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SPECIFICITY STUDIES ON HUMAN INTESTINAL ALKALINE PHOSPHATASE*

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SUMMARY

Studies were conducted on an alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) purified from human intestinal mucosa using substrates recorded as specific for the specific phosphatases. The data obtained from Michaelis constant determinations, mixed-substrate experiments, response to specific inhibitors, nonspecific inhibition by 6 M urea and heat treatment, indicated that at the physiological pH (in which range these studies were performed), the alkaline phosphatase can perform efficiently the functions attributed to specific phosphatases.

INTRODUCTION

Intestinal mucosa is a rich source of alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) activity. This raises questions as to the role of this enzyme in this location. The most obvious function of alkaline phosphatase in the intestinal mucosa is providing inorganic phosphate for metabolic processes. It has been suggested that the brush border alkaline phosphatase activity is digestive in function[1], and that it may be implicated in transport of hexoses by a mechanism involving their sequential phosphorylation and dephosphorylation[2]. It is significant that in the brush border region of the epithelial cells of the intestinal mucosa, the following enzymatic activities have been localized: Inorganic pyrophosphatase (EC 3.6.1.1), ATPase (EC 3.6.1.3), D-glucose 6-phosphatase (EC 3.1.3.9), D-glucose 1-phosphatase (EC 3.1.3.10) and D-fructose 1,6-diphosphatase (EC 3.1.3.11).

In view of the abundance of alkaline phosphatase in intestinal mucosa, it is conceivable that this enzyme might perform the functions attributed to specific phosphatases.

Although alkaline phosphatase is assayed at an alkaline pH, which is done as a matter of convenience, in an intracellular milieu it is hard to visualize a role for this enzyme at a pH, not far from neutrality.

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The object of this study was to determine the specificity of alkaline phosphatase under assay conditions approximating the biological pH range using substrates available for specific phosphatases, and applying criteria available to test the identities of two or more enzymatic activities[3,4].

MATERIALS

The alkaline phosphatase utilized in this study was extracted from human intestinal mucosa with "Genetron-113" (trichlorotrifluoroethane). The enzyme activity released by this solvent into the aqueous phase was purified utilizing Sephadex gel permeation (Sephadex G-200) and anion exchange (Sephadex A-50) chromatography[5].

The following chemicals and biologicals were products of Sigma Chemical Co., St. Louis, Mo.: ATP, disodium salt from equine muscle, and D-glucose 1-phosphate were both designated crystalline grade. D-fructose 1,6-diphosphate, sodium salt, D-serine, L-serine, D-phenylalanine, L-phenylalanine, phosvitin from egg vitellin, Tris–HCl (Trizma HCl) and Tris (Trizma base) were all Sigma reagent grade.

From Schwartz Mann, Orangeburg, New York, N.Y. the disodium salts of *p*-nitrophenyl phosphate and D-glucose 6-phosphate were obtained.

DL-*O*-Phosphoserine was purchased from Calbiochem, Los Angeles, California, and sodium pyrophosphate and urea from J. T. Baker Chemical Co., Phillipsburg, N.J.

METHODS

Enzyme assays

When *p*-nitrophenyl phosphate was used as a substrate for the enzyme, the release of *p*-nitrophenol was measured by its absorbance at 410 nm[6], in 0.1 M Tris buffer at pH values of 7.4 for mixed-substrate experiments, experiments with urea, heat treatment studies and in the determination of Michaelis constants.

When D-glucose 6-phosphate, D-glucose 1-phosphate, D-fructose 1,6-diphosphate, DL-*O*-phosphoserine, phosvitin, sodium pyrophosphate and ATP were the substrates for the enzyme the release of inorganic phosphate was followed at 660 nm[7] in 0.1 M Tris buffer at pH values of 7.4 for mixed-substrate experiments, experiments with urea, heat treatment studies and in the determination of Michaelis constants, with the exception of phosvitin, for which Michaelis constants were not evaluated.

Enzyme activity is expressed in units/ml. This represents μmoles of product released per min per ml. The spectrophotometric measurements were performed on a Bausch and Lomb Spectronic 20 Spectrophotometer.

