Potent inhibition of membrane-bound rat intestinal alkaline phosphatase by a new series of phosphate analogues

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The inhibition by phosphonates and phosphate analogues of the alkaline phosphatase activity of rat intestinal brush-border membrane vesicles was studied at pH 7.5 and 30°C. Phenylene-1,3-diphosphonate, 2,6-dinitrophenylphosphonate and phosphonoacetaldehyde were found to be competitive inhibitors, with K_i values in the range 16–80 μM . Adenosine 5'-[β -thio]diphosphate and adenosine 5'-[γ -thio]triphosphate are also very potent inhibitors, with K_i values of approx. 10 μM . The inhibition produced by these thiophosphates was mainly competitive but with a slight non-competitive element. Adenosine 5'-[β -imido]triphosphate is also a competitive inhibitor of the alkaline phosphatase, but oxidation of the ribose moiety of this compound with NaIO_4 results in an active-site-directed irreversible inhibitor that could be of general use in studies of the mechanism of action of this enzyme.

In an investigation of the part that the enzyme alkaline phosphatase (EC 3.1.3.1) might play in the transport of inorganic phosphate across the plasma membrane of the mammalian small intestine, we sought a series of specific inhibitors of the enzyme. Our aim was to obtain examples of potent inhibitors whose action on alkaline phosphatase could be characterized to provide data for the interpretation of results arising from studies of P_i transport.

The present paper reports the findings made with several new inhibitors of alkaline phosphatase. For this study we decided that it was important to look at the properties of the enzyme by using the brush-border membrane-vesicle preparations that are also used in transport experiments, rather than work with a highly purified form of the enzyme that is free of any constraints that might arise from the interactions of the enzyme molecule with other components of the membrane.

The accompanying paper (Shirazi *et al.*, 1981) assesses the role of alkaline phosphatase in membrane phosphate transport.

Abbreviations used: [β -imido]ATP, adenosine 5'-[β -imido]triphosphate; [β -methylene]ATP, adenosine 5'-[β -methylene]triphosphate; [β -imido]GTP, guanosine 5'-[β -imido]triphosphate; [β -thio]ADP, adenosine 5'-[β -thio]diphosphate; [γ -thio]ATP, adenosine 5'-[γ -thio]triphosphate; Hepes 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

Experimental

Membrane-bound alkaline phosphatase

Brush-border membrane vesicles from fresh rat small intestine were prepared by modifications of techniques described by Kessler *et al.* (1978) and Lücke *et al.* (1978). Full details of the method are given in the accompanying paper (Shirazi *et al.*, 1981). The rats, of the Wistar strain, had approx. 100g body wt. each and were obtained from our own inbred colony. The vesicle preparation was used directly as the source of alkaline phosphatase. It could be stored at -20°C in sterilized 10mM-Hepes/Tris buffer, pH 7.5, containing 100mM-mannitol for several weeks without loss of activity or noticeable change in properties.

The specific activity of alkaline phosphatase was 0.54 unit/mg of protein, when assayed at pH 8.9 (10mM-Tris/HCl buffer) at 30°C with 0.5mM-*p*-nitrophenyl phosphate as substrate. The specific activity at pH 7.5 was 0.28 unit/mg of protein. One unit results in the formation of 1 μmol of product/min.

Assay of alkaline phosphatase

All assays were performed at 30°C with 10min incubation periods. The substrate was *p*-nitrophenyl phosphate (diTris salt) at a concentration in the range 0.1–0.5 mM, depending on the experiment, and

the reaction mixture was buffered at pH 7.5 with 10 mM-Tris/HCl buffer. The total reaction mixture was 2 ml and the assay was started by the addition of 10 μ l of the vesicle preparation (50 μ g of protein). After termination by the addition of 1 ml of 0.5 M-NaOH, the liberated *p*-nitrophenol was determined at 400 nm ($\epsilon_{400} = 18.8 \text{ M}^{-1} \cdot \text{cm}^{-1}$). The release of *p*-nitrophenol was linear with respect to time throughout the 10 min incubation period.

Determination of the free phosphate concentration present in the substrate (Chen *et al.*, 1956) showed that such contamination was less than 1%. Thus the substrate was used directly without further purification.

To study the effects of the inhibitors on the kinetics of the enzyme, solutions of the inhibitors were adjusted to the pH of the assay system and included in reaction mixtures at the required concentrations.

