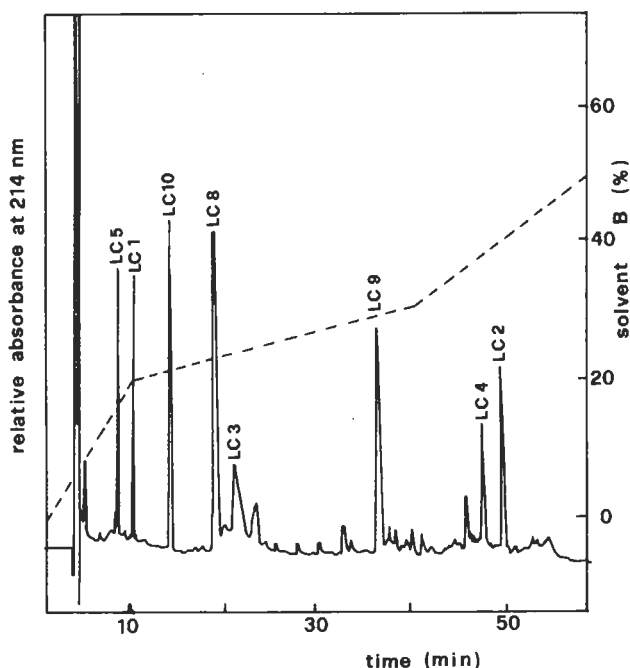


Fig. 2. The HPLC separation of endoproteinase Lys-C peptides from 10 nmol of Cm-AcP1. Column: Vydac, 5 μ m, 4.6 \times 250 mm. Solvent A, 10 mM TFA in water, solvent B, 10 mM TFA in acetonitrile. Flow rate 1.0 ml/min. (—) absorbance; (---) elution gradient.

Table IV. Amino Acid Composition of LC-, AN-, and SP-Peptides from Cm-AcP1^a

		Position	Yield (%)
LC1	Ser _{0.8} , Glu _{1.0} , Gly _{0.9} , Ala _{1.1} , Val _{0.8} , Lys _{0.8}	1–6	90
LC2	Cys _{1.9} , Asp _{1.0} , Ser _{2.1} , Glu _{1.0} , Pro _{1.0} , Gly _{1.1} , Ala _{2.0} , Val _{2.8} , Ile _{1.8} , Leu _{2.0} , Phe _{2.0} , Lys _{1.0} , Arg _{1.9}	7–28	60
LC3	Cys _{0.9} , Asp _{7.6} , Thr _{2.9} , Ser _{3.1} , Glu _{3.0} , Pro _{1.9} , Gly _{2.0} , Ala _{2.0} , Val _{3.0} , Met _{0.9} , Ile _{1.0} , Leu _{1.0} , Tyr _{2.0} , Lys _{2.0} , Arg _{2.1} , Trp _{0.8}	29–65	38
LC4	Cys _{0.8} , Asp _{6.5} , Thr _{2.0} , Ser _{0.9} , Glu _{4.1} , Gly _{1.0} , Ala _{2.0} , Met _{1.8} , Ile _{3.7} , Leu _{3.1} , Tyr _{0.9} , Phe _{2.0} , Lys _{1.0} , His _{3.0} , Arg _{3.9}	66–102	42
LC5	Asp _{1.0} , Ser _{1.0} , Glu _{1.1} , Val _{0.9} , Lys _{1.0}	103–107	82
LC8	Asp _{1.0} , Ser _{1.0} , Glu _{1.9} , Pro _{1.0} , Gly _{1.0} , Ile _{1.0} , Leu _{1.9} , Tyr _{0.9} , Lys _{1.0}	113–123	88
LC9	Cys _{2.8} , Asp _{4.1} , Ser _{0.9} , Glu _{4.9} , Pro _{1.0} , Gly _{1.1} , Val _{2.0} , Ile _{2.0} , Leu _{2.0} , Tyr _{2.9} , Phe _{1.0} , Lys _{1.0} , Arg _{1.0}	124–150	65
LC10	Glu _{0.9} , Ala _{1.0} , Leu _{1.0} , Phe _{0.9} , Lys _{1.0}	151–155	85
AN1	Cys _{1.8} , Asp _{1.0} , Ser _{2.7} , Glu _{1.1} , Pro _{1.0} , Gly _{2.0} , Ala _{2.0} , Val _{2.8} , Ile _{1.9} , Leu _{2.0} , Phe _{1.0} , Lys _{1.0} , Arg _{0.9}	1–22	38
AN2	Thr _{1.0} , Glu _{1.1} , Ala _{1.1} , Val _{1.9} , Leu _{1.0} , Phe _{1.0} , Lys _{1.0} , Arg _{0.9}	23–31	43
AN3	Asp _{2.0} , Ser _{0.9} , Glu _{1.0} , Val _{1.2}	32–36	64
AN4	Asp _{1.7} , Ile _{1.0} , Arg _{1.0} , Trp _{0.9}	37–41	71
AN5	Asp _{1.0} , Thr _{1.9} , Ser _{2.0} , Ala _{2.0} , Tyr _{1.1}	42–49	55
AN8	Asp _{1.0} , Thr _{0.9} , Ala _{1.0} , Phe _{2.0}	81–85	46
AN9	Cys _{1.0} , Asp _{1.0} , Met _{0.7} , Ile _{0.9} , Leu _{1.0} , Tyr _{0.9}	86–91	22
AN10	Asp _{2.0} , Ser _{0.9} , Glu _{1.1} , Leu _{1.0} , Arg _{1.0}	92–97	40
AN11	Cys _{0.9} , Asp _{4.0} , Ser _{1.9} , Glu _{2.1} , Gly _{1.0} , Ala _{1.1} , Val _{1.1} , Ile _{1.1} , Leu _{3.2} , Tyr _{0.9} , Lys _{4.0} , Arg _{1.0}	98–119	38
AN12	Asp _{1.0} , Glu _{2.9} , Pro _{0.9} , Ile _{1.9} , Leu _{1.0} , Lys _{1.0}	120–128	43
AN13	Asp _{2.0} , Pro _{1.1} , Gly _{1.0} , Tyr _{2.0}	129–134	57
AN17	Thr _{0.9} , Glu _{1.1} , Lys _{1.0} , His _{0.9}	154–157	27
SP2	Cys _{1.8} , Asp _{1.0} , Ser _{2.8} , Glu _{1.0} , Pro _{0.9} , Gly _{2.1} , Ala _{1.0} , Val _{2.9} , Ile _{2.0} , Leu _{1.9} , Phe _{0.9} , Lys _{1.0} , Arg _{1.0}	3–23	51
SP3	Asp _{1.0} , Thr _{0.9} , Glu _{1.0} , Ala _{1.0} , Val _{1.9} , Leu _{1.1} , Phe _{0.9} , Lys _{1.0} , Arg _{1.0}	24–33	42
SP6	Cys _{0.8} , Asp _{2.9} , Thr _{0.9} , Glu _{1.0} , Ala _{1.0} , Met _{0.8} , Ile _{1.0} , Leu _{1.1} , Tyr _{0.9} , Phe _{1.9}	81–93	58
SP8	Asp _{4.8} , Ser _{1.9} , Glu _{4.1} , Pro _{1.8} , Gly _{2.1} , Ile _{2.0} , Leu _{2.9} , Tyr _{2.9} , Phe _{1.0} , Lys _{1.0}	115–139	62
SP9	Cys _{2.7} , Glu _{3.0} , Ala _{1.0} , Val _{1.9} , Leu _{2.0} , Tyr _{0.9} , Phe _{0.9} , Lys _{1.0} , Arg _{1.0}	140–154	56