Kinetic assays

Michaelis constants were determined from a Woolf[8] plot, of kinetic data for each of the following substrates: *p*-nitrophenyl phosphate, DL-*O*-phosphoserine, D-glucose 6-phosphate, D-glucose 1-phosphate, D-fructose 1,6-diphosphate, sodium pyrophosphate and ATP. 0.1 M Tris–HCl (pH 7.4) was the buffer used in these studies.

Inhibitor studies with L-phenylalanine were performed in 12.5 mM borate buffer, pH 7.8. D-Phenylalanine, which was not inhibitory was used as a control. Inhibition by L-phenylalanine was computed by comparing enzyme activity in presence

of L-phenylalanine, with enzyme activity in presence of noninhibitory D-phenylalanine The extent of inhibition of enzyme at 5, 10, 15 and 20 mM L-phenylalanine concentrations in 12.5 mM borate buffer (pH 7.8) was studied with each of the following substrates: *p*-nitrophenyl phosphate, DL-*O*-phosphoserine, D-glucose 6-phosphate, D glucose 1-phosphate, D-fructose 1,6-diphosphate, sodium pyrophosphate, phosvitin and ATP. The type of inhibition was determined from enzyme activity values obtained with various concentrations of each of the substrates containing 10 mM D-phenylalanine as compared with the values obtained in presence of 10 mM L-phenylalanine.

Similar experimental format was used when inhibition of enzyme activity by L-serine, in presence of non-inhibitory D-serine was studied in 12.5 mM borate buffer pH 7.8, using *p*-nitrophenyl phosphate, and DL-*O*-phosphoserine as substrates for the enzyme.

Mixed-substrate experiments

The buffer used in mixed-substrate experiments was 0.1 M Tris–HCl, pH 7.4 The ratio of the affinities of the enzyme for two substrates in mixed-substrate experiments was determined as follows: First, the substrate concentration at which each of these substrates attained their maximum velocity (V) was determined. The enzyme activity values (μmoles/min per ml) at this substrate concentration was noted. With one substrate (*p*-nitrophenyl phosphate) enzyme activity was monitored by measuring both the release of *p*-nitrophenol and inorganic phosphate. With the other substrates which were being assayed for their competition with *p*-nitrophenyl phosphate for the enzyme, enzyme activity was monitored by measuring the release of inorganic phosphate.

An equimolar mixture of *p*-nitrophenyl phosphate and another substrate was mixed at concentrations sufficient for each of the substrates to attain V when assayed individually, and enzyme activity in the presence of this mixed-substrate was monitored by measuring release of *p*-nitrophenol and inorganic phosphate.

Experiments with urea

A 0.5-ml aliquot of enzyme was mixed with 4.5 ml of 6 M urea in 0.1 M Tris–HCl buffer, pH 7.4, and incubated at 37 °C for 30 min. For control under the same conditions urea was substituted with 4.5 ml of 0.1 M Tris–HCl buffer, pH 7.4. After 30 min, 0.2-ml aliquots from urea treated and control tubes were withdrawn and the ratio of enzyme activity before and after treatment with urea was evaluated with all the eight substrates used in this study.

Heat treatment studies

A 0.5-ml aliquot of enzyme in 4.5 ml 0.1 M Tris–HCl buffer, pH 7.4, was incubated at 55 °C for 1 h. At the end of this period the tube containing the heated enzyme is cooled under cold tap water, and then left to come to room temperature. Subsequently, 0.2-ml aliquots were withdrawn and enzyme activity was determined with all the eight substrates used in this study. Enzyme activity of the unheated enzyme served as a control.

RESULTS

Table I lists the Michaelis constants obtained with the various substrates. The K_m values obtained with DL-*O*-phosphoserine, sodium pyrophosphate, and ATP are of the same order of magnitude as that obtained with *p*-nitrophenyl phosphate. The K_m values obtained with D-glucose 1-phosphate, and D-glucose 6-phosphate were similar and represented nearly a 19-fold increase over the K_m value obtained with *p*-nitrophenyl phosphate.

TABLE I

A COMPARISON OF MICHAELIS CONSTANTS OBTAINED WITH THE VARIOUS SUBSTRATES

Values of Michaelis constant (K_m) for the purified enzyme were calculated from the Woolf plots of the kinetic data obtained with the substrates listed below. 0.1 M Tris–HCl (pH 7.4) was the buffer used in these kinetic studies.

