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REACTION OF α-MANNOSIDASE FROM *PHASEOLUS VULGARIS* WITH GROUP-SPECIFIC REAGENTS

ESSENTIAL CARBOXYL GROUPS

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Summary

When the pK_m of α-mannosidase was determined at different pH values, the results indicated that ionizable groups with pK values of approx. 3.8 and 5.7 could be essential. Modification with carbodiimide or Woodward's Reagent K abolished the enzyme activity. The substrate analogue, α-methyl-D-mannoside, protected the enzyme against inactivation. Incorporation of a ^{14}C-labeled nucleophile reagent in the presence or absence of the analogue suggested that 2—4 carboxyl groups were protected. Exchange studies indicated that the essential Zn^{2+} could be bound to such groups.

There was no indication that hydroxyl groups, sulphydryl groups, guanidino groups or amino groups take part in the catalytic activity.

Introduction

α-Mannosidase from *Phaseolus vulgaris* is a glycoprotein of molecular weight approx. 220 000 and is composed of two similar subunits. The enzyme contains two essential zinc atoms per molecule [1,2]. In a previous paper, this author reported that tryptophan appears to be located in or near the active site [3].

In the present paper, further investigations on the amino acids associated with the enzyme activity are reported. By kinetic analysis and chemical modification studies employing selective reagents, evidence for essential carboxyl groups was obtained. Such groups seem to bind zinc to the protein.

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Materials

Glycine ethyl ester and diisopropyl fluorophosphate were supplied by Koch Light Laboratories. 1-Ethyl-3(3-dimethylaminopropyl)carbodiimide was obtained from Calbiochem. Diethyl pyrocarbonate was purchased from Aldrich and Methylene Blue from Bio-Rad Laboratories. *N*-Ethyl-5-phenylisoxazolium-3′-sulphonate (Woodward's Reagent K), 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent), *p*-chloromercuribenzoic acid, *N*-acetylimidazole, butan-2,3-dione, and phenylglyoxal were supplied by Sigma Chemicals. All other chemicals were reagent grade.

[1-^{14}C]Glycine ethyl ester (35 Ci/mol) was purchased from New England Nuclear Corporation. $^{65}ZnCl_2$ (2.0 Ci/g) and [1-^{14}C]hexadecane (1.1 μCi/g) were obtained from the Radiochemical Centre, Amersham, U.K.

Methods

Preparation of α-mannosidase. α-Mannosidase I was purified from extracts of *P. vulgaris* by an immunoadsorbent method described previously [4].

Protein determination. The protein concentration of α-mannosidase solutions was determined from the absorbance at 280 nm, assuming $E^{1\%}_{1cm} = 11.1$ [1].

Enzyme assay and reaction rate studies. Enzyme activity was assayed using *p*-nitrophenyl-α-D-mannopyranoside as substrate [2]. Initial velocity studies were performed between pH 2.6 and 6.5 in 0.1 M sodium citrate, sodium acetate, sodium phosphate and borax-succinic buffers. The results were fitted to double reciprocal plots for determination of Michaelis constants, (K_m). pK_m was plotted vs. pH to indicate ionizable groups at the active site of the free enzyme and in the enzyme · substrate complex [6]. Determination of these pK values at different temperatures was used to calculate the heat of ionization, ΔH, of such groups [7].

Radioactive measurements. Radioactivity was measured in a Packard Tri-Carb scintillation counter. Samples were counted in Dimilume® scintillation liquid. Counting efficiencies were determined using [1-^{14}C]hexadecane as standard. ^{65}Zn was determined by crystal scintillation counting in a Packard spectrometer.

Radioactive exchange studies. 4 μM α-mannosidase in 0.05 M sodium citrate buffer (pH 5.0) was incubated with 1 mM $^{65}Zn^{2+}$ for 18 h at room temperature. After extensive dialysis by ultrafiltration against 0.05 M Tris-HCl buffer (pH 7.5), the enzyme preparations were subjected to ultracentrifugation in 5—20% sucrose gradients. The fractions were assayed for radioactivity and enzyme activity.

