Biochimica et Biophysica Acta, 438 (1976) 153—158

BBA 67825

IMPORTANCE OF SH GROUPS IN CATALYSIS BY BOVINE BRAIN ACID PHOSPHATASE

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(Received October 28th, 1975)

Summary

The rate of inactivation of acid phosphatase (EC 3.1.3.2) from bovine brain by dithiobis-(2-nitrobenzoic acid) (Nbs_2) is identical to the rate of titration of one of the two SH groups of this enzyme. The rate of inactivation of the enzyme by Nbs_2 is pH dependent and, at 300 mM NaCl, can be described by the reaction of a single SH group of pK 8.4. At low ionic strength the pK determined from the $k_{inactivation}$ vs. pH profile is 7.7 and the results deviate markedly from the predicted values at pH values $\leqslant 6$. The decrease of V upon addition of salts is paralled by the decrease of inactivation rate by Nbs_2. The relevance of SH groups in catalysis by bovine brain acid phosphatase is discussed in terms of these data.

Introduction

We have recently shown that acid phosphatase (EC 3.1.3.2) purified from bovine brain [1] has two SH groups and that blocking these groups with SH inhibitors completely abolishes the activity of the enzyme [2]. These results, together with several reports that other acid phosphatases of low molecular weight are extremely sensitive to inhibition by SH reagents [3—8] indicate that SH groups might be involved in catalysis by the low molecular-weight acid phosphatases.

The results presented in this report support this expectation. In addition of its bearing to the understanding of the mode of action of acid phosphatases, these results show that acid phosphatase ** contains a SH group with high reactivity at low pH and very sensitive to the increase in salt concentration.

Abbreviation: Nbs_2, 5,5'-dithiobis-(2-nitrobenzoic acid).

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** Throughout this paper acid phosphatase, unless specifically stated, refers to bovine brain acid phosphatase of low molecular weight [2].

Experimental Procedures

All reagents were analytical grade; deionized, twice distilled water was used throughout. Acid phosphatase was purified and concentrated as described previously [2]. Enzyme activity was measured using *p*-nitrophenyl phosphate (Sigma Chem. Co.) as substrate in a reaction mixture containing 100 μmol of citrate (K^+) buffer, pH 5.0, and 2 μmol of *p*-nitrophenyl phosphate in 2.0 ml. The reaction was started by the addition of enzyme, carried on at 37°C for 2 min and stopped with 1.0 ml of 1.0 M NaOH. The *p*-nitrophenoxide was measured at 405 nm in a Zeiss PMQ II spectrophotometer. Protein was measured by the modified Folin method [9] using crystalline serum albumin (Sigma Chem. Co) as standard. The purified enzyme (specific activity 25 units/mg) is stable for months when stored in a solution containing 2.0 M $(NH_4)_2SO_4$, 1 mM P_i and 1 mM dithiothreitol (Sigma Chem. Co.). Before use the enzyme was desalted and freed from dithiothreitol by passing a 0.2-ml aliquot of the concentrated stock solution through a column (0.7 × 18 cm) of BioGel P-2 equilibrated with 10 mM potassium citrate buffer pH 5.0, containing 1 mM EDTA. Buffers used were: potassium citrate (pH 5—6); Tris/maleate (pH 6—7.4); Tris · HCl (pH 7.4—8.8) and glycine/NaOH (pH 8.8—10). Inactivation by 5,5′-dithiobis-(2-nitrobenzoic acid) (Nbs_2) (BDH Biochemicals) was determined by incubating the enzyme with $2 \cdot 10^{-4}$ M Nbs_2 (unless stated) in a final volume of 300 μl at 15°C. 50-μl aliquots were taken at appropriate times and the residual activity measured in the standard assay. The apparent first-order constants for inactivation were calculated from the slopes of the log activity vs. time plots. The inactivation constant was determined at 25°C for all pH values under 6.5. This was judged necessary because of the slow reaction obtained at 15°C at these pH values. When these rate constants were corrected to 15°C using the activation energy obtained for the fastest runs, the rate constants were coincident with those previously obtained at 15°C. Titration by Nbs_2 was carried out essentially as described by Ellman [10] at 15°C. The extinction coefficient of the 2-nitro-5-mercaptobenzoate was corrected when necessary using the $pK = 5.1$ [11]. The stability of the enzyme under all conditions used was determined by preincubation of the enzyme, in a reaction mixture that contained all additions except Nbs_2. Aliquots were then assayed at times that always exceeded the time required for complete inactivation by Nbs_2. The enzyme was found to be stable in all cases. Ionic strength was increased by the addition of NaCl. When the reaction rate was measured at pH values over 7, the *p*-nitrophenoxide was measured continuously in a Gilford recording spectrophotometer. Rates were taken as the linear initial parts of the product vs. time curves. K_m and V were determined by the Lineweaver and Burk [12] method. All lines were calculated by simple regression in a Hewlett-Packard model 10 calculator and had a correlation better than 0.999.

