Common-type acylphosphatase: steady-state kinetics and leaving-group dependence

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A number of acyl phosphates differing in the structure of the acyl moiety (as well as in the leaving-group pK_a of the acids produced in hydrolysis) have been synthesized. The K_m and V_{max} values for the bovine common-type acylphosphatase isoenzyme have been measured at 25 °C and pH 5.3. The values of k_{cat} differ widely in relation to the different structures of the tested acyl phosphates: linear relationships between $\log k_{cat}$ and the leaving group pK_a , as well as between $\log k_{cat}/K_m$ and the leaving-group pK_a , were observed. On the other hand, the K_m values of the different substrates are very close to each other, suggesting that the phosphate moiety of the substrate is the main chemical group interacting with the enzyme active site in the formation of the enzyme–substrate Michaelis complex. The enzyme does not catalyse transphosphorylation between substrate and concentrated nucleophilic acceptors (glycerol and methanol); nor

does it catalyse $H_2^{18}O$ –inorganic phosphate oxygen exchange. It seems that no phosphoenzyme intermediate is formed in the catalytic pathway. Furthermore, during the enzymic hydrolysis of benzoyl phosphate in the presence of ^{18}O -labelled water, only inorganic phosphate (and not benzoate) incorporates ^{18}O , suggesting that no acyl enzyme is formed transiently. All these findings, as well as the strong dependence of k_{cat} upon the leaving group pK_a , suggest that neither a nucleophilic enzyme group nor general acid catalysis are involved in the catalytic pathway. The enzyme is competitively inhibited by Pi, but it is not inhibited by the carboxylate ions produced during substrate hydrolysis, suggesting that the last step of the catalytic process is the release of Pi. The activation energy values for the catalysed and spontaneous hydrolysis of benzoyl phosphate have been determined.

INTRODUCTION

Acylphosphatase is a low-molecular-mass enzyme that catalyses the hydrolysis of the carboxy-phosphate bond, and it is widespread in all vertebrate tissues [1]. There is considerable evidence to suggest that the enzyme is involved in the control of ion-pump activities, since it is able to hydrolyse the aspartyl-phosphate bonds that are produced during the action of membrane Na^+ , K^+ ([2], and citations herein), and Ca^{2+} -pumps ([3], and citations herein). The enzyme is also implicated in the control of glycolytic flow, since it hydrolyses 1,3-bisphosphoglycerate, releasing Pi and maintaining ADP concentrations at levels suitable to sustain high glycolytic flow [4,5]. Previous papers demonstrated that the thyroid hormones enhance acylphosphatase expression [6,7]. These findings suggest that part of the excess of heat production in hyperthyroidism is caused by the increased levels of acylphosphatase, which has a role in a futile cycle involving 1,3-bisphosphoglycerate [8] and in the uncoupling of Na^+ , K^+ and Ca^{2+} -pumps [2,3].

Two acylphosphatase isoenzymes are expressed in animals in a tissue-specific manner [9,10], which are named the muscle type (MT) and the organ common (or erythrocyte) type (CT), since the former is highly expressed in skeletal muscle and heart, whereas the latter is expressed in all tissues [10], although its expression is particularly high in erythrocytes, brain and testis. Both isoenzymes have been isolated from a number of vertebrate tissues and sequenced ([11], and citations herein). The three-

dimensional structure of muscle isoenzyme has been determined by NMR techniques ([12], and citations herein). Recently, crystals of the CT-isoenzyme have been produced [13] and the three dimensional structure of this isoenzyme has been determined by X-ray crystallography [14]. The overall structure of CT-acylphosphatase (a basic protein that consists of 98 amino-acid residues) reveals a very compact protein, consisting of five-stranded mixed-sheet with two helices running parallel to the sheet. The sheet is slightly curved with a right-handed twist, and the helices interact with one side of the sheet forming a compact core [14]. Site-directed mutagenesis experiments (performed with MT-acylphosphatase) have suggested that Arg-23 and Asn-41 are essential residues [15,16], Arg-23 being involved in the binding of the substrate phosphate moiety [14]. This paper deals with steady-state kinetic studies performed on the CT-acylphosphatase isoenzyme.

MATERIALS AND METHODS

Materials

The CT-isoenzyme was purified from bovine testis as previously described [11]. $[^{18}O]$ water at 97% isotope enrichment was purchased from Cambridge Isotope Laboratories. $[^{32}P]Pi$ (8500 Ci/mmol) was purchased from NEN. All other reagents were the purest commercially available.

Abbreviations used: BCA, bicinchoninic acid; MT, muscle type; TMS, trimethylsilyl; CT, organ common type; PTPase, phosphotyrosine protein phosphatase.

