Kinetic Effects of Inorganic Pyrophosphate Analogs on Several Inorganic Pyrophosphate Hydrolyzing Enzymes†

Susan J. Kelly, Fred Feldman, James W. Sperow, and Larry G. Butler*

ABSTRACT: Inorganic pyrophosphate analogs in which the bridge oxygen is replaced by nitrogen (imidodiphosphate) or carbon (methylene diphosphonate) elicit kinetic responses which vary from complete interchangeability of imidodiphosphate and PP_i by microsomal inorganic pyrophosphatase to total lack of recognition of the analogs by a maize leaf inorganic pyrophosphatase. Nonspecific alkaline phosphatases from *Escherichia coli* and from calf intestine both hydrolyze imidodiphosphate, but at much lower rates than they hydrolyze PP_i. Hydrolysis of 4-nitrophenyl phosphate by both these enzymes was competitively inhibited at relatively high inhibitor concentrations by both imidodiphosphate and methylene diphosphonate. Yeast inorganic pyrophosphatase does not hydrolyze imidodiphosphate; this enzyme is competitively inhibited by imidodiphosphate but not by methylene diphosphonate. The results are consistent with the previously known differences in the geometric parameters of PP_i and the analogs. The analogs distinguish subtle differences in specificity of the yeast and maize leaf enzymes, and provide further evidence for the remarkable similarity of the *E. coli* and intestinal alkaline phosphatases.

The enzymic hydrolysis of inorganic pyrophosphate can be considered to be the simplest possible model of biological utilization of high-energy phosphates. A variety of enzymes which differ considerably in their catalytic properties and mechanisms of action can catalyze this reaction. These PP_i-hydrolyzing enzymes range from nonspecific alkaline phosphatases (EC 3.1.3.1) which are Zn^{2+}-containing metalloenzymes operating through a covalent phosphoryl intermediate attached at a serine residue (Reid *et al.*, 1969; Fernley and Bisaz, 1968) to highly specific inorganic pyrophosphatases (EC 3.6.1.1) which require added divalent metal ions for activity, and which may operate by a concerted mechanism (Bennett *et al.*, 1973; Cooperman and Chin, 1973b; Sperow *et al.*, 1973). Other PP_i-hydrolyzing enzymes, with properties which do not correspond to either of these groups, are known; an example employed in this work is the inorganic pyrophosphatase (glucose-6-phosphatase) of rat liver microsomes (EC 3.1.3.9) which has a mechanism involving a covalent phosphorylhistidine intermediate (Feldman and Butler, 1972).

As an adjunct to our investigations of these PP_i-hydrolyzing enzymes, we have compared their kinetic responses to PP_i analogs in which the bridge oxygen atom is replaced by nitrogen or carbon. Nucleoside di- and triphosphate analogs of this type have been used in studies of several different enzymes (Coulomb *et al.*, 1969; Yount *et al.*, 1971; Chou and Singer, 1971; Hegyvary and Post, 1971; Eckstein *et al.*, 1971; Milner and Wood, 1972), but the corresponding PP_i analogs have been tested only with inorganic pyrophosphatase from yeast (Negi *et al.*, 1972; Cooperman and Chin, 1973a; Sperow *et al.*, 1973). In this paper we described kinetic studies using imidodiphosphate and methylene diphosphonate, the imido and methylene analogs of PP_i, respectively, with several other enzymes which hydrolyze PP_i: *Escherichia coli* (Anderson and Nordlie, 1967) and intestinal (Fernley and Bisaz, 1968) alkaline phosphatase and microsomal inorganic pyrophosphatase (Nordlie and Arion, 1964). Comparison of the responses of these enzymes and those previously reported for inorganic pyrophosphatase of yeast (Sperow *et al.*, 1973) and maize leaf (Bennett *et al.*, 1973) reveals a somewhat variable pattern of interaction of the analogs with the different enzymes.

Methods

Methylene diphosphonate was obtained from Miles Laboratories; imidodiphosphate was synthesized (Nielsen *et al.*, 1961) from diphenylimidodiphosphoric acid produced from diphenyl phosphoramidate (Kirsanov and Zhmurova, 1958) which was prepared by the method of Stokes (1893). The preparation and assay of rat liver microsomal inorganic pyrophosphatase (Feldman and Butler, 1972) and *E. coli* alkaline phosphatase (Sperow and Butler, 1971) were as previously described.

