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EFFECT OF SULFHYDRYL REAGENTS ON THE ACTIVITY OF HISTIDINOLPHOSPHATASE FROM *SALMONELLA TYPHIMURIUM* AND BAKERS' YEAST

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SUMMARY

Inhibition of histidinolphosphatase (EC 3.1.3.15) from either a mutant of *Salmonella typhimurium* or bakers yeast strongly suggests the involvement of a sulfhydryl group in the catalytic activity. Inhibition by micromolar concentrations of *p*-chloromercuribenzoate is noncompetitive with the yeast enzyme, but the mercurial acts as a competitive inhibitor of the *Salmonella* enzyme. Inhibition can be protected against by histidinol. Ethylenimine inhibits yeast phosphatase activity in nearly a first order fashion, while the reaction with *p*-chloromercuribenzoate is distinctly biphasic. Other reagents, such as iodoacetate, iodoacetamide and *N*-ethylmaleimide, that react with sulfhydryl groups also inactivate. The *Salmonella* enzyme is inhibited by 5,5′-dithiobis-(2-nitrobenzoic acid) more rapidly at pH 7 than at pH 8.5; addition of $MnCl_2$ changes the kinetics of activation at various pH values. The similarity in the involvement of a sulfhydryl in the inactive site of the phosphatases strengthens the suggestion that the two enzymes are evolutionarily close, even though the *Salmonella* enzyme is a bifunctional enzyme in wild-type strains having imidazoleglycerolphosphate dehydratase activity in addition to phosphatase activity.

INTRODUCTION

Phosphatases from several sources catalyzing the hydrolysis of phosphate monoesters have been studied by a variety of techniques. The mechanism of action of *Escherichia coli* alkaline phosphatase has been probed using both kinetic and chemical methods (for review see Reid and Wilson, ref. 1). A phosphoryl enzyme is known to be formed as an intermediate during catalysis [2] and phosphoserine has been isolated from the enzyme [3, 4]. Observations on mammalian alkaline phosphatases are also consistent with the general mechanism proposed for *E. coli* alkaline phosphatase [5]. In contrast to the involvement of serine in alkaline phosphatase, some phosphatases from mammalian sources are inhibited by reagents that react with sulfhydryl groups, thus implicating cysteine as being necessary for catalytic

Abbreviations: DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); BIP, DL-α-bromo-β-(5-imidazolyl)-propionic acid.

activity. Prostatic acid phosphatase is inactivated by *p*-chloromercuribenzoate and metal ions such as Cu^{2+} and Fe^{3+} [6]; however, it is also inhibited by diisopropylfluorophosphate. Due to the complexity of the latter reaction, serine was not thought to be necessarily implicated [7].

Nordlie [8] has reviewed the evidence that suggests involvement of a sulfhydryl group in the active site of glucose-6-phosphatase [9]. Other evidence indicates that this enzyme is a metalloenzyme which can be inhibited by metal-chelating agents [10].

Histidinolphosphatase (L-histidinol-phosphate phosphohydrolase, EC 3.1.3.15) has been recently purified from baker's yeast [11] and from a strain of *Salmonella typhimurium* with a nonsense lesion [12]. In both cases, two forms of the enzyme were isolated, but with the *S. typhimurium* enzymes, it is highly likely that proteolysis is the reason for the multiple forms. If the phosphatase from the subregion [13] of the bifunctional *hisB* gene (which also possesses imidazoleglycerolphosphate dehydratase activity) is related by the evolutionary process to the yeast phosphatase as suggested by Millay and Houston [11], then similar amino acid residues within the catalytic sites should be demonstrable. This study shows that both are inhibited by sulfhydryl reagents.

MATERIALS AND METHODS

Iodoacetic acid was obtained from Eastman Organic Chemicals. Histidinol dihydrochloride and sodium *p*-chloromercuribenzoate were purchased from Sigma. Ethylenimine was procured from Matheson, Coleman and Bell, and *N*-ethylmaleimide from Mann Research Laboratories. Pierce Chemical Co. provided 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), and L-histidinol-phosphate was obtained from Cyclo Chemical Co. All solutions of these chemicals were made fresh as needed. DL-α-Bromo-β-(5-imidazolyl)-propionic acid was synthesized by the procedure of Yankeelov and Jolley [14], or purchased from Pierce Chemical Co. *p*-[^{14}C]Chloromercuribenzoate was from New England Nuclear.

