Studies on synthesis and degradation rates and some molecular properties of guinea-pig muscle acylphosphatase

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Acylphosphatase (acylphosphate phosphohydrolase, EC 3.6.1.7) was purified from guinea-pig muscle by a procedure involving immuno-affinity chromatography and a subsequent ion-exchange chromatography. This purification technique gave an overall yield of about 60% and permitted the isolation of three molecular forms with acylphosphatase activity, with a distribution greatly resembling those found in horse and turkey muscle. The main form appears to be very similar to the corresponding form in horse and turkey muscle, as indicated by amino acid composition, end-group analysis, the presence of glutathione as a mixed disulphide in almost the same stoichiometric ratio and kinetic analysis. From turnover data, the main form of acylphosphatase in guinea-pig muscle exhibits a degradation constant of 0.10 day^{-1} , corresponding to a half-life of 6.8 days. These values are very close to those found for muscle total soluble proteins.

Acylphosphatase (EC 3.6.1.7) is a widespread hydrolase that specifically acts on the carboxyl phosphate bond of acyl phosphates. Acylphosphatase can act on important physiological substrates such as carbamoyl phosphate (Grisolia *et al.*, 1958; Ramponi *et al.*, 1961), 3-phosphoglyceroyl phosphate (Ramponi *et al.*, 1967), γ -glutamyl phosphate, β -aspartyl phosphate and succinoyl phosphate (Berti *et al.*, 1977). Furthermore, it can hydrolyse the phosphorylated intermediate of $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -dependent ATPase, both in sarcoplasmic-reticulum vesicles and in their pepsin digest (Stefani *et al.*, 1981). The real function of acylphosphatase is still debated. Its pH optimum is about 5, but at neutral pH it retains half of its activity. From subcellular-fractionation and cytochemical studies (Hernández-Yago *et al.*, 1978), muscle acylphosphatase appears to be localized in the cytoplasm, in proximity to sarcoplasmic reticulum.

On present knowledge, acylphosphatase seems to have a regulatory function, controlling the concentrations of highly reactive compounds such as acyl phosphates. The possible role of this enzyme

has been discussed in reviews by Grisolia & Hood (1972) and Ramponi (1975, 1976).

In our laboratory, acylphosphatase was obtained as a pure protein from horse muscle and characterized with regard to various functional and structural properties (Ramponi *et al.*, 1969). Studies on the primary structure led us to establish the complete amino acid sequence of this enzyme, which consists of a peptide chain of 98 amino acid residues with an acetyl group blocking the *N*-terminus and a tyrosine residue at the *C*-terminus. This polypeptide chain is linked with glutathione by an S–S bond in position 21. The M_r of the protein calculated from this sequence is 11365 (Cappugi *et al.*, 1980).

Antibodies to horse muscle acylphosphatase were obtained from immunized rabbits and purified from globulins by affinity chromatography (Berti *et al.*, 1980). These antibodies were bound to a Sepharose 4B matrix, and allowed us to purify the enzyme in a simpler and faster way than by the classical procedure.

The enzyme obtained by the two different procedures had the same specific activity. However, that purified by immuno-affinity chromatography consisted of three forms, the most abundant of which (about 60% of total activity) appears to correspond to the previously isolated enzyme in terms of M_r ,

Abbreviations used: SDS, sodium dodecyl sulphate; dansyl, 5-dimethylaminonaphthalene-1-sulphonyl; Mes, 4-morpholine-ethanesulphonic acid.

value, electrophoretic mobility and amino acid composition. A second form, present only in small amount, showed the same M_r but slightly different amino acid composition. The third form is characterized by almost double the M_r value and, from preliminary results, may be considered a dimeric form of the most common form (Manao *et al.*, 1983). Similar results were also obtained for turkey muscle acylphosphatase (Stefani *et al.*, 1982). These findings indicated the occurrence, *in vivo*, of multiple molecular forms of acylphosphatase, the less common of which were probably lost during the classical procedure of isolation. As a result, it seemed worthwhile to isolate acylphosphatase from various organisms by the immunochemical procedure, taking advantage of possible cross-reaction with the antibodies against horse muscle enzyme.

To this end, the present paper reports the purification of acylphosphatase from guinea-pig (*Cavia porcellus*) muscle and a study of its properties and rates of synthesis and degradation.

Experimental

Materials and animals

Sephacrose 4B (CNBr-activated) and CM-Sephadex C25 were purchased from Pharmacia, Uppsala, Sweden. AG50 1X8 mixed-bed resin was obtained from Bio-Rad; ^{14}C -labelled L-amino acid mixture in aqueous solution (CFB 104) was from The Radiochemical Centre, Amersham, Bucks., U.K. Aquassure, from New England Nuclear, was used as scintillation liquid. Benzoyl phosphate was synthesized as described by Camici *et al.* (1976). All other chemicals were of analytical grade.

