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INHIBITION OF HORSE MUSCLE ACYLPHOSPHATASE BY PYRIDOXAL 5'-PHOSPHATE

GIAMPIETRO RAMPONI, GIAMPAOLO MANAO, GUIDO CAMICI and GRAHAM F. WHITE*

Institute of Biological Chemistry, University of Florence, V. le G. Morgagni 50, 50134 Florence (Italy)

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Summary

It has been shown that horse muscle acylphosphatase is inhibited by pyridoxal 5'-phosphate and that the inhibition is pH dependent, reversible and competitive with respect to substrate binding.

Spectral analysis on the EI complex demonstrates the presence of a Schiff base.

Reduction of the pyridoxal 5'-phosphate-inhibited enzyme with sodium borohydride, followed by amino acid analysis, produces a diminution of the free lysine peak and the appearance of a new peak corresponding to ϵ-pyridoxyllysine.

The results suggest that there is at least one NH_2-lysyl residue of horse muscle acylphosphatase at or near the active site of the enzyme.

Introduction

Acylphosphatase (acylphosphate phosphohydrolase, E.C. 3.6.1.7) acts specifically by catalyzing the hydrolysis of the carboxy-phosphate bond of acyl phosphates, according to reaction I.

$$\text{Acylphosphate} + H_2O \rightarrow \text{carboxylate} + \text{orthophosphate} \qquad \text{(I)}$$

The enzyme is widely distributed in animal tissue (for example, liver [1,2], brain [3] and erythrocytes [4]) although muscle has received most attention as a rich and convenient source [5—8].

In addition to simple aliphatic [9] and aromatic [10] acylphosphates, 1,3-diphosphoglycerate [11] and carbamyl phosphate [12,13] can act as sub-

* Present address: Department of Biochemistry, University College, P.O. Box 78, Cardiff, South Wales, U.K.

strates for acyl phosphatase. A possible physiological role for acylphosphatase activity may therefore be one of regulation of metabolic pathways involving 1,3-diphosphoglycerate and carbamyl phosphate (e.g. the glycolytic pathway and pyrimidine biosynthesis) by its ability to hydrolyze these substrates. Acylphosphatase could also control acylation and carbamylation of proteins [14,15] by regulating the levels of reactive acyl and carbamyl phosphates.

Amino acid analyses of acylphosphatase purified from both horse [5] and rabbit [7] muscle show a relative abundance of lysine residues. This fact prompted us to determine whether these residues were important in the catalytic action of the enzyme. Pyridoxal 5′-phosphate has been used extensively for studying the role of lysyl ϵ-amino groups in the action of a number of enzymes [16–21]. Modification of lysyl ϵ-amino groups occurs by formation of a Schiff base with the aldehydic group of pyridoxal 5′-phosphate.

This communication (a) reports experiments on the effect of pyridoxal 5′-phosphate on the catalytic activity of acylphosphatase and (b) demonstrates that the amino acid residues, with which the pyridoxal 5′-phosphate interacts are lysine residues.

Materials and Methods

Enzyme. Acylphosphatase from horse skeletal muscle was purified as previously described [5]. After dissolving the lyophilized protein in buffer (1000 units of activity [5] per ml of 0.1 M acetate buffer, pH 5.3, or 0.05 M 3,3-dimethylglutarate buffer, pH 6.4 and 7.6, or 0.05 M Tris · HCl buffer, pH 8.8), the solutions were stored at 0°C. Higher dilutions were avoided as these lead to loss of activity.

Substrate. Dilithium benzoyl phosphate was prepared by the method of Ramponi et al. [10] and was >98% pure on the basis of phosphate content, acyl content and carboxy-phosphate bond analysis. This substrate was used to assay acylphosphatase activity by a continuous spectrophotometric method [10]. Kinetic measurements of benzoyl phosphate hydrolysis were made at 25° C in a Gilford Model 2400 automatic recording spectrophotometer.

