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A New Acylphosphatase Isoenzyme from Human Erythrocytes: Purification, Characterization, and Primary Structure†

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ABSTRACT: A new acylphosphatase from human erythrocytes was isolated by an original purification procedure. It is an isoenzyme of the well-characterized human skeletal muscle acylphosphatase. The erythrocyte enzyme shows hydrolytic activity on acyl phosphates with higher affinity than the muscle enzyme for some substrates and phosphorylated inhibitors. The sequence was determined by characterizing the peptides purified from tryptic, peptic, and *Staphylococcus aureus* V8 protease digests of the protein, and it was found to differ in 44% of the total positions as compared to the human muscle enzyme. About one-third of these differences are in the form of strictly conservative replacements. The protein consists of 98 amino acid residues; it has an acetylated NH_2-terminus and does not contain cysteine: Ac-Ala-Glu-Gly-Asn-Thr-Leu-Ile-Ser-Val-Asp-Tyr-Glu-Ile-Phe-Gly-Lys-Val-Gln-Gly-Val-Phe-Phe-Arg-Lys-His-Thr-Gln-Ala-Glu-Gly-Lys-Lys-Leu-Gly-Leu-Val-Gly-Trp-Val-Gln-Asn-Thr-Asp-Arg-Gly-Thr-Val-Gln-Gly-Gln-Leu-Gln-Gly-Pro-Ile-Ser-Lys-Val-Arg-His-Met-Gln-Glu-Trp-Leu-Glu-Thr-Arg-Gly-Ser-Pro-Lys-Ser-His-Ile-Asp-Lys-Ala-Asn-Phe-Asn-Asn-Glu-Lys-Val-Ile-Leu-Lys-Leu-Asp-Tyr-Ser-Asp-Phe-Gln-Ile-Val-Lys-OH.

Acylphosphatase was first described as acetylphosphatase by Lipmann (1946). The enzyme is a small protein (M_r 11 000–12 000) characterized by a specific phosphomonohydrolase activity. It catalyzes the hydrolysis of compounds with the general structure R–COO–PO_3^{2-}, such as 1,3-diphosphoglycerate and carbamyl phosphate (Ramponi, 1975), succinyl phosphate (Berti et al., 1977), and the β-aspartyl phosphates that are present in phosphorylated intermediates

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of the sarcoplasmic reticulum Ca^{2+},Mg^{2+}-dependent ATPase (Stefani et al., 1981) and Na^+,K^+-dependent ATPase (Hokin et al., 1965). Benzoyl phosphate and *p*-nitrobenzoyl phosphate are synthetic substrates useful for measuring the enzyme's activity by continuous optical tests (Ramponi et al., 1966a). The enzyme shows negligible activity on phosphate monoesters; thus, it differs substantially from alkaline phosphatases (Sperow and Butler, 1971) and from acid phosphatases (low molecular weight) (Ramponi et al., 1980; Taga & Van Etten, 1982) that exhibit phosphomonohydrolase activities on both phosphate monoesters and acyl phosphates.

Acylphosphatase has now been studied in a variety of vertebrate species or tissues (Ramponi, 1975; Camici et al., 1984, 1986; Manao et al., 1984, 1985; Liguri et al., 1984; Mizuno et al., 1984). The amino acid sequence of the skeletal muscle enzyme has been defined in several vertebrate species: horse (Cappugi et al., 1980), turkey (Camici et al., 1983), rabbit (Manao et al., 1985; Kizaki et al., 1985), pig (Mizuno et al., 1985), calf (Camici et al., 1986), and man (Manao et al., 1984). We have further demonstrated that the enzyme from horse heart (Stefani et al., 1985) is structurally the same protein as that from horse skeletal muscle.

