ENZYMIC HYDROLYSIS OF PHOSPHONATE ESTERS

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Summary - Enzymes capable of hydrolyzing the 4-nitrophenyl monoester of phenylphosphonic acid, which has no isosteric analog in nature, to 4-nitrophenol and phenylphosphonate have been detected in plants, animals, and microorganisms. The mucosa of vertebrate small intestine is an especially rich source of this activity, which is also present in commercial preparations of alkaline phosphatase. Small but clear-cut differences in the properties of this enzyme and alkaline phosphatase suggest that the two activities do not reside in the same protein.

In this paper we demonstrate that 4-nitrophenyl phenylphosphonate (1) is hydrolyzed by enzymes widely distributed in nature, describe some of the characteristics of this enzyme from a particularly rich source, calf intestinal mucosa, and compare it with alkaline phosphatase from the same source.

MATERIALS AND METHODS

Reagents - 4-Nitrophenyl phenylphosphonate was synthesized as described (1); this material is now available from Regis Chemical Company, Morton Grove, Illinois. Bis-(4-nitrophenyl)phosphate (Aldrich Chemical Company) was purified by hydrolysis of contaminating 4-nitrophenyl phosphate ($< 1\%$) catalyzed by purified E. coli alkaline phosphatase, extraction of the remaining diester into benzene, and isolation as the ammonium salt (1).

Enzyme sources - Wheat germ acid phosphatase was obtained from Pentex, Inc., calf intestinal alkaline phosphatase from P-L Biochemicals (Lot PA-2). E. coli alkaline phosphatase (with further purification by the method of Sperow and Butler (2)) and chicken intestinal alkaline phosphatase from Worthington Biochemical Corporation, and all other preparations from Sigma Chemical Company. Tissues for the distribution study were obtained from local sources.

Enzymic hydrolysis of 4-nitrophenyl esters was quantitated spectrophotometrically by the appearance of the 4-nitrophenolate ion at 400 nm (ε=18,320 M^{-1} cm^{-1}, pK 7.15) (3). Standard assay conditions for phosphonate esterase[1], alkaline phosphatase, and phosphodiesterase were 1 mM 4-nitrophenyl phenylphosphonate, 4-nitrophenyl phosphate, bis-(4-nitrophenyl)phosphate, respectively, in 0.1 M Tris, pH 9.0 (pH 8 for phosphonate esterase), at 30°C.

RESULTS

Distribution of phosphonate esterase activity. Several readily available phosphatases hydrolyze 4-nitrophenyl phenylphosphonate. The activity is shown in

[1]As employed here, "phosphonate esterase" refers solely to hydrolysis of monoesters, not diesters, of phosphonic acids.

Table I, along with the corresponding activity toward phosphate mono- and diesters. Under these conditions, phosphonate esterase activity is greater than phosphodiesterase activity, but somewhat less than phosphomonoesterase activity. The activity is greater at pH 8.0 than at pH 5.0.

The distribution of phosphonate esterase activity was determined in a variety of sources using the standard assay. As shown in Table II, significant activity is detected in all samples tested. An especially rich source of the enzyme is vertebrate intestinal mucosa; the highest activity is present in those sections closest to the stomach (Table III). Other experiments demonstrated that the stomach itself has much lower activity. Phosphonate esterase activity of the intestine is generally somewhat lower in the young (8-12 weeks) than in the mature (1-3 years) rabbit. Similar patterns have been observed with alkaline phosphatase (4,5) and phosphodiesterase I (6).

Properties of phosphonate esterase from calf intestinal mucosa.

Molecular weight. As estimated by gel filtration (7) of commercial (P-L Biochemicals) calf intestinal alkaline phosphatase, the molecular weight of phosphonate esterase and alkaline phosphatase was 107,000 and 90,000 respectively (data not shown). The activities of the two enzymes eluted in fractions which were clearly different. Values of 100,000 (8) and 140,000 (9) for the

Table I

Enzymic Phosphonate Esterase Activity of Several Commercial Phosphatase Preparations

