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STEADY STATE KINETICS AND EFFECT OF SH INHIBITORS ON ACID PHOSPHATASE FROM BOVINE BRAIN

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Summary

1. Product inhibition studies and transphosphorylation to methanol using two different substrates indicate that acid phosphatase from bovine brain forms a phosphoryl enzyme and that the phosphorylation step can not be rate limiting.

2. Acid phosphatase from bovine brain is inhibited by 5,5′-dithiobis-(2-nitrobenzoic acid); this inhibition can be counteracted by inorganic phosphate.

Incubation of the enzyme with *p*-nitrophenyl phosphate in the presence of p-chloromercuribenzoate leads, initially, to a higher degree of inhibition than that found with the same concentration of inhibitor in the absence of substrate. Both the titration by 5,5′-dithiobis-(2-nitrobenzoic acid) and inhibition by *p*-chloromercuribenzoate are consistant with the presence of 2 SH groups per mol of enzyme.

Introduction

The widespread occurrence of acid phosphatase (orthophosphoric monoester hydrolase (E.C. 3.1.3.2.) has led to a series of studies on the distribution, substrate specificity and mechanism of this enzyme (for review see ref. 1).

Recently, acid phosphatases from rat liver [2], human prostate [3] and wheat germ [4], were shown to form a phosphorylated intermediate in the reaction pathway catalyzed by these enzymes. This finding confirms earlier kinetic data obtained with other acid phosphatases [1].

Red cell [5] and bovine brain [6] acid phosphatases are markedly inhibited by low concentrations of heavy metals; the latter enzyme can be complete-

Abbreviations: PCMB, *p*-chloromercuribenzoate; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); Nph-*P*, *p*-nitrophenyl phosphate; phenyl-*P*, phenyl phosphate; Nph, *p*-nitrophenol; R-*P*, phosphomonoester.

ly inhibited by PCMB and this inhibition can be reversed by mercaptoethanol [6]. Prostatic acid phosphatase is partially inhibited by blocking the SH groups with Ag^+ [7]. Thus the importance of cysteine in the activity of the enzyme has been suggested for acid phosphatases from different sources.

It is becoming apparent that there may be unifying features behind the variety of kinetic properties that have been described [1] in many partially purified preparations of this enzyme.

In this paper we present a steady state analysis of the kinetics of acid phosphatase from bovine brain*, and, using external acceptors it is shown that the kinetics are consistant with the formation of a phosphoryl enzyme. It is also reported that acid phosphatase requires free SH groups for catalysis and that both substrate and P_i modify the reactions of inhibitors with the SH groups of the enzyme.

Experimental procedures

DTNB, Tris and PCMB were obtained from B.D.H. Ltd. *p*-Nitrophenyl phosphate and phenyl phosphate, purchased from Sigma Chem. Co., contained less than 1% free P_i and were used without further purification; crystalline bovine serum albumin was obtained from the same firm. Methanol, ethanol and 1-propanol were from Merck A.G. All other reagents were analytical grade; deionized, twice distilled water was used throughout.

Purified acid phosphatase was obtained from bovine brain according to a published procedure [6]. The enzyme was concentrated by pressure ultrafiltration using a AMICOM UM-2 membrane. Enzyme solutions containing 0.5—1.0 mg of protein per ml (spec. act. 25—30 units/mg) were stored at 0—4°C. The activity of this enzyme preparation was tested daily using the standard assay (vide infra) with *p*-nitrophenyl phosphate as substrate. Under these conditions the preparation is completely stable for one month.

Protein concentration was determined by a modification of the Folin method [8] using crystalline bovine serum albumin as standard.

Assay of enzyme activity

The activity of acid phosphatase was determined using either *p*-nitrophenyl phosphate (Nph-*P*) or phenyl phosphate (phenyl-*P*) as substrate. When Nph-*P* was used as substrate the reaction mixture contained, unless otherwise stated: potassium acetate buffer pH 5.0 (final concentration 0.05 M), 2 μmol of Nph-*P* and enzyme in a total volume of 2.0 ml. The solutions were equilibrated at 37°C; the reaction started by the addition of enzyme and stopped after two minutes of incubation. A unit (U) of enzyme activity is defined as the amount of enzyme that releases 1 μmol of *p*-nitrophenol per min under those conditions. Specific activity is given as units/mg of protein. When the product to be measured was *p*-nitrophenol the reaction was stopped with 1.0 ml of 1.0 M NaOH and readings were made at 405 nm in a Zeiss PMQ II spectrophotometer using an extinction coefficient of 18, 300 $cm^{-1} \cdot M^{-1}$.