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Acyl phosphates

Acyl phosphates having differing acyl groups were synthesized. Benzoyl phosphate and 2-methoxybenzoyl phosphate were prepared as previously described [17,18]. Acetyl phosphate, propionyl phosphate, and butyryl phosphate were synthesized as follows: 40 mmol of each acyl anhydride were slowly added to a mixture of 40 mmol of phosphoric acid dissolved in 48 ml of 30% (v/v) pyridine, previously chilled in ice. The mixture was stirred for about 1 h, then 120 mmol of LiCl dissolved in 12 ml of water was added. The acyl phosphates were precipitated by adding cold ethanol or, in some cases, ethanol–acetone mixtures. Phenylacetyl and *p*-nitrobenzoyl phosphates were prepared using a similar but slightly modified method, since the corresponding anhydrides were not commercially available. Anhydrides were then synthesized from free acid and acyl chlorides as follows: 40 mmol of each free acid and 40 mmol of triethylamine were dissolved in 8 ml of anhydrous tetrahydrofuran. The solutions were chilled in ice, and 40 mmol of the corresponding acyl chloride was slowly added under stirring. The precipitates formed during the reactions were discarded by filtration. The acid anhydrides in the mixture were concentrated under vacuum and added to the phosphate–pyridine mixture as described for acetyl phosphate, propionyl phosphate and butyryl phosphate synthesis. Product yields ranging from 30% to 50% were obtained. Some acyl phosphates, such as acetyl phosphate, propionyl phosphate and butyryl phosphate, were purified by repeated fractional precipitation with ethanol, whereas phenylacetyl phosphate and *p*-nitrobenzoyl phosphate were purified by preparative reversed-phase chromatography using a C18 preparative bulk-packing phase (55–105 μ m, Waters, U.S.A.) following the procedure previously described for the purification of 2-methoxybenzoyl phosphate [18]. The final products were analysed for acyl phosphate bond content using the hydroxylamine–ferric chloride photometric method and calibration curves described in the following section [19]. The free Pi content in the acyl phosphates was assayed by the method of Baginski et al. [20]. Benzoyl [32 P]phosphate was synthesized as previously described [21] using [32 P]Pi and benzoic anhydride.

Enzyme assay

The 2-methoxybenzoyl and benzoyl phosphatase activities were determined by continuous spectrophotometric assays as previously described [18,22]. *p*-Nitrobenzoyl phosphatase activity was assayed by a similar method following the time-dependent absorbance change owing to the absorption difference between the substrate and its hydrolysis product at 313 nm. Acetyl, propionyl, butyryl and phenylacetyl phosphatase activities were determined using the method of Lipmann and Tuttle [19], with minor modifications. In brief, this method is based on the fast reaction of acyl phosphates with a neutralized solution of hydroxylamine to form acyl-hydroxamate derivatives, which were then reacted with the ferric ion in acidic medium to produce ferric–hydroxamic acid complexes that were measured photometrically. The calibration curves were determined using the corresponding acid anhydride or acyl chloride standard solutions (anhydrides or acyl chlorides were dissolved in anhydrous acetonitrile). The substrates were dissolved in 0.1 M sodium acetate buffer, pH 5.3, and the reaction was started by adding a catalytic amount of acylphosphatase. The final test volume was 0.5 ml and the incubation was performed at 25 °C. The enzymic catalysis was blocked at different times by adding 0.25 ml of a 4 M hydroxylamine solution (adjusted to pH 7.0 with NaOH). After 15 min, 0.4 ml of a 0.23 M FeCl₃ solution containing 3.75 M HCl and 1.15 M trichloroacetic acid was added and the

absorbance was read at 510 nm 15 min later. Controls without enzyme were prepared and incubated as described. The $V_{\max}$ and K_m (means $\pm$ S.E.) were calculated by fitting the initial rate data to the Michaelis–Menten equation with the non-linear regression program Fig. P (Biosoft). All initial rate measurements were carried out at least in triplicate.

Protein assay

Protein concentration was determined by the bicinchoninic acid (BCA) kit method (Sigma), using BSA as standard.

Benzoyl phosphate enzymic hydrolysis in [18 O]water

Benzoyl phosphate (1 mM final concentration) was dissolved in [18 O]water, and pH was adjusted to 5.5. A small amount of acylphosphatase was then added, and the mixture was incubated for 40 min at room temperature to achieve complete hydrolysis. A portion of 50 μ l was withdrawn, transferred in a screw-cap conical vial and dried. Successively, sample derivatives were obtained for GLC–MS analysis, as described below.