The present paper reports an original purification technique, a kinetic characterization, and the complete amino acid sequence of the enzyme from human erythrocytes. This latter enzyme differs substantially from that of skeletal muscle in its kinetic properties and primary structure. The human skeletal muscle and erythrocyte enzymes are isoenzymes. The length of their polypeptide chains is identical, but there are 44% differences in their amino acid positions; both enzymes show an acetylated NH_2-terminus. They appear to be the products of a common ancestral gene, whose duplication and successive evolutionary divergence gave two distinct but related genes. Thus, the two human isoenzymes represent an example of protein differentiation (Schultz & Schirmer, 1979).

Materials and Methods

Acetyl phosphate carbamyl phosphate, and glyceraldehyde 3-phosphate diethyl acetal (monobarium salt) were purchased from Sigma Chemical Co. 1,3-Diphosphoglycerate was synthesized and purified according to Manao et al. (1977). Benzoyl phosphate was prepared according to Camici et al. (1976). All other chemicals were the best commercially available.

Protein concentration was determined by the biuret method (Beisenherz et al., 1953) using bovine serum albumin as the standard.

Acylphosphatase Activity. The hydrolytic activity of acylphosphatase on 1,3-diphosphoglycerate, carbamyl phosphate, and acetyl phosphate was assayed by incubating the enzyme at 25 °C in the presence of various concentrations of substrates in 0.1 M acetate buffer, pH 5.3. Control tubes containing substrate but no enzyme were incubated simultaneously. The orthophosphate released after different time intervals was determined (Baginsky et al., 1967). Hydrolysis of 1,3-diphosphoglycerate was also measured as described by Negelein (1965). Acylphosphatase activity was assayed routinely by using benzoyl phosphate as substrate (Ramponi et al., 1966b).

Polyacrylamide Gel Electrophoresis. Electrophoretic analysis of the purified enzyme was carried out by the methods of Panyim and Chalkley (1969) and Dunker and Rueckert (1969).

Amino Acid Analyses, NH_2- and COOH-Terminal Analyses, Sequence Determination, and Fast Atom Bombardment Mass Spectrometry. These were carried out as previously described by Camici et al. (1983). Phenylthiohydantoin (PTH)[1] derivatives were analyzed according to Manao et al. (1985), except that the analyses of PTH–Arg and PTH–His were performed by HPLC using a small column of Aquapore RP-300 (10 μm, 4.6 × 30 mm). The elution employed the isocratic mode (90% 0.04 M acetate buffer, pH 4.40, and 10% methanol at a flow rate of 1 mL/min).

Enzymatic Digestions. Before every enzymatic digestion, the protein solution in a screw-capped vial was immersed for 3–5 min in a boiling water bath and chilled in ice. Tryptic digestion was carried out as previously described by Camici et al. (1983). Peptic digestion was performed under conditions previously described (Camici et al., 1983) except that the enzyme was dissolved in 5% acetic acid containing 0.01 M HCl. Digestion with *Staphylococcus aureus* V8 protease was carried out by incubating for 21 h at 37 °C 50 nmol of enzyme dissolved in 0.1 mL of 0.05 M phosphate buffer, pH 7.8, containing 2 mM EDTA. Thermolytic digestion was done as previously described (Camici et al., 1983) but without urea.

Separation of Peptides. Peptides were separated by HPLC using a Beckman instrument with an Aquapore RP-300 column. Peptides were eluted from the column with gradients of acetonitrile containing 10 mM TFA as indicated in the figure legends.

Enzyme Isolation. Recently outdated blood or concentrated erythrocytes from a human blood bank were used routinely for enzyme purification. Four liters of blood or 2 L of concentrated erythrocytes was usually processed. Preliminary assays on freshly drawn blood had shown no significant differences in acylphosphatase activity content as compared to outdated blood samples. All operations were carried out at 0–4 °C unless otherwise stated.

The material was centrifuged at 500*g* for 15 min; then plasma and buffy coat were removed by suction. The packed cells were washed (1:3 v/v) with 0.15 M NaCl containing 0.1% (w/v) glucose. The cells were suspended by repeated inversions and centrifuged at 400*g* for 10 min. This procedure was repeated twice, the supernatant and upper layer of the erythrocytes being discarded each time.