White male guinea pigs, weighing 400–500 g, were obtained from S. Morini, S. Polo d'Enza, Italy. Animals were fed on a solid diet (21.53% protein content) prepared by Dottori Piccioni, Brescia, Italy.

Anti-(horse muscle acylphosphatase) antibodies were obtained and purified as reported by Berti *et al.* (1980).

Methods

Acylphosphatase activity measurements. Acylphosphatase was assayed continuously at 283 nm with benzoyl phosphate as substrate. The unit of activity is defined as the amount of the enzyme that liberates 1 μmol of benzoate/min at 25°C and pH 5.3 (Ramponi *et al.*, 1966).

Protein determination. This was done by the biuret reaction, by the method of Beisenherz *et al.* (1953), slightly modified: 1.5 ml of the biuret reagent was added to 0.05–0.20 mg of protein in a final volume of 2.0 ml. The A_{546} of the samples was

determined after 10 min. Crystalline bovine serum albumin was used as the reference standard. Protein concentration of the purified enzyme was estimated spectrophotometrically at 280 nm.

Amino acid determination. This was done by the ninhydrin method of Garrels *et al.* (1972), with tyrosine as a standard.

Polyacrylamide-gel electrophoresis. Polyacrylamide-gel electrophoresis of the purified enzyme was performed by the method of Panyim & Chalkley (1969). The M_r of the enzyme was determined by SDS/polyacrylamide-gel electrophoresis as described by Dunker & Rueckert (1969).

Preparation of the immunoabsorbent. Purified antibodies were bound to CNBr-activated Sepharose 4B previously washed with 1.0 M-HCl and prehydrolysed by incubation for 30 min in 0.1 M- NaHCO_3 buffer, pH 8.7, containing 0.5 M-NaCl, at room temperature. Antibody binding was performed by incubating the resin with the immunoglobulins in a ratio of 5 mg of protein/ml of gel. The mixture was incubated in 0.1 M- NaHCO_3 buffer, pH 8.7, containing 0.5 M-NaCl, at 4°C with continuous shaking overnight. The reaction was terminated by washing the resin with the incubation buffer; this was followed by a wash cycle repeated three times and performed with 0.1 M-sodium acetate buffer, pH 4.0, containing 1.0 M-NaCl, and 0.1 M-sodium borate buffer, pH 9.0, containing 1.0 M-NaCl. The residual active groups of the resin were finally completely blocked by incubation with 1.0 M-ethanolamine at room temperature for 30 min.

Amino acid analysis. Analysis was performed by the procedure of Spackman *et al.* (1958), in a Carlo Erba 3A29 Amino Acid Analyser, equipped with a Spectra-Physics SP4100 integrator. Cysteine plus cystine was independently determined as cysteic acid in a performic acid-oxidized protein sample (Lee & Drescher, 1979). Tryptophan was determined by the method of Edelhoch (1967). Values for serine and threonine were corrected as described by Moore & Stein (1963).

N- and C-terminal analysis. The N-terminal residues were determined by the dansyl procedure reported by Gray (1972) and by the Edman-degradation technique as described by Tarr (1975). C-Terminal analysis was performed by hydrazinolysis. Technical details of these analyses are reported in another paper from our laboratory (Manao *et al.*, 1983).

Determination of glutathione and free thiol groups. The presence of glutathione and its stoichiometry in the enzyme were determined by a procedure of isotopic dilution, after the cleavage of the S-S bridge by oxidation with performic acid and the separation of glutathione (as sulphonic acid) by high-voltage electrophoresis (Manao *et al.*, 1983).

Free thiol groups were determined by the spectrophotometric method of Ellman (1959).

Administration of the radioisotope. Acylphosphatase turnover was determined by a technique of continuous administration of ^{14}C -labelled amino acids. Animals were individually kept in cages and, after 24h starvation, they received each day 25g of the solid diet and 12.5×10^6 d.p.m. of the ^{14}C -labelled amino acid mixture dissolved in their drinking water. At intervals after being placed on this treatment, groups of three animals were decapitated between 07:00 and 07:15h. Muscles were removed from back and hind legs and prepared for radioactivity counting.

Preparation and counting of samples. Muscle was weighed and homogenized (1:4, w/v) in 20mM-triethanolamine/HCl buffer, pH 7.2, containing 0.154M-NaCl. The enzyme was isolated by the same steps described for the purification procedure. Samples of the purified enzyme were freeze-dried, dissolved in 1 ml of water and added to 10ml of the scintillation liquid. Protein concentration was estimated spectrophotometrically and results were expressed as d.p.m./pmol of enzyme.