Inorganic phosphate–medium water 18 O exchange

Mes (10 mM final concentration) and Pi (100 mM final concentration) were dissolved in water and the pH was adjusted to 5.5 with NaOH. Then 100 μ l samples were withdrawn, transferred to a small screw-cap conical vial and dried. The residue was dissolved in 100 μ l of [18 O]water, and 1 μ l of acylphosphatase (15 units; the unit is defined as the amount of enzyme that catalyses the hydrolysis of 1 μ mol of benzoyl phosphate at 25 °C and pH 5.3) was added. The mixture was incubated at room temperature. Aliquots of 1 μ l were withdrawn at various incubation times and diluted with 100 μ l of acetonitrile contained in a small screw-cap conical vial. The mixture was dried, and derivatives of inorganic phosphate were formed for GLC–MS analysis, as described below. In order to check the performance of the method, a parallel experiment with calf intestine alkaline phosphatase was performed [in this experiment, the pH of the incubation mixture (100 μ l) was adjusted to 9.0, and 5 enzyme units were added (the unit is defined as the amount of enzyme that catalyses the hydrolysis of 1 μ mol of *p*-nitrophenyl phosphate at 25 °C and pH 9.8)]. It is well known that alkaline phosphatase forms a covalent enzyme–phosphate intermediate during its catalytic process before releasing Pi [23], and thus is certain to catalyse the [18 O]water–inorganic phosphate oxygen exchange.

Trapping experiments

The trapping experiments were performed using a method similar to those described by Guan and Dixon [24], Pot et al. [25] and Cirri et al. [21] for trapping phosphoenzyme intermediates of rat phosphoprotein tyrosine phosphatase-1 (PTPase-1), leucocyte-antigen-related PTPase (LAR) and low M_r PTPase, respectively. Briefly, the method consists of a rapid mixing of the enzyme with a 32 P-labelled substrate followed by rapid denaturation with SDS. The enzyme (10–20 μ g in 2 μ l) was quickly mixed with 18 μ l of benzoyl-[32 P]phosphate (20 mM) dissolved in 0.1 M sodium acetate buffer, pH 5.3. Immediately after, 20 μ l 0.125 M Tris/HCl buffer, pH 6.8, containing 4% SDS and 20% (v/v) glycerol was rapidly added with vigorous mixing. The sample was then analysed by SDS/PAGE without heating and gels were analysed by autoradiography for 2 and 4 day exposures.

PAGE analysis

PAGE was performed according to the method of Laemmli [26].

GLC-MS analysis

Samples containing Pi and/or benzoate were dried using a Savant vacuum drier apparatus. The residues were dissolved in 20 μ l acetonitrile containing 0.01 M HCl and subsequently in 30 μ l *N*-methyl-*N*-trimethylsilyl trifluoroacetamide containing 2% (w/w) trimethylchlorosilane. The mixtures were incubated at 60 °C for 30 min to obtain the trimethylsilyl (TMS) derivatives. Analyses were performed with a GLC-MS system from Hewlett Packard. The system consists of a gas-chromatography model 5890, series II, and a mass-detector model MSD 5971 A, using an electronic gun at 70 eV. The GLC analyses were performed on an SPB 1 (Supelco) capillary column [30 m $\times$ 0.25 mm i.d., 0.25 μ m phase film, using helium as carrier gas, 103.4 kPa. Temperature programme: 80 °C (1 min hold) to 120 °C at 5 °C/min, then to 300 °C at 30 °C/min; splitter, 1:30; scan range, *m/z* 70–350; scan rate, 2.5 scan/sec].

RESULTS

Michaelis-Menten parameters and structure-activity relationships

The analysis was performed on a number of acyl phosphates that differ from each other in the structure of the acyl group. These include benzoyl-, 2-methoxybenzoyl-, *p*-nitrobenzoyl-, phenylacetyl-, acetyl-, propionyl- and butyryl-phosphates. K_m and V_{max} values relative to the above substrates, as well as the leaving-group pK_a , k_{cat} , and k_{cat}/K_m values, are shown in Table 1. It can be seen that acyl phosphates exhibit K_m values very close to each other, suggesting that the substrate phosphate moiety is the main chemical group involved in binding at the active site of acylphosphatase. Furthermore, we found that the V_{max} values of the acyl phosphates tested differ greatly from each other. The plot of the log of k_{cat} versus the differing substrate leaving-group pK_a values (Figure 1A) shows that a linear relationship exists between these two parameters; this suggests that the pK_a of the leaving group strongly affects the rate of acylphosphatase catalysis. This can be described by the equation:

$$\log k_{cat} = -1.38 \pm 0.15 \text{ p}K_a^{\text{XOH}} + 8.72 \pm 0.67 \quad (n = 7; r = 0.97)$$

where pK_a^{XOH} is the pK_a of the leaving group XOH. The plot of $\log k_{cat}/K_m$ versus pK_a (see Figure 1B) is similar to the plot of $\log k_{cat}$ versus pK_a (Figure 1A), since the K_m values of the various substrates are very close to each other. The relationship between $\log k_{cat}/K_m$ versus leaving-group pK_a can be described by the equation:

$$\log k_{cat}/K_m = -1.44 \pm 0.22 \text{ p}K_a^{\text{XOH}} + 9.7 \pm 0.95 \quad (n = 7; r = 0.95)$$