Erythrocytes were then lysed by addition (1:3 v/v) of cold, bidistilled water, the mixture being continually stirred. After 30 min, the pH was adjusted to 6.8, and, while manually stirring, cold ethanol and chloroform were slowly added to final concentrations of 20% (v/v) and 5% (v/v), respectively. Sixty minutes later, the denaturated hemoglobin was removed by centrifugation at 550*g* for 30 min in polyethylene bottles. The supernatant was adjusted to pH 5.8, centrifuged at 35000*g* for 30 min, and applied to a CM Sephadex C-25 column (2.5 × 40 cm) previously equilibrated with 10 mM acetate buffer, pH 5.8, containing 20% (v/v) ethanol. The resin was then washed with 500 mL of equilibrating buffer, followed by 500 mL of 10 mM acetate buffer, pH 5.8, in order to remove ethanol. Elution was achieved by applying a 0.005–0.6 M NaCl linear gradient in 10 mM acetate buffer, pH 5.8 (Figure 1A). Fractions with acylphosphatase activity were collected and concentrated by ultrafiltration on a YM2 Amicon membrane to a final concentration of about 10 mg/mL. The sample was applied to a Sephadex G-75 column (1.5 × 90 cm) equilibrated and eluted with 0.1 M NaCl in 50 mM acetic acid. The elution pattern of the gel filtration chromatography

[1] Abbreviations: TFA, trifluoroacetic acid; SDS, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography; PTH, phenylthiohydantoin; FAB, fast atom bombardment; CPY, carboxypeptidase Y; T, tryptic peptides; Sp, *S. aureus* V8 protease peptides; Th, thermolytic peptides; EDTA, ethylenediaminetetraacetic acid.

Table I: Main Purification Steps of Acylphosphatase from Human Erythrocytes

step	total act. (units)[a]	total protein (mg)	total volume (mL)	sp act. (units/mg)	yield (%)
(1) erythrocyte lysate	30000	638400	6000	0.047	100
(2) supernatant after ethanol/chloroform treatment	10980	1188	5400	6.81	36
(3) active peak from ionic exchange chromatography	6600	66	80	100	22
(4) active peak from gel filtration chromatography	4800	1.56	40	3000	16
(5) active peak from reverse-phase HPLC	3600	0.48	2	7500	12

[a] Units are expressed as micromoles of benzoyl phosphate released per minute at 25 °C and pH 5.3.