^a The digestions were performed on 10 nmol of Cm-AcP-1. The values are expressed as molar ratios. Values for Ser and Thr were corrected for 15% and 5% destruction, respectively. Values of contaminating amino acids at a level of less than 15% are not reported. Cys was determined as Cm-Cys. Yield was calculated as moles obtained from moles of digested protein.

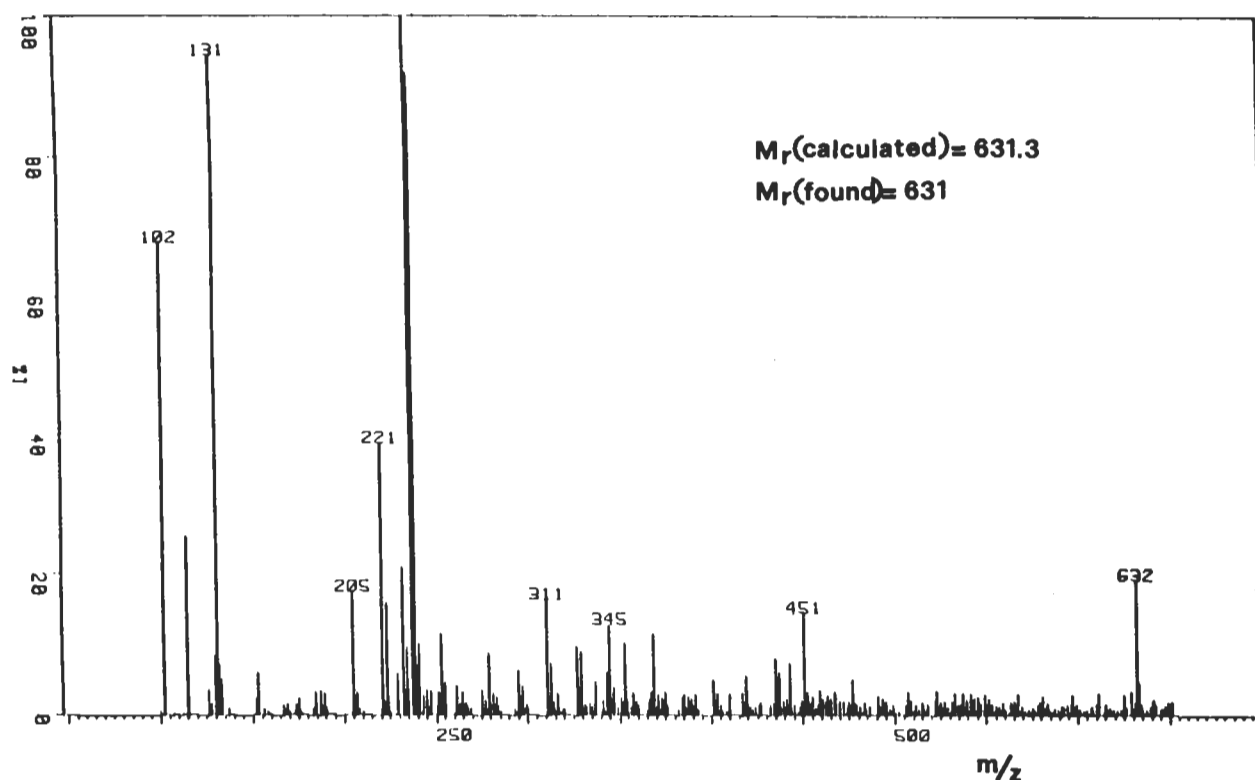


Fig. 3. Positive FAB mass spectrum of LC1.

gave only Glu, thus indicating that the sequence was X-Ala-Glu. The nature of the acylating group was deduced by combining the data from amino acid analysis and the M_r determined by mass spectrometry of LC1 peptide. Its sequence was Ac-Ala-Glu-Val-Gly-Ser-Lys. LC-peptides were ordered analyzing two additional peptide series produced by the digestion of Cm-AcP1 with Asp-N endoproteinase and *S. aureus* V8 protease.

