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THE INHIBITION OF ALKALINE PHOSPHATASE BY PERIODATE AND PERMANGANATE

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SUMMARY

Periodate and permanganate strongly inhibit alkaline phosphatase in barbital buffer at concentrations as low as 10^{-7} M. Oxidation of the enzyme does not appear to be involved. Periodate is a reversible competitive inhibitor, whereas permanganate is an irreversible inhibitor. Enzyme inhibited by permanganate is completely restored with low concentrations of reducing agents. Periodate and phosphate bind simultaneously but antagonistically to the enzyme.

INTRODUCTION

Alkaline phosphatase (orthophosphoric-monoester phosphohydrolase, EC 3.1.3.1) is a metalloprotein (Zn) catalyzing the hydrolysis and transphosphorylation of phosphate monoesters. Aside from inorganic phosphate there are few good inhibitors of the enzyme. Although several non-substrate inhibitors such as the phenyl phosphonates and the phenyl phosphinates are effective, their K_i values are about two orders of magnitude higher than the K_i for inorganic phosphate [1]. Following a suggestion of Benisek [2], Sorenson, in some preliminary experiments in this laboratory, found that permanganate was an effective inhibitor of alkaline phosphatase.

The present study reports that permanganate and periodate are potent inhibitors of alkaline phosphatase. Neither chromate nor perchlorate inhibited the enzyme. Thus the effectiveness of permanganate and periodate as inhibitors does not appear to be related to their oxidizing ability and further evidence on this point will be presented. It is also reported that periodate binds simultaneously and anticooperatively with phosphate even though both are competitive with the substrate, *p*-nitrophenyl phosphate. The kinetic observations with periodate are simple and show that periodate is a reversible competitive inhibitor. The kinetic observations with permanganate are more complicated in that there is a time course for inhibition and inhibition is irreversible.

MATERIALS AND METHODS

Enzyme and reagents

The enzyme used in the bulk of the experiments was the partially pure (2000

μmoles/min per mg enzyme) enzyme isolated from *Escherichia coli*, strain CW 3747, according to the method of Neu and Heppel [3] and assayed under the conditions of Malamy and Horecker [4]. The enzyme was extensively dialyzed against 0.01 M barbital buffer at pH 8.6.

The *p*-nitrophenyl phosphate was supplied by the Aldrich Chemical Co., $KMnO_4$ and $NaHPO_4$ by the J. T. Baker Chem. Co., the KIO_4 and Na_2CrO_4 from Matheson, Coleman and Bell, and the $NaClO_4$ and Tris from the G. Frederick Smith Chem. Co., and Sigma Chem. Co., respectively.

Enzyme assay

p-Nitrophenyl phosphate was used as substrate in 0.01 M barbital buffer, pH 8.6, 25 °C. A Zeiss PMQ II Spectrophotometer fitted with a log converter and recorder was used to follow the increase in absorbance at 400 nm attending the hydrolytic release of *p*-nitrophenol. A 1- or 5-cm-pathway cuvette was used in combination with different absorbance spans according to the circumstances. Thus for low substrate concentrations a 5-cm cuvette was used with an absorbance span of 0–0.1.

Periodate inhibition

Periodate is a potent inhibitor of the hydrolysis of *p*-nitrophenyl phosphate by alkaline phosphatase in 0.01 M barbital buffer at all pH values studied from 6.8 to 9.6.

The data in Fig. 1 show that the inhibition is competitive between periodate and the ester substrate. The K_i derived from these plots is $5 \cdot 10^{-8}$ M at pH = 8.6, 2 orders of magnitude smaller than the K_i for inorganic phosphate, under the same conditions. The extent of inhibition is the same regardless of the order of addition of