The enzyme from strain TA387 *(his01242 hisB2133)* of *S. typhimurium* was purified by a procedure developed in this laboratory [12]. The enzyme was separated into two fractions (A and B) during the last step of purification involving preparative polyacrylamide gel electrophoresis [15]. Fraction B, the major component, was used for the experiments reported here. This enzyme had a molecular weight of about 52 000 and showed a single band on Ornstein–Davis analytical polyacrylamide gel electrophoresis. Subunits of 18 000 and 16 500 were observed upon gel electrophoresis in sodium dodecylsulfate. The purified enzyme was dialyzed against 0.05 M Tris–HCl, pH 8.5, to remove mercaptoethanol and stored at 4 °C. The purification of yeast phosphatase has been described; the enzyme after urea gel electrophoresis was used [11].

Histidinolphosphatase activity of strain TA387 enzyme was determined at 37 °C as described except that the concentration of triethanolamine was increased to 125 mM. An extinction coefficient of $2.60 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ at 820 nm was used to calculate the concentration of phosphate hydrolyzed from substrate. The yeast phosphatase was assayed by the procedure of Millay and Houston [11], except that 200 mM triethanolamine was used.

Chemical modification experiments were carried out either by adding the

reagents directly to the assay media and the phosphatase reaction initiated by the addition of enzyme or by first incubating the enzyme for various times with modifying reagents and adding samples of this mixture to a tube containing histidinol phosphate at 37 °C. In the latter experiments, the assay solution contained specific quantities of mercaptoethanol at sufficient concentrations to terminate the reaction with sulfhydryl groups on the enzyme. Controls were employed utilizing buffer in the place of modifying reagents.

RESULTS

Reaction with p-chloromercuribenzoate

At low concentrations, *p*-chloromercuribenzoate is recognized as a highly specific chemical reagent for thiol groups [16]. The enzyme from strain TA387 is quite sensitive to very low concentrations of *p*-chloromercuribenzoate (Fig. 1). In these studies, the reaction was initiated by addition of enzyme and the level of phosphatase activity is shown after 8 min of reaction during which the enzyme had an opportunity to react either with substrate or *p*-chloromercuribenzoate. It is clear that protection was afforded by the inclusion of the competitive inhibitor, histidinol [12,17], at a concentration about 4 times its K_i value. It was also apparent that 1 mM $MnCl_2$ (Fig. 1; closed symbols) had no effect on the course of *p*-chloromercuribenzoate inhibition with or without histidinol.

Several experiments demonstrated that mercurial inhibition was reversible. The *S. typhimurium* phosphatase was incubated with 0.27 μM *p*-chloromercuribenzoate in 0.1 M triethanolamine hydrochloride, pH 7.5, at 0 °C and 75-μl samples