In addition, Cm-AcP1 was treated with BNPS-skatole to cleave the chain at the sole Trp present in the molecule. The protein has an acylated NH_2 -terminus and so this treatment generates a single free α - NH_2 -group (i.e., that of the amino acid following Trp). Thus, the Edman degradation performed directly on the cleavage mixture gives a single amino acid sequence. Figures 4 and 5 show the HPLC separations of AN- and SP-AcP1 peptides, respectively. Table IV reports the amino acid compositions of the purified AN and SP peptides. AN17 overlaps LC10 and SP9 and gives the information necessary to complete the COOH-terminal sequence.

The above data are sufficient to reconstruct the entire primary structure of AcP1 (see Fig. 6). The

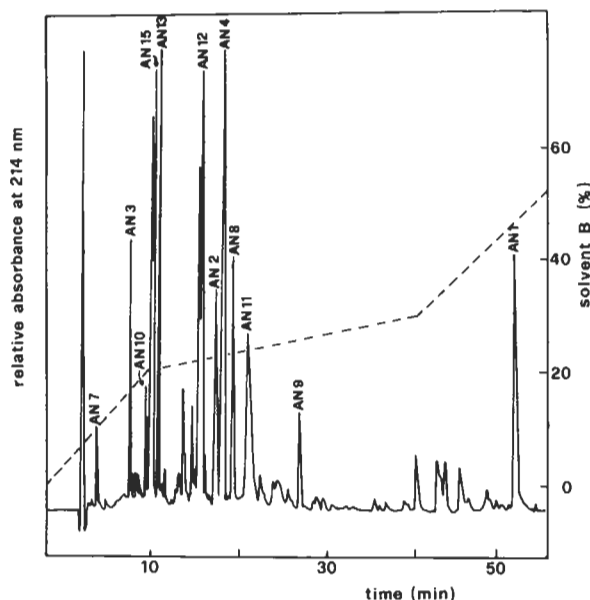


Fig. 4. The HPLC separation of endoproteinase Asp-N peptides of Cm-AcP1. Column: Aquapore RP300, 7 μm , 4.6 \times 250 mm. The overlapped peptides were rechromatographed at different conditions. All other details are the same as in Fig. 2.

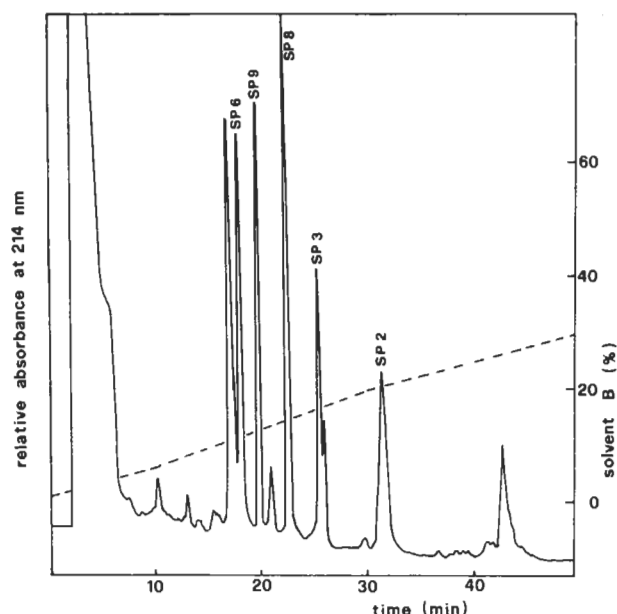


Fig. 5. The HPLC separation of *S. aureus* protease peptides of Cm-AcP1. Column, Aquapore RP 300, 7 μ m, 4.6 \times 250 mm; solvent A, 0.02 M ammonium bicarbonate, pH 7.0; solvent B, acetonitrile. Flow rate, 1.5 ml/min. All details are the same as in Fig. 2.

protein consists of a single polypeptide chain 157 residues long. It is acetylated at the NH₂-terminus and His is the COOH-terminal residue.

The sequence of AcP2 isoenzyme was obtained by analyzing two series of peptides (LC and AN) and by the sequence analysis performed after CNBr cleavage. Since AcP2 contains a sole Met residue it gave a single sequence starting from the residue that follows Met. This happens because the protein has an acylated amino terminus that is not available for Edman degradation. Figures 7 and 8 show the HPLC separation of LC and AN peptides obtained from AcP2 while Table V shows their amino acid compositions. Figure 9 shows the sequence of AcP2 deduced from all the information we found. AcP2 consists of 157 amino acid residues and is NH₂-acetylated as AcP1. The molecular weights calculated from the sequence are 18,062 for AcP1 and 17,848 for AcP2.