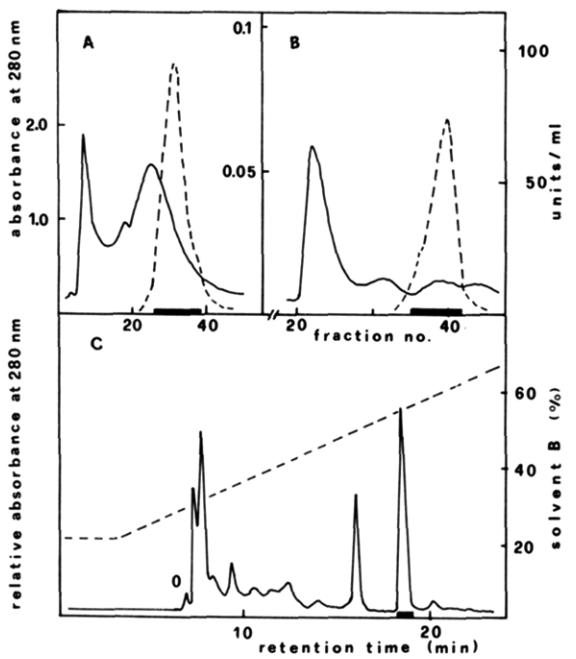

FIGURE 1: (A) Elution pattern of acylphosphatase activity and proteins from a CM Sephadex C-25 column (2.5 cm × 40 cm). Elution was performed by changing the NaCl concentration from 0.005 to 0.6 M in 600 mL of 10 mM acetate buffer, pH 5.5; 6-mL fractions were collected. (B) Elution pattern of acylphosphatase activity and proteins from a Sephadex G-75 column (1.5 cm × 90 cm). The eluent was 0.1 M NaCl in 50 mM acetic acid; 6-mL fractions were collected: (—) absorbance; (---) activity. (C) Reversed-phase HPLC of acylphosphatase peak from Sephadex G-75. The column was an Aquapore RP-300 (0.46 cm × 25 cm), 10 μm. Solvent A was 10 mM TFA in water; solvent B was 10 mM TFA in acetonitrile. Flow rate, 1.5 mL/min. (—) Absorbance, (---) elution gradient. Fractions indicated by solid bars were pooled.

is shown in Figure 1B. Fractions with acylphosphatase activity were collected and concentrated by ultrafiltration. The last purification step was a reverse-phase chromatography, performed on a Beckman instrument equipped with an Aquapore RP-300 column (Brownlee Labs. Inc.). Proteins were eluted with a linear gradient of acetonitrile containing 10 mM TFA, as illustrated in Figure 1C.

Results and Discussion

Recently we used immunoaffinity chromatography for the purification of skeletal muscle acylphosphatase from several vertebrate species (Manao et al., 1983, 1984, 1985; Camici et al., 1984; Liguri et al., 1984). Unfortunately, anti-skeletal muscle acylphosphatase antibodies do not cross-react with erythrocyte acylphosphatase. Thus, we devised an original procedure to purify the human erythrocyte enzyme. This new strategy is summarized in Table I, while panels A–C of Figure 1 show the chromatographic separations carried out at steps 3–5, respectively. The preparation yielded a pure protein as demonstrated by polyacrylamide gel electrophoresis (Figure 2). With regard to the thermal stability, 90% of our preparation's activity is destroyed when it is heated for 10 min at 37 °C over a pH range of 5.3–7.4.

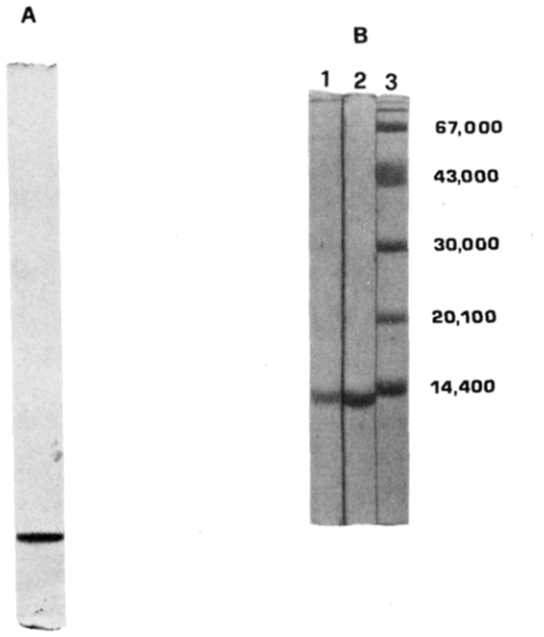

FIGURE 2: Electrophoretograms of purified human erythrocyte acylphosphatase. (A) Nondenaturating polyacrylamide gel electrophoresis of erythrocyte enzyme; 10 μg of protein was applied; (B1 and B2) SDS–polyacrylamide gel electrophoresis of human muscle acylphosphatase and human erythrocyte acylphosphatase, respectively; 10 μg of proteins was applied to each tube; (B3) standard proteins.