Samples from the pH 7.2 supernatants were centrifuged at 100000g for 60 min in a Spinco L50 centrifuge, and the supernatant was used to determine the specific radioactivity of total soluble proteins and free amino acids. Protein concentration was measured by the biuret method. Then 9 ml of 0.306M-trichloroacetic acid was added to 0.5 ml of the supernatant plus 0.5 ml of water. The mixture was centrifuged at 3000g for 10 min. The precipitate was washed with 2×5 ml of diethyl ether and finally dissolved in 2 ml of 0.2M-NaOH. This solution, neutralized with 0.4 ml of 1M-HCl, was transferred, in 1 ml portions, to counting vials containing 10 ml of the scintillation liquid. Specific radioactivity of total soluble proteins was expressed as d.p.m./mg of protein.

For measurement of the specific radioactivity of free amino acids, the supernatants from trichloroacetic acid precipitation were extracted with 2×25 ml of diethyl ether. Portions (0.5 and 1.0 ml) from each aqueous sample were added to 10 ml of the scintillation liquid and used for radioactivity counting. The concentration of amino acids was determined by the ninhydrin method, and specific radioactivity was expressed as d.p.m./ μmol of amino acid.

Results

Acylphosphatase purification and characterization

The whole enzyme purification procedure was performed at 0–4°C. Guinea-pig muscle (average 110g) from back and hind legs was homogenized (1:4, w/v) in 20mM-triethanolamine/HCl buffer,

Fig. 1. Elution profile from immunoaffinity column A_{280} (—) was monitored with a flow-cell. Eluate fractions (4 ml) were collected and assayed for acylphosphatase activity (●). The column was first washed with 20mM-triethanolamine/HCl buffer, pH 7.2 (A), and then eluted with 0.2M-acetic acid (B).

pH 7.2, containing 0.154M-NaCl, in a Waring Blendor for 90s. The homogenate was centrifuged for 30 min at 8000g in a Beckman J21 centrifuge. The supernatant was filtered through glass fibre and adjusted to pH 4.8 with 1M-HCl. Then it was centrifuged for 15 min at 8000g. The precipitate was discarded, the supernatant was adjusted to pH 8.0 by addition of 2M-NaOH and stirred for 30 min at 4°C. This material was centrifuged for 90 min at 30000g. The supernatant from this centrifugation was applied to a chromatographic column (2.5 cm $\times$ 4.0 cm) filled with the immuno-adsorbent that had been equilibrated with 20mM-triethanolamine/HCl buffer, pH 7.2, containing 0.154M-NaCl. To remove non-specifically adsorbed proteins, the column was washed with the equilibrating buffer until the A_{280} of the effluent was the same as that of the buffer. The subsequent equilibration with 20mM-triethanolamine/HCl, pH 7.2, without NaCl was performed to lower the ionic strength of the eluate. The enzyme was eluted from the immuno-adsorbent with 0.2M-acetic acid at a flow rate of 50 ml/h. The A_{280} of the effluent was monitored, and the fractions corresponding to the A_{280} peak were collected. Fig. 1 shows the elution pattern of the enzyme.

The last purification step was accomplished by ion-exchange chromatography. The sample was adjusted to pH 6.5 by addition of 1M-NaOH and diluted 1:5 with 20mM-Mes buffer, of the same pH. This solution was introduced into a column of CM-Sephadex C25 (1.5 cm $\times$ 50 cm) that was equilibrated with the same buffer. The column was washed with 500 ml of the equilibrating buffer and developed with 500 ml of a 0–0.5M-NaCl linear gradient in 20mM-Mes buffer, pH 6.5. The column

effluent was collected in 5 ml fractions. The elution profile (Fig. 2) showed the presence of three peaks. Fractions corresponding to each peak were pooled and assayed for acylphosphatase activity. Each peak was enzymically active, with the same specific activity, indicating the presence of three molecular forms of acylphosphatase in guinea-pig muscle, here named GP1, GP2 and GP3 according to the order of elution from CM-Sephadex.

Table 1 reports the various steps of the purification procedure. The major form, GP1, was characterized with respect to some molecular and kinetic properties, as well as to its rates of synthesis and degradation in muscle *in vivo*.