* Unless specifically stated the term acid phosphatase used throughout this manuscript refers to acid phosphatase from bovine brain.

P_i was determined after stopping the reaction with 0.8 ml of acid molybdate followed by 0.2 ml of the reducing reagent [9]. After 20 min at room temperature, readings were made at 660 nm.

With phenyl-*P* as substrate the reaction conditions were similar to those described for Nph-*P* except that the substrate concentration used was 5 mM and the incubation time was four min. Phenoxide was measured at 287 nm using a molar extinction coefficient of 2600 $cm^{-1} \cdot M^{-1}$ [10].

Under all conditions used in this work product formation varies linearly with time and the rate varies linearly with enzyme concentration. All assays were done in duplicate, and blanks containing no enzyme were used.

Maximum velocity (V) and Michaelis constants (K_m) were determined using Lineweaver Burk plots [11] that were calculated by regression analysis in a Hewlett Packard Model 10 Calculator.

Concentrated stock alcohol solutions were prepared by weight and kept in stoppered flasks at 4 °C.

Titration of SH groups with DTNB

Unless otherwise stated all conditions were those described by Ellman [12]. The absorbance at 412 nm was followed at room temperature (22 ± 1 °C) in a Cary 14 spectrophotometer. Reaction mixtures contained 0.1 M Tris · HCl buffer (pH 8.0), 0.2 mM DTNB and enzyme; a reference cell containing no enzyme was used in all cases.

Inactivation by PCMB or DTNB

The reaction mixture usually contained potassium succinate buffer (50 mM, pH 6.0), PCMB or DTNB and enzyme in a total volume of 0.2 ml. The reaction proceeded at 37 °C and was started by the addition of enzyme. Unless otherwise stated, 30 µl aliquots were removed for the assay of residual activity in a final vol. of 2.0 ml.

Effect of substrate on the rate of inactivation by PCMB

The reaction mixture contained potassium succinate buffer (50 mM, pH 6.0), 3 µmol of Na^+ *p*-nitrophenyl phosphate, PCMB and 0.022 mg of enzyme in a total volume of 0.2 ml. The reaction was started by the addition of enzyme and incubated at 37 °C. Before the addition of enzyme both the reaction mixture and the enzyme were allowed to reach 37 °C (usually 3 min). At selected time intervals 30 µl aliquots were removed and pipetted into 1.0 ml of ice cold 1.0 M NaOH. After dilution to 3.0 ml the absorbance was determined at 400 nm in a Zeiss PMQ II spectrophotometer.

Results

The rate data obtained varying the concentration of *p*-nitrophenyl phosphate, either alone or in the presence of P_i or *p*-nitrophenol, is presented in Fig. 1 (a and b). The Michaelis constant, inhibition constants, and other pertinent kinetic constants that can be calculated from the data; following the treatment and nomenclature presented by Hsu et al. [13]; are shown in