Table II: Kinetic Parameters of Acylphosphatase from Human Erythrocytes and Skeletal Muscle

	erythrocyte enzyme	skeletal muscle enzyme
pH optimum	5.5	5.3
K_m on benzoyl phosphate (mM)	0.15	0.81
K_m on acetyl phosphate (mM)	2.90	6.20
K_m on 1,3-diphosphoglycerate (mM)	0.55	0.62
K_m on carbamyl phosphate (mM)	5.42	4.20
V_{max} on benzoyl phosphate[a]	7500	3650
V_{max} on acetyl phosphate[a]	35	33
V_{max} on 1,3-diphosphoglycerate[a]	6900	1570
V_{max} on carbamyl phosphate[a]	22	7
K_i by 2,3-diphosphoglycerate[b] (mM)	0.41	3.74
K_i by phosphoenolpyruvate[b] (mM)	0.38	2.23
K_i by orthophosphate[b] (mM)	0.30	2.72
K_i by chloride[b] (mM)	51.70	42.10

[a] Micromoles per milligram of enzyme per minute at pH 5.5 and 25 °C. [b] Competitive type inhibition; benzoyl phosphate as substrate.

The properties of this new acylphosphatase were compared with those of the related isoenzyme from human skeletal muscle (Table II). Several kinetic parameters indicated that the erythrocyte enzyme had a higher affinity for most substrates and also for some phosphorylated competitive inhibitors than does the muscle enzyme. The erythrocyte enzyme's low K_i values for orthophosphate, 2,3-diphosphoglycerate, and phosphoenolpyruvate suggested that these compounds may