Bovine alkaline phosphatase was obtained from fresh intestine by a solubilization and extraction procedure utilizing solutions containing 9% (v/v) *n*-butyl alcohol, analogous to the procedure of Morton (1954). The fraction obtained between 40 and 60% saturated $(NH_4)_2SO_4$ was dialyzed twice against 0.03 M Tris, pH 7.6. The enzyme was then chromatographed twice on DEAE-cellulose. The first column was run in 0.05 M Tris and the enzyme was eluted by the addition of 0.1 M NaCl to the buffer; the second column was run in 0.03 M Tris and the enzyme was eluted by a linear NaCl gradient from 0 to 0.06 M. The enzyme was further purified by affinity chromatography on phenylalanyl-Sepharose according to Doellgast and Fishman (1973). Hydrolytic activity toward PP_i and imidodiphosphate coincided with hydrolytic activity toward 4-nitrophenyl phosphate in the elution pattern. Protein concentration was estimated by ultraviolet absorption, assuming $A_{280} = 1.0$ for a solution containing 1 mg of protein ml^{-1}. The specific activity (V_m) of this preparation toward 4-nitrophenyl phosphate (Sigma) was 250 μmol min^{-1}

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TABLE I: Kinetic Parameters for Alkaline Phosphatases.[a]

	E. coli			Intestinal		
	V_{max} (μmol min^{-1} mg^{-1})	K_m (mM)	K_I (mM)	V_{max} (μmol min^{-1} mg^{-1})	K_m (mM)	K_I (mM)
4-Nitrophenyl phosphate	66.7	0.036		71.4	0.044	
PP_i	40.9	0.28		44.8	0.33	0.27
Imidodiphosphate	1.8	0.28		2.5	0.28	
Methylene diphosphonate			1.2			1.5

[a] Conditions for assays were given under Methods. K_I values were measured against 4-nitrophenyl phosphate.

mg^{-1} when measured under optimal conditions of 1 M Tris, pH 9.0, at 30°.

Kinetic assays of the alkaline phosphatases were run in 1 M Tris, pH 8.0, 30°. Breakdown of 4-nitrophenyl phosphate was quantitated for nitrophenol production by continuous spectrophotometric monitoring of the appearance of the 4-nitrophenolate ion at 400 nm (ϵ 18,320 M^{-1} cm^{-1}, pK = 7.15) (Kezdy and Bender, 1962) and for phosphate production by a modification of the Fiske–SubbaRow assay (1925). Under the conditions of our assay the ratio of moles of 4-nitrophenol released to moles of phosphate released is 3.4:1 for the intestinal enzyme and 2.4:1 for the bacterial enzyme. As predicted by Hinberg and Laidler (1972), the K_m values observed by the two techniques were the same within experimental error. Hydrolysis of imidodiphosphate and of PP_i was also quantitated by the modification of the Fiske–SubbaRow assay; the observed rate of phosphate production was corrected for transfer to Tris using the above ratios. Imidodiphosphate breakdown in the acidic molybdate reagent, which was faster than that observed with PP_i, was corrected by extrapolation to 0 time exposure to the reagent and by appropriate control samples. Jencks and Gilchrist (1965) have shown that phosphoramidate is visualized by this assay; therefore, corrected for phosphate transfer to Tris, the assay measures the production of 2 mol of phosphate for every mol of imidodiphosphate hydrolyzed. Possible complications by competition of phosphoramidate, produced by the enzyme, with imidodiphosphate for the enzyme active site were minimized by measuring only initial rates, which were linear with time, so that relatively little phosphoramidate was present. Other controls indicated that no hydrolysis of methylene diphosphonate occurred either enzymatically or in the assays for P_i.

Results

Microsomal inorganic pyrophosphatase was found to be an effective catalyst for imidodiphosphate hydrolysis; under standard conditions imidodiphosphate and PP_i were hydrolyzed at similar rates. Values for kinetic parameters for imidodiphosphate hydrolysis in 0.1 M cacodylate, pH 6.0, were: V_{max}, 63 nmol min^{-1} per mg of protein, and K_m, 0.38 mM. Because values of kinetic parameters for this enzyme depend upon whether and how the enzyme is activated by detergent and/or alkali treatment (Stetten and Burnett, 1967), quantitative comparisons between different preparations are not very meaningful. These values are consistent with those obtained for PP_i under similar conditions (Nordlie *et al.*, 1968), however, and suggest that imidodiphosphate is approximately as effective as PP_i as a substrate for this enzyme. The other PP_i analog tested, methylene diphosphonate, cannot serve as substrate because of its stable P–C bonds; in concentrations up to 2 mM, it did not inhibit hydrolysis of 2.5 mM PP_i by this microsomal enzyme.

Both intestinal and *E. coli* alkaline phosphatase also hydrolyze imidodiphosphate; relevant kinetic parameters are shown in Table I. PP_i and 4-nitrophenyl phosphate are hydrolyzed at a comparable rate, which is much greater than the rate of hydrolysis of imidodiphosphate. All three analogs are competitive inhibitors of 4-nitrophenyl phosphate hydrolysis (some K_I values are not shown because they were obtained under different conditions). The K_I values for methylene diphosphonate are significantly larger than the K_m values for imidodiphosphate and PP_i, which are similar. In 0.1 M Tris, pH 9, there is good agreement between the values for K_m and K_I for imidodiphosphate.