Preparation	Specific activity (nmoles min^{-1} mg^{-1})[a]					
	pH 5.0			pH 8.0		
	R'POR	POR	R'OPOR	R'POR	POR	R'OPOR
Calf Intestinal Alkaline Phosphatase (P-L)	87	210	12.7	355	917	41.5
Calf Intestinal Alkaline Phosphatase (Sigma)	10	155	3.1	36	621	6.4
Chicken Intestinal Alkaline Phosphatase	18	54	6.7	32	150	16.5
E. coli Alkaline Phosphatase	16	536	5.6	41	1728	0 11.1
Wheat Germ Acid Phosphatase	6	187	1.5	12	40	1.9

[a]Specific activity is expressed per mg dry weight and was determined in 0.05 M acetate, pH 5.0, and in 0.05 M Tris, pH 8.0, in 1 mM substrate as follows: R'POR, 4-nitrophenyl phenylphosphonate; POR, 4-nitrophenyl phosphate; R'OPOR, bis-(4-nitrophenyl)phosphate.

Table II

Enzymic Phosphonate Esterase in Several Plant and Animal Tissues

Source	Activity[a] (n moles $min^{-1}g^{-1}$)
Red Algae (Porphyridium cruentum)	5
Yeast (Saccharomyces cerevisiae)	10
Mushroom (Agaricus campestris)	32
Corn (Zea mays), leaf tissue	28
Spinach (Spinacia oleracea), leaf tissue	37
Enteric bacterium (Escherichia coli)	33
Flesh fly (Sarcophaga bullata Parker), pupal stage	8
Toad (Xenopus laevis), small intestinal mucosa	1020
Chicken (Gallus domesticus), small intestinal mucosa	1770
Rabbit (Oryctolagus cuniculus), small intestinal mucosa	1420

[a]Weighed tissue was homogenized in 0.1 M Tris, pH 8.0, in a Waring blendor, centrifuged, and 0.1 ml of the supernatant assayed under standard conditions. Activity is expressed per gram fresh or thawed weight of tissue.

molecular weight of calf intestinal alkaline phosphatase have been reported.

Isoelectric point. On isoelectric focusing large losses of both activities were observed, presumably due to metal chelation (see later section) by ampholyte, and/or to the low pH (10). Addition of 0.6 mM Zn^{2+} and adjustment to neutral pH resulted in recovery of up to 20% of the original activity. As shown in Figure 1, one region of phosphonate esterase activity (pI 4.5) and two peaks (pI 4.4; 4.8) of alkaline phosphatase activity were observed.

Effect of digestive enzymes. Incubation of 10 mg of the P-L intestinal preparation for 32 hours with 1 mg of pronase in 0.05 M Tris, pH 9.0, resulted in loss of 60% of the phosphonate esterase activity and only 10% of the alkaline phosphatase activity. Either 1 mM L-phenylalanine or 5 mM P_i protected the alkaline phosphatase against inactivation, but neither protected the phosphonate esterase. Control samples without pronase were fully active over this time period. The catalytic activity and electrophoretic mobility of neither enzyme was affected by incubation of 1.0 mg of the intestinal preparation with 0.1 mg neuraminidase, cellulase, bee venom, hyaluronidase, or pancreatin in 1.0 ml of 0.05 M cacodylate, pH 7.0, for 36 hours.

Catalytic properties. At 1 mM 4-nitrophenyl phenylphosphonate in 0.1 M

Table III

Distribution of Phosphonate Esterase in Rabbit Intestinal Mucosa

Tissue	Specific Activity (nmoles $min^{-1}g^{-1}$)[a]	
	Young	Mature
Duodenum	1300	2200
Jejunum	1600	1800
Ileum	1000	600
Caecum	100	200
Colon	100	200

[a]Frozen rabbit intestinal scrapings (Pel-Freeze Biologicals, Rogers, Arkansas) designated young (8-12 weeks) or mature (1-3 years) and were stored frozen until use. Specific activity was determined under standard conditions and is expressed per g thawed weight of mucosa.

Tris acetate, maximal enzyme-catalyzed production of 4-nitrophenol is observed in the pH range 7.5-8.0. Phosphonate esterase activity is unstable below pH 6, and is quite stable from pH 6 to pH 9. Aromatic esters of both aliphatic and aromatic phosphonic acids are substrates; aliphatic esters are hydrolyzed only very slowly, if at all.