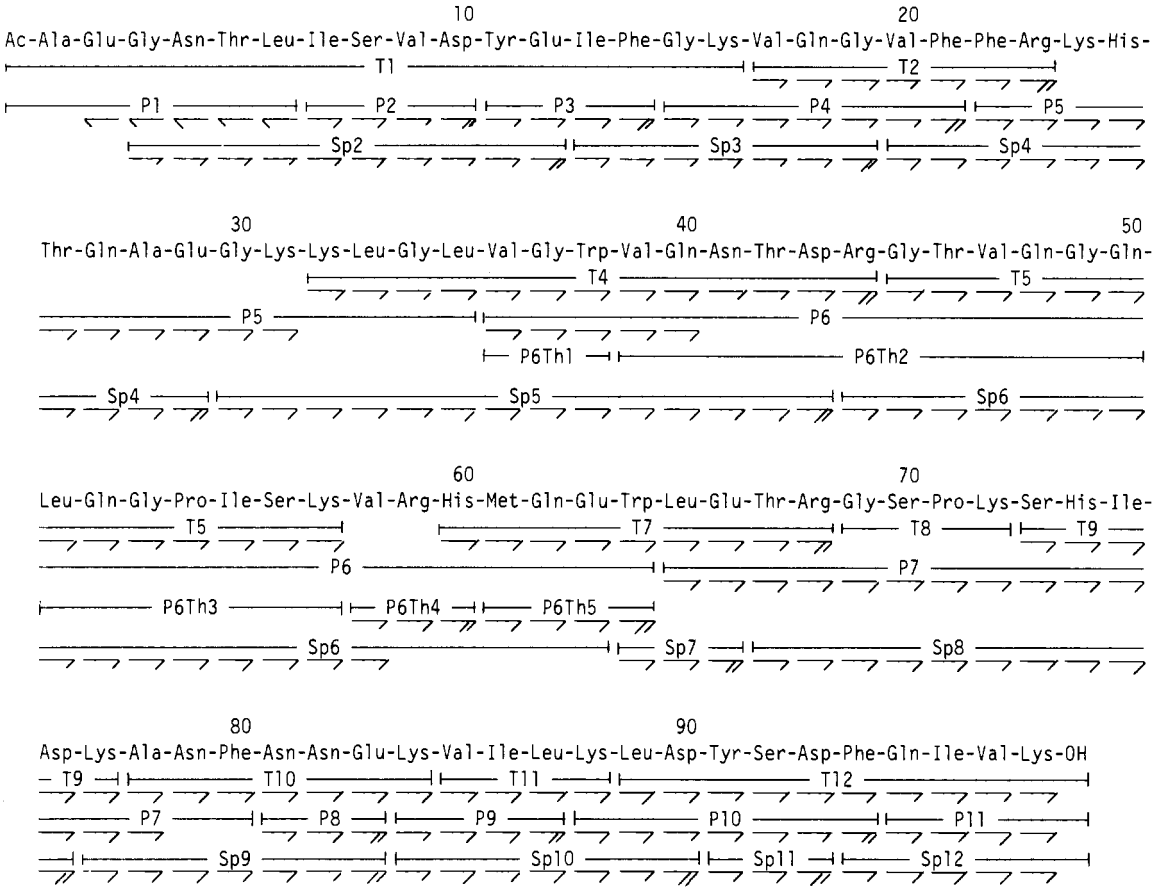

FIGURE 3: Amino acid sequence of human erythrocyte acylphosphatase. The figure summarizes the peptides used to reconstruct the complete sequence. The symbols for the sequence results are as follows: (—) NH_2-terminus and subsequent residues revealed by manual Edman degradation, PTH-derivatives being identified by HPLC; (⇀) residues identified as free amino acids after Edman degradation of the penultimate residue of the peptide; (—) carboxypeptidase Y time-course analysis; Ac represents acetyl.

exert a regulatory role on the hydrolytic activity of acylphosphatase in vivo.

The primary structure of human erythrocyte acylphosphatase is presented in Figure 3. The determination of the complete covalent structure was based on the isolation (Figures S-1–S-4) and determination of the amino acid composition (Tables S-I–S-III) and sequences of fragments obtained from digestion of the enzyme with trypsin, pepsin, thermolysin, and *S. aureus* V8 protease (supplementary material; see paragraph at end of paper regarding supplementary material).

The data sufficed to reconstruct the sequence by overlapping the various peptide types. Moreover, the NH_2- and the COOH-terminus results (Table III) agreed very well with the proposed structure.

The structure of the NH_2-terminus was determined from both P1 and Sp2 as follows: (i) P1 is Edman negative; its structure was studied by time-course analysis of the amino acids released upon CPY treatment; the results indicate that Leu is the COOH-terminal residue, whereas Ala, which is not released as a free amino acid, precedes the other five residues. Furthermore, these data indicate that Glu is at position 2. Comparison of these results with the sequence found for Sp2 (Figure 3) justifies the sequence assigned to the NH_2-terminus of the enzyme. (ii) The FAB/mass spectrum (Figure 4) demonstrated that the observed molecular weight of the peptide agreed with the *N*-acetyl derivative of a peptide whose composition corresponded to that of P1.

As shown in Table III, the amino acid composition of the erythrocyte acylphosphatase compared favorably with that calculated from the sequence analysis. The enzyme consists of 98 amino acid residues and has a molecular weight of 11 165.

Table III: Amino Acid Composition and Terminal Residues of Erythrocyte Acylphosphatase[a]

amino acid	
Asp + Asn	10.4 (10)
Thr	4.7 (5)
Ser	5.3 (5)
Glu + Gln	14.5 (14)
Pro	2.0 (2)
Gly	10.3 (10)
Ala	3.2 (3)
Val	7.9 (9)
Met	1.1 (1)
Ile	5.9 (6)
Leu	7.0 (7)
Tyr	2.1 (2)
Phe	4.9 (5)
Lys	10.2 (10)
His	3.2 (3)
Arg	4.0 (4)
Trp	2.0 (2)
total residues	(98)

NH_2-terminus: Edman, none
NH_2-terminus: dansyl, none
COOH-terminus: carboxypeptidase B, Lys

[a] Acid hydrolyses were performed on the enzyme at 110 °C for 24 and 72 h in duplicate samples. The values of Thr and Ser were obtained by extrapolation to zero time of hydrolysis. Trp was determined as described (Camici et al., 1983). Numbers in parentheses are derived from the sequence (Figure 3).