Reversibility of inhibition

Portions (1–7 μ l) of a concentrated solution of the inhibitor were added to 0.5 ml of the brush-border membrane-vesicle suspension in 100 mM-mannitol/10 mM-Hepes/Tris buffer, pH 7.5 containing about 2.5 mg of protein. This procedure enabled the required concentration of the inhibitor to be achieved without disturbing the osmolarity of the medium. The controls contained 1–7 μ l of the Hepes/Tris buffer. During incubation at room temperature 0.1 ml samples were removed at appropriate time intervals for separation of the protein fraction from the bulk of the inhibitor by rapid gel filtration. The samples were tested for alkaline phosphatase activity and protein concentration (Lowry *et al.*, 1951) before and after gel filtration. The technique involving the use of a combination of centrifugation and gel filtration described by Penefsky (1977) was used for the rapid gel filtration. The method uses Sephadex G-25 (fine grade) contained in disposable plastic syringes that can be inserted in centrifuge tubes. After Step 1 of the method given by Penefsky (1977), 0.2 ml of sterile 10 mM-Tris/HCl buffer, pH 7.5, was applied to the column, which was then centrifuged for 90 s at 900 rev./min (approx 100 g) in an MSE bench centrifuge, and the effluent was discarded. The sample (0.1 ml) was then applied and treated exactly as described by Penefsky (1977). The buffer was sterilized by passage through a Millipore filter of pore size 0.22 μ m. Preliminary tests with mixtures of 10 mM-dichromate and 1% (w/v) Blue Dextran indicated complete separation of high-molecular-weight and low-molecular-weight components under the conditions employed. With a syringe of 1 ml capacity efficient separation is achieved provided that the load does not exceed 0.1 ml in volume.

Kinetic data

Inhibition data were analysed by weighted regression analysis (Wilkinson, 1961) of fits to $1/v$ -versus-[I] plots (Dixon & Webb, 1964) and $[S]/v$ -versus-[I] plots (Cornish-Bowden, 1974) by using a program written for the Harris S125 computer at Chelsea College. K_i values were obtained from calculation of the points of intersection of the $1/v$ -versus-[I] plots. K_m values were obtained from weighted regression analysis of $1/v$ -versus- $1/[S]$ plots (Cleland, 1967).

Preparation of oxidized nucleotide

Oxidation of [β -imidol]ATP with 10% molar excess of NaIO₄ was performed exactly as described by Lowe *et al.* (1979a). The concentration of the product was determined from the absorbance at 260 nm.

Materials

The diTris salt of *p*-nitrophenyl phosphate was obtained from Calbiochem (Bishops Cleeve, U.K.) and the various nucleotides were bought from Boehringer Corp. (London W.5, U.K.).

Results and discussion

Inhibition by phosphonates

Of a number of compounds tested, the three phosphonates with the greatest inhibitory effect on alkaline phosphatase are shown in Fig. 1. Phosphonates were found to be classical competitive inhibitors, and a representative set of results obtained with compound (I) is shown in Fig. 2. The parallel $[S]/v$ -versus-[I] plots (Cornish-Bowden, 1974) distinguish between competitive and mixed inhibition that cannot be resolved by $1/v$ -versus-[I] plots alone. The K_i values (μ M) for compounds (I), (II) and (III) are respectively 16, 78 and 47 at pH 7.5 (mean value of three determinations in each case; the range of the estimates was 5–10% of the mean). The K_m value for *p*-nitrophenyl phosphate under these conditions is $103 \pm 6 \mu$ M.

No time-dependency of the inhibition could be demonstrated, and the inhibition was reversible by centrifugation-gel filtration. Thus the aldehyde group in compound (III), which could be envisaged in some circumstances to interact covalently with the enzyme protein, was without effect in this instance.

The inhibition characteristics of the phosphonates match those reported by Kochman *et al.* (1964) and Landt *et al.* (1978), who also studied the effect of various phosphonates on intestinal alkaline phosphatase. These previous studies led to the conclusion that aromatic phosphonates bind to the enzyme particularly well compared with their aliphatic homologues. We did not find this to be

Fig. 1. Inhibitors of rat intestinal alkaline phosphatase

(I) Phenylene-1,3-diphosphonate; (II) 2,6-dinitrophenylphosphonate; (III) phosphonoacetaldehyde; (IV) oxidized [β - γ -imido]ATP.

Fig. 2. Inhibition of alkaline phosphatase by phenylene-1,3-diphosphonate

The inhibition was studied at pH 7.5 at 30°C with *p*-nitrophenyl phosphate as substrate and with 50 μ g of an intestinal brush-border membrane-vesicle preparation as the source of alkaline phosphatase. The concentration of substrate was fixed at 0.2 mM (■), 0.1 mM (△) and 0.05 mM (▲). For full details see the text. In (a) the results are presented in the form of $1/v$ -versus-[I] plots and in (b) as $[S]/v$ -versus-[I] plots. The lines were fitted to the experimental points by weighted regression analysis.

necessarily so, since phosphonoacetaldehyde (III) is midway between compounds (I) and (II), both aromatic compounds, in its potency. Indeed, in the study by Landt *et al.* (1978), phosphonoacetic acid

was found to be as inhibitory as many of the aromatic phosphonates they tested. Thus, although large aromatic side chains do seem to have some influence on substrate and substrate-analogue bind-

ing (Szajn *et al.*, 1977; Woodroffe, 1978), other factors also seem to be involved. It could be that in the case of acetaldehyde and acetate derivatives the electronegativity of the carbonyl or the carboxy group results in attraction by the positive charge density assumed to be present at the active site of alkaline phosphatase (Landt *et al.*, 1978; Woodroffe & Butterworth, 1979).