Results

The pH profile of the inactivation rate constants of acid phosphatase by (excess) Nbs_2 is sensitive to ionic strength (Fig. 1). The rate is first order in enzyme and first order in Nbs_2 (not shown) under the conditions used. These

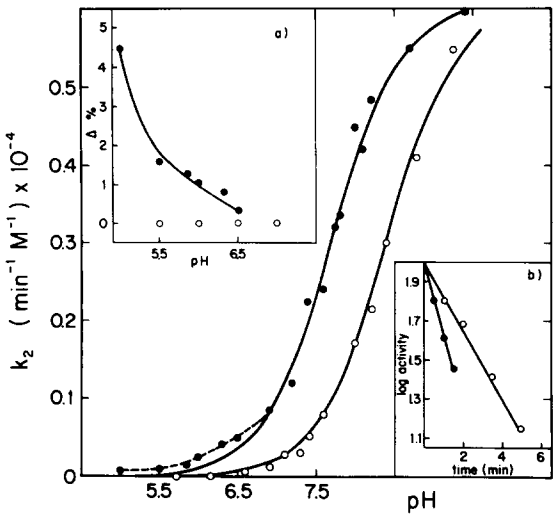

Fig. 1. Plots of the apparent second-order rate constants for the reaction of inactivation of acid phosphatase by Nbs_2 vs. pH ●, experimental points with 20 mM buffers (see Experimental Procedures for details); ○, experimental points with 20 mM buffers with 300 mM added NaCl. Solid lines are theoretical based on Eqn. 1, see text. Inset a), (k_2 experimental $- k_2$ theoretical/k_2 experimental) for conditions with (○) and without (●) NaCl. Inset b), typical first-order plots at pH 8.0 with (○) and pH 8.1 without NaCl (●).

experiments were carried out at only two different ionic strengths because the kinetic effects of ionic strength in this interval were shown to vary linearly with $\sqrt{\mu}$ [13]. The solid lines of Fig. 1 were calculated according to

$$k_{calc} = k_{max} \frac{K}{K + H^+} \quad (1)$$

where k_{max} is the rate constant at the plateau and K, in each case, is the acid dissociation constant.

The reactivity differences can be correlated with a shift of the apparent pK of one SH group from 7.7 (at 0.02 M buffer) to 8.4 (at 0.32 M total ionic strength). At pH values below 6.5 and in the absence of NaCl the experimental points deviate from the calculated curve (inset Fig. 1).

Acid phosphatase has a total of two SH groups per mol of enzyme [2]. The titration rate of these groups was determined at different pH values at both high and low ionic strength. An example of these experiments is presented in Fig. 2. One of the titration process has a rate constant equal to the inactivation rate constant obtained in the same titration experiment.

The sensitivity of one of the titration processes to changes in ionic strength is parallel to the changes in inactivation rate constants (Table I). At pH 6.0 one rate constant for titration is easily obtained and the enzyme can be titrated, or inactivated, only at low ionic strength (Table I, Fig. 1).

The increase in ionic strength decreases the reaction rate of the acid phosphatase-catalyzed reaction at any pH and only V is affected at pH 8 [13]. When the rate constants for inactivation by Nbs_2 and the V are plotted against $\sqrt{\mu}$ all points fall in the same line (Fig. 3). This is a strong evidence that the

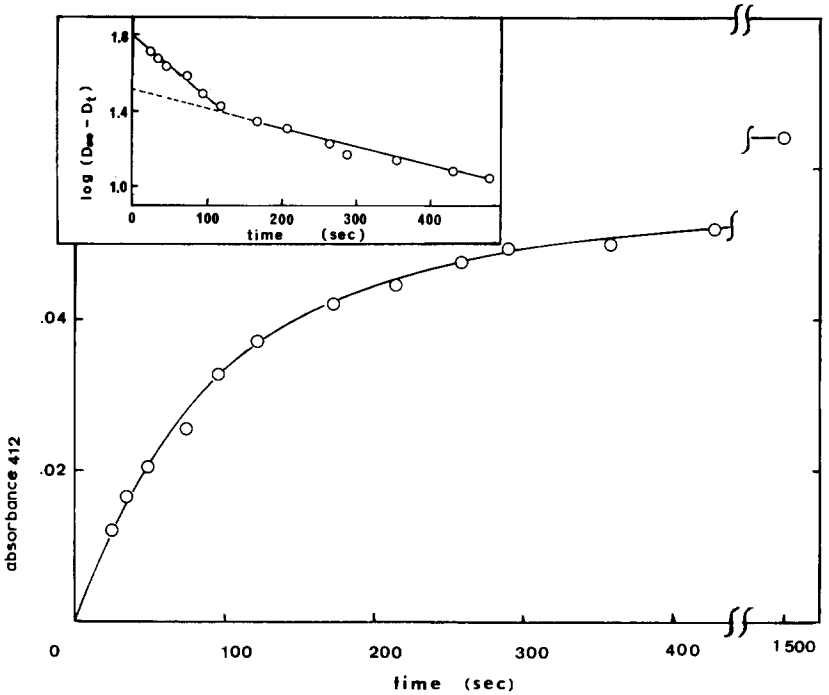

Fig. 2. Titration of acid phosphatase by Nbs_2. See Experimental Procedures for details. 30 μg of acid phosphatase were titrated with $5.5 \cdot 10^{-5}$ M Nbs_2 at pH 9.2 (300 mM NaCl) in 1.0 ml. The rate constant for the faster reaction was calculated from the difference between the observed and extrapolated values (inset).