The protein lacks cysteine whose presence has been detected in a strictly conserved sequence stretch in the skeletal muscle

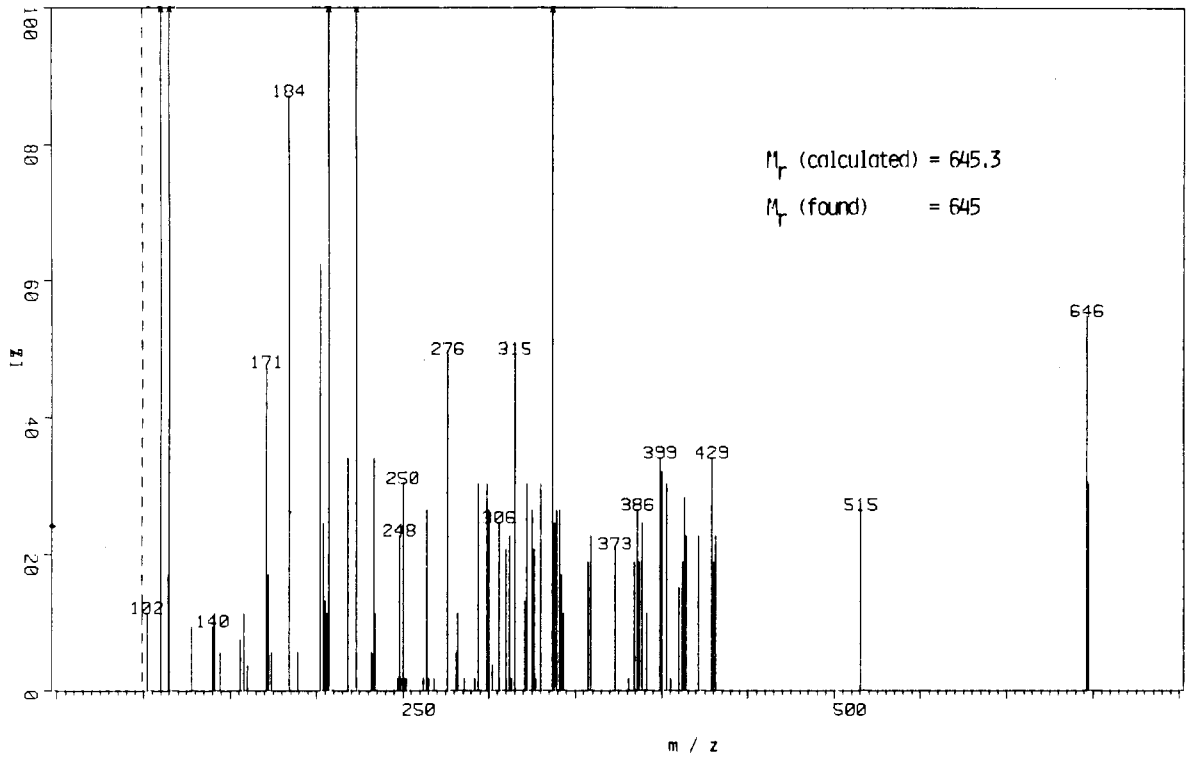

FIGURE 4: Positive FAB mass spectrum of P1.

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                                        10                                  20
Erythrocyte          Ac-Ala-Glu-Gly-Asn-Thr-Leu-Ile-Ser-Val-Asp-Tyr-Glu-Ile-Phe-Gly-Lys-Val-Gln-Gly-Val-Phe-Phe-Arg-Lys-His-
Skeletal muscle      ..-Ser-Thr-Ala-Gln-Ser-...-Lys-... ... ... ... ...-Val-... ...-Arg-... ... ... ...-Cys-... ...-Met-Tyr-

                                30                                  40                                  50
Thr-Gln-Ala-Glu-Gly-Lys-Lys-Leu-Gly-Leu-Val-Gly-Trp-Val-Gln-Asn-Thr-Asp-Arg-Gly-Thr-Val-Gln-Gly-Gln-
...-Glu-Asp-...-Ala-Arg-...-Ile-...-Val-... ... ... ...-Lys-... ...-Ser-Lys-... ... ...-Thr-... ...