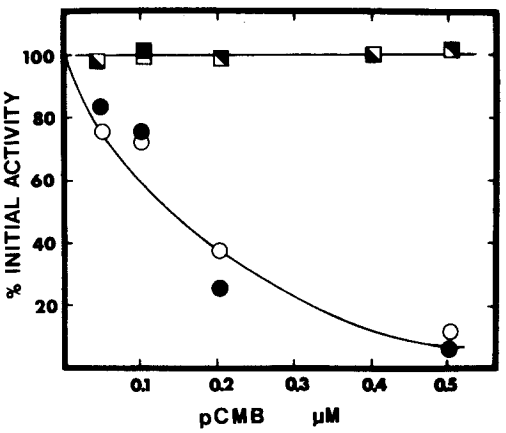

Fig. 1. Inhibition of strain TA387 histidinolphosphatase by *p*-chloromercuribenzoate. Portions of a 1.14-μM stock solution of *p*-chloromercuribenzoate were added to histidinol phosphate (5.3 mM final concentration) in a final concentration of 100 mM triethanolamine hydrochloride, pH 7.5, at 37 °C. 5 μl of phosphatase (Fraction B) were added to make a total of 95 μl and incubated for 8 min before termination with 200 μl of ascorbate–molybdate reagent. The absorbance at 820 nm was compared to blanks with water in place of enzyme. Some assays, indicated by the squares, were carried out with 79 μM histidinol in addition to *p*-chloromercuribenzoate. Assays indicated by circles are without histidinol. All assays were in triplicate and the closed symbols indicate assays containing 1 mM $MnCl_2$ and open symbols were assays without Mn^{2+}.

were withdrawn and assayed for 20 min at pH 7.5 against 6.25 mM histidinol phosphate. A rapid loss of 50% of the activity was observed within 2 min of *p*-chloromercuribenzoate treatment followed by a slower loss of activity until only 1% remained after 120 min of *p*-chloromercuribenzoate treatment. The inclusion of 6.25 mM mercaptoethanol in the assay completely reversed the inhibition to control level. The diluted control lost 20% of initial activity in 120 min. Enzyme treated with 1.14 μM *p*-chloromercuribenzoate at pH 7.5 at 0 °C was completely inactivated within 2 min, but could be restored to control level of activity by inclusion of 40 mM mercaptoethanol in the assay.

A Lineweaver–Burk plot of the *S. typhimurium* phosphatase activity assayed in the absence of mercaptoethanol, but in the presence or absence of *p*-chloromercuribenzoate, indicated that the inhibition by *p*-chloromercuribenzoate is competitive with substrate and can be protected against by high histidinol phosphate concentration (Fig. 2). Other experiments using higher *p*-chloromercuribenzoate concentration are consistent with this interpretation except that some deviation from linearity occurred at very low histidinol phosphate concentrations. Ample time (20 min) was afforded for reaction with the mercurial which would be irreversible under these conditions. An inhibition constant of 0.05 μM was calculated for *p*-chloromercuribenzoate from Fig. 2.

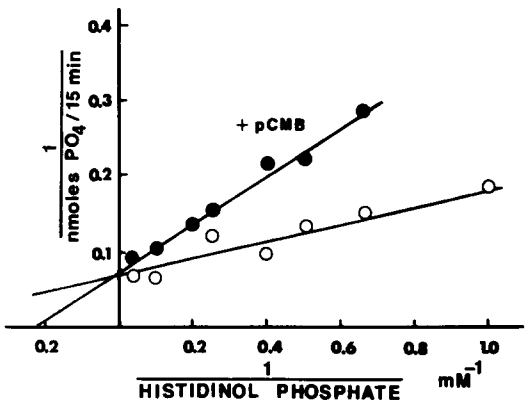

Fig. 2. Competitive inhibition by *p*-chloromercuribenzoate. Duplicate assays of *S. typhimurium* phosphatase at 37 °C were made in the absence (○) and presence (●) of 0.10 μM *p*-chloromercuribenzoate using 1–10 mM histidinol phosphate at pH 7.5.

Mercurial inhibition of yeast histidinolphosphatase

Yeast phosphatase was also shown to be sensitive to *p*-chloromercuribenzoate, but high concentrations were needed to inhibit the enzyme (Fig. 3). Kinetic analysis by a Lineweaver–Burk plot, shown in the inset, illustrates the point that *p*-chloromercuribenzoate is a noncompetitive inhibitor of the yeast histidinolphosphatase. The results shown here were obtained using very short assays (30 s), but identical results were found if a longer 20-min assay was employed using diluted enzyme.