3.3. Comparison of Secondary Structure Predictions

We have analyzed the hydrophobicity and charged residue profiles together with α -helix, β -

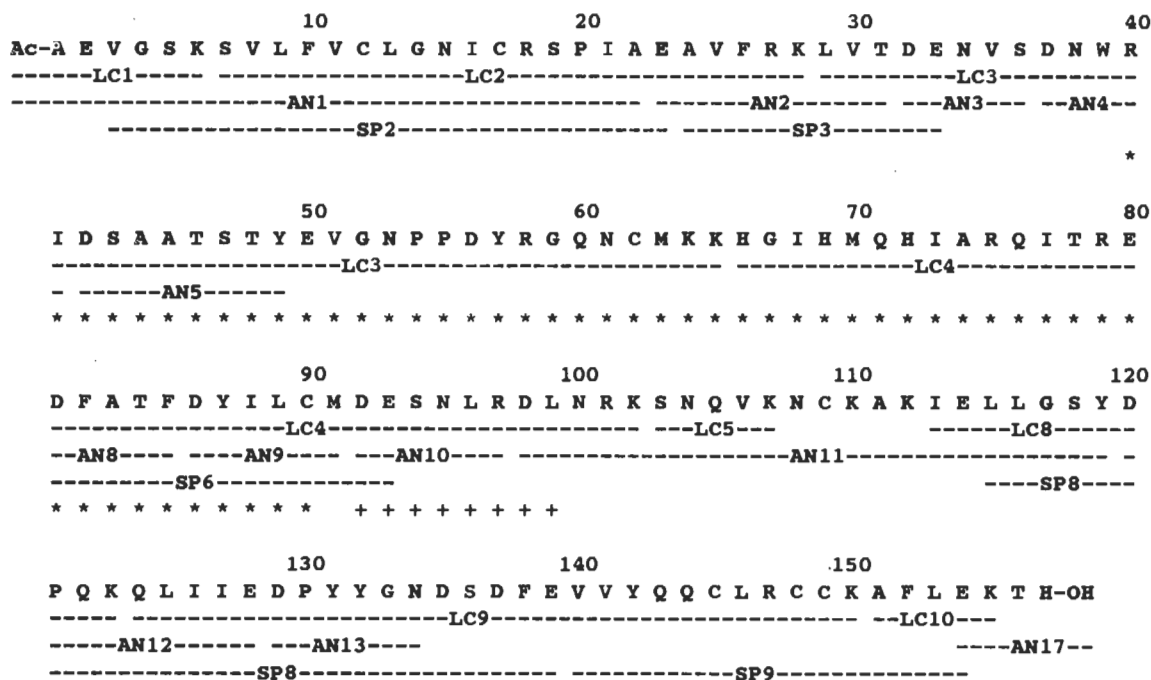


Fig. 6. The complete amino acid sequence of rat liver AcP1. LC, endoproteinase Lys-C peptides; AN, endoproteinase Asp-N peptides; SP, *S. aureus* protease peptides. (*), indicates the sequence results obtained on BNPS skatole cleaved protein. (+), indicates the sequence results obtained on CNBr cleaved protein. Ac, acetyl.

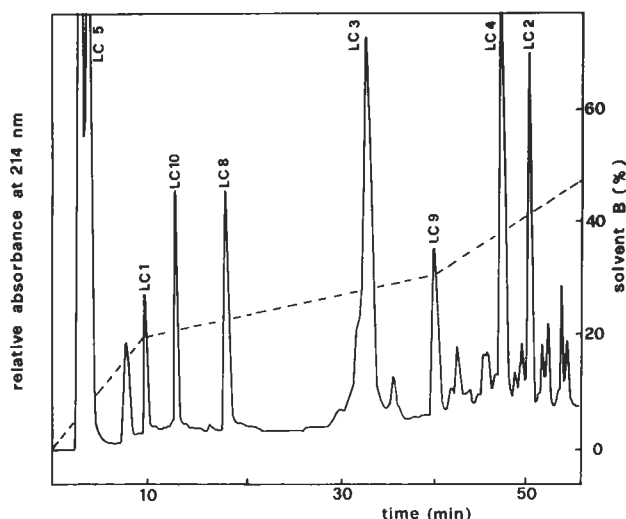


Fig. 7. The HPLC separation of endoproteinase Lys-C peptides from AcP2. Flow rate 1.2 ml/min. Other details are the same as in Fig. 4.

sheet, and reverse turn propensity plots of AcP1 and AcP2 to estimate if the different sequences of the two isoenzymes cause variations either in the secondary structure predictions and on the hydrophobicity and charged residue profiles. These analyses, performed by the method of Novotny and Auffray (1984), are reported in Fig. 10. It can be seen that the β -sheet profile in the 59–61 zone is slightly higher for AcP2 than for AcP1. Furthermore, little α -helix propensity