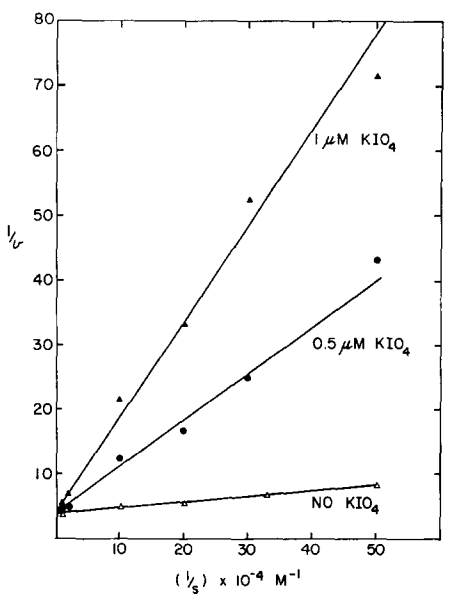

Fig. 1. Double-reciprocal plots of initial velocity versus *p*-nitrophenyl phosphate concentration in the presence of KIO_4. Reaction conditions: $2.9 \cdot 10^{-9}$ M alkaline phosphatase. Reaction medium: 0.01 M barbital buffer, pH 8.6, at 25 °C. Initial velocity was obtained using the 0.1 absorbance scale on the Zeiss PMQ II Spectrophotometer with a chart speed of 4 inches/min, 5-cm cuvettes.

inhibitor and substrate and is independent of the length of time that enzyme and inhibitor are preincubated before the addition of *p*-nitrophenyl phosphate. Dilution experiments also attest to the reversibility of the interaction of periodate and alkaline phosphatase. For example, at 10^{-4} M periodate and 10 μM *p*-nitrophenyl phosphate there was no detectable enzyme activity. Nonetheless, when enzyme ($1.2 \cdot 10^{-6}$ M) was incubated with $4 \cdot 10^{-4}$ M and $2 \cdot 10^{-4}$ M periodate for 4 min and then diluted 400 X into 10 μM *p*-nitrophenyl phosphate, 25 and 45% enzyme activity was measured. These observations are just what would be expected from Fig. 1 if periodate is a reversible inhibitor. Thus periodate is a reversible competitive inhibitor and the most potent known for alkaline phosphatase. Full enzyme activity can be restored immediately during an assay by the addition of $5 \cdot 10^{-6}$ M final concentration ascorbic acid. Similarly inhibition can be prevented by the prior addition of ascorbic acid. In these experiments ascorbic acid simply removes periodate by reduction.

The plot of reciprocal velocity versus phosphate concentration in the presence of a fixed concentration of periodate and *p*-nitrophenyl phosphate (Fig. 2) yields a straight line [5]. A family of such lines corresponding to different periodate concentrations intersects at a common point below the abscissa. In this type of plot parallel lines are obtained if the two inhibitors, periodate and phosphate, bind in a mutually exclusive fashion. If they bind independently, the intersection occurs on the X axis at an abscissa value of $-K_i$ for phosphate, otherwise below or above the X axis at an abscissa value of $-\alpha K_i$ where α is the factor by which the dissociation constant for

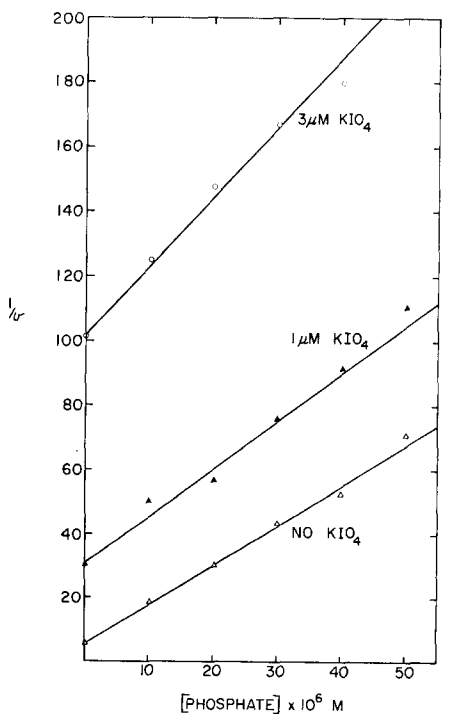

Fig. 2. Theorell-Yonetani plot of initial velocity versus phosphate concentration at varying concentrations of KIO_4 and fixed 5 μM *p*-nitrophenyl phosphate concentration. Reaction conditions and medium as in Fig. 1.

periodate is changed by the prior binding of phosphate (and the factor by which the dissociation constant for phosphate is changed by the prior binding of periodate). Thus $\alpha = \infty$ for mutually exclusive binding (parallel lines), $\alpha = 1$ for independent binding and $\alpha < 1$ or $\alpha > 1$ for cooperative or antagonistic binding. In our case α is about 7 for periodate. The interference is that periodate and phosphate can bind simultaneously but antagonistically (anticooperatively).