Other reagents. 3,3-Dimethylglutaric acid and pyridoxal 5′-phosphate were obtained from Fluka AG (Switzerland), *N*-carbobenzoxy lysine from Sigma (U.S.A.). Pyridoxamine 5′-phosphate · HCl was from K & K Laboratories (U.S.A.), pyridoxine 5′-phosphate was synthesized by reducing pyridoxal 5′-phosphate with $NaBH_4$. All other reagents, of the highest purity available, were purchased from either British Drug Houses Ltd (England), E. Merck AG (Germany) or C. Erba (Italy).

Preparation and analysis of pyridoxyl-labelled acylphosphatase. 6.7 mg of pure acylphosphatase (about 0.67 μmol) dissolved in 18 ml of 0.05 M 3,3-dimethylglutarate buffer, pH 7.6, and 4.5 ml of 4 mM pyridoxal 5′-phosphate (18 μmol) dissolved in the same buffer, were mixed together. The reaction mixture was incubated at 25° C for 30 min and then reduced by slow addition of sodium borohydride (100-fold in excess of pyridoxal 5′-phosphate). The reduction was carried out in a pH stat apparatus to maintain pH value around 7.6. The mixture was then dialyzed for 3 days against water, changing the water two times every day.

Amino acid analysis of the modified enzyme was performed by the method of Spackman et al. [22] using a Beckman Unichrom analyzer equipped with a micro cuvet. The protein was first hydrolyzed in 6 M HCl at 110° C for 24 h. Synthesis of ϵ-pyridoxyllysine for use as a chromatographic standard was acheived by the method of McKinley-McKee and Morris [21], starting from *N*-carbobenzoxy lysine.

Results

Pre-incubation of acylphosphatase with pyridoxal 5′-phosphate. In order to determine whether pyridoxal 5′-phosphate has any effect on acylphosphatase activity, experiments were performed in which the enzyme was first incubated with various pyridoxal 5′-phosphate concentrations, and then assayed after various intervals, in solutions of benzoyl phosphate containing the same pyridoxal 5′-phosphate concentration as the pre-incubation mixture. The experimental details and results are presented in Fig. 1a. Pyridoxal 5′-phosphate produces a decrease in activity which, as can be seen at the lower concentrations, is time dependent. The inhibition is well established after 2 min and always reaches a maximum after 15 min. Fig. 1a also shows that, under the same conditions, pyridoxal itself, pyridoxamine 5′-phosphate and pyridoxine 5′-phosphate have no effect on acylphosphatase activity.

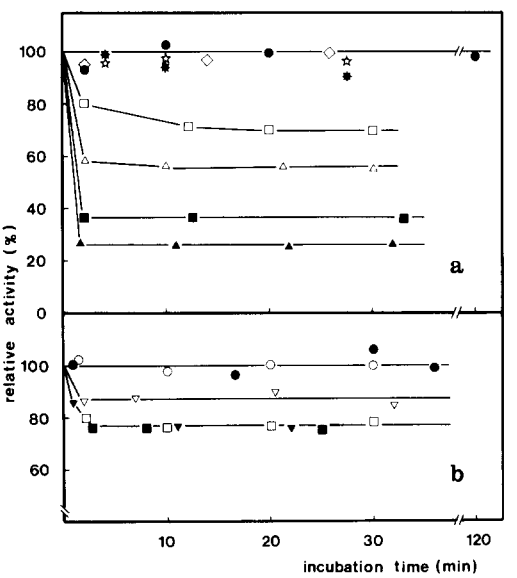

Fig. 1. The inhibition of acylphosphatase by pyridoxal 5′-phosphate. (a) 0.05 M 3,3-dimethylglutarate buffer, pH 7.6. (b) 0.1 M acetate buffer, pH 5.3. Aliquots of enzyme solution (20 μl, 1000 units/ml in the appropriate buffer) were treated with 5-μl samples of various pyridoxal 5′-phosphate solutions (1—10 mM) in the same buffer. After incubation at 25° C, 1 μl samples were withdrawn at the indicated intervals and assayed for activity at 283 nm in solutions containing 0.4 mM benzoyl phosphate, and buffer and pyridoxal 5′-phosphate as in the preincubation mixture. The residual activity was calculated as a percentage of that obtained by incubation in the absence of pyridoxal-5′-phosphate. ● + ○, controls before and after other tests; ◇, pyridoxal = 2.0 mM; ★, pyridoxamine 5′-phosphate = 0.8 mM; ☆, pyridoxine 5′-phosphate = 0.8 mM; pyridoxal 5′-phosphate concentrations: ▽ 0.02 mM; ▼, 0.1 mM; □, 0.2 mM; △, 0.4 mM; ■, 0.8 mM; ▲, 2.0 mM.