Polyacrylamide-gel-electrophoretic patterns of forms GP1 and GP3 are shown in Fig. 3. Fig. 3(a) shows the electrophoretograms obtained in the presence of 4M-urea, from which the very similar mobility and homogeneity of the samples may be observed. From Fig. 3(b), showing the electro-

Fig. 2. *Elution profile from CM-Sephadex C25*
Elution was performed with a linear gradient from 0 to 0.5M-NaCl in 20mM-Mes buffer, pH 6.5. Further details are given in the text. Three peaks of A_{280} (—) and of acylphosphatase activity (●) were obtained, termed respectively GP1, GP2 and GP3 in order of elution.

phoretograms obtained in the presence of SDS, a M_r value of about 12000 may be calculated for GP1 and of about 24000 for GP3.

With respect to the kinetic properties, the main form of guinea-pig muscle acylphosphatase had an optimum pH of 5.15, with benzoyl phosphate as the substrate. Apparent K_m values of 0.57 and 1.08 mM were calculated for benzoyl phosphate and acetyl phosphate respectively. Both phosphate and chloride competitively inhibited benzoyl phosphate hydrolysis, with apparent K_i values of 1.73

Fig. 3. *Polyacrylamide-gel electrophoresis of GP1 and GP3*

(a) Electrophoresis in 4M-urea of GP1 (1) and GP3 (2). (b) Electrophoresis in 0.1% SDS of GP3 (1) and GP1 (3) and standard proteins (4). The samples on gels (1) and (3) were preincubated in 4M-urea and 1% SDS; the samples on gels (2) and (4) were preincubated in the same medium containing also 1% 2-mercaptoethanol. Each gel, except for gel (4), was loaded with 10 μ g of protein.

Table 1. *Summary of the purification of acylphosphatase from guinea-pig skeletal muscle*

Sample	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)
pH 7.2 supernatant	450	3500	9700	0.36	100
pH 4.8 supernatant	400	3300	6600	0.50	94
pH 8.0 supernatant	380	3200	5250	0.61	91
Euate from affinity chromatography	25	2950	1.34	2200	84
First peak from CM-Sephadex C25 (GP1)	40	2280	0.63	3600	65
Second peak from CM-Sephadex C25 (GP2)	30	100	0.03	3300	2.8
Third peak from CM-Sephadex C25 (GP3)	40	195	0.06	3500	5.6

Table 2. *Physicochemical data for acylphosphatase main forms from guinea-pig, horse and turkey muscle*
Apparent M_r was measured by SDS/polyacrylamide-gel electrophoresis. Apparent pH optimum and K_m were measured with benzoyl phosphate as substrate.

Acylphosphatase main form	Apparent M_r	Specific activity (units/mg)	Apparent pH optimum	K_m (mM)	K_i (P _i) (mM)	K_i (Cl ⁻) (mM)
Guinea pig (GP1)	12000	3600	5.15	0.57	1.73	29.1
Horse (Hol)	12600	3800	5.3	2.0	1.70	40.0
Turkey (T1)	13400	3500	5.3	1.0	2.8	40.0

Table 3. *Amino acid composition of acylphosphatase main forms from guinea-pig (GP1), horse (Hol) and turkey (T1) muscle*

Values are expressed as mol of residue per mol of enzyme

	GP1	Hol	T1
Lysine	11.0	8.6	8.8
Histidine	0.7	0.0	0.2
Arginine	5.0	7.0	7.0
Tryptophan*	2.9	1.8	2.0
Aspartic acid	8.2	8.2	8.1
Threonine†	5.7	5.6	7.3
Serine†	13.8	12.8	12.3
Glutamic acid	11.2	10.9	12.7
Proline	3.1	3.2	3.0
Glycine	10.6	9.6	11.3
Alanine	5.2	3.4	5.1
Cystine (half)‡	1.8	2.1	1.6
Valine	11.0	11.2	10.0
Methionine	2.1	2.0	1.9
Isoleucine	4.4	3.0	2.0
Leucine	4.0	3.0	5.0
Tyrosine	3.6	4.1	3.1
Phenylalanine	3.3	4.0	4.8

* Determined as described by Edelhoch (1967).

† Values were corrected as described by Moore & Stein (1963).

‡ Cystine was determined as cysteic acid.

and 29.1 mM respectively. These kinetic data are reported in Table 2, in comparison with the same parameters of the corresponding forms of the enzyme from horse and turkey muscle.

Table 3 shows the amino acid composition of the main form of acylphosphatase from guinea-pig, horse and turkey muscle.

Hydrazinolysis of form GP1 indicated the presence of tyrosine and glycine as C-terminal residues. The dansyl procedure revealed only glutamic acid as the N-terminal residue. By the Edman degradation no N-terminal residue was found in GP1, nor were free thiol groups detected in this enzyme. These data suggest the presence of glutathione linked as a mixed disulphide to the main polypeptide chain, and that the N-terminal residue of the main chain is blocked, as was previously demonstrated for horse muscle acylphosphatase (Ramponi *et al.*, 1971; Cappugi *et al.*, 1976). In

Fig. 4. Time courses of labelling with (a) ^{14}C -labelled amino acids of guinea-pig muscle free amino acid pool, and (b) incorporation of ^{14}C -labelled amino acids into the main form of acylphosphatase (■) and into the soluble proteins of guinea-pig muscle (●). For details see the text.

fact, glutathione (γ -Glu-Cys-Gly), containing γ -linked glutamate, can give a dansyl derivative (dansyl-Glu), but not an Edman reaction. Furthermore glycine, the C-terminal residue of glutathione, was obtained by hydrazinolysis. The presence of glutathione was demonstrated by the procedure described under 'Methods' and a glutathione/enzyme molar ratio of 0.83 was calculated.

Synthesis and degradation constants

Experimental data were analysed as described by Fritz *et al.* (1969). The degradation rate constant (k_d) was obtained from the slope of the straight line resulting from plotting $\ln(P_{\text{max}}^* - P^*)$ versus time, where P_{max}^* = d.p.m./mol of protein at maximum labelling and P^* = d.p.m./mol of protein at any time between the time at which the amino acid pool has reached constant specific radioactivity and the time at which the protein pool has reached constant specific radioactivity. Half-lives were obtained from the relationship: $t_{1/2} = \ln 2/k_d$.

Table 4. *Turnover constants for acylphosphatase and total soluble proteins*

For details see the text. [P] = protein concentration; k_s = synthesis rate constant; k_d = degradation rate constant.

Proteins	[P]		k_s	k_d	Half-life (days)
	(mg/g)	(pmol/g)	(pmol/g per day)	(day ⁻¹)	
Acylphosphatase (main form)	5.13×10^{-3}	4.46×10^2	45	0.10	6.9
Total soluble proteins	84.18	1.68×10^6	1.7×10^5	0.10	6.9

During continuous administration of the radioisotope, radioactivity in the free amino acid pool reached a constant value 14 days after the animals were placed on the diet (Fig. 4a). Fig. 4(b) shows that the rate of incorporation of ¹⁴C-labelled amino acids into muscle soluble proteins and the main form of acylphosphatase (GP1) reaches saturating values after 38 days. Consequently, rate constants were calculated from the data collected during this time interval.

Table 4 shows rate constants for the synthesis (k_s) and degradation of total soluble proteins and of GP1 in guinea-pig muscle, in addition to half-lives for these proteins. The rate constants were calculated by assuming an average M_r of 50000 for soluble muscle proteins and by using the measured concentration (mg/g wet wt. of tissue).

Discussion

The availability of specific anti-(horse muscle acylphosphatase) antibodies allowed us to purify this enzyme in a very good yield from guinea-pig muscle, owing to its cross-reactivity with these antibodies. By this technique we demonstrated the presence of multiple molecular forms of acylphosphatase in guinea-pig muscle, with a distribution that greatly resembles that in horse muscle (Manao *et al.*, 1983) and in turkey muscle (Stefani *et al.*, 1982), although only about half of the total acylphosphatase activity is present with respect to horse tissue. From the reported results, the main form of acylphosphatase in guinea-pig muscle appears to be very similar to the corresponding form in horse and turkey muscle, in both its functional and structural properties. Glutathione is present in almost the same stoichiometric ratio, indicating that this tripeptide is conserved in the structure of acylphosphatase from different species. A comparison of the amino acid compositions of guinea-pig, horse and turkey enzyme indicates differences of about 14% and 15% respectively. However, such differences should not affect the active site of the enzyme, since their main kinetic parameters are similar, nor should they affect antigenic determinants, owing to the high binding efficiency of these enzymes with the same immunoabsorbent. End-

group analysis further supports the homologies between the three enzymes.

Rate constants for synthesis and degradation were obtained with the assumption (Fritz *et al.*, 1969) that, if any protein is present in a tissue at a constant concentration, the synthesis and degradation rates must be equal. Furthermore, assuming that synthesis is zero order and degradation is first order, the rate of synthesis must be equal to k_s and the rate of degradation to the product of k_d multiplied by the steady-state protein concentration. This is shown by the data of Table 4. Apparent rate constants for synthesis are not directly comparable because, for soluble proteins, they reflect the synthesis of many individual proteins. However, values of k_d indicate that the main form of acylphosphatase, in guinea-pig muscle, has the same half-life as total soluble proteins of this tissue.

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