Inclusion of 2.0 mM Cd^{2+}, Ni^{2+}, Sr^{2+}, Mg^{2+}, or Mn^{2+} in the standard assay mixture has no effect on the phosphonate esterase activity; Zn^{2+} or Ba^{2+} inhibit to an extent of less than 10%, and Cu^{2+} inhibits approximately 25%. Ca^{2+} gives a slight activation of less than 10%. Inclusion of 0.33 mM EDTA in the assay results in time-dependent loss of activity; 10 minutes after exposure of the enzyme to the chelator less than 5% of the original activity remains. Addition of 3.3 mM Zn^{2+} to the EDTA-inactivated enzyme results in a rapid recovery of activity; the final rate is approximately 90% of the rate obtained at this Zn^{2+} concentration with enzyme never exposed to EDTA. Other metal ions restore activity to the EDTA-inactivated enzyme more slowly and to a lesser extent, or not at all.

L-phenylalanine, which is a stereospecific uncompetitive inhibitor of intestinal alkaline phosphatase (11), also inhibits phosphonate esterase activity. Hydrolysis of 2 mM 4-nitrophenyl phenylphosphonate in the standard assay was inhibited 50% by 10 mM L-phenylalanine.

DISCUSSION

It has been considered that biological activity of phosphonates is due to

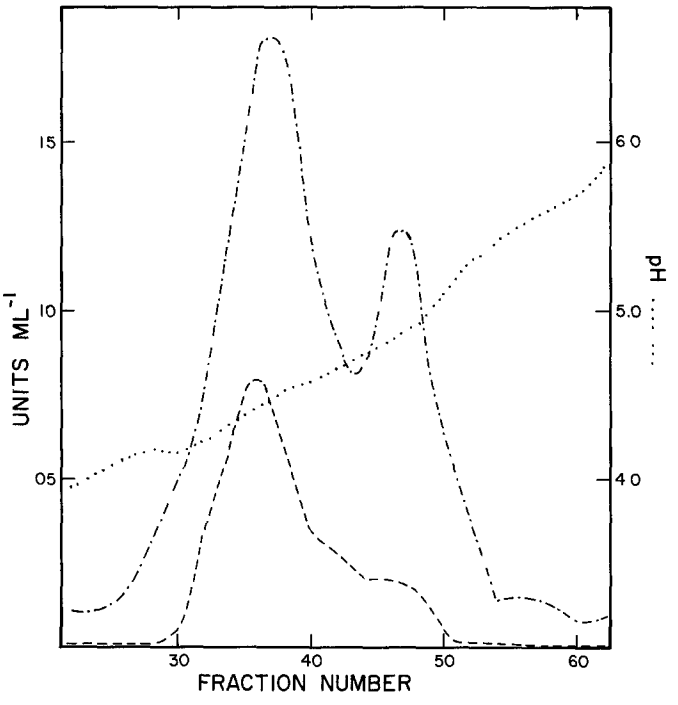

Figure 1. Determination of Isoelectric Point. Isoelectric focusing of 14 mg of calf intestinal alkaline phosphatase (P-L Biochemicals) was performed in pH 3-6 ampholyte according to the method of Bennett et al. (14), for 62 hours at 400 volts. Fractions of 1.5 ml were collected at a rate of 1.5 ml per minute regulated by a peristaltic pump. After the pH was measured (·····), 100 µl 1.0 M Tris, pH 8.0, and 10 µl 0.1 M $ZnCl_2$ were added to each fraction. Following a 24 hour incubation at 4°C, the phosphonate esterase (---) and alkaline phosphatase (-·-·-) activities were measured under standard conditions.

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their similarity to naturally occurring phosphates (12). The evidence presented here shows for the first time that phosphonate compounds which have no isosteric phosphate analog in living organisms (e.g. esters of phenylphosphonic acid) are nevertheless substrates for enzymes widely distributed throughout nature. Although some of the properties of phosphonate esterase are similar to those of alkaline phosphatase, the differences are significant and rule out the possibility that a single protein bears both enzymic activities. The broader distribution for this activity than that of biological phosphonates themselves (13) suggests that the role of the enzymes is not limited to phosphonate metabolism.

Subsequent investigations have shown that the phosphonate esterase activity described here is almost certainly due to a phosphodiesterase enzyme. These results will be described in a later publication.

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