Figure 1 Brønsted plots for k_{cat} at pH 5.3 and 25 °C (A) and for k_{cat}/K_m at pH 5.3 and 25 °C (B)

The experimental points refer to acetyl phosphate, propionyl phosphate, butyryl phosphate, phenylacetyl phosphate, benzoyl phosphate, 2-methoxybenzoyl phosphate and *p*-nitrobenzoyl phosphate. The leaving-group pK_a values are reported in Table 1.

These linear free-energy relationships (Brønsted correlations) provide information about the rate-limiting step and the nature of the transition state. Thus, since the kinetic parameter k_{cat} monitors the limiting step of the catalytic pathway, Figure 1(A) demonstrates that the transition-state structures of the limiting step of all tested substrates are very similar and that the enzyme utilizes a common mechanism for leaving-group pK_a values ranging from 3.41 to 4.87. A similar conclusion can be deduced from Figure 1(B); in fact, since we excluded the possibility that intermediates other than the Michaelis enzyme-substrate complex are formed (see Scheme 2, and the following paragraphs), it is likely that the limiting step in the catalytic pathway of acylphosphatase is also monitored by the kinetic parameter k_{cat}/K_m . The observed $\beta_{\text{leaving group}}$ value of -1.38 is close to that observed for the uncatalysed hydrolysis of phosphate ester

Table 1 Kinetic parameters for the enzymic hydrolysis of acyl phosphates at pH 5.3 and 25 °C

The initial rate measurements were carried out at least in triplicate.

	K_m (mM)	Relative V_{max}	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}mM^{-1}$)	Leaving-group pK_a	K_i (mM)
Acetyl phosphate	0.20 ± 0.04	1.00	181 ± 5	905	4.75	
Propionyl phosphate	0.30 ± 0.04	0.65	117 ± 2	390	4.87	
Butyryl phosphate	0.15 ± 0.02	0.42	77 ± 2	513	4.81	
Phenylacetyl phosphate	0.23 ± 0.03	2.12	384 ± 6	1670	4.28	
Benzoyl phosphate	0.11 ± 0.01	8.42	1524 ± 12	12700	4.19	
2-Methoxybenzoyl phosphate	0.15 ± 0.01	8.05	1457 ± 10	9713	4.09	
<i>p</i> -Nitrobenzoyl phosphate	0.20 ± 0.03	49.82	9018 ± 32	45090	3.41	
Inorganic phosphate						0.64 ± 0.11

Figure 2 Suggested transition state for the enzyme-catalysed hydrolysis of benzoyl phosphate

R-O⁻ indicates the benzoate ion.

dianions ($\beta_{\text{leaving group}} = -1.2$, Kirby and Varvoglis [27]); it is also consistent with a highly dissociative transition state (Figure 2), and with the fact that the leaving group departs as an anion.

The high sensitivity to leaving-group dependence ($\beta_{\text{leaving group}} = -1.38$) indicates that no general acid catalysis is involved in the acyl phosphate enzymic hydrolysis. In fact, when general acid catalysis occurs (as described for the aryl phosphate hydrolysis catalysed by the low- M_r PTPase), low sensitivity to leaving-group dependence has been observed ($\beta_{\text{leaving group}} = -0.27$, Wu and Zhang [28]). Our findings also agree with those of Satchell et al. [29], who, studying the dependence of k_{cat} of acylphosphatase from chicken muscle on pH, found that k_{cat} is not dependent upon pH in the range 4–10. In fact, they suggested that a group with a $\text{p}K_a = 7.9$, assigned to the free enzyme, is involved only in substrate and in phosphate binding, and so does not act as a general acid in the catalysis.