Substrate	$K_m \times 10^{-2}$ *(mM)*
p-Nitrophenyl phosphate	1.3
DL-*O*-Phosphoserine	1.3
Sodium pyrophosphate	1.3
ATP	1.3
D-Fructose 1,6-diphosphate	5.7
D-Glucose 1-phosphate	24.7
D-Glucose 6-phosphate	25.0

Fig. 1 is a plot of percentage of enzyme activity remaining against the inhibitor (L-phenylalanine) concentration in mM, when substrates listed in the legend, as well as sodium pyrophosphate, phosvitin, and ATP were used in the study.

Half-maximal inhibition of the enzyme with *p*-nitrophenyl phosphate, D-fructose 1,6-diphosphate, DL-*O*-phosphoserine, phosvitin, sodium pyrophosphate and ATP as substrates was attained at an L-phenylalanine concentration of 10 mM (Half-

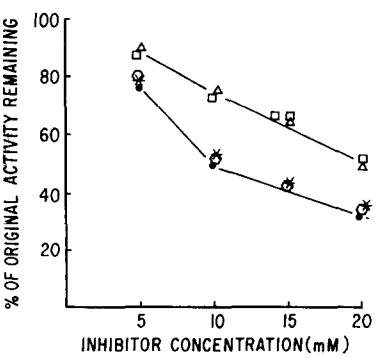

Fig. 1. Inhibition studies with L-phenylalanine in 12.5 mM borate buffer (pH 7.8). % of original enzyme activity (the ordinate) measured with various substrates at inhibitor (L-phenylalanine) concentrations of 5, 10, 15 and 20 mM (abscissa). Curves identical with the bottom curve in the figure were also obtained with 1 mM ATP, 1 mM sodium pyrophosphate and phosvitin (150 mg/100 ml solution in borate buffer, pH 7.8). Substrates: □—□, glucose 6-phosphate (2 mM); △—△, glucose 1-phosphate (2 mM); ●—●, *p*-nitrophenyl phosphate (1 mM); ○ — ○, fructose 1,6-diphosphate (2 mM); ×—×, *O*-phosphoserine (1 mM).

maximal inhibition is defined as the concentration of inhibitor required to reduce the enzyme activity to half the value it was without inhibitor.) However, when D-glucose 6-phosphate and D-glucose 1-phosphate were the substrates, the half-maximal inhibition of the enzyme was reached at an inhibitor concentration of 20 mM.

Fig. 2 is a plot of percentage of enzyme activity remaining against the inhibitor (L-serine) concentration in mM, when either *p*-nitrophenyl phosphate or DL-*O*-phosphoserine were the substrates. Half-maximal inhibition of the enzyme with either substrate was reached at an inhibitor (L-serine) concentration of 20 mM.

The nature of inhibition of enzyme by either L-phenylalanine or L-serine was uncompetitive[10].

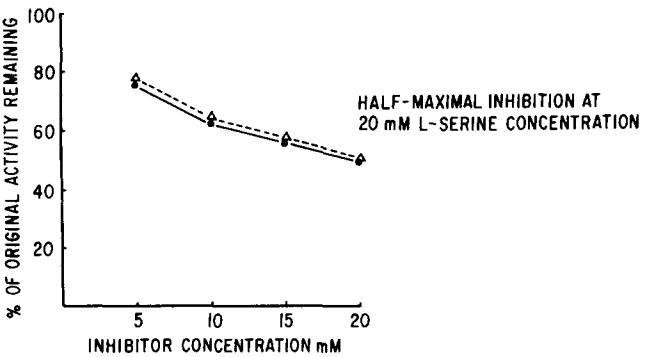

Fig. 2. Inhibition studies with L-serine in 12.5 mM borate buffer (pH 7.8). The ordinate indicates the percentage of the original enzyme activity remaining. The abscissa indicates inhibitor (L-serine) concentration in mM. Inhibition of the enzyme activity with L-serine was studied with 1 mM DL-*O*-phosphoserine (△---△) and 1 mM *p*-nitrophenyl phosphate (●—●).