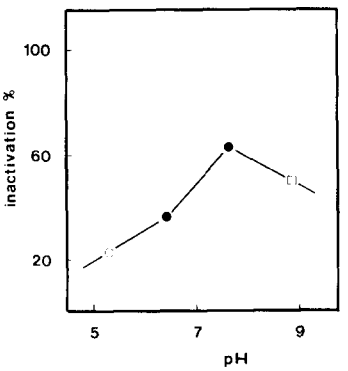

Fig. 2. The effect of pH on pyridoxal 5′-phosphate inhibition of acylphosphatase. ○, 0.1 M acetate buffer; ●, 0.05 M 3,3-dimethylglutarate buffer; □, 0.05 M Tris · HCl buffer. Enzyme was incubated with 0.8 mM pyridoxal 5′-phosphate at 25° C for 20 min and assayed.

Similar experiments at pH 5.3 produced the results shown in Fig. 1b.

The effect of pH. The effect of pH on the extent of inactivation is shown in Fig. 2 and reaches a maximum at about pH 7.6.

Reversibility of the pyridoxal 5′-phosphate inhibition of acylphosphatase. A 22-μl sample of acylphosphatase solution (1000 units/ml at pH 7.6) was preincubated at 25° C with 3 μl of 5 mM pyridoxal 5′-phosphate in the same buffer (or 3 μl of buffer alone for the control), giving a final pyridoxal 5′-phosphate concentration of 0.6 mM (zero for control). After 15 min, attempts were made to reverse the resulting inhibition by (a) treatment with lysine (b) dilution. Experimental details and results are shown in Table I. Preincubation of the enzyme with 0.6 mM pyridoxal 5′-phosphate produces about 60% inactivation as expected (line 3). Although free lysine has very little effect on the

TABLE I

REVERSIBILITY OF THE PYRIDOXAL 5′-PHOSPHATE INHIBITION OF ACYLPHOSPHATASE

Acylphosphatase was preincubated with 0.6 mM PL-5′-*P* at pH 7.6 as described in the text. (a) Treatment with lysine: 5 μl samples of preincubated mixtures (test and controls) were diluted with 1 μl of 0.6 mM PL-5′-*P* in buffer pH 7.6 (1 μl buffer alone for control), containing sufficient lysine to give a final concentration of 25 mM. At the indicated intervals 1 μl aliquots were assayed for activity in 2.5 ml samples of 0.4 mM benzoyl phosphate in buffer, supplemented with PL-5′-*P* and lysine at the same concentrations as in the final incubation mixture. (b) Dilution: a 5 μl sample of preincubated test mixture was treated with 55 μl of buffer alone, giving 5/60 dilution. Compared to the control (5 μl of buffer preincubated enzyme treated with 1 μl of buffer, giving 5/6 dilution), this is a 10-fold dilution. 10 μl samples of this diluted solution were assayed as before in 0.4 mM benzoyl phosphate containing 5/60 × 0.6 = 0.05 mM PL-5′-*P*.

Preincubation	Treatment	% residual activity after treatment for		
		2	10	20 min
Enzyme alone	None	100	100	100
Enzyme alone	25 mM lysine	91	93	99
Enzyme + 0.6 mM PL-5′-*P*	None	42	44	43
Enzyme + 0.6 mM PL-5′-*P*	25 mM lysine	54	72	81
Enzyme alone	10-fold dilutn	101	88	72
Enzyme + 0.6 mM PL-5′-*P*	10-fold dilutn	88	75	68