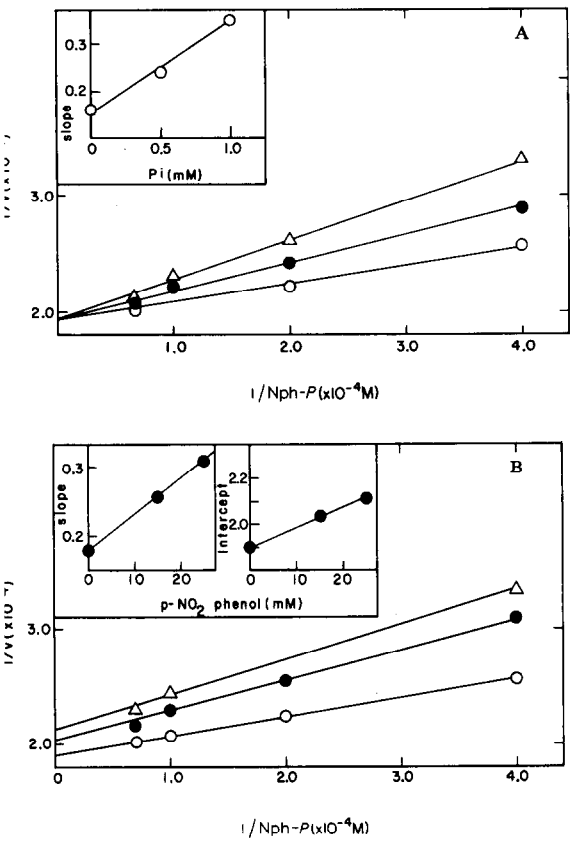


Fig. 1. Reciprocal plot of the inhibition of *p*-nitrophenyl phosphate hydrolysis by P_i and *p*-nitrophenol. Conditions of the assay are described in Experimental Procedures. 5 μg of enzyme were used per tube, rate is expressed as concentration of *p*-nitrophenol produced per min under the conditions of the assay. The points represent the average of at least 5 independent experiments, with a maximum deviation of 5%. A, P_i concentration: ○———○, none; ●———●, 0.5 mM; △———△, 1.0 mM. B, *p*-nitrophenol concentration: ○———○, none; ●———●, 15 mM; △———△, 25 mM.

Table I. Both products inhibit linearly, P_i being competitive and *p*-nitrophenol non-competitive inhibitors, respectively.

Another efficient substrate for this enzyme is phenyl phosphate. The K_m for this substrate is 1 mM, more than one order of magnitude higher than that of *p*-nitrophenyl phosphate, but the V for both substrates is identical.

TABLE I

KINETICS PARAMETERS CALCULATED FROM THE DATA SHOWN IN FIG. 1

For details, see text.

K_a	8.8×10^{-5} M
K_{ip}	2.10×10^{-1} M
K_i	7.6×10^{-4} M
K_{ia}	5.4×10^{-4} M
K_{eq}	≤0.296 M

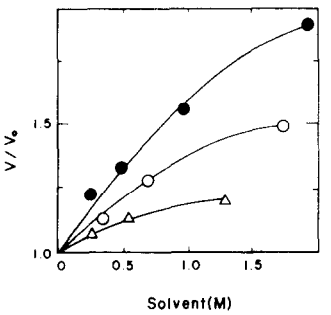


Fig. 2. Effect of methanol, ethanol and propanol on the rate of hydrolysis of *p*-nitrophenyl phosphate. Conditions of the assay are described in Experimental Procedures. V_0 is the rate of *p*-nitrophenol release measured in the absence of alcohol and V is the rate measured at the indicated alcohol concentrations. ●———●, methanol; ○———○, ethanol; △———△, propanol.

The rate of decomposition of Nph-*P*, as measured by *p*-nitrophenol release s increased by methanol, ethanol and propanol, the effect being greatest for methanol (Fig. 2). The initial rate of *p*-nitrophenol release from Nph-*P* increases up to 2-fold and then it seems to level off after 2 M methanol.

Although methanol increases the rate of liberation of *p*-nitrophenol (or henol) from *p*-nitrophenyl (or phenyl) phosphate, there is little effect on the ate of release of free P_i under the same conditions. The difference between the otal *p*-nitrophenol (or phenol) concentration produced in the decomposition f either substrate and the P_i concentration is a measure of the amount of nethyl phosphate formed under steady state conditions. The ratio between the mount of phenol and P_i formed under the same conditions can be used to stimate the extent of transphosphorylation. This has been used as an estimation of transphosphorylation by alkaline phosphatase [14]. This ratio has been letermined both for Nph-*P* and phenyl-*P* at pH 5.0 at different methanol concentrations (Table II). The transphosphorylation conditions were similar to

TABLE II

EFFECT OF METHANOL ON THE RATIO OF ROH/P_i (TRANSPHOSPHORYLATION RATIO)

The conditions of the incubation and the methods for the determination of P_i, *p*-nitrophenol and phenol re described in Experimental Procedures. In order to minimize the error in the determination of products the analyses were conducted as follows: at each methanol concentration a reaction mixture of 3–4 times the usual volume was prepared and equilibrated at 37 °C. The reaction was started by addition of nzyme and stopped after 2 min (or 4 min for phenyl phosphate) with NaOH. An aliquot was used for the etermination of *p*-nitrophenol (or phenol) and, after careful neutralization, another aliquot was used for he determination of P_i. The results represent the average of 5 independent determinations, ± standard eviation.