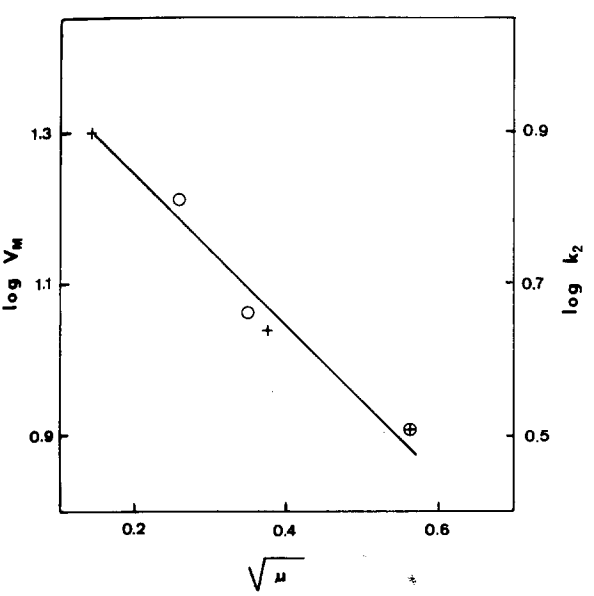

Fig. 3. Effect of ionic strength on the apparent inactivation constant (k_2) (○) by Nbs_2 and on log V (+) at pH 8.0. μ represents ionic strength; the increase being obtained by adding NaCl.

TABLE I

REACTION RATE OF Nbs_2 WITH ACID PHOSPHATASE

For details see Experimental Procedures.

Conditions	k_2 ($min^{-1} \cdot M^{-1} \times 10^{-4}$)		
	Inactivation	Titration	Titration
pH 9.2 + 0.3 M NaCl	0.51 ± 0.02	0.52 ± 0.06	0.28 ± 0.02
pH 8.8 + 0.3 M NaCl	0.43 ± 0.04	0.38 ± 0.04	0.26 ± 0.03
pH 8.8	0.57 ± 0.03	0.56 ± 0.06	0.28 ± 0.03
pH 6.0 *	0.085 ± 0.008	0.105 ± 0.011	—
pH 6.0 + 0.3 M NaCl *	Stable	—	—

* At 25°C.

effect of ionic strength on V is related to the effect of ionic strength on the decrease of reactivity of an essential SH group.

Discussion

The involvement of one SH group in catalysis by acid phosphatase is strongly indicated on different grounds. Titration rates are identical with inactivation rates, showing that blocking this group abolishes enzymatic activity. The effect of ionic strength on titration rate constants, at different pH values, is paralleled by the effect on inactivation. The diminishment of V by an increase in salt concentration can be correlated, directly, with the decrease of reactivity of one essential SH group.

The $k_{2\ \text{inactivation}}$ vs. pH profile for the inactivation reaction of acid phosphatase and Nbs_2 at $\mu = 0.32$ reflects a simple enzyme inhibitor system in which the dissociation of a single SH group determines the rate of the second order reaction. The pK obtained is comparable to reported values [14].

The $k_{2\ \text{inactivation}}$ vs. pH profile obtained at low ionic strength differs from this simple behavior. Firstly, the pK obtained at 0.02 M ionic strength is lower than expected for a SH group. The experimental points deviate considerably from the calculated curve at pH values lower than 6.5 in a pH-dependent manner. This constitutes an indication of an interaction involving one of the SH groups of acid phosphatase with a second ionizable group. In papain, a similar increase in reactivity has been ascribed to a SH-histidine interaction [15]. The involvement of histidine in acid phosphatase has been suggested from the photoxidation pH profiles [16]. In other acid phosphatases there is direct evidence for the participation of a histidine residue in catalysis [17,18].

From the data presented we may conclude that one of the SH groups of acid phosphatase is involved in an ionic-strength sensitive interaction with another group, or groups, that affect its reactivity. The nature of this group(s) and the type of involvement of this interaction in catalysis is under investigation.

Acknowledgements

This work was supported, in part by the Fundação de amparo à Pesquisa do Estado de Sao Paulo (Projeto BIOQ/FAPESP). H.M.S.B., who is a predoctoral fellow of FAPESP, wishes to thank that organization for financial support.

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