A time course of inactivation showed a definitely biphasic curve similar to the *S. typhimurium* enzyme and at high concentrations of *p*-chloromercuribenzoate

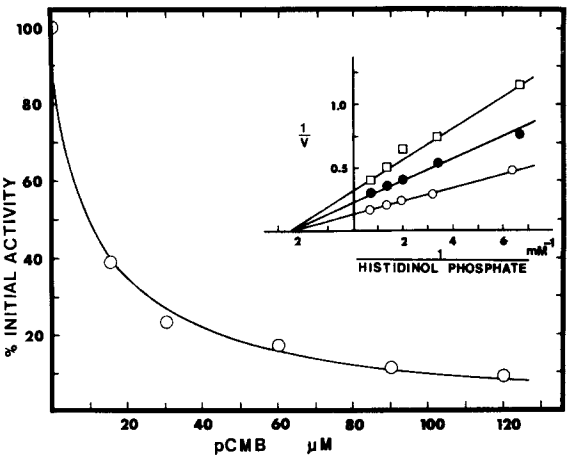

Fig. 3. Inhibition of yeast histidinolphosphatase by *p*-chloromercuribenzoate. Enzyme was preincubated 10 min at 37 °C with the indicated concentrations of *p*-chloromercuribenzoate in 50 μl solution of 100 mM triethanolamine hydrochloride, pH 8.5. Enzyme assay was initiated by addition of 10 μl of 10 mM histidinol phosphate and terminated after 30 s. The inset shows a double-reciprocal plot of *p*-chloromercuribenzoate inhibition. The enzyme was assayed at various concentrations of histidinol phosphate at 37 °C in 100 mM triethanolamine hydrochloride, pH 8.5. Reactions proceeded 30 s before termination. The plots show the results without *p*-chloromercuribenzoate (○), 70 μM *p*-chloromercuribenzoate (●), and 210 μM *p*-chloromercuribenzoate (□). The units of velocity are nmoles phosphate released per 30 s.

(150 μM), a substantial part of the activity was lost within 1 min, with a slower rate of loss in the next 20 min. If treated enzyme was then dialyzed overnight against 2000 vol. of 50 mM Tris–HCl at pH 8.5 containing 10 mM mercaptoethanol, about 70% of the activity could be recovered. Addition of a large amount (to 130 mM) of mercaptoethanol to the assay solution after 60 min *p*-chloromercuribenzoate inactivation could also partially reactivate the enzyme, but to a lesser extent (about 50%).

When the extent of *p*-chloromercuribenzoate incorporation was determined using *p*-chloromercuri[^{14}C]benzoate, 4.5 moles of *p*-chloromercuri[^{14}C]benzoate were incorporated per mole of protein compared to 4.0 moles when 20 mM histidinol was included. The modified protein was separated from excess *p*-chloromercuri[^{14}C]benzoate by a Sephadex G-50 column and the protein concentration was determined using fluorescamine.

Effect of ethylenimine on phosphatase activity

Ethylenimine was introduced as a reagent specific for sulfhydryl sidechains in proteins by Raferty and Cole [18]. The modification converted cysteine into acid-resistant *S*-aminoethylcysteine, and the usefulness of the reagent in producing new trypsin-susceptible sites on proteins was pointed out. Ethylenimine was found to strongly inhibit the yeast phosphatase, 98% inactivation being obtained after 20 min with 5 mM reagent at pH 8.5. If the yeast enzyme was incubated with *p*-chloromercuribenzoate (15 μM) until a level of about 60% inactivation was reached then exposed to 2.3 mM ethylenimine (Fig. 4A), the loss of most of the residual activity was seen in a manner typical of ethylenimine action. If the enzyme so treated was