Methanol (M)	Substrate	
	Phenyl phosphate (Phenol/P_i)	*p*-Nitrophenyl phosphate (*p*-Nitrophenol/P_i)
None	0.97 ± 0.03	1.01 ± 0.01
1.5	1.26 ± 0.03	1.27 ± 0.04
2.0	1.56 ± 0.06	1.57 ± 0.02
3.0	1.94 ± 0.10	2.14 ± 0.01

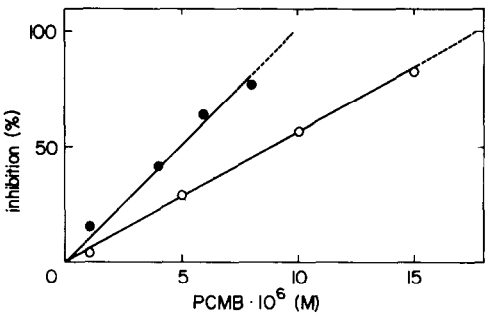


Fig. 3. Effect of preincubation with PCMB on acid phosphatase activity. ○———○, 20 or ●———●, 10 μg of acid phosphatase were preincubated with the indicated PCMB concentrations and assayed as described in Experimental Procedures. The percentage of final inhibition is plotted against PCMB concentration in the preincubation. See text for further details.

those used in the determination of initial reaction rates in order to avoid possible complications arising from inhibition by products. It can be appreciated that the mean transphosphorylation ratio is similar at three concentrations of methanol suggesting that for both substrates the distribution of products upon transphosphorylation is identical.

Acid phosphatase rapidly loses activity when preincubated with PCMB, and a plateau of residual activity is reached before 2 min when the preincubation is carried out as described in Experimental Procedures. A linear plot is obtained by plotting the percentage of final inhibition against the PCMB concentration (Fig. 3). The extrapolation of the lines in Fig 3 to complete inhibition shows that two equivalents of PCMB per 13 000 g of enzyme are necessary to obtain total loss of activity. When the partially inhibited enzyme is assayed the release of *p*-nitrophenol is linear during the time of the standard assay.

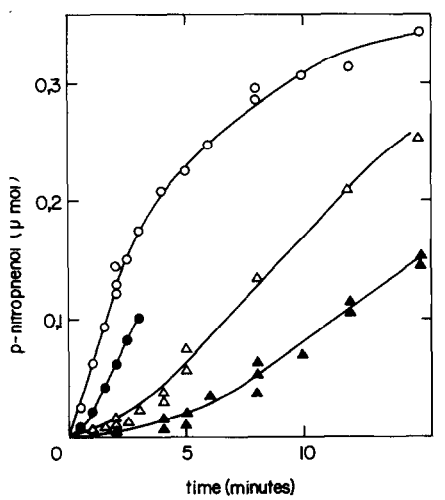
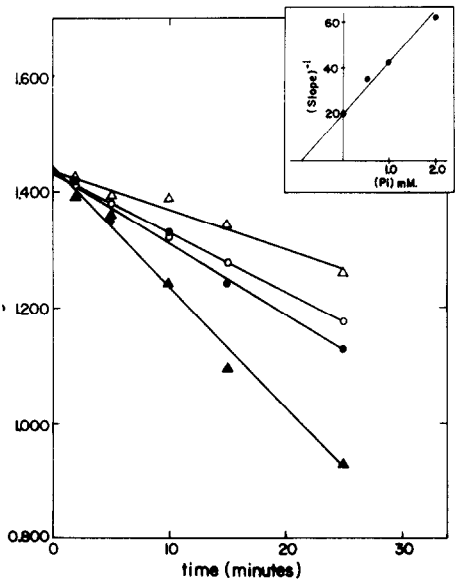


Fig. 4. Effect of substrate on the inhibition of acid phosphatase by PCMB. Details are described under Experimental Procedures. PCMB concentration (μM); ○———○, none (3); ●———●, 5 (1); △———△, 10 (3); ▲———▲, 15 (3). The number in brackets shows the number of experiments presented in the figure.