Ultracentrifugation. Density gradient ultracentrifugation was performed in a Spinco Model L 2-65B ultracentrifuge in a SW 65 rotor spinning at 32 000 rev./min for 17 h at 5° C. The gradients were drained through an ISCO-6 optical unit connected to a UA-5 absorbance monitor.

Modification studies. Photo-oxidation of the enzyme using Methylene Blue as sensitizing dye was carried out according to Westhead [8]. The reagent was added to 3 μM enzyme in 0.05 M sodium phosphate buffer (pH 6.5) to a final

concentration of 0.005%. The solution was flushed with O_2 before irradiation. A reflector lamp fitted with a 250-W tungsten bulb was used at a distance of approx. 15 cm. The sample was stirred vigorously while the temperature was kept at 5°C. A control sample was kept in the dark. Aliquots were assayed for enzyme activity and amino acid composition. The sample was dialysed before amino acid analysis. Photo-oxidation was also carried out in 0.1 M sodium phosphate buffer at different pH values from 5.0 to 8.0.

Reaction with diethylpyrocarbonate was performed as described by Ludewig et al. [9]. The reagent was dissolved in ethanol shortly before use and kept at 0°C. Carbethoxylation of the enzyme was done at pH 6.1 in 0.1 M sodium phosphate buffer or at pH 7.5 in 0.1 M Tris-HCl buffer, both containing 0.1 M NaCl. The reaction was carried out while stirring at 0°C. Light absorption was followed at 242 nm. The number of carbethoxylated histidyl residues was calculated assuming a molar extinction coefficient of carbethoxyhistidine at pH 6.1 of 32000 $M^{-1} \cdot cm^{-1}$ [10] and a molecular weight of 220 000 for the dimeric α-mannosidase.

Reaction with Woodward's Reagent K was essentially performed as described by Pétra [11]. 47 mM reagent was freshly prepared in 1 mM HCl. To an enzyme solution of 3 μM α-mannosidase in 0.05 M 2(*N*-morpholino)ethanesulphonate (MES) buffer (pH 4.2)/1 M NaCl, the reagent solution was added under vigorous stirring at room temperature to a final concentration of 2.2 mM. The pH was kept constant by addition of 0.02 N NaOH. Aliquots were removed for determination of enzyme activity. Parallel reactions were performed in the presence of α-methyl-D-mannoside or α-methyl-D-galactoside.

Reaction with water soluble carbodiimide was performed according to Hoare and Koshland [12]. The reaction buffer used was 0.1 M MES (pH 4.2)/1 M NaCl. To a 4 μM solution of α-mannosidase was first added glycine ethyl ester to 20 mM and then 1-ethyl-3(3-dimethylaminopropyl)carbodiimide to a final concentration of 2 mM. The reaction mixture was stirred at room temperature. Parallel reactions were performed in the presence of α-methyl-D-mannoside or α-methyl-D-galactoside. When modification with [1-^{14}C]glycine ethyl ester was performed, the reaction was quenched by addition of 1 M sodium acetate buffer (pH 5.0) [13]. The solution was then exhaustively dialysed in Collodion bags against 0.1 M sodium acetate (pH 5.0) before the radioactivity was assayed.

Reaction with 5,5'-dithiobis(2-nitrobenzoic acid) was carried out as described by Habeeb [14]. The reagent was added to 5 μM α-mannosidase in 0.1 M sodium phosphate buffer (pH 7.0) to a concentration of 2 mM.

Reaction with *p*-chloromercuribenzoic acid was performed according to Riordan and Vallee [15]. 1 mM reagent was reacted with 2 μM α-mannosidase in 0.1 M sodium phosphate buffer (pH 7.0) or in 0.1 M sodium acetate buffer (pH 4.5).