The value of K_i varies markedly in the pH range 6.8 to 9.1 (Table I). Binding increased with increasing pH which is opposite to the direction of either phosphate or substrate "binding" (K_m) in this pH range [1].

TABLE I

K_i VALUES AT DIFFERENT pH VALUES

Variation of the dissociation constant for the binding of periodate by alkaline phosphatase as a function of pH. These values were obtained from double-reciprocal plots similar to those shown in Fig. 1 for pH 8.6. The reaction medium for other pH values was the same as in Fig. 1 except for pH 6.8 in which the buffer was 0.01 M maleate instead of 0.01 M barbital.

pH	$K_i \times 10^{-7}$ M
6.8	4.9
7.6	1.4
8.6	0.5
9.1	0.5

Permanganate inhibition

Our observations with permanganate are more complicated than with periodate. Enzyme at low concentration ($3 \cdot 10^{-9}$ M) preincubated with low permanganate ($2 \cdot 10^{-7}$ M) in a 5-cm path length cuvette, showed no enzyme activity when substrate (10^{-3} M final concentration) was added after 15 s and only 7% after 5 s. This observation indicates a half time of less than 0.03 min. A further study of the inhibition process with varying inhibitor concentrations in which the enzyme was added directly to a solution of substrate (5 μM) and permanganate in 0.01 M barbital buffer, pH 8.6, so that enzyme was exposed simultaneously to substrate and inhibitor showed that permanganate, even at concentrations as low as 10^{-7} M behaved like an "irreversible" inhibitor of the enzyme. There was a continual decline in activity until after 5 min little or no enzyme activity remained (Fig. 3). The addition of fresh substrate had no effect whereas the addition of a fresh aliquot of enzyme resulted in renewed hydrolysis of *p*-nitrophenyl phosphate until the added enzyme was inactivated. In these experiments the release of *p*-nitrophenol was recorded continuously. The decline of the reaction rate gave approximately linear first order plots. The half time in 5 μM substrate was about 0.8 min and did not change rapidly with permanganate concentration.

The prior or simultaneous addition of *p*-nitrophenyl phosphate in high concentration completely protected the enzyme against inactivation by permanganate. Experiments were carried out in which 0.01 M Tris, 10^{-4} M inorganic phosphate, or $5 \cdot 10^{-5}$ M periodate were added to the enzyme before the addition of permanganate (10^{-6} M) and then assayed for enzyme activity by adding high concentrations of *p*-nitrophenyl phosphate (10^{-3} M) at times up to 15 min later. Controls contained no permanganate. These experiments showed that Tris, phosphate and periodate com-

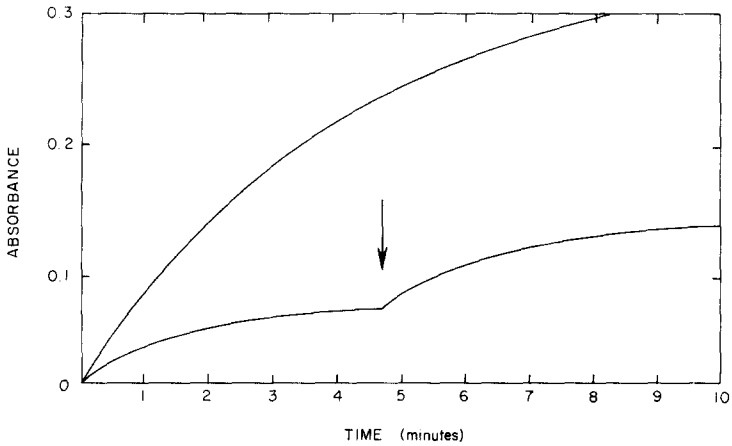

Fig. 3. Absorbance versus time plot in the presence of $KMnO_4$. Upper curve (control), 5 μM *p*-nitrophenyl phosphate in 0.01 M barbital buffer pH 8.6 at 25 °C, $2.9 \cdot 10^{-9}$ M enzyme. Lower curve, control in presence of 10^{-6} M $KMnO_4$; ↓, addition of fresh aliquot of enzyme to cuvette $2.9 \cdot 10^{-9}$ M final concentration. Reaction medium and conditions: as in Fig. 1. Trace obtained using 0.5 absorbance scale with 1 inch/min chart speed. The curvature in the control is caused by substrate exhaustion, 5-cm cuvettes.