                                        60                                  70
Leu-Gln-Gly-Pro-Ile-Ser-Lys-Val-Arg-His-Met-Gln-Glu-Trp-Leu-Glu-Thr-Arg-Gly-Ser-Pro-Lys-Ser-His-Ile-
Val-... ... ...-Glu-Asp-... ...-Asn-Ser-...-Lys-Ser-... ...-Ser-Lys-Val-... ... ...-Ser-...-Arg-...

                                80                                  90
Asp-Lys-Ala-Asn-Phe-Asn-Asn-Glu-Lys-Val-Ile-Leu-Lys-Leu-Asp-Tyr-Ser-Asp-Phe-Gln-Ile-Val-Lys-OH
...-Arg-Thr-... ...-Ser-... ... ...-Thr-...-Ser-... ...-Glu-... ...-Asn-...-Ser-...-Arg-Tyr-..
```

FIGURE 5: Alignment of acylphosphatase sequences from human erythrocyte and skeletal muscle.

enzymes of all mammalian species studied to date. The skeletal muscle enzyme from birds, such as turkey (Camici et al., 1983) and duck (unpublished data), also shows cysteine in the homologous polypeptide stretch. Furthermore, the enzyme from human erythrocytes contains three histidine residues whereas no other acylphosphatases from either skeletal muscle or heart contain histidine (Cappugi et al., 1980; Camici et al., 1983; Manao et al., 1984, 1985; Kizaki et al., 1985; Mizuno et al., 1985; Stefani et al., 1985).

A comparison of the primary structures of human erythrocyte and skeletal muscle acylphosphatases is presented in Figure 5. The amino acid substitutions account for 44% of the total residues, while the length of the polypeptide chains is identical and the NH_2-terminus is acetylated in both isoenzymes. Only about one-third of these substitutions are strictly conservative. The COOH-terminal residue, which is always tyrosine in all known isoenzymes from skeletal muscle, is instead lysine in that from erythrocytes.

Diederich and Grisolia (1969) purified and characterized a calf brain acylphosphatase. Some features of this enzyme bear a striking resemblance to our erythrocyte isoenzyme, but it differs markedly from the skeletal muscle enzyme. Both calf brain and human erythrocyte enzymes have similar molecular weights, lysine as the COOH-terminal residue, and a blocked NH_2-terminus. Their amino acid compositions are also quite similar; the minor discrepancies may be due to the species difference.

Recent findings from our laboratory indicate that the human erythrocyte enzyme does not cross-react with anti-skeletal muscle acylphosphatase antibodies. Studies are in progress to establish if brain acylphosphatase is structurally related to the erythrocyte enzyme.

A specific acylphosphatase from human erythrocytes was purified 900-fold by Rakitzis and Mills (1969). These authors observed no hydrolysis of carbamyl phosphate, which is only a competitive inhibitor, while our enzyme catalyzes the hydrolysis of this compound. It appears reasonable that Rakitzis and Mills observed no hydrolysis considering the difference in the relative V_{max} for 1,3-diphosphoglycerate and benzoyl phosphate vs. carbamyl phosphate (Table II). In fact, the K_i reported by those authors is close to the K_m found for our enzyme.

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Supplementary Material Available

Four figures related to the HPLC separation of tryptic, *S. aureus* V8 protease, peptic, and thermolytic subfragments and three tables giving their amino acid compositions (7 pages). Ordering information is given on any current masthead page.

Registry No. $PhCO_2PO_3H_2$, 6659-26-3; $AcOPO_3H_2$, 590-54-5; $NH_2CO_2PO_3H_2$, 590-55-6; $H_2O_3POCH_2CH(OH)CO_2PO_3H_2$, 1981-49-3; $CO_2HCH(OPO_3H_2)CH_2OPO_3H_2$, 138-81-8; PO_4^{3-}, 14265-44-2; Cl^-, 16887-00-6; phosphoenolpyruvate, 138-08-9; acyl phosphatase, 9012-34-4; acyl phosphatase (human erythrocyte), 104848-58-0.

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