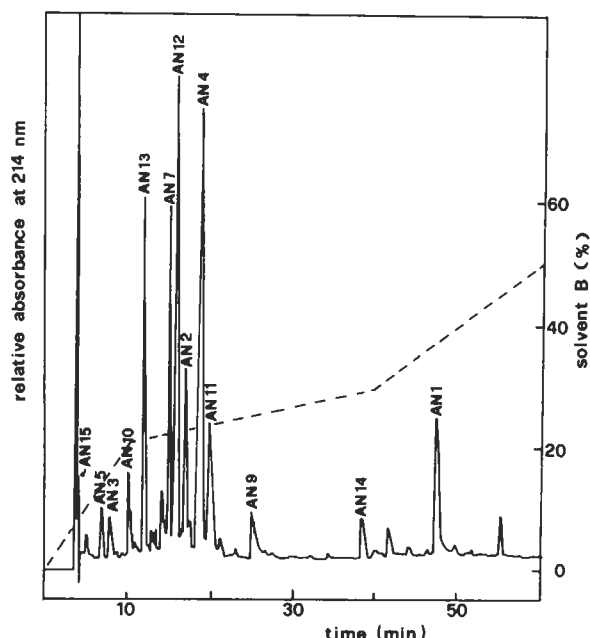


Fig. 8. The HPLC separation of endoproteinase Asp-N peptides from AcP2. Details are the same as in Fig. 4.

peaks are present at 42 and 63 positions in AcP1, while these are not present in AcP2. On the other hand, the hydrophobicity profile comparison indicates that AcP2 is more hydrophylic than AcP1 in the 69–75 region, and more hydrophobic in the 59–63 zone. Some little differences in the charged residue

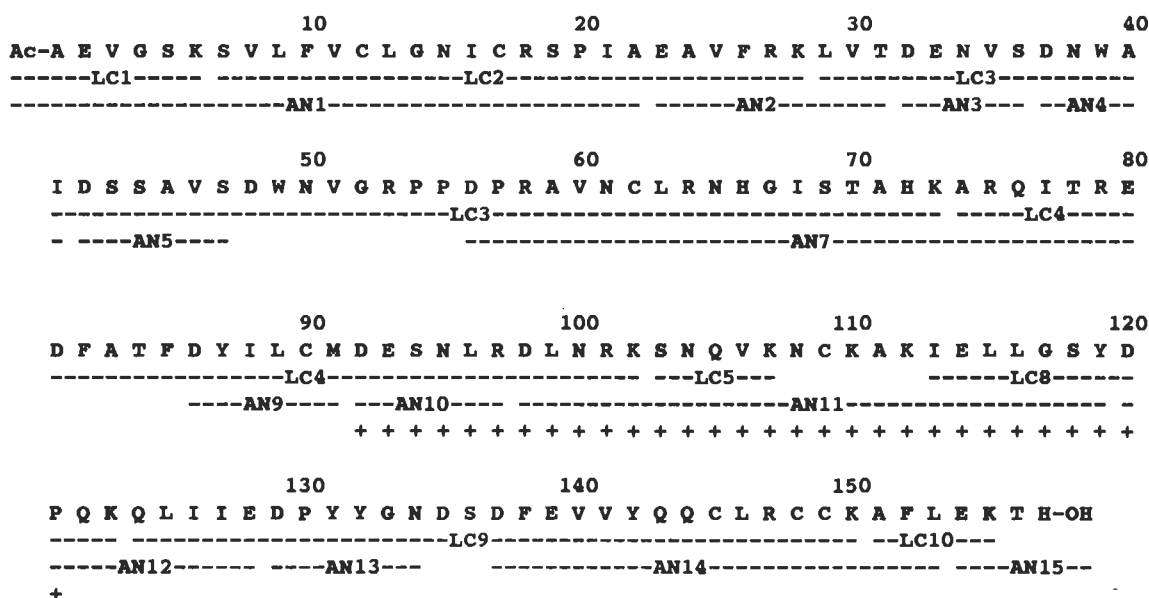


Fig. 9. The complete amino acid sequence of rat liver AcP2. Details are the same as in Fig. 6.