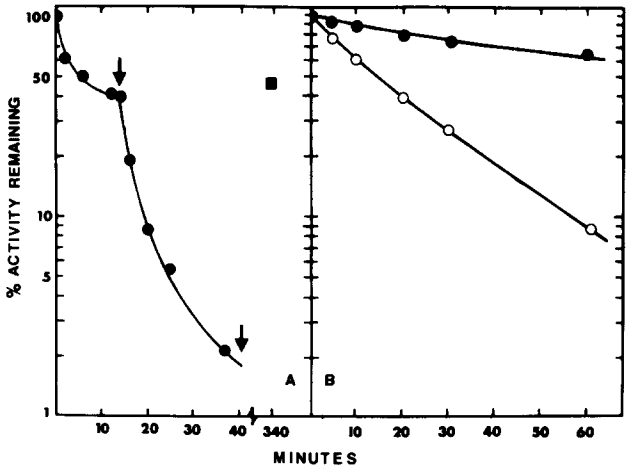

Fig. 4. Protection from ethylenimine inactivation by histidinol and *p*-chloromercuribenzoate. (A) Yeast phosphatase was incubated with 15 μM *p*-chloromercuribenzoate at pH 8.5 at 25 °C until residual activity was about 40% of initial activity, then at 15 min ethylenimine was added to 2 mM final concentration and inhibition allowed to proceed further. At 40 min, dialysis of the reaction solution was started against 2 l of 0.05 M Tris–HCl, pH 8.5, with 0.01 M mercaptoethanol. After 5 h, the activity of the enzyme was tested again (■). (B) Yeast phosphatase was incubated at 25 °C with 2 mM ethylenimine in the absence (O) and presence (●) of 20 mM histidinol. Incubation was in 100 mM triethanolamine hydrochloride, pH 8.5. Samples of 3 μl were taken at indicated times and assayed for 30 s as described in text.

then dialyzed overnight against 50 mM Tris–HCl with 10 mM mercaptoethanol, the recovery of about 46% of the activity was seen. As the ethylenimine reaction was not reversible, this represented 77% of expected regain if *p*-chloromercuribenzoate acted at the same site as did the ethylenimine and protected the group against reaction with ethylenimine. This is approximately the same percent recovery obtained by dialysis of inactive *p*-chloromercuribenzoate-treated enzyme. It could also be shown (Fig. 4B) that the rate of inhibition by 2 mM ethylenimine could be reduced by the presence of 20 mM histidinol in the reaction. Histidinol was shown to substantially inhibit hydrolysis of histidinol phosphate at this concentration [11].

Treatment of the *S. typhimurium* phosphatase at 0 °C with 95 mM ethylenimine in 0.10 M Tris–HCl had little effect on activity over 20 min of incubation. At this time, control enzyme had lost 1 to 2% of the initial activity and treated enzyme had lost 20%. Enzyme treated in the presence of 75 μM histidinol had the same level of activity as control enzyme. Thus, protection was afforded by inhibitor, but inactivation was relatively slow compared to other reagents. Inactivation by ethyleneimine was also observed at 25 and 37 °C, but controls were also less stable at these temperatures.

Inhibition by iodoacetate

The effect of millimolar concentrations of iodoacetate was tested and the expected loss of activity was observed with increasing concentration of iodoacetate (Fig. 5A). When the *S. typhimurium* enzyme was incubated for various times with different concentrations of iodoacetate and then assayed in the presence of mercapto-

ethanol to quench the alkylation, a pseudo first-order loss of activity was observed (Fig. 5B). No difference was observed if the mercaptan was added first and then the substrate 5 min later. Controls also lost activity at this temperature, but at a substantially slower rate. Intermediate temperatures were also used. The reaction was temperature dependent since little inhibition occurred when alkylation was performed at 0 °C, where controls were stable.

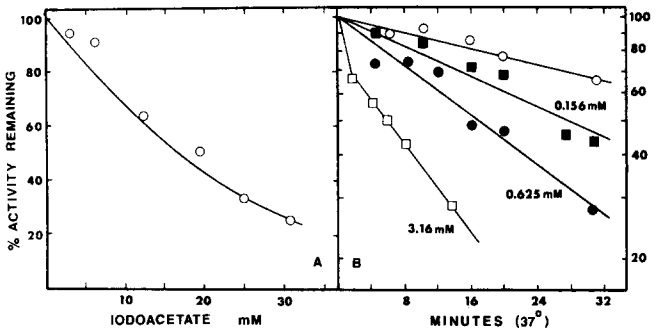

Fig. 5. Effect of iodoacetate on *Salmonella* phosphatase activity. (A) Strain TA 387 phosphatase was assayed for 8 min at 37 °C against 5.8 mM histidinol phosphate including various concentrations of iodoacetate in 0.10 M triethanolamine hydrochloride at pH 7.5. (B) Enzyme was incubated with 0.156 mM (■), 0.625 mM (●) and 3.16 mM (□) iodoacetate for various times in 0.1 M triethanolamine hydrochloride, pH 8.5, at 37 °C and 75-μl samples were withdrawn and added to 10 μl of 50 mM histidinol phosphate containing 0.5 M mercaptoethanol at 37 °C. A control (○) with water in place of iodoacetate was used.