Reaction with diisopropyl fluorophosphate was performed by the method of Cohen et al. [16]. 3 μM enzyme in 0.05 M sodium phosphate buffer (pH 7.0) was incubated at room temperature with 0.5 or 3 mM diisopropyl fluorophosphate. To assure adequate reaction conditions, thrombin was assayed as a control. Its coagulant ability was completely abolished.

Reaction with *N*-acetylimidazole was carried out according to Riordan and

Vallee [17]. 5 μM enzyme in 0.05 M Tris-HCl buffer (pH 7.4) or in 0.05 M sodium acetate buffer (pH 5.5) was incubated at room temperature with 2 mM reagent.

Reaction with acetic anhydride was performed either in half-saturated sodium acetate (pH 8.5) [18] or in 0.05 M Tris-HCl buffer (pH 7.5) [17], at concentrations of 2 μM enzyme and 0.8 mM acetic anhydride.

Reaction with butan-2,3-dione was carried out at room temperature in 0.1 M sodium borate buffer (pH 8.0) [19]. 4 μM enzyme solution was incubated with 4 mM reagent.

Reaction with phenylglyoxal was carried out in 0.1 M sodium bicarbonate buffer (pH 8.0) at room temperature. 4 μM enzyme was incubated with 4 mM reagent [20].

Results and Discussion

The effect of pH on the enzyme activity

Plots of pK_m versus pH were employed to obtain information on the ionizable groups participating in the enzyme reaction [6]. The plots curved near pH 3.3, 3.8 and 5.7 (Fig. 1). Similar results were obtained in sodium citrate, sodium acetate, sodium phosphate and borax-succinic buffers. Since the substrate does not ionize in this pH region, the bending points seem due to ionizable groups in the enzyme or in the enzyme · substrate complex. The point near pH 3.8 probably belongs to a group in the free enzyme and could well indicate a carboxyl group. The pK value at pH 3.3 is most likely attributable to the ionization of the same group in the combined state [6]. This group, which seems still free in the complex, undergoes an acid shift as the enzyme combines with the substrate. The point at pH 5.7 may be ascribed to a group in the free enzyme. This group seems involved in the binding of the substrate. The pH value suggests either an imidazole group with a low pK value, or a carboxyl group with a high pK value.

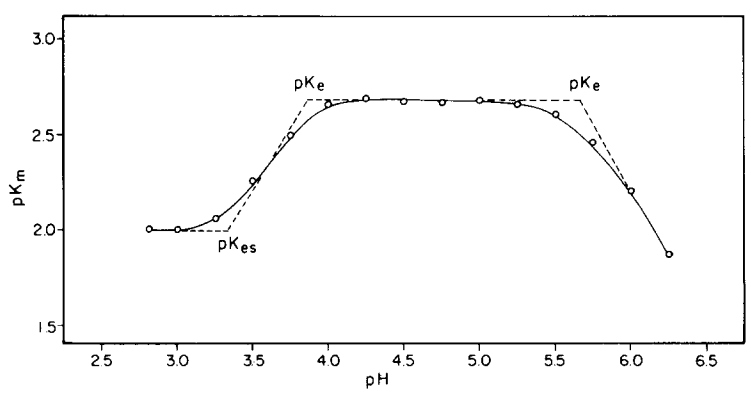

Fig. 1. Effect of pH on the pK_m of α-mannosidase. K_m values were derived from double-reciprocal plots of initial velocity of the enzyme between pH 2.6 and 6.5 in 0.05 M sodium citrate buffer. *p*-Nitrophenyl-α-D-mannopyranoside was the substrate. pK_m is the negative logarithm to the Michaelis-Menten constant. pK_e and pK_{es} indicate the pK values of essential ionizable groups in the free enzyme and in the enzyme · substrate complex, respectively [6].