Inhibition by benzoyl phosphate hydrolysis products

Inhibition by reaction products (benzoate and Pi) was measured in the assay buffer at 25 °C. Preliminary inhibition tests with differing benzoate and Pi concentrations were performed. These show that in the absence of benzoate, Pi inhibits acylphosphatase-catalysed hydrolysis of benzoyl phosphate, whereas benzoate concentrations up to 5 mM, in the absence of Pi, shows no inhibition. The K_i value relative to Pi, and the type of inhibition, were determined by measuring the initial rates of benzoyl

Figure 3 Inhibition of CT-acylphosphatase by Pi (Lineweaver-Burk plot)

The initial rates were measured at pH 5.3 and 25 °C, using benzoyl phosphate as substrate. The inhibitor concentrations were: (●), no inhibitor; (○), 0.5 mM Pi; (▲), 0.75 mM Pi; (△), 1 mM Pi; (■), 1.25 mM Pi. Inset: replot of $K_{m(\text{obs})}$ values against Pi concentrations.

phosphate hydrolysis at concentrations ranging from 0.2 to 5 mM, in the presence of four different Pi concentrations. The data were fitted according to Lineweaver and Burk with a linear regression program (Fig. P, Biosoft). All graphs gave identical V_{max} values, indicating that the inhibition is competitive (Figure 3). The apparent K_m values were replotted against Pi concentrations obtaining a straight line that intersects the abscissa at a point corresponding to $-K_i$ ($K_i = 0.64 \pm 0.11$).

Phosphotransfer reaction between substrate and some nucleophilic acceptors

The phosphotransfer reaction was tested measuring the release of Pi and benzoate from benzoyl phosphate enzymically hydrolysed in the presence of 1–2 M glycerol or methanol. In all cases, the molar ratios of Pi/benzoate were close to one, suggesting that no transphosphorylation occurred.

H_2^{18}O -inorganic phosphate oxygen exchange experiments

As described in Materials and methods, these experiments were performed with both acylphosphatase and alkaline phosphatase. This latter enzyme catalyses the H_2^{18}O -inorganic phosphate oxygen exchange, since its catalytic pattern involves the formation of an enzyme-phosphate covalent complex [23] that is successively hydrolysed to produce free enzyme and Pi; thus this experiment is a good control on the performance of this technique. In fact, Figure 4(F) shows that alkaline phosphatase is able to catalyse the incorporation of ^{18}O atoms into the phosphate group when incubated with Pi and H_2^{18}O [the incorporation of ^{18}O was time-dependent (results not shown); all possible isotopomers are present after 20 h incubation]. In contrast, acylphosphatase is not able to catalyse the oxygen exchange between H_2^{18}O and inorganic phosphate (the spectrum reported in Figure 4E is identical with that of the control experiment with Pi and H_2^{18}O , but without enzyme), suggesting that no enzyme-phosphate covalent complexes are formed.

Trapping experiments

In the autoradiography analysis (4 days exposure) no ^{32}P -labelled enzyme was detected. Thus the formation of a covalent phosphoenzyme in the acylphosphatase reaction is unlikely.

The enzymic hydrolysis of benzoyl phosphate in H_2^{18}O

Experiments of benzoyl phosphate hydrolysis in H_2^{18}O may provide information related to the catalytic pathway. We considered Scheme 1:

Scheme 1

Figure 4 GLC-MS analyses

Panel A, GLC separation of the TMS derivatives of benzoic acid (peak 1) and Pi (peak 2). The chromatography was performed with an SPB 1 (Supelco) capillary column (30 m $\times$ 0.25 mm ID, 0.25 μ m phase film), using helium as carrier gas. Details are described in the text. M indicates the molecular ion. Panels B and C show the mass spectra of peak 1 ($M = 194$ m/z ; $M-CH_3 = 179$ m/z) and peak 2 ($M = 314$ m/z ; $M-CH_3 = 299$ m/z), respectively. Panel B also represents the mass spectrum of peak 1 obtained in the GLC analysis of the products of benzoyl phosphate enzymic hydrolysis performed in $H_2^{18}O$. Panel D shows the mass spectrum of peak 2 as obtained in the analysis of the products of benzoyl phosphate enzymic hydrolysis performed in $H_2^{18}O$ ($M = 316$ m/z and $M-CH_3 = 301$ m/z indicate the presence of the tri-TMS- $P^{18}O_3$ isotopomer). Panel E, inorganic phosphate-medium water ^{18}O exchange experiment performed with acylphosphatase: this panel shows the mass spectrum of peak 2 as obtained in the analysis of the mixture containing Pi, $H_2^{18}O$ and acylphosphatase; the incubation was carried out at room temperature for 120 h at pH 5.5 ($M = 314$ m/z and $M-CH_3 = 299$ m/z indicate the presence of the tri-TMS- $P^{16}O_4$ isotopomer). Samples incubated for 24, 48 and 96 h (not reported) give identical spectra. Panel F, inorganic phosphate-medium water ^{18}O exchange experiment performed with alkaline phosphatase. The mass spectrum of peak 2 as obtained in the analysis of the mixture containing Pi, $H_2^{18}O$ and alkaline phosphatase is shown; the mixture was incubated at pH 9.8 and room temperature for 20 h [M peaks at 314, 316, 318, 320, 322 m/z and peaks ($M-CH_3$) ions at 299, 301, 303, 305, 307 m/z all indicate the presence of the five possible tri-TMS-phosphate isotopomers]. Additional analyses were performed using identical sample mixtures incubated with alkaline phosphatase for 48 and 150 h. After 120 h, the mass spectrum (not reported) corresponds mainly to that of the tri-TMS- $P^{18}O_4$ ($M = 322$ m/z and $M-CH_3 = 307$ m/z). Panel G, mass spectrum of peak 2 from the control sample; the control was performed incubating 100 mM Pi in $H_2^{18}O$ at pH 5.5 and room temperature for 120 h. This spectrum demonstrates that the spontaneous exchange process is negligible.