Discussion

We have previously reported that imidodiphosphate is not a substrate and neither imidodiphosphate nor methylene diphosphonate are inhibitors of PP_i hydrolysis by maize leaf

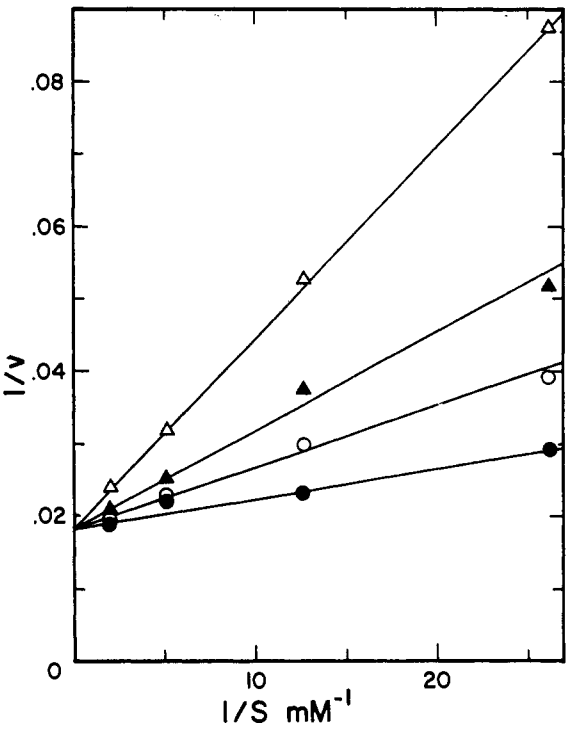

FIGURE 1: Inhibition by methylene diphosphate of 4-nitrophenyl phosphate hydrolysis by *E. coli* alkaline phosphatase. Methylene diphosphonate was present at concentrations of 0 (●), 1.19 (○), 2.38 (▲), and 4.76 (△) mM. Velocity is expressed as μmol of 4-nitrophenol released per min per mg of protein.

TABLE II: Summary of Kinetic Interactions of PP_i Analogs with Five PP_i-Hydrolyzing Enzymes.

Enzyme	Interaction			
	Substrate		Competitive Inhibitor	
	Imidodiphosphate	Methylene Diphosphonate	Imidodiphosphate	Methylene Diphosphonate
Microsomal inorganic pyrophosphatase	Good	No		No
Intestinal alkaline phosphatase	Poor	No	Yes	Yes
E. coli alkaline phosphatase	Poor	No	Yes	Yes
Maize leaf inorganic pyrophosphatase	No	No	No	No
Yeast inorganic pyrophosphatase	No	No	Yes	No

inorganic pyrophosphatase (Bennett *et al.*, 1973), and neither imidodiphosphate (Sperow *et al.*, 1973) nor *N*-3-phosphorylhistidine (Feldman and Butler, 1972) are substrates for yeast inorganic pyrophosphatase. With the latter enzyme, methylene diphosphonate and other phosphonate analogs of PP_i are not inhibitory under conditions of optimal activity with Mg^{2+} as the activating ion, but imidodiphosphate is a competitive inhibitor (K_I = 62 μM) of PP_i hydrolysis (Sperow *et al.*, 1973).

The kinetically observed interactions of these five enzymes with the PP_i analogs are summarized in Table II. The responses range from virtually complete interchangeability with PP_i by microsomal pyrophosphatase to apparent complete lack of interaction with the maize leaf pyrophosphatase. In general, the kinetic effects are consistent with the known properties of the PP_i analogs. Thus, only one of the enzymes is insensitive to imidodiphosphate, but three are essentially insensitive to methylene diphosphonate, which geometrically resembles PP_i less closely than does imidodiphosphate (Larsen *et al.*, 1969). Furthermore, for those enzymes which are affected by both imidodiphosphate and methylene diphosphonate, kinetic parameters indicate the former interacts more strongly with the enzymes.

The hydrolytic activity of the alkaline phosphatases toward the imidodiphosphate could be due to traces of a contaminating enzyme in one or both of the preparations, but the *E. coli* enzyme appeared to be homogeneous (Sperow and Butler, 1971), and hydrolytic activity toward both imidodiphosphate and PP_i comigrated with activity toward 4-nitrophenyl phosphate during purification of the intestinal enzyme, which suggests that all three activities are due to the same enzyme protein. This is also consistent with the observed competitive inhibition of 4-nitrophenyl phosphate hydrolysis by the analogs with these enzymes. The catalytic activity of *E. coli* enzyme toward P–N bonds has previously been reported (Yount *et al.*, 1971a,b; Snyder and Wilson, 1972).