Table V. Amino Acid Composition of LC- and AN-Peptides from Cm-AcP2^a

		Position	Yield (%)
LC1	Ser _{1.0} , Glu _{1.0} , Gly _{1.0} , Ala _{0.9} , Val _{0.9} , Lys _{1.0}	1-6	90
LC2	Cys _{1.9} , Asp _{1.0} , Ser _{1.9} , Glu _{1.0} , Pro _{0.9} , Gly _{1.1} , Ala _{2.2} , Val _{2.8} , Ile _{1.8} , Leu _{2.0} , Phe _{2.1} , Lys _{1.0} , Arg _{1.9}	7-28	72
LC3	Cys _{0.9} , Asp _{9.6} , Thr _{1.9} , Ser _{4.7} , Glu _{1.2} , Pro _{2.9} , Gly _{2.1} , Ala _{3.7} , Val _{5.2} , Ile _{2.0} , Leu _{2.0} , Lys _{0.9} , His _{2.0} , Arg _{2.9} , Trp _{1.8}	29-73	88
LC4	Cys _{0.7} , Asp _{5.8} , Thr _{1.8} , Ser _{1.3} , Glu _{3.3} , Ala _{1.7} , Met _{0.7} , Ile _{2.0} , Leu _{3.0} , Tyr _{1.0} , Phe _{2.2} , Lys _{1.0} , Arg _{3.7}	74-102	85
LC5	Asp _{1.1} , Ser _{1.1} , Glu _{0.9} , Val _{1.0} , Lys _{1.0}	103-107	45
LC8	Asp _{1.0} , Ser _{1.0} , Glu _{2.0} , Pro _{0.9} , Gly _{1.0} , Ile _{1.0} , Leu _{1.9} , Tyr _{0.9} , Lys _{1.1}	113-123	75
LC9	Cys _{2.7} , Asp _{4.0} , Ser _{1.2} , Glu _{5.1} , Pro _{1.0} , Gly _{1.2} , Val _{1.8} , Ile _{1.6} , Leu _{2.0} , Tyr _{2.7} , Phe _{1.2} , Lys _{1.1} , Arg _{0.8}	124-150	34
LC10	Glu _{1.0} , Ala _{0.8} , Leu _{0.9} , Phe _{0.9} , Lys _{1.0}	151-155	79
AN1	Cys _{1.9} , Asp _{1.0} , Ser _{3.1} , Glu _{1.1} , Pro _{0.9} , Gly _{2.1} , Ala _{1.9} , Val _{3.0} , Ile _{1.9} , Leu _{2.0} , Phe _{1.0} , Lys _{1.0} , Arg _{0.9}	1-22	75
AN2	Thr _{1.0} , Glu _{1.1} , Ala _{1.0} , Val _{1.9} , Leu _{1.0} , Phe _{1.1} , Lys _{1.0} , Arg _{1.0}	23-31	86
AN3	Asp _{1.8} , Ser _{1.1} , Glu _{1.0} , Val _{0.9}	32-36	90
AN4	Asp _{2.0} , Ala _{1.0} , Ile _{0.9} , Trp _{0.8}	37-41	56
AN5	Asp _{1.0} , Ser _{2.9} , Ala _{1.0} , Val _{0.8}	42-47	90
AN7	Cys _{0.8} , Asp _{2.7} , Thr _{1.9} , Ser _{1.3} , Glu _{2.1} , Pro _{1.0} , Gly _{1.0} , Ala _{2.9} , Val _{1.0} , Ile _{1.9} , Leu _{1.0} , Lys _{1.1} , His _{1.9} , Arg _{4.1}	56-80	63
AN9	Cys _{0.9} , Asp _{1.0} , Met _{0.8} , Ile _{1.0} , Leu _{1.0} , Tyr _{0.8}	86-91	44
AN10	Asp _{1.9} , Ser _{1.0} , Glu _{1.1} , Leu _{0.9} , Arg _{1.0}	92-97	72
AN11	Cys _{1.0} , Asp _{4.3} , Ser _{2.0} , Glu _{2.3} , Gly _{1.1} , Ala _{1.2} , Val _{1.0} , Ile _{1.0} , Leu _{3.3} , Tyr _{1.2} , Lys _{3.9} , Arg _{1.1}	98-119	80
AN12	Asp _{1.0} , Glu _{3.1} , Pro _{1.1} , Ile _{1.7} , Leu _{1.0} , Lys _{1.0}	120-128	74
AN13	Asp _{2.0} , Pro _{0.9} , Gly _{1.1} , Tyr _{1.9}	129-134	66
AN14	Cys _{2.8} , Asp _{1.0} , Glu _{3.1} , Ala _{1.0} , Val _{2.0} , Leu _{1.9} , Tyr _{1.0} , Phe _{2.1} , Lys _{1.0} , Arg _{1.0}	137-153	78
AN15	Thr _{1.0} , Glu _{1.1} , Lys _{1.0} , His _{0.9}	154-157	76

^a Details are the same as in Table IV.

profiles can also be observed. The small secondary structure propensity differences present in the 40-75 region suggest that AcP1 and AcP2 retained very similar conformations. Nevertheless, the little observed hydrophilicity and charged residue profile variations, could be the basis of the different kinetic properties of the two isoenzymes. In fact, as described above, these have different affinity for L-phosphotyrosine and pyridoxal 5'-phosphate and different sensitivity to purine compounds activation.

3.4. Homology of AcP1 and AcP2 with other Phosphatases

AcP1 and AcP2 show no global homology with the main family of phosphotyrosine protein phosphatases such as PTPase 1B from human placenta, CD45 and LAR (Tonks *et al.*, 1989). In addition, it is worth noting that we did not find any homology with alkaline phosphatases or intermediate and high *M_r* acid phosphatases.

A comparison of the primary structures of AcP1 and AcP2 with those of other related phosphatases (human erythrocyte B_{fast} and B_{slow} acid phosphatase isoenzymes and bovine low *M_r* cytosolic acid phosphatase) is presented in Fig. 11. Erythrocyte B_{fast} and B_{slow} sequences have been very recently determined in our (Manao *et al.*, 1991) and another laboratory (Dissing *et al.*, 1991). The N-terminus of all these phosphatases (Ala) is acetylated. Only the bovine liver enzyme has Arg as the C-terminal amino acid, whereas all the others have His at the C-terminus. The five isoenzymes have 8 cysteine residues, all conserved in the same sequence sites. We have previously demonstrated that all are present in the free thiol form in the native bovine liver enzyme (Camici *et al.*, 1989). Furthermore, we have found that both Cys12 and Cys17 are essential for the catalytic function of the bovine liver enzyme. The conservation of these two cysteines in all rat AcP1 and AcP2, human erythrocyte B_{fast} and B_{slow}, and bovine low *M_r* isoenzymes confirm our finding on the essential role of both Cys12 and Cys17 in the active site. It is interesting to note that the major human placenta phosphotyrosine protein phosphatase (PTPase 1B), which is inhibited by thiol modifying reagents (Tonks *et al.*, 1988), contains a 226-231 sequence (Charbonneau *et al.*, 1989) that is homologous to the 12-17 sequence of all phosphatases presented in Fig. 11.