pletely protected the enzyme from inactivation by permanganate. Although Tris does react with permanganate, as measured by a decline in $A_{526\ nm}$, an absorption peak for permanganate, the reaction is too slow to account for the ability of Tris to protect the enzyme. In no case did these protectants restore activity once it had been lost. This underscores the "irreversible" nature of the permanganate inhibition process. By way of contrast the reducing agents ascorbic acid (5 μM), hydrazine sulfate (a "speck"), and Mn^{2+} (5 μM) not only protected the enzyme but also rapidly restored complete activity to the inactivated enzyme. These reductants "protect" by reducing the inhibitor prior to the addition of enzyme. They restore activity either by reducing enzyme bound permanganate as well as free permanganate or by reducing oxidized enzyme. This question will be discussed later.

Neither ClO_4^-, nor $CrO^2{}_4^-$ at 10^{-6} M concentration had any effect on the enzymic activity. At 10^{-4} M, ClO_4^- inhibited the enzyme slightly.

DISCUSSION

With K_i about two orders of magnitude lower than for inorganic phosphate, periodate is the most potent reversible competitive inhibitor of alkaline phosphatase known. Periodate does not serve as an oxidant in inhibiting alkaline phosphatase. This is shown by the fact that the effects of periodate are reversible and depend only upon the final concentrations of periodate present during the assay. Thus prior incubation with high concentrations of periodate does not inhibit the enzyme if the periodate concentration is lowered by dilution during the assay. Similarly prolonged preincubation does not show any progressive increase in inhibition, only the final concentration of periodate counts. The fact that inhibition is released by reductants only indicates that periodate is being removed. We should also note that inhibition is "immediate" when periodate is added to an ongoing assay.

The Theorell–Yonetani plots [5] indicate that a ternary complex can be formed between enzyme, phosphate and periodate. The formation of such a ternary complex indicates that phosphate binds at a different site than periodate. The formation of a ternary complex between enzyme, phosphate and a phosphonate had been deduced by Halford [6] on the basis of spectral observations.

Although our observations clearly indicate that periodate does not function as an oxidant in the periodate inhibition of alkaline phosphatase the situation with permanganate is not clear. We observe a time course of inhibition with permanganate. The order of addition of inhibitor and substrate is important. Enzyme activity can be restored only by reducing agents. These observations all suggest that permanganate is functioning as an oxidant, yet they are not inconsistent with the slow formation of a very stable addition complex between permanganate and the enzyme. A very stable addition complex would dissociate very slowly.

Some evidence against the "oxidant theory" and in support of the "complex theory" can be found in the nature of the reductants that were used. In the "oxidant theory" the reductant would have to reduce the "oxidized enzyme". Ascorbate is a well known but mild reductant. It does not, for example, reduce disulfide linkages. The situation is a little more extreme in that even Mn^{2+} restores activity and Mn^{2+} can reduce only powerful oxidants such as MnO_4^- and it does not appear likely that the "oxidized enzyme" would be so potent an oxidizing agent. Yet we cannot completely rule out an oxidative mechanism for the inhibition of alkaline phosphatase by permanganate.

The four anions, IO_4^-, MnO_4^-, ClO_4^- and CrO_4^{2-} are similar in size and shape [7] and except for differences in electrical charge might a priori be considered as possible phosphate analogues. Despite structural similarities chromate and perchlorate do not affect the enzyme at least at low concentration. Even periodate which does inhibit the enzyme does not function as a phosphate analogue because it binds at a different site than phosphate.

These structural similarities refer to the solid state and the explanation for the very different effects of perchlorate and periodate on the enzyme may be a reflection of the very different structures in water at slightly alkaline pH. In contrast to perchlorate, periodate forms polybasic anions in water, the most prominent of which is $H_3IO_6^{2-}$ at slightly alkaline pH [8]. It is possible that this ion is the actual inhibitor.

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