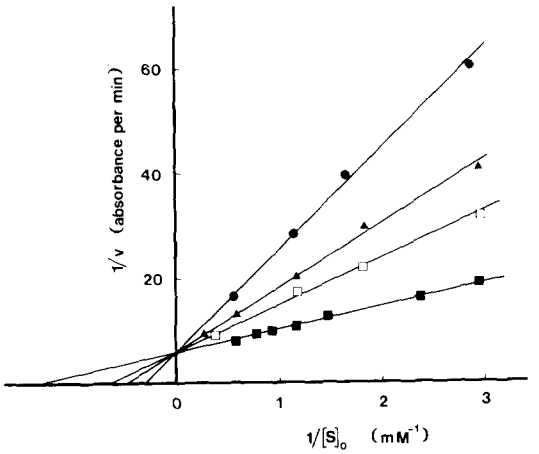

Fig. 3. Lineweaver-Burk plots for acylphosphatase at various pyridoxal 5′-phosphate concentrations. 8-μl samples of enzyme solution (1000 units/ml, pH 7.6) and 2-μl samples of pyridoxal 5′-phosphate solutions (0—5 mM, pH 7.6) were mixed and incubated for 15 min. For each pyridoxal 5′-phosphate concentration in the preincubation mixture, quartz spectrophotometer cells (10 mm) were prepared containing buffer (pH 7.6), various benzoyl phosphate concentrations from 0.34 mM to 3.40 mM, and pyridoxal 5′-phosphate at the same concentration as in the preincubation mixture in a total volume of 2.5 ml. 1 μl samples of pyridoxal 5′-phosphate-preincubated enzyme were added to these solutions and the initial velocity of benzoyl phosphate hydrolysis (v, absorbance units/min) determined at 283 nm. Pyridoxal 5′-phosphate concentrations: ■, zero; □, 0.25 mM; ▲, 0.50 mM; ●, 1.00 mM.

normal enzyme activity (line 2), addition of lysine to pyridoxal 5′-phosphate-inhibited enzyme produces a time-dependent reversal of the inhibition (line 4); free lysine competes with the enzyme for the pyridoxal 5′-phosphate. Dilution of pyridoxal 5′-phosphate-preincubated enzyme (43% of control activity) produces an initial re-activation to 88% of the control (line 6). Thereafter, the activity falls in a fashion, parallel to that of a similarly diluted control, without pyridoxal 5′-phosphate (line 5). These results demonstrate reversibility of the pyridoxal 5′-phosphate inhibition of acylphosphatase.

Type of inhibition and inhibition constant. The type of inhibition produced by pyridoxal 5′-phosphate and K_i value were determined by initial velocity measurements at a series of benzoyl phosphate and pyridoxal 5′-phosphate concentrations as described in the legend to Fig. 3. The results are plotted according to Lineweaver and Burk [23]. The common intercept on the $1/v$ axis indicates that the inhibition of acylphosphatase by pyridoxal 5′-phosphate is purely competitive with respect to benzoyl phosphate. The K_i value obtained from the linear [24] plot of apparent K_m against pyridoxal 5′-phosphate concentration was 0.32 mM.

Identification of enzyme-pyridoxal 5′-phosphate complex: absorbance spectrum. Reaction mixture containing acylphosphatase and pyridoxal 5′-phosphate in 0.05 M 3,3-dimethylglutarate, buffer pH 7.6, showed absorbance bands at 430 and 341 nm (Fig. 4). Absorbance maxima in the 410—430 nm region are typical of protein-pyridoxal 5′-phosphate complexes, and are attributed to the presence of a protonated Schiff base. The 341 nm band has been attributed to an unsubstituted aldimine [25—27]. The acylphosphatase-pyridoxal 5′-phosphate complex reduced by $NaBH_4$ had an absorbance maximum (Fig. 4) at 320 nm as expected [28,29].