The bovine liver enzyme contains only 2 His residues, whereas both AcP2 and B_{fast} have 3 His residues and both AcP1 and B_{slow} contain 4 His residues. AcP2, B_{slow}, and the bovine liver enzyme contain a sole Met residue, while both AcP1 and B_{fast} have 3 Met residues. Met91, His66, and His72 are conserved in all isoenzymes. The degree of sequence homology of these various phosphatases is presented in Table VI. Homology among these enzymes range from 77.7 to 93.0%. Although the homology among these isoenzymes is generally high, there is a very peculiar difference between AcP1 and AcP2 and also between B_{fast} and B_{slow}. In fact, most of the sequence (1-39 and 74-157 regions) of AcP1 is identical to that of AcP2.

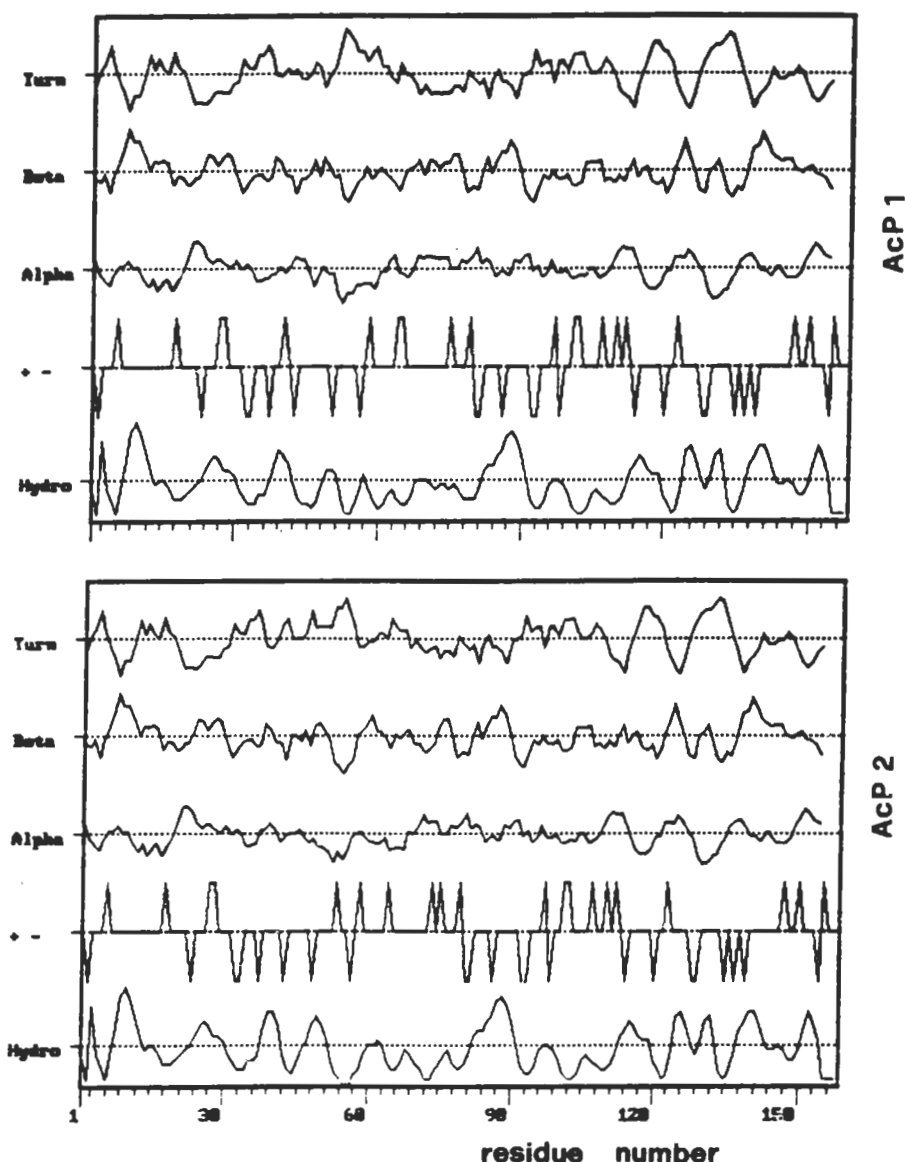


Fig. 10. α -Helix, β -sheet, and β -turn propensity plots and hydrophobic and charged residues profiles of AcP1 and AcP2. The graphs were obtained using the PC/GENE program, release 6.5 (IntelliGenetics, Inc., California).

Table VI. Homology of the Primary Structures of Various Mammalian Phosphatases (%)

	AcP2 (rat)	B _{fast} (human)	B _{slow} (human)	Low <i>M_r</i> AP (bovine)
AcP1 (rat)	89.2	87.3	80.9	77.7
AcP2 (rat)		79.6	89.2	86.6
B _{fast} (human)			87.3	80.9
B _{slow} (human)				93.0

Nevertheless, in the 40–73 zone, the sequence of AcP1 differs greatly from that of AcP2 in about 50% of the positions. Some replacements are not conservative. A similar picture is present also in the human erythrocyte B_{fast}–B_{slow} pair as underlined by Dissing *et al.* (1991). The sequence identity between AcP1 and AcP2 in the 40–73 region is 50%, and between B_{fast} and B_{slow} is 41.2%. Otherwise, the sequence identity

	10	20	30	40	50
AcP1	Ac-AEVGSKSVL	FCVLGNICRSP	IAEAVFRKLV	TDENVSDNHR	ISDAATSTYEVGN
AcP2	--	-----	-----	A--S-V-DWN--R	
Bfast	--QAT-----	-----	Q-I-E--V-----G--I--		
Bslow	--QAT-----	-----	Q-I-E--V--G-V-DWN--R		
Low Mr AP	--QVT-----	-----	Q-I--V--G-V-DWN--R		