When acid phosphatase is incubated with PCMB in the presence of substrate, the kinetic of *p*-nitrophenol release is not linear. As shown in Fig. 4 the beration of product with no inhibitor is linear up to 2 min, however in the resence of inhibitor there is an apparent "lag phase" in the liberation of -nitrophenol. In this initial part the degree of inhibition is higher than that btained with the same concentration of PCMB in the absence of substrate. ıfter this initial period the liberation of product with time becomes linear and he inhibition is comparable to that obtained in the absence of substrate.

In titration experiments with DTNB (8 runs) a value of 2.07 ± 0.2 SH roups per 13 000 g of enzyme was found. Details of the titration procedure re described in Experimental Procedures. The titration experiments were also epeated with 0.01 M sodium dodecylsulfate using an extinction coefficient of 2 600 [15] for the released thiophenoxide. Although the rate of thiophenoxde release increases in these experiments the total number of SH groups was ound to be 2.0 ± 0.18 (4 experiments).

Preincubation of acid phosphatase with DTNB leads to a decrease of the nzyme activity which can be counteracted by P_i (Fig. 5). When the inverse of he slopes for inactivation are plotted against P_i concentration, a linear plot is btained (inset Fig. 5). This treatment is valid when the $E \cdot P_i$ complex does ot react with the inhibitor or when the rate constant of the reaction is negligile when compared to that of the free enzyme. Essentially similar methods ave been published [16]. The dissociation constant of P_i was found to be 0.74 nM, which compares well with 0.70 mM, which was the measured K_i of phosphate for acid phosphatase at the same pH.

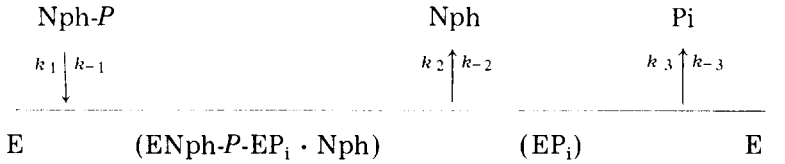


ig. 5. Effect of phosphate on the inhibition of acid phosphatase by DTNB. 10 μg of enzyme were reincubated at pH 6 as indicated in Experimental Procedures with 0.02 μmol of DTNB and, variable P_i. i concentration in preincubation (mM): △———△, 2; ○———○, 1.0; ●———●, 0.5; ▲———▲, none. At ıtervals, 30 μl aliquots were removed and assayed for activity as indicated in Experimental Procedures. ctivity is expressed as (Log A_{400} − 2). All lines were calculated by linear regression. Inset shows the eciprocal value of the slopes against P_i concentration.

Discussion

The steady state analysis of the kinetics of bovine brain acid phosphatase reveals a Uni-Bi [13] pathway for the hydrolysis of *p*-nitrophenyl phosphate.

P_i, a well known competitive inhibitor in other acid phosphatases [1] is the last product to leave the enzyme after each catalytic cycle in acid phosphatase from bovine brain. The minimal scheme representing this type of reaction pathway is



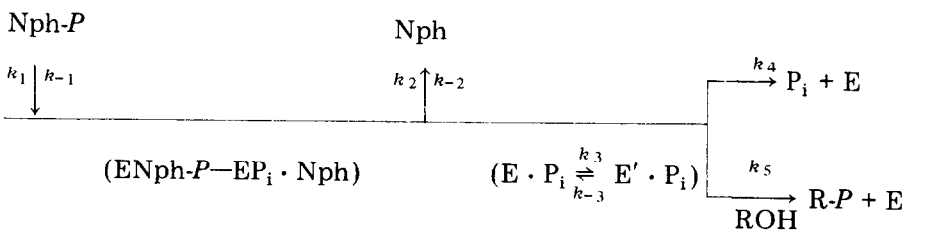
(1)

The assumed minimal value for the ΔG^0 of hydrolysis of *p*-nitrophenyl phosphate leads to K_{eq} = 158 M for this reaction and following the kinetic analysis [13] of the system represented in Scheme 1, $K_{eq} \leqslant 0.296$ M. This type of discrepancy between the observed and calculated K_{eq}, is found in other acid phosphatases and has led [13], to the conclusion that one of the possible descriptions of the reaction pathway includes the isomerization of the (Enzyme · P_i) complex.