The yeast enzyme was much less reactive towards iodoacetate and iodoacetamide, reaching 50% activity in a first-order reaction only after about 50 min at 25 °C using 85 mM iodoacetamide at pH 8.5. Iodoacetate was less effective, giving only 15% inactivation after 2 h of treatment at pH 8.5 using 85 mM iodoacetate at 25 °C.

Effect of N-ethylmaleimide

Various concentration of *N*-ethylmaleimide up to 45 mM were incubated with the *S. typhimurium* phosphatase in 0.1 M triethanolamine hydrochloride at pH 7.5, 0 °C, for 10 min and then assayed at 37 °C for 10 min. Little loss of activity was found under these conditions even if the enzyme was treated at 37 °C. The yeast phosphatase was equally resistant to inactivation by *N*-ethylmaleimide and inhibition only occurred if the enzyme was treated with the reagent in 8 M urea. It has been shown that the yeast enzyme is capable of recovering full activity after short exposures to 8 M urea [11]. A plot of time versus log fraction of activity remaining in the presence of 20 mM *N*-ethylmaleimide at pH 8.0 in 8 M urea at 25 °C showed a biphasic curve, with a fast inactivation to about 30% residual activity in less than 5 min, then a relatively slower rate over the next hour. The rate of inactivation was greatly reduced at pH 7.5. Previous experiments have shown that potassium cyanate in 8 M urea inactivates yeast phosphatase [11]. High concentrations (0.5 M) of cyanate are needed to produce 60% inactivation in 5 min at 25 °C, pH 8.5, if urea is not used. Subsequent inactivation proceeds at a much lower rate.

Reaction with DTNB

Ellman's reagent [19] is a highly specific reagent developed for reaction with sulfhydryl groups. Reaction of strain TA387 enzyme with this reagent proved to be both temperature and pH dependent. Neither enzyme was inhibited by the nitrothiolate ion which is released from Ellman's reagent. Rapid inactivation was induced by 2 mM DTNB at 37 °C compared to the much slower rate of inhibition at 25 °C under otherwise identical conditions. 50% of the activity was lost in less than 1 min at 37 °C compared to about 60 min at 25 °C. However, controls at 37 °C lost considerable activity, 50% in 20 min. It would be expected that the sulfhydryl anion is the reactive species in the disulfide interchange. However, the pH dependence of the reaction (Fig. 6A) was contrary to what one might expect according to the proposed mechanism of reaction involving the sulfhydryl anion [20]. The inclusion of $MnCl_2$ changed this somewhat as illustrated in Fig. 6B. Controls were activated by Mn^{2+} about 20–30%. Under these conditions, DTNB at pH 8 showed the best inhibition of phosphatase activity and considerably slower inactivations were seen in the pH 7, 7.5 and 8.5. When the increase in absorbance at 412 nm due to DTNB reaction was followed at 30 °C at pH 8 without Mn^{2+}, it was found that in 1 h, when the enzyme