Tables IIa and IIb present results obtained in mixed-substrate experiments. In these experiments *p*-nitrophenyl phosphate is referred to as "Substrate 1". The other substrates chosen to study competition with *p*-nitrophenyl phosphate for the enzyme are designated "Substrate 2". By comparing the release of *p*-nitrophenol from *p*-nitrophenyl phosphate alone (Substrate 1) with release of *p*-nitrophenol from the mixed substrate "the percent recovery of products" was computed in the 5th column of Tables IIa and IIb. The 5th column also tabulates the percent recovery of inorganic phosphate, which is done by averaging the release of inorganic phosphate from Substrate 1 and Substrate 2 and comparing it with that released from the mixed substrate.

Data presented in Table IIa indicate that competition between *p*-nitrophenyl phosphate (Substrate 1) and substrates: *O*-phosphoserine, fructose 1,6-diphosphate, phosvitin, ATP and sodium pyrophosphate for the enzyme was similar. Thus the mixed-substrate velocity monitored by the release of *p*-nitrophenol falls to nearly one-half the value obtained with Substrate 1 (*p*-nitrophenyl phosphate).

However, substrates listed in Table IIb are not quite as efficient in lowering the mixed-substrate velocity as substrates listed in Table IIa. When glucose 1-phosphate was included in the mixed-substrate with *p*-nitrophenyl phosphate the rate of decomposition of the latter was 69% of the original value. When glucose 6-phosphate was

TABLE IIa

MIXED-SUBSTRATE EXPERIMENTS

Product	*Enzyme activity (μmoles/min per ml) measured by release of products*			*Recovery of products (%)*
	Substrate 1	*Substrate 2*	*Mixed-substrate*	
	p-Nitrophenyl phosphate (1 mM)	*O-phosphoserine (1 mM)*	*Substrates 1 + 2*	
p-Nitrophenol	13.6	—	6.7	49
Inorganic phosphate	13.0	13.1	13.0	100
	p-Nitrophenyl phosphate (1 mM)	D-*Fructose 1,6-diphosphate (2 mM)*	*Substrates 1 + 2*	
p-Nitrophenol	12.1	—	6.5	54
Inorganic phosphate	11.6	10.8	11.3	100
	p-Nitrophenyl phosphate (1 mM)	*Phosvitin (150 mg/100 ml)*	*Substrates 1 + 2*	
p-Nitrophenol	11.8	—	6.1	52
Inorganic phosphate	10.9	4.2	7.3	97
	p-Nitrophenyl phosphate (1 mM)	*ATP (1 mM)*	*Substrates 1 + 2*	
p-Nitrophenol	17.8	—	9.4	53
Inorganic phosphate	17.0	18.2	17.2	98
	p-Nitrophenyl phosphate (1 mM)	*Sodium pyrophosphate (1 mM)*	*Substrates 1 + 2*	
p-Nitrophenol	17.6	—	9.1	52
Inorganic phosphate	16.7	18.1	17.8	100

TABLE IIb

MIXED-SUBSTRATE EXPERIMENTS

Product	*Enzyme activity (μmoles/min per ml) measured by release of products*			*Recovery of products (%)*
	Substrate 1	*Substrate 2*	*Mixed-substrate*	
	p-Nitrophenyl phosphate (1 mM)	D-*Glucose 1-phosphate (2.5 mM)*	*Substrates 1 + 2*	
p-Nitrophenol	12.4	—	8.6	69
Inorganic phosphate	12.0	11.1	11.4	99
	p-Nitrophenyl phosphate (1 mM)	D-*Glucose 6-phosphate (1mM)*	*Substrates 1 + 2*	
p-Nitrophenol	17.9	—	13.6	76
Inorganic phosphate	16.9	17.5	17.0	99

the mixed-substrate with *p*-nitrophenyl phosphate the rate of decomposition of the latter was 76% of the original value.

Table III compares results obtained in urea and heat treatment studies. Ratio of enzyme activity before and after treatment with urea was similar with substrates listed. Enzyme activity was unaffected by heat treatment at 55 °C for 1 h.