External acceptors have been used with hydrolytic enzymes for the kinetic demonstration of the existance of covalent intermediates in their reaction pathways [1,14]. Our results with acid phosphatase from bovine brain show that there is a marked transphosphorylation to methanol using either *p*-nitrophenyl or phenyl phosphate as substrates, as indicated by the fact that in both cases the rate of phenol (or *p*-nitrophenol) release is increased, while the rate of P_i production is not substantially affected.

Other acid phosphatases show rate enhancements by the addition of external acceptors [1] and this phenomena is observed also with alkaline phosphatase from *Escherichia coli*, an enzyme known to catalyze covalently the hydrolysis of phosphomonoesters [14]. Prostatic acid phosphatase can also transphosphorylate to ethanol or ethanolamine [3]. In this case, however, it would seem that, for ethanolamine, at least, there is no rate increase produced by the addition of the acceptor.

In order to explain the rate increase produced by the addition of alcohols the following simplified scheme can be applied.



(2)

In the case of bovine brain acid phosphatase there is, at least, one isomerization step included in E′, as discussed above.

Furthermore, the transphosphorylation ratio (P_i/phenol or P_i/*p*-nitrophenol) is identical, for both substrates at any of the methanol concentrations used, indicating strongly that a phosphoryl enzyme is indeed consistant with all the kinetic data (Scheme 2).

As the V for phenyl- and *p*-nitrophenyl phosphate is identical, despite their large differences in K_m and chemical reactivity [17], the rate limiting step of the acid phosphatase-catalyzed hydrolysis is the same for both substrates and this step, presumably, can not be the phosphorylation of the enzyme. The rate increase obtained by increasing the methanol concentration is, most probably, due to a rate limiting dephosphorylation that is relieved by transphosphorylation to methanol. This effect leads to the conclusion that k_4 must be smaller than k_5 (Scheme 2). The leveling effect obtained when increasing the methanol concentration can be due either to a simple solvent effect on the enzyme or to a combination of solvent and kinetic effects.

The importance of SH groups for acid phosphatase catalysis has been suggested by various inhibition experiments in acid phosphatases from several sources [5—7], but the involvement of SH groups in catalysis itself remains unclear.

Both the titration with DTNB and the inhibition by preincubation with PCMB suggest that beef brain acid phosphatase has two free SH groups that are important for catalytic activity, based on an apparent molecular weight of 43 000 [6].

It had been shown that PCMB also inhibits the enzyme activity [6]. The PCMB inhibited enzyme shows normal kinetics. The reaction of PCMB with the enzyme, under the conditions here described is too fast to permit accurate measurements of the rate of inactivation.

P_i a well known [6] competitive inhibitor protects the enzyme against inhibition with DTNB during preincubation. As the calculated association constant for protection and the K_i coincide, either the SH groups are near the active site or P_i stabilizes a conformation of the enzyme that does not react with DTNB.

Substrate does not protect the enzyme against the inhibition caused by PCMB. Furthermore, there occurs initially an increase in the inhibition caused by PCMB. This effect can be rationalised as a change in the stoichiometry of the inhibition caused by the exposure of one of the SH groups in the presence of substrate. The exposure of a previously less reactive group in the presence of substrate has been demonstrated with NADase [18]. The apparent reactivation could occur by an internal transfer of the inhibitor from one SH group to another. This type of transfer has been documented before to explain the results of DTNB inhibition on α-amylase [19].

The proposed explanation or the direct involvement of the groups in catalysis is difficult to prove indirectly. Structural studies in progress will help to establish the validity of the proposed scheme. The slow rate of recovery of enzyme activity in the PCMB-substrate incubations will be of use in order to clarify further this phenomenon.

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