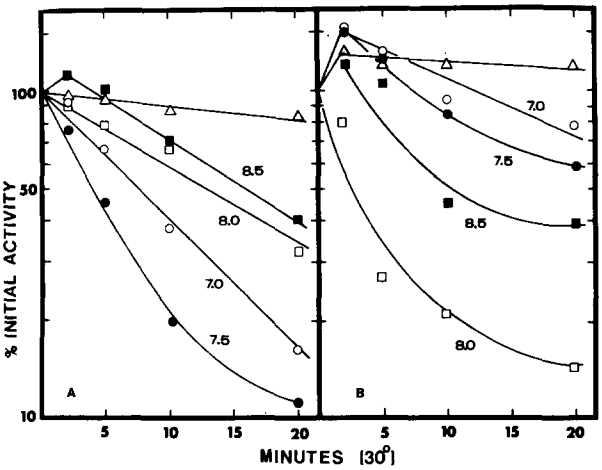

Fig. 6. Effect of pH and Mn^{2+} on inactivation of *Salmonella* phosphatase by DTNB. Enzyme was treated at 30 °C with 2.0 mM DTNB at pH 7.0 (○), pH 7.5 (●), pH 8.0 (□) and pH 8.5 (■). Samples (5 μl) were withdrawn at intervals and added to 75 μl of 4.2 mM histidinol phosphate in 0.5 M triethanolamine, pH 7.5, at 37 °C and assayed for 8 min. Controls were performed throughout at all pH values. These were essentially identical and are represented by a single line (△). (A) Reaction performed without Mn^{2+}. (B) Reaction performed with 1 mM $MnCl_2$.

was completely inactived, 1.6 sulfhydryl groups were modified per absorbance unit of the enzyme at 280 nm after the enzyme was dialyzed extensively to remove mercaptoethanol.

First-order loss of activity at 0 °C observed with 2 mM DTNB was completely prevented by the inclusion of 75 μM histidinol when the treated enzyme was assayed by dilution into substrate. $MnCl_2$ (1 mM) did not change the level of protection. Furthermore, the inhibition by DTNB alone was reversible. Overnight dialysis at 4 °C of a DTNB-inactivated sample of enzyme against 0.05 M Tris–HCl–0.01 M

mercaptoethanol at pH 7.5 results in an average recovery of about 60% of the initial activity. When the enzyme was reacted for 1 h at pH 8 with Ellman's reagent in the presence of 75 μM histidinol, only 0.7 sulfhydryl group was modified per unit absorbance at 280 nm with less than 10% loss of activity.

The yeast phosphatase is inactivated by 1 mM DTNB at pH 7.5 with a half-time of inactivation of about 55 min at 25 °C. Due to the small quantities of protein available, it was difficult to determine the number of reacting groups. However, when 60% of the activity was abolished, approx. 0.5 thiol had reacted with DTNB.

Effect of 2-bromo-3-imidazole propionic acid

Jolley and Yankeelov have described the synthesis of a new reagent, α-bromo-β-(5-imidazolyl)-propionic acid (BIP) which is specific for cysteinyl residues on proteins [21]. Little alkylation occurs at other potentially reactive side chains. Since this reagent incorporated at least some of the features present in the substrate, histidinol phosphate, it was tested for its ability to inactivate the phosphatase. However, the specificity of the *S. typhimurium* enzyme appears to be fairly narrow since as small a change as removal of the $-CH_2-OH$ from histidinol changes the K_i nearly 200-fold, from 52 μM to 10 mM [16].

The *S. typhimurium* enzyme was incubated with various concentrations of BIP as described in Fig. 7A and tested for activity. Preincubation at 0 °C resulted in no inhibition; however, a short time (2 min) at 37 °C was sufficient to substantially decrease the level of phosphatase activity. Overnight incubation of the enzyme with 20 mM BIP at 4 °C showed no inhibition if the substrate was added at 0 °C and then brought to 37 °C rather than first warmed and then histidinol phosphate added. If the inhibitor at a concentration of 20 mM BIP was incubated at 37 °C with strain TA387 phosphatase and then samples removed for assay, a first-order loss of activity