The most specific of these PP_i-hydrolyzing enzymes is the preparation from maize leaves. Although this enzyme is weakly inhibited by relatively high concentrations of phosphoenolpyruvate, no other phosphorus-containing compound has been found to affect the rate of PP_i hydrolysis or serve as substrate (Bennett *et al.*, 1973). The least specific of these enzymes with respect to imidodiphosphate is the microsomal pyrophosphatase. It has previously been reported that phosphoramidate (Parvin and Smith, 1969) and carbamyl phosphate (Lueck and Nordlie, 1970) are good substrates for this enzyme.

The use of analogs that differ from PP_i only slightly in bond lengths and angles (Larsen *et al.*, 1969) reveals subtle differences in details of specificity of enzymes which otherwise appear to be rather similar, such as the yeast and maize leaf pyrophosphatases. On the other hand, the two nonspecific alkaline phosphatases interact with these analogs in a remarkably similar manner, as revealed by their virtually identical values for K_m and K_I, and by their similar ratios of values for V_m. Thus, these studies point out a close correspondence in the detailed mechanism of these two enzymes from unrelated sources.

When these alkaline phosphatases were assayed with concentrations of the PP_i analogs and other inhibitors two–three orders of magnitude below their K_i values, a small but reproducible *stimulation*, rather than inhibition, of 4-nitrophenol release was observed. These effects, and their implications for the mechanism of action of these enzymes, will be described in a subsequent publication.

Added in Proof

Since this manuscript was submitted it has come to our attention that Kiellstrom and Bishop (1970) have previously reported that yeast inorganic pyrophosphatase, as well as the corresponding enzymes from rabbit liver and from *Aspergillus*, are not inhibited by methylene disphosphonate. This is entirely consistent with our observations.

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Structures and Serological Activities of Three Oligosaccharides Isolated from Urines of Nonstarved Secretors and from Secretors on Lactose Diet†

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ABSTRACT: Normal food intake or lactose diet was found to induce an excretion of fucose-containing oligosaccharides in urines of ABO(H)-secretors. The urines of A- and B-secretors were found to contain the trisaccharides 3-*O*-(2-acetamido-2-deoxy-α-D-galactopyranosyl)-2-*O*-(α-L-fucopyranosyl)-D-galactose and 3-*O*-(α-D-galactopyranosyl)-2-*O*-(α-L-fucopyranosyl)-D-galactose, respectively. The urines of the O(H)-secretors contained the disaccharide 2-*O*-α-L-fucopyranosyl-D-galactose. The three oligosaccharides represent nonreducing terminals of the A, B, and H(O) antigenic determinants which was confirmed by inhibition studies in the appropriate precipitin systems. The structures of the isolated oligosaccharides were established by sugar analysis, methylation analysis, optical rotation, and alkaline degradation studies.

Fractionation by gel chromatography of urinary ultrafiltrates from starved secretors revealed 6-deoxyhexose (fucose) patterns characteristic of each ABO blood group (Lundblad, 1966). Lactodifucotetraose was found to be the characteristic compound in O(H)-secretor urine (Lundblad, 1968; Björndal and Lundblad, 1970) and two blood-group-active pentasaccharides accounted for the main difference between the 6-deoxyhexose patterns from A- and B-secretors (Lundblad, 1967; Björndal and Lundblad, 1970; Lundblad and Svensson, 1973b). After normal food intake the excretion of 6-deoxyhexose was significantly increased and gel chromatographic fractionation revealed the presence of additional low molecular weight 6-deoxyhexose-containing compounds (Lundblad, 1966). We now report a closer examination of these 6-deoxyhexose-containing components and demonstrate that food intake as well as lactose ingestion induces an endogenous formation of oligosaccharides characteristic of the blood group of the individual.

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Materials and Methods

Urine. Nine healthy males, divided equally between blood groups A, B, and O, gave urine specimens at 2-hr intervals in the afternoon, following a normal meal. Similar samples were collected after a 24-hr starvation. Two individuals, an A- and an O(H)-secretor, were starved for 24 hr and then given 100 g of lactose *per os.* Three consecutive samples of urine were then collected as before. This experiment was repeated with the A-secretor, but 100 g of sucrose was substituted for lactose.

Preservation. Bacterial growth was prevented by the addition of phenylmercuric nitrate (30 ml of saturated solution/l.).

Antisera. Human anti-A blood-grouping serum (lot A 8356-2) and human anti-B blood-grouping serum (lot B 93481) were purchased from Ortho Diagnostics.