To elucidate the nature of these groups, the heat of ionization was determined at the different pK values. While carboxyl groups have ΔH values of approx. ± 1500 cal $\cdot$ mol^{-1}, the ΔH for imidazole is given as approx. 7500 cal $\cdot$ mol^{-1} [6]. The ΔH for the groups with pK values of 3.8 and 5.7 was found to be 1900 cal $\cdot$ mol^{-1} and 2500 cal $\cdot$ mol^{-1}, respectively. However, these figures should be interpreted with care, as only small differences in the measured values will affect considerably the calculated ΔH values.

Thus, the kinetic data suggest that carboxyl groups take part in the enzyme reaction. The question whether imidazole groups also participate must be left open, although most of the evidence seems against.

Modification of carboxyl groups

Treatment of α-mannosidase at pH 4.2 with carbodiimide in the presence of glycine ethyl ester resulted in a rapid decline in enzyme activity (Fig. 2). The rate and extent of inactivation obtained with carbodiimide alone were essentially the same as in the presence of the nucleophile. As the K_m and pH optimum of partially-inactivated α-mannosidase did not differ from the values of the native enzyme, while V was reduced, the remaining activity seems due to residual unmodified enzyme rather than to altered molecules with distinct kinetic properties [21].

In the presence of 100 mM and 200 mM α-methyl-D-mannoside the rate of inactivation was reduced.

Carbodiimide might react with several functional groups of amino acids in proteins; the most likely ones, besides carboxyl groups, are the side chains of tyrosine, serine and cysteine [22]. The reaction at pH 4.5 should normally limit the modification to carboxyl groups. Moreover, tyrosine residues in proteins can be regenerated from their corresponding derivatives by exposure to hydroxylamine. Such treatment of modified α-mannosidase did not regenerate the enzyme activity. Sulphydryl groups cannot be demonstrated in α-mannosidase [1]. Neither specific sulphydryl reagents nor the serine reagent diisopropyl fluorophosphate had any effect on the enzyme activity (see below).

As an additional control, α-mannosidase was exposed to Woodward's Reagent K, which under mild conditions and at pH below 4.7 reacts selectively with carboxyl groups [11,23]. The reagent abolished α-mannosidase activity in a manner similar to carbodiimide. Modification of carboxyl groups thus seems to inactivate α-mannosidase. Inactivation was lower in the presence of α-methyl-D-mannoside, consistent with the results from the carbodiimide modification. This protection of activity by a substrate analogue could indicate that the carboxyl groups might be located in the active site region. However, the observations could also be explained by allosteric effects.

Changes in molecular structure are often experienced when proteins are modified. When double diffusion experiments with anti-α-mannosidase antiserum versus native α-mannosidase and enzyme modified with carbodiimide or Woodward's Reagent K were set up, no spur formation could be detected. Nor was there any difference between the ultracentrifugational patterns of native and modified enzyme. These results suggest that no major conformation change has occurred after carboxyl modification.