	60	70	80	90	100
AcP1	PPDYRGQNCMKKHGIHMQHIA	RGITRED	FATFDYIL	CMDES	NLRDLNRKSNQV
AcP2	--P-AV--LRN--STA-K-----				
Bfast	-----S--R--P-S-V--K-----				
Bslow	S--P-AVS-LRN--TA-K--K-----				
Low Mr AP	S-NP-AVS-LRN--NTA-K--V-K--V-----				

	110	120	130	140	150
AcP1	KNCKAKI	ELLGSYDPQKQLI	IEDPPYGHDS	DFEVVYQCLRCCKAFLEKTH	
AcP2	-----	-----	-----	-----	
Bfast	-T-----	-----	T-----V--R--A-		
Bslow	-T-----	-----	T-----V--R--A-		
Low Mr AP	--R-----	-----	A--T--V--R--V-R		

Fig. 11. Alignment of various mammalian low M_r phosphatases. AcP1 and AcP2 are from rat liver; B_{fast} and B_{slow} are from human erythrocytes [Dissing *et al.* (1991), Manao *et al.* (1991)]; low M_r AP is from bovine liver [Camici *et al.* (1989)].

between AcP1 and B_{fast} is 76.5% in this region, while that between AcP2 and B_{slow} is 85.3%. In this zone, bovine liver low M_r acid phosphatase is more similar to AcP2 and B_{slow} than to AcP1 and B_{fast} isoenzymes.

In conclusion, two types of low M_r acid phosphatases (which have PTPase activity) are present in mammalian tissues. One is constituted by AcP1 and B_{fast} (type 1), while the other is constituted by AcP2, B_{slow}, and bovine liver enzyme (type 2). The differences between the type 1 and type 2 isoenzymes clearly indicate that they are the product of two different, but related mRNAs. These different mRNAs probably arise from a single gene by an alternative splicing mechanism, as it was suggested by Dissing *et al.* (1991) for the human red cell isoenzymes. The only other alternative is that two genes exist, one encoding for AcP1 and the other for AcP2. Experiments (using degenerate polynucleotide probes constructed on the amino acid sequence basis) are presently underway in our laboratory to isolate the cDNAs which code for the two isoenzymes. These will be used for screening genomic libraries in order to isolate the natural gene(s) and to study the AcP1 and AcP2 mRNAs production mechanism.

4. CONCLUSION

It is well known that various growth factor receptors and several retroviral oncogenes have phosphotyrosine kinase activity. Under physiological conditions, growth factors receptors are autoactivated by Tyr-phosphorylation in the presence of growth fac-

tors, and can then phosphorylate target proteins on tyrosine residues. With reference to the growth factors signal transduction, these are the fundamental steps necessary for the induction of cell proliferation.

Rat liver AcP1 and AcP2 are low M_r acid phosphatases that possess PTPase activity. In fact, they are able to dephosphorylate the cytoplasmic domain of human erythrocyte band 3 and angiotensin I, both phosphorylated on tyrosine using calf thymus phosphotyrosine protein kinase P40 (unpublished results). Various authors have previously demonstrated that cytosolic low M_r acid phosphatases have very specific PTPase activity against some tyrosine phosphorylated protein substrates (Chernoff and Lee, 1985; Boivin and Galand, 1986; Whaeed *et al.*, 1988; Ramponi *et al.*, 1989; Zhang and Van Etten, 1990). Both AcP1 and AcP2 are inhibited by vanadate (Fujimoto *et al.*, 1988) and this is of particular interest since Klarlund (1985) demonstrated that NRK-1 cells become transformed when treated with this strong PTPase inhibitor. The amino acid sequences of rat liver AcP1 and AcP2, human erythrocyte B_{fast} and B_{slow}, and bovine liver low M_r acid phosphatase demonstrate that there is no homology between this enzyme family and the receptor like PTPases LAR, CD45, human placenta PTPase 1B, and rat brain PTPase-1 (Guan *et al.*, 1990). Furthermore, AcP1 and AcP2 are distinct from rat liver PTPase 1 and PTPase-2, quite recently purified by Hiraga and Tsuike (1991), since these last isoenzymes have higher molecular weights (67 and 36 kD, respectively).

Two queries arise from our results: (i) why are there two low M_r cytosolic phosphotyrosine protein phosphatases in mammalian tissues; and (ii) what is their function. These questions merit additional work to clarify whether the two isoenzymes have a different cellular physiological function. Our preliminary results indicate that some functional differences exist between AcP1 and AcP2. We confirm Fujimoto *et al.*'s findings that the two isoenzymes have different affinity for L-phosphotyrosine (Fujimoto *et al.*, 1988). We also found that AcP2 has a K_i for pyridoxal 5'-phosphate which is two orders of magnitude lower than AcP1, and that only AcP2 is activated by purine compounds, especially cGMP. In our opinion, the data on L-phosphotyrosine affinity is extremely important since it indicates a possible difference in action on the part of the two isoenzymes toward Tyr-phosphorylated proteins. The differences between AcP1 and AcP2 in the 40–73 region could be the structural basis of the different properties of the two isoenzymes. The data on activation and inhibition suggest

that only AcP2 is regulated by physiological compounds such as purine derivatives or pyridoxal 5'-phosphate, whereas AcP1 is not affected by these compounds.

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