where E is the enzyme, BP is benzoyl phosphate $E \cdot BP$ is the Michaelis enzyme-substrate complex, E-P is an enzyme-phosphate covalent complex, E-B is a covalent enzyme-benzoate complex (i.e. an acyl-enzyme), P-OH is phosphate B-OH is benzoate, and the reaction profiles 1-4 describe the possible mechanisms of the catalytic acylphosphatase action. Figure 4(D) reports the GLC-MS analyses of the benzoyl phosphate enzymic hydrolysis products formed in the presence of $H_2^{18}O$. It can be seen that ^{18}O has been incorporated only into inorganic phos-

phate (see the mass peak at $m/z = 316$ corresponding to the tri-TMS- $P^{18}O_3$ derivatives) and not in benzoate (see the mass peak at $m/z = 194$ corresponding to TMS-benzoate, Figure 4(B)), suggesting that both mechanism 1 and 4 of Scheme 1 should be excluded. Thus the results of the experiments reported in Figures 4(B) and 4(D) demonstrate that the water attack is directed against the phosphorus atom of the substrate; nevertheless, this finding gives us no possibility of discriminating between mechanisms 2 and 3 of Scheme 1. These last two mechanisms differ

Figure 5 Determination of activation energies (Arrhenius plots) for the enzyme-catalysed hydrolysis (A) and for spontaneous hydrolysis (B) of benzoyl phosphate at pH 5.3

k' is the apparent first-order kinetic constant.

from each other, since mechanism 2 proposes the water attacks the substrate phosphorus in the enzyme–substrate Michaelis complex, whereas mechanism 3 describes a mechanism that involves the formation of an enzyme–phosphate covalent complex subsequently attacked by H_2^{18}O to form free enzyme and $[\text{O}^{18}]\text{Pi}$.

The activation energies of non-catalyzed and catalyzed benzoyl phosphate hydrolysis

The temperature-dependence of the spontaneous hydrolysis of benzoyl phosphate was studied. Benzoyl phosphate (5 mM) was dissolved in 0.1 M sodium acetate buffer, pH 5.3, and aliquots were incubated at different temperatures in the range 14–65 °C. At various incubation times, aliquots of the mixtures were withdrawn and the benzoyl phosphate concentration remaining was determined by the hydroxylamine–ferric chloride reagent [19]. The temperature-dependence of the acylphosphatase-catalysed reaction was determined measuring V_{max} at different temperatures in the range 17–37 °C. The Arrhenius plots (Figures 5A and 5B) gave straight lines, enabling us to calculate the activation energies of both catalysed (27.1 ± 0.8 kJ/mol) and uncatalysed (104.7 ± 8.2 kJ/mol) benzoyl phosphate hydrolysis reactions at pH 5.3.

DISCUSSION

The results reported in this paper demonstrate that the substrate binds to the enzyme predominantly through its phosphate moiety. In fact, substrates differing in the acyl moiety structure elicit very close K_m values. All findings enable us to propose the following scheme (Scheme 2) for the benzoyl phosphate hydrolysis catalysed by the CT-acylphosphatase isoenzyme:

Scheme 2

where E is acylphosphatase, B-P is benzoyl phosphate, $\text{E} \cdot \text{B-P}$ is a Michaelis enzyme–substrate complex, B-OH is benzoate and $\text{E} \cdot \text{Pi}$ is the Michaelis enzyme–inorganic phosphate complex.