Inhibition by nucleotides

The nucleotide derivatives tested are listed in Table 1, together with a summary of their inhibitory

Table 1. Inhibition of alkaline phosphatase by nucleotides

The inhibition was studied at pH 7.5 at 30°C with *p*-nitrophenyl phosphate as substrate. For full details see the text.

Compound	K_i (μM)
[$\beta\gamma$ -Imido]ATP	68 (± 2)
[$\beta\gamma$ -Methylene]ATP	22 (± 4)
[$\beta\gamma$ -Imido]GTP	80 (± 12)
[β -Thio]ADP	10 (± 2)
[γ -Thio]ATP	5 (± 0.7)

properties. Most of the nucleotides had similar potency towards alkaline phosphatase in that they acted as competitive inhibitors with K_i values in the range approx. 60–80 μM . Two nucleoside thiophosphorates were tested. These were derivatives of ADP and ATP in each of which the terminal phosphate group is replaced by a phosphorothioate. These derivatives, [β -thio]ADP and [γ -thio]ATP, inhibited very strongly, with K_i values of 10 μM . The inhibition was largely of competitive type, although there was evidence of a degree of 'mixed' behaviour in that calculated fits to $[S]/v$ -versus-[I] plots of inhibition data exhibited a slight degree of convergence. Also, a degree of non-linearity became evident at higher concentrations of inhibitor (Fig. 3). The inhibition was reversible.

Competitive inhibition by orthophosphorothioates has been reported by Eckstein & Sternbach (1967) and by Fernley (1973). The latter showed that orthophosphorothioate was a substrate for human placental alkaline phosphatase, although activity with the equivalent phosphate compound was about 300 times greater. Similar findings were made with the *Escherichia coli* enzyme (Mushak & Coleman, 1972). Since inorganic and nucleoside

Fig. 3. Inhibition of alkaline phosphatase by [β -thio]ADP

The inhibition was studied under the same conditions as those given in Fig. 2. The concentration of substrate was fixed at 0.2 mM (■), 0.1 mM (△) and 0.05 mM (▲). In fitting the lines to the experimental points, the data at the highest inhibitor concentrations were ignored in the regression analysis.

pyrophosphates are relatively good substrates for alkaline phosphatase (Fernley & Walker, 1967; Eaton & Moss, 1967; Butterworth, 1968; Horder, 1975), it is no surprise to find that nucleoside phosphorothioates are powerful inhibitors of rat intestinal enzyme. The apparent deviation from competitive behaviour may be a property of the replacement of an oxygen atom by a sulphur atom, but could also be connected with the pyrophosphate group on these compounds. Although, as mentioned above, various pyrophosphates are substrates for alkaline phosphatase, they also exhibit strong substrate inhibition (Wass & Butterworth, 1971), the cause of which has not been completely explained. Also, Fernley & Walker (1967) showed that ATP exhibited a degree of mixed behaviour when tested as an inhibitor of orthophosphate hydrolysis. Such behaviour may be a complicating factor in interpretation of the results we obtained with these compounds.

An active-site-directed inhibitor of alkaline phosphatase appears to be realized in the form of oxidized [$\beta\gamma$ -imido]ATP (compound IV in Fig. 1). Incubation of alkaline phosphatase with the oxidized compound results in a time-dependent loss of activity (Fig. 4) that is irreversible by rapid gel filtration. [$\beta\gamma$ -Imido]ATP itself is a reversible competitive inhibitor ($K_i = 69 \mu\text{M}$), so that it is probable

that the oxidized compound does combine with a group at, or close to, the active centre. Lowe *et al.* (1979b) believe that oxidized nucleotides such as oxidized [$\beta\gamma$ -imido]ATP react with a free amino group to inactivate mitochondrial ATPase. These authors showed that borohydride reduction is not necessary to stabilize the inhibitory-protein adduct, and suggested that, on reaction with protein, phosphate elimination occurred, leaving a conjugated aldehyde that would form a very stable Schiff base with amino groups (Lowe *et al.*, 1979b).

Conclusions

Our results confirm the opinion of Landt *et al.* (1978) that certain phosphonates are exceptionally good inhibitors of intestinal alkaline phosphatase. We find, however, that phosphorothioate derivatives of nucleoside pyrophosphates are even stronger inhibitors, and appear to be exceeded in potency only by inorganic compounds such as vanadates and permanganates (McComb *et al.*, 1979). Oxidized nucleotides such as oxidized [$\beta\gamma$ -imido]ATP could have general use in mapping the residues adjacent to the active site of phosphate-hydrolysing enzymes of various specificities.

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Fig. 4. Time-dependence of the inhibition of alkaline phosphatase by oxidized [$\beta\gamma$ -imido]ATP

The membrane-vesicle preparation was preincubated at 20°C with [$\beta\gamma$ -imido]ATP and oxidized [$\beta\gamma$ -imido]ATP at a concentration of 1.83 mM before withdrawal. At the times shown, of samples for assay of residual enzymic activity. For full details see the text. The zero-time samples were obtained immediately after mixing of the enzyme and the reagents.

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