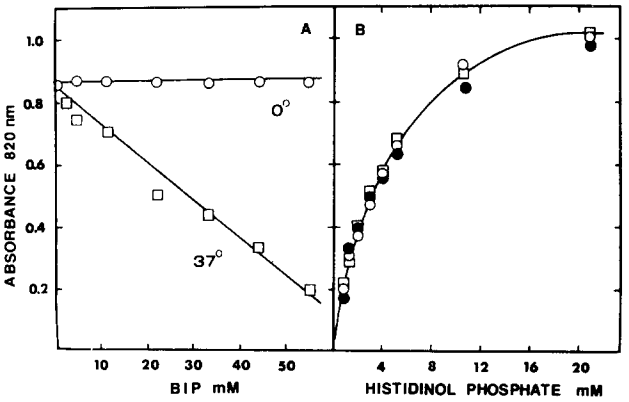

Fig. 7. Effect of DL-α-bromo-β-(5-imidazolyl)-propionate. (A) Strain TA387 enzyme was incubated for 1 h at 0 °C with different concentrations of BIP at pH 7.5 in 0.25 M triethanolamine hydrochloride. 5 μl of 100 mM histidinol phosphate were added to 75 μl of enzyme at 0 °C (O), then transferred to a 37 °C bath and terminated after 8 min with 200 μl ascorbate–molybdate. Another 75-μl sample (□) was first raised to 37 °C for 2 min after 1 h at 0 °C and then 5 μl of histidinol phosphate added for an additional 8 min at 37 °C. All assays were done in triplicate. (B) Enzyme was assayed at various concentrations of histidinol phosphate containing no BIP (○), 10.5 mM BIP (●) or 26.3 mM BIP (□). The reaction was carried out at pH 7.5 and was initiated by addition of enzyme.

was observed to the 10% activity level with a half-time of about 4 min. However, the alkylating compound does not appear to be an inhibitor at pH 7.5 as indicated by its effect on enzyme velocity in Fig. 7B. This reaction was initiated by addition of enzyme to a histidinol phosphate solution containing BIP. Even though the reaction with BIP was strongly temperature dependent, substrate was able to protect against inactivation at 37°. The K_m histidinol phosphate calculated from these data using a Lineweaver–Burk plot is 5.0 mM.

When the yeast phosphatase was incubated with 20 mM BIP at pH 8.5, no appreciable inactivation occurred after 40 min at 0,25 or 37°.

DISCUSSION

The inhibition of histidinolphosphatase from *S. typhimurium* and bakers yeast by a variety of reagents known to react with sulfhydryl groups strongly suggests that such a group(s) is required for activity. The participation of this sulfhydryl group in the catalytic site, as opposed to its involvement in holding together some crucial tertiary structure, is suggested by several facts. The inhibition of the *S. typhimurium* phosphatase by *p*-chloromercuribenzoate is competitive, i.e. inhibition can be prevented by high concentrations of histidinol phosphate. Furthermore, the evidence that histidinol, a strong competitive inhibitor, protects from inactivation, also suggests that the involvement of the sulfhydryl group in catalysis is a more direct one. Similar evidence for the direct participation of a sulfhydryl group is available for glucose-6-phosphatase [8]. In the latter case, it could also be shown that *p*-chloromercuribenzoate was a competitive inhibitor. Regeneration of high levels of activity after *p*-chloromercuribenzoate treatment indicated the lack of any irreversible inactivation. The number of sulfhydryl groups is not known, nor is it known whether there are disulfide bonds in the enzyme.

The noncompetitive *p*-chloromercuribenzoate inhibition pattern of the yeast phosphatase indicates the existence of at least some differences within the active site compared to the *S. typhimurium* enzyme. This could conceivably be due to the inherent differences of reactivity of the sulfhydryl groups between the two species which are reflected both in the *p*-chloromercuribenzoate inhibition data and the much higher specific activity of the yeast enzyme. Evidence was presented that ethylenimine and *p*-chloromercuribenzoate reacted at the same sites, since the portion of enzyme inactivated by *p*-chloromercuribenzoate before partial inactivation by ethylenimine could be recovered with the same efficiency as *p*-chloromercuribenzoate-treated enzyme. Thus, *p*-chloromercuribenzoate reaction "protects" from ethylenimine inactivation. Other variations between the rates of inactivation of the yeast and *S. typhimurium* enzymes were seen when sulfhydryl reagents such as iodoacetate, *N*-ethylmaleimide and ethyleneimine were employed.

Even though Mn^{2+} is not required for the expression of activity, it does have substantial effects on the pH profile of mutant phosphatase enzymes from *S. typhimurium*. However, Mn^{2+} has no effect on the course of *p*-chloromercuribenzoate inhibition, but changes were noted using DTNB as an inactivating reagent which may indicate Mn^{2+}-induced changes in the enzyme [22].

ACKNOWLEDGEMENTS

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