The number of modified residues was determined by reaction with carbodi-

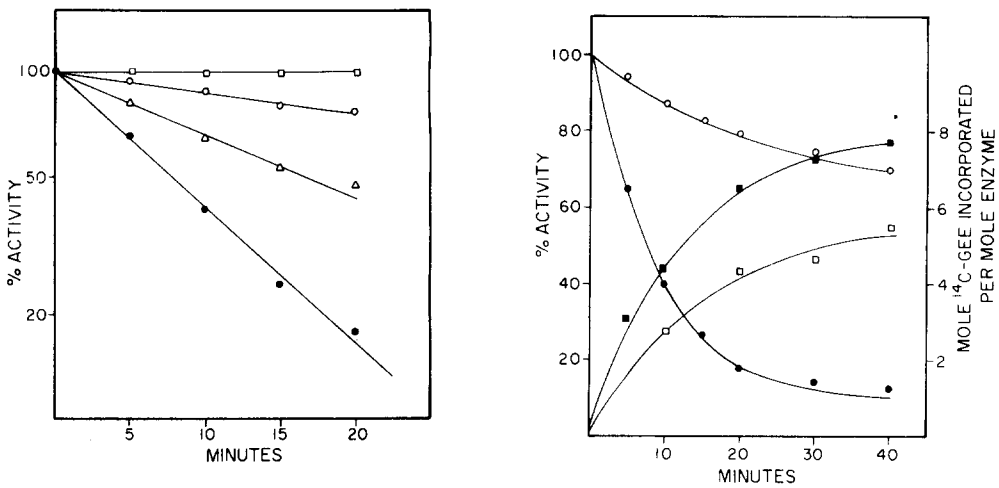

Fig. 2. The effect of carbodiimide on α-mannosidase activity in the presence or absence of α-methyl-D-mannoside. 4 μM of α-mannosidase in 0.1 M MES buffer (pH 4.2)/1 M NaCl was incubated with 20 mM glycine ethyl ester and 2 mM carbodiimide. ●———●, α-mannosidase; △———△, α-mannosidase in the presence of 100 mM α-methyl-D-mannoside; ○———○, α-mannosidase in the presence of 200 mM α-methyl-D-mannoside; □———□, control (no reagent added).

Fig. 3. Correlation between enzyme activity and incorporated radioactivity in the reaction of α-mannosidase with carbodiimide and [1-^{14}C]glycine ethyl ester. 4 μM of α-mannosidase in 0.1 M MES buffer (pH 4.2)/1 M NaCl was incubated with 20 mM [1-^{14}C]glycine ethyl ester and 2 mM carbodiimide. The reaction was quenched with 1 M sodium acetate buffer (pH 5.0). Exhaustive dialysis against 0.1 M sodium acetate buffer (pH 5.0) was performed before the radioactivity was assayed. ●———●, α-mannosidase activity in the absence of α-methyl-D-mannoside. ○———○, α-mannosidase activity in the presence of 200 mM α-methyl-D-mannoside; ■———■, radioactivity incorporated in the absence of α-methyl-D-mannoside; □———□, radioactivity incorporated in the presence of 200 mM α-methyl-D-mannoside.

imide in the presence of [1-^{14}C]glycine ethyl ester. Fig. 3 shows the results obtained in the presence or absence of α-methyl-D-mannoside. Enzyme activity decreased with increasing incorporation of radioactivity. α-Methyl-D-mannoside seemed to prevent both loss of enzyme activity and incorporation of ^{14}C-label. When 13% of the activity remained in the unprotected enzyme and the protected sample had approx. 70% activity left, there was a difference of 2.3 mol incorporated [1-^{14}C]glycine ethyl ester between protected and unprotected α-mannosidase. The results indicate that there may be approx. 8 carboxyl groups of similar reactivity and that 2—4 of them are protected by α-methyl-D-mannoside. The specificity of the reaction is remarkable, as α-mannosidase contains a large number of free carboxyl groups [1]. A similar high reactivity of essential carboxyl groups has been shown for other enzymes [24—26]. It is also interesting to note that the carbohydrate-binding capacity of the lectins, concanavalin A [27], wheat germ agglutinin [28] and α-D-galactopyranosyl-binding lectin [13], was greatly reduced after modification with carbodiimides.

Modification of histidyl groups

Since the ionization curves left some uncertainty as to whether imidazole groups take part in the enzymatic process and such groups have been shown to

bind zinc in other proteins such as carboxypeptidase A [29], attempts were made to modify the histidyl residues.

Photo-oxidation using Methylene Blue is widely used for this. However, oxidation of other residues might also occur, especially of those of tryptophan, tyrosine, cysteine and methionine. The pH dependence of the reaction should give some hints, as imidazole groups are less susceptible to oxidation in the protonated state [8]. When α-mannosidase was exposed to photo-oxidation, only 15% of the enzyme activity was left after 20 min and α-methyl-D-mannoside offered some protection (Fig. 4A). Amino acid analysis showed that enzyme with 25% residual activity had one-third of the histidyl residues destroyed, while the tyrosine content seemed unchanged. The absorption at 280 nm was reduced, with 16% and 11% after photo-oxidation of unprotected and protected enzyme, respectively. This corresponds to oxidation of approx. 7 and 4 tryptophan residues [3,30]. The rate of inactivation was higher at pH 7.0 than at pH 5.0 (Fig. 4B). The decline in enzyme activity, therefore, seems due to oxidation of essential tryptophan residues rather than to modification of histidine [31].