The prior release of benzoate in the substrate hydrolysis step and the formation of the $\text{E} \cdot \text{Pi}$ Michaelis complex are suggested by both the observed competitive inhibition of Pi and the absence of benzoate inhibition. The formation of a benzoyl enzyme covalent complex is excluded, since the enzymic hydrolysis of benzoyl phosphate, performed in the presence of H_2^{18}O , did not produce $[\text{O}^{18}]\text{benzoic acid}$, but produced $[\text{O}^{18}]\text{Pi}$ instead, indicating a water attacks at the phosphorus atom of the substrate. Thus the hydrolysis of acyl phosphates catalysed by acylphosphatase can be described as an ordered uni-bi system.

Further findings, such as the inability of the enzyme to catalyse either the transphosphorylation from substrate to nucleophilic compounds or the H_2^{18}O –Pi oxygen exchange, together with the inability to trap an enzyme–phosphate covalent intermediate and the strong dependence of k_{cat} on the leaving-group $\text{p}K_a$, all suggest that no phosphoenzyme is formed in the catalytic pathway. Although neither us nor others have produced data on the stereochemistry of the acylphosphatase reaction at the phosphorus atom, our results agree well with the three dimensional structure (determined by X-ray crystallography) of the CT-acylphosphatase active site. No nucleophilic residues have been found in the active site environment [14]. On the contrary, acid/alkaline phosphatases and PTPases have a different mechanism, since they form covalent phosphoenzyme intermediates during their catalytic processes [23]. The active sites of these enzymes contain nucleophilic residues such as serine (alkaline phosphatase), histidine (acid phosphatase), and cysteine (PTPase). Instead, other enzymes, such as inositol monophosphatase, inositol polyphosphate 1-phosphatase and fructose 1,6-bisphosphatase, utilize an activated water molecule rather than an amino acid nucleophile to attack the substrate phosphorus atom [30–33]. Leech et al. [34], studying the kinetic mechanism of inositol monophosphatase, found results very similar to those we obtained with acylphosphatase. In fact, inositol monophosphatase is unable to catalyse transphosphorylation and no covalent phosphoenzyme intermediate was trapped. Furthermore, inositol is a poor enzyme inhibitor and inositol monophosphatase does not catalyse H_2^{18}O –Pi oxygen exchange in the absence of inositol. These three phosphatases differ from acylphosphatase, since they are magnesium-dependent and inhibited by lithium. With reference to inositol monophosphatase, most of the inositol 1-phosphate binding energy is contributed by the phosphate–magnesium interaction (there are two magnesium ions involved in this reaction mechanism) [33]. In the mechanism of CT-acylphosphatase, which does not involve metal ions, the substrate phosphate moiety contributes most of the binding energy and makes extensive contacts with the guanidinium group of Arg-23, forming additional hydrogen bonds to the backbone-amide groups of Phe-21, Arg-23 and Lys-24 [14]. Other differences refer to the water molecule activation: in inositol monophosphatase, the site-1 magnesium contributes to the water molecule activation [33], whereas in acylphosphatase, this role is performed by Asn-41 [14].

We suggest that the limiting step of the catalytic pathway is the hydrolysis of acyl phosphate in the enzyme active site. In fact, if

E·Pi accumulates, we would expect quite similar k_{cat} values for substrates with differing structures, since they give the common E·Pi intermediate in their enzymic hydrolysis pathways. In the case of alkaline phosphatase, almost identical k_{cat} values (measured at pH ≥ 8) for a wide variety of substrates were observed (in the catalytic pathway of this enzyme, the dissociation of E·Pi is rate-limiting [35]). Thus k_{cat} (whose value is $1.524 \times 10^3 \text{ s}^{-1}$) corresponds to k_2 in the catalytic process, since the value of k_1 is probably $> 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$, as commonly observed for the formation of complexes between enzymes and small molecule ligands.

The pronounced dependencies of k_{cat} and k_{cat}/K_m on the leaving-group $\text{p}K_a$ suggest that the transition-state structure of the benzoyl-phosphate-catalysed hydrolysis is highly dissociative, and therefore the enzyme uses no general acid catalytic group to assist the leaving-group release in the transition state. We also found that *p*-nitrophenyl phosphate is very slowly hydrolysed by acylphosphatase with the same mechanism as acyl phosphates (unpublished results). The poor activity of the enzyme on this aryl phosphate is certainly owing to difficulties in the release of the phenolate ion in the transition state without the assistance of a general acid group. On the other hand, only high- M_r aryl phosphates (protein phosphotyrosines) are formed in biological systems, and their hydrolysis is catalysed by the large family of PTPases, which have a common catalytic mechanism and use a general acid (an aspartic residue) to donate a proton to the leaving phenolate group [36–40]. Thus it appears that acylphosphatase possesses a very high specificity for acylphosphates.