TABLE III

TREATMENT WITH 6 M UREA AND HEAT TREATMENT (55 °C FOR 1 h)

Substrate	*Enzyme activity (μmoles/min per ml)*			
	Before urea treatment	*After urea treatment*	*Before heat treatment*	*After heat treatment*
p-Nitrophenyl phosphate	20.1	10.5	21.7	21.7
D-Fructose 1,6-diphosphate	18.8	9.7	19.1	18.9
D-Glucose 1-phosphate	21.3	10.7	21.9	22.0
D-Glucose 6-phosphate	22.2	11.6	22.1	22.2
DL-*O*-Phosphoserine	18.3	9.4	19.9	19.7
ATP	18.2	9.4	19.7	19.8
Sodium pyrophosphate	22.0	11.3	22.3	22.0
Phosvitin	5.4	2.8	5.1	5.1

DISCUSSION

The fact that L-phenylalanine, a specific inhibitor of alkaline phosphatase[11], inhibits the enzyme regardless of the substrates used in this study, and that L-serine, a specific inhibitor of phosphoserine phosphatase (EC 3.1.3.3)[12], inhibits the enzyme with either DL-*O*-phosphoserine or *p*-nitrophenyl phosphate as substrate, indicates that the same active center in the enzyme molecule is involved in the hydrolysis of substrates used in this study. The same conclusion can be drawn from data obtained in mixed-substrate experiments, non-specific inhibition studies with urea, and heat treatment studies. The differential behavior of substrates listed in Table IIb in regard to their competition with *p*-nitrophenyl phosphate for the enzyme and in inhibition studies with L-phenylalanine requiring a greater concentration of this inhibitor to achieve half-maximum inhibition can be attributed to the differences in Michaelis constants for the enzyme obtained with these substrates, as opposed to those obtained with substrates listed in Table IIa.

Although there is a difference in competition between *p*-nitrophenyl phosphate and substrates listed in Tables IIa and IIb for the enzyme the release of inorganic phosphate in these mixed-substrate experiments is constant for both groups. Had there been no competition between substrates in these experiments, the mixed-substrate velocity as measured by release of inorganic phosphate would have been additive.

The pH value of 7.4 used in measurement of Michaelis constants, mixed-substrate experiments, experiments with urea and heat treatment is close to the pH prevailing in a biological milieu. This fact, and the abundance of alkaline phosphatase in human intestinal mucosa, could, by results obtained in this study, implicate the enzyme in functions that have been ascribed to specific phosphatases (EC numbers 3.6.1.1, 3.6.1.3, 3.1.3.9, 3.1.3.10, 3.1.3.11, 3.1.3.3 and 3.1.3.16). The results of this study are consistent with reports in literature demonstrating the identity of alkaline phosphatase (EC 3.1.3.1), the inorganic pyrophosphatase (EC 3.6.1.1) and the ATPase (EC 3.6.1.3) activities[13–15].

REFERENCES

1 Rothstein, A., Meier, C. R. and Scharff, G. T. (1953) *Am. J. Physiol.* 173, 41

2 Miller, D and Crane, R. K. (1961) *Biochim. Biophys. Acta* 52, 281.
3 Dixon, M. (1938) *Enzymologia* 5, 198.
4 Dixon, M. and Webb, C. E. (1964) *Enzymes*, 2nd edn, pp. 202–203, Academic Press, New York
5 Narayanan, S. and Appleton, H. D. (1972) *Clin. Chem.* 18, 548.
6 Garen, A. and Levinthal, C. (1960) *Biochim. Biophys. Acta* 38, 470.
7 Fiske, C. H. and SubbaRow, Y. (1925) *J. Biol. Chem.* 66, 375.
8 Woolf, B. (1932) quoted in *Allgemeine Chemie der Enzyme* (Haldane, J. B. S. and Stern, K. G. eds) p. 119, Dresden and Leipzig Steinkopff Verlag
9 Lineweaver, H. and Burk, D. (1934) *J. Am. Chem. Soc.* 56, 658.
10 Ebersole, E. R., Guttentag, C. and Wilson, P. W. (1943) *Arch. Biochem.* 3, 399.
11 Fishman, W. H., Green, S. and Inglis, N. H. (1962) *Biochim. Biophys. Acta* 62, 363.
12 Borkenhagen, L. F. and Kennedy, E. P. (1959) *J. Biol. Chem.* 234, 849.
13 Eaton, R. H. and Moss, D. W. (1967) *Biochem. J.* 102, 917.
14 Moss, D. W. and Walli, A. K. (1969) *Biochim. Biophys. Acta*, 191, 476.
15 Holdsworth, E. S. (1970) *J. Membrane. Biol.* 3, 43.