When ethylformylation with the more specific histidyl reagent, diethylpyrocarbonate, was performed at a molar ratio of reagent to enzyme of 100 : 1, an increase in absorption at 242 nm was observed, indicating formation of *N*-carbethoxyhistidine residues. The enzyme activity did not decrease. At 10-times higher concentrations of diethylpyrocarbonate, the enzyme activity decreased to approx. 45% after 20 min and the substrate analogue had no protective effect. Such a slow decrease in enzyme activity at very high concentrations of

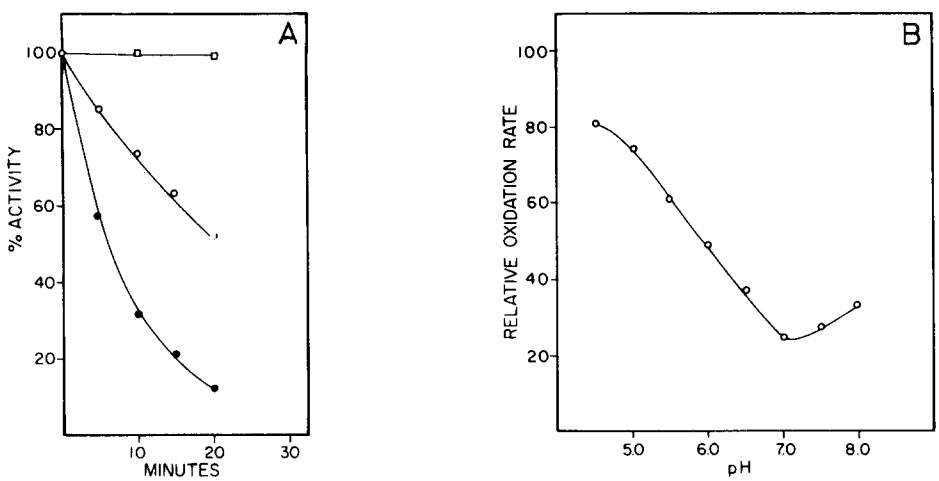

Fig. 4. A. Time-course of photo-oxidation of α-mannosidase in the presence of Methylene Blue. Incubation was performed with 3 μM enzyme in 0.05 M sodium phosphate buffer (pH 6.5)/0.005% Methylene Blue. The incubation mixtures were thoroughly flushed with O_2 before irradation, which was carried out at a distance of approx. 15 cm for 15 min, using a reflector lamp fitted with a 250-W tungsten bulb. ●——●, α-mannosidase; ○——○, α-mannosidase in the presence of 150 mM α-methyl-D-mannoside, □——□, control (no irradation). B. Effect of pH on the photo-oxidation of α-mannosidase. Incubation was performed in 0.05 M sodium phosphate buffer, pH 5.0 to 8.0. Experimental conditions as under A. The relative oxidation rate signifies the percentage of initial enzyme activity lost during oxidation.

modifying reagent is probably due to random modification leading to conformational changes in the enzyme. This was supported by the observation of spurs between the inactivated enzyme preparation and the native enzyme when tested against anti-α-mannosidase antiserum.

These experiments consequently indicate that histidine residues are not involved in determining the α-mannosidase activity.

Reaction with selective reagents for other amino acid residues

The effect of some other compounds on the enzyme activity is summarized in Table I. As reported earlier, cysteine was not detected in this α-mannosidase [3]. To exclude the possibility of the amino acid being present at subdectectable levels, the effect of Ellman's reagent and *p*-chloromercuribenzoic acid on the enzyme activity was tested. Neither reagent had any inhibitory effect. Diisopropyl fluorophosphate, which inactivates many enzymes by phosphorylation of active serine, also did not inhibit the enzyme. Further, treatment with *N*-acetylimidazole, which acetylates tyrosine residues, had no effect. Acetic anhydride (which, in half-saturated sodium acetate, has been shown to acetylate amino groups) left approx. 70% activity after 20 min. The presence of the substrate analogue offered no protection and the modified enzyme preparation showed spur formation with native α-mannosidase, when tested by double diffusion against anti-α-mannosidase antiserum. The inactivation seems therefore due to conformational changes. Phenylglyoxal or butan-2,3-dione, which primarily modify guanidino groups, did not affect the α-mannosidase activity. Thus, there was no indication for residues of cysteine, serine, tyrosine, arginine or amino groups participating in the enzymatic action of α-mannosidase.

Studies on the binding of zinc in α-mannosidase

α-Mannosidase is inactivated by exposure to EDTA at pH 4.5 (its pH optimum) and is immediately reactivated by the addition of Zn^{2+} [2]. Based on the present knowledge of functional groups in the enzyme, additional experiments were carried out to obtain more information on the interaction of the metal ion with the enzyme.

α-Mannosidase was incubated with 1 mM EDTA in sodium citrate/phosphate buffer at different pH values. A plot of the activity vs. pH resulted in a sigmoid

TABLE I

EFFECTS OF SELECTIVE REAGENTS ON α-MANNOSIDASE ACTIVITY

2—5 μM enzyme was incubated with the different reagents for 3 h.

Reagent	Ratio of inhibitor to enzyme (mol/mol enzyme)	Activity remaining (%)
Diisopropyl fluorophosphate	1000	>95
5,5'-Dithiobis(2-nitrobenzoic acid)	400	>95
p-Chloromercuribenzoic acid	500	>95
N-acetylimidazole	400	>95
Acetic anhydride	400	68
Butan-2,3-dione	1000	>90
Phenylglyoxal	1000	>90

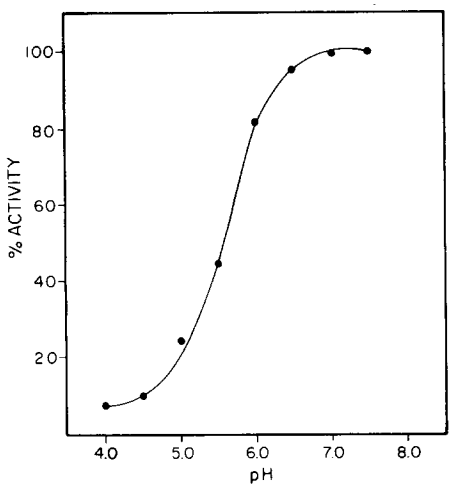

Fig. 5. The effect of pH on EDTA treatment of α-mannosidase. α-Mannosidase, 3 μM, was incubated with 1 mM EDTA in 0.05 M sodium citrate/phosphate buffer from pH 4.0 to 7.5 at room temperature for 15 h.

curve, with the lowest residual activity at pH 4.0 and an inflection at pH 5.6 (Fig. 5). The enzyme was inactivated at pH 4.5 also in the absence of EDTA, but at a slower rate. The presence of 1 mM Zn^{2+} or 100 mM α-methyl-D-mannoside protected the enzyme from inactivation. The lack of demonstrable changes in conformation, the immediate reactivation on the addition of Zn^{2+} [2], as well as the protection by the substrate analogue, argue for Zn^{2+} participating in the catalytic action. The metal seems bound to a group with pK value around 5.6.

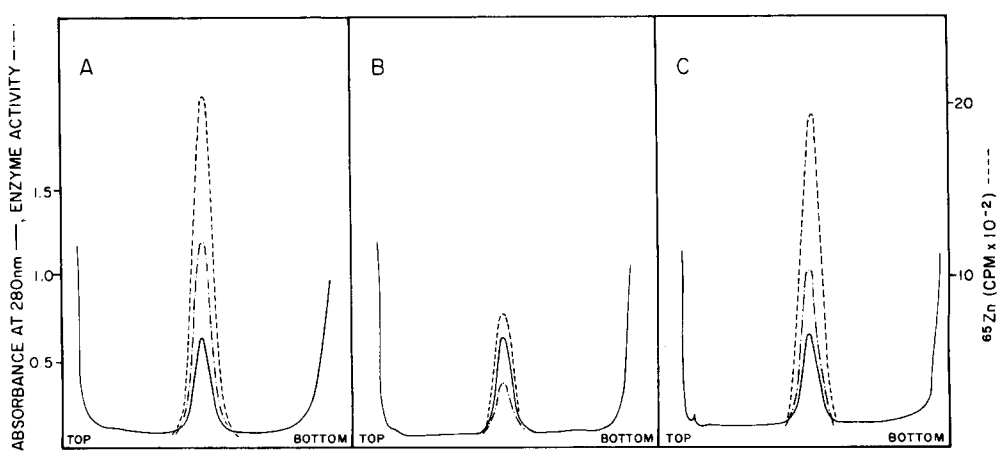

Fig. 6. Density gradient ultracentrifugation of native and modified α-mannosidase after incubation with ^{65}Zn. 4 μM α-mannosidase in 0.05 M sodium citrate buffer (pH 5.0) was incubated in 1 mM $^{65}Zn^{2+}$ for 18 h at room temperature. After thorough dialysis the sample was run in a 5—20% sucrose gradient at 5°C and 32 000 rev./min for 17 h in a SW 65 rotor. A. Native α-mannosidase. B. α-Mannosidase modified to 30% residual activity with carbodiimide and glycine ethyl ester prior to exposure to ^{65}Zn. C. α-Mannosidase modified with carbodiimide and glycine ethyl ester in the presence of 200 mM α-methyl-D-mannoside, treated as under B. ——— absorbance at 280 nm; ·——· α-mannosidase activity; - - - - - - radioactivity.

The binding of Zn^{2+} after modification of carboxyl groups was then studied. Native α-mannosidase was exposed to radioactive Zn^{2+} solution for 18 h at room temperature and subjected to ultracentrifugation after exhaustive dialysis. Parallel experiments were performed with enzyme preparations inactivated with carbodiimide in the presence or absence of α-methyl-D-mannoside to 85% and 30% residual activity, respectively, and dialysed to remove the reagents prior to exposure to radioactive Zn (Fig. 6). The enzyme which had been modified by carbodiimide in absence of substrate analogue showed a considerably lower incorporation of ^{65}Zn than did the native enzyme. The protected enzyme contained only slightly less radioactivity than the unmodified α-mannosidase. The ratio of enzyme activity to radioactivity was the same in all preparations. It therefore seems that Zn^{2+} is bound to the enzyme by carboxyl groups, which have been shown to be ligands for this metal ion in other proteins [29,32,33]. Zn^{2+} is probably bound to the carboxyl group with pK around 5.7, although interaction also with the carboxyl group pK 3.8 or other groups cannot be excluded.

Conclusion

Determination of pK values as well as heat of ionization indicated the presence of essential carboxyl groups in α-mannosidase. Modification studies of the enzyme in the presence or absence of a substrate analogue support these assumptions. Incorporation of radioactive glycine ethyl ester suggested that 2—4 carboxyl groups might take part in the catalytic function. Zn^{2+} being an essential ion for α-mannosidase seems bound by carboxyl groups. Although imidazole is a possible ligand for Zn^{2+}, the bulk of evidence was against the presence of histidine in the active site region. Neither do cysteine, serine, tyrosine, arginine or amino groups seem to participate in the enzymatic function of α-mannosidase.

Previous results indicate that α-