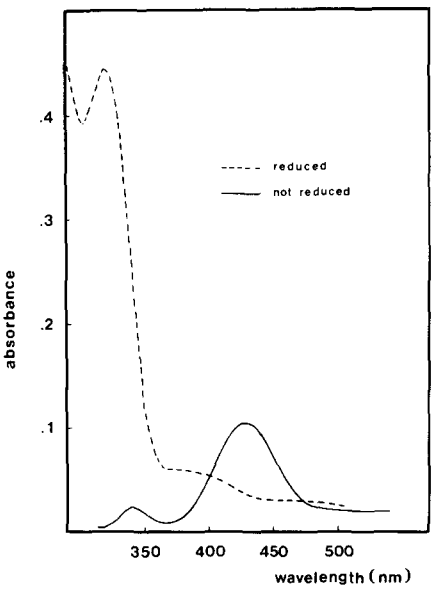

Fig. 4. The absorbance spectra of enzyme-pyridoxal 5′-phosphate and reduced complexes. The absorbance of a solution containing 0.3 mg per ml of acylphosphatase, 0.8 mM pyridoxal 5′-phosphate in 0.05 M 3.3-dimethylglutarate buffer, pH 7.6, was read against 0.8 mM pyridoxal 5′-phosphate in the same buffer. The solutions were incubated at room temperature for 15 min before spectra were measured (solid line; light-path 10 mm). A solution containing the reduced complex, 0.23 mg of protein per ml in 0.1 M phosphate buffer, pH 8.0, was read against the same buffer (broken line; light-path 5 mm). Spectra were obtained with a Beckman DK-1A spectrophotometer.

TABLE II

AMINO ACID COMPOSITION OF NATIVE AND PYRIDOXYL-ACYLPHOSPHATASE

Amino acid	No. of residues/mol of protein	
	Native acyl-phosphatase [5]	Pyridoxyl-acyl-phosphatase
Lys	8	6.4
Arg	5	5.0
Asp	6	6.5
Thr	5	4.5*
Ser	10	8.5*
Glu	9	9.3
Pro	3	2.7
Gly	7	7.3
Ala	3	2.8
Cystine/half	2	0.9*
Val	8	8.2
Met	2	1.3
Ile	2	2.4
Leu	3	2.6
Tyr	3	3.0
Phe	3	2.7

* Uncorrected for acid decomposition.

Identification of ϵ-pyridoxyllysine residues in the modified enzyme. ϵ-Amino groups of lysine produce a Schiff base with pyridoxal 5′-phosphate. Subsequent treatment with sodium borohydride results in reduction of the Schiff base to a secondary amine and acid hydrolysis of the modified enzyme gives ϵ-pyridoxyllysine residues. On amino acid analysis, ϵ-pyridoxyllysine appeared as single peak between lysine and histidine, but partially overlapping the histidine peak, in general agreement with other work [18,21]. Amino acid analysis of pyridoxyl-acylphosphatase showed that the modified protein has essentially the same composition as native acylphosphatase, except for a diminution of the lysine peak and the appearance of a new peak between the lysine and histidine positions. The position of this new peak corresponds precisely to that of synthetic ϵ-pyridoxyllysine. Results of the amino acid analysis of modified and native acylphosphatase are shown in Table II. Besides the loss of lysine residues, both cysteine and methionine values appear lower than in the native enzyme. The value for half-cystine is only approximate because the peak for free cystine in the normal analysis is small and therefore difficult to quantitate. Despite these minor changes in S-containing amino acids, it appears that the main site of pyridoxal 5′-phosphate action is at ϵ-amino groups of lysine residues in acylphosphatase.

Discussion

The results reported above show that pyridoxal 5′-phosphate produces pH-dependent inhibition of horse muscle acylphosphatase activity by its interaction with ϵ-amino groups of lysine residues. At pH 7.6, the inhibition is reversed by the addition of lysine or by dilution, and is purely competitive with respect to substrate binding (K_i = 0.32 mM). Since no inhibition was observed when pyridoxal was incubated with the enzyme, the phosphate group of pyridoxal 5′-phosphate appears to play an essential role in the inhibition process. Acylphosphatase acts on a number of acylphosphates, is strongly inhibited by inorganic and other phosphates [30,31], and probably has some binding sites for the phosphate group [32]. Since pyridoxal 5′-phosphate analogues, as pyridoxamine 5′-phosphate and pyridoxine 5′-phosphate show no inhibition under the same experimental conditions as before, it is therefore possible that the phosphate group in pyridoxal 5′-phosphate facilitates inhibition by directing the molecule to the active site, whereupon a Schiff base is formed between the active site lysine and the inhibitor.

The spectral data of EI complex, the amino acids analysis of pyridoxyl-enzyme and the purely competitive type of inhibition, suggest that there is at least one lysine residue at the active site of acylphosphatase, which is susceptible to interaction with pyridoxal 5′-phosphate.

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