The Gibbs free energies relative to both the limiting step of the enzymic and the spontaneous reaction pathways were calculated according to the transition-state theory:

$$\Delta G^\ddagger = -RT \ln (kh/kT) \quad (5)$$

at 25 °C and pH 5.3, and values of 54.9 kJ/mol and 100.8 kJ/mol were obtained, respectively. The rate constants (k) used for the $\Delta G^\ddagger$ calculations were k_{cat} for the enzyme-catalysed reaction, and the apparent first-order rate constant for the spontaneous benzoyl phosphate hydrolysis reaction (both measured at 25 °C and pH 5.3).

The value of $\Delta G^\ddagger = 54.9 \text{ kJ/mol}$ differs from that of E_a [27.1 kJ/mol as calculated from the Arrhenius plot of the enzyme-catalysed reaction (Figure 5A)]. However, recognizing that the two thermodynamic parameters are related by:

$$\Delta G^\ddagger = \Delta H^\ddagger - T\Delta S^\ddagger \quad (6)$$

and that $\Delta H^\ddagger \cong E_a$, it follows that: (i) this difference is due to the entropy term $T\Delta S$ and (ii) that ΔS has a negative sign. Thus the formation of the transition state during benzoyl phosphate hydrolysis in the active site of acylphosphatase is accompanied by a strong reduction in the entropy of the hydrated enzyme–transition-state complex with respect to that of the hydrated enzyme–substrate Michaelis complex. On the other hand, the E_a value for the spontaneous benzoyl phosphate hydrolysis (104.7 kJ/mol, pH 5.3) is close to the value of $\Delta G^\ddagger = 100.8 \text{ kJ/mol}$ calculated from the apparent first-order non-enzymic hydrolysis rate constant (measured at pH 5.3 and 25 °C) and from equation (5). Considering that $\Delta H^\ddagger \cong E_a$, we deduce that ΔS relative to the transition state of the uncatalysed reaction has a positive sign. The observed entropy reduction in the formation of the transition state of the limiting step of the enzyme-catalysed reaction is probably due to the stabilization of the transition state by the active-site environment or, alternatively, by rearrangement of the solvent structure in the enzyme hydration shell. The former hypothesis agrees with the observed

$\beta_{\text{leaving group}}$ value of -1.38 (calculated from the free-energy plot reported in Figure 1A), which is slightly higher than that observed for non-enzymic hydrolysis of phosphate dianions ($\beta_{\text{leaving group}} = -1.2$, see Kirby and Varvoglis [27]). Hollfelder and Herschlag [41] reported that the values of $\beta_{\text{leaving group}}$ are rendered more negative in low dielectric media because electrostatic interactions are less dampened. Thus acylphosphatase may catalyse the hydrolysis of acyl phosphates using the strategy of the stabilization (in the active-site environment) of the same transition state that is formed during the non-enzymic hydrolysis reaction. In addition, the high negative $\beta_{\text{leaving group}}$ values taken from Figures 1(A) and 1(B) exclude the possibility that metal ions are involved in the leaving-group departure; in fact, it has been suggested that the co-ordination of the leaving oxygen with a metal ion at the active site of an enzyme in the transition state has little dependence in the Brønsted-type correlations [42,43].

The enzyme is able to decrease strongly the Gibbs free energy of activation in the benzoyl phosphate hydrolysis reaction with respect to that of the uncatalysed reaction. Thus acylphosphatase causes a 1.1×10^8 -fold increase in the benzoyl phosphate hydrolysis rate with respect to that of the uncatalysed hydrolysis at 25 °C and pH 5.3 (Table 1 and Figure 5B).

Acylphosphatase is an evolutionarily conserved protein [11], a property generally associated with important cell functions. The understanding of its mechanism at the molecular level may help in designing and constructing specific enzyme inhibitors useful in revealing the true substrates of acylphosphatase in different cell types, and therefore to discover its function. The tissue-specific expression of the two isoenzymes suggests different functions, or, alternatively, a different regulation of their expression. This latter hypothesis is supported by experiments on cell differentiation [44–46]. In particular, a very recent report on the differentiation of the K562 cell line when stimulated by phorbol myristate acetate (which induces megakaryocytic differentiation), or by aphidicolin or hemin (which stimulate erythrocytic differentiation) has demonstrated that whereas the MT isoform showed an average 10-fold increase independently of the differentiating agent used, only hemin treatment caused a similar increase of the CT isoform, suggesting a different role for the two isoenzymes in the cell [46]. Finally, the demonstration that the enzyme tertiary structure is identical to that of the RNA-binding domain of nuclear RNA-binding proteins [47,48], and that the enzyme possesses nucleolytic activity [49], together with the observation that the CT isoenzyme is able to migrate into the nucleus during apoptosis [50], all suggest that the enzyme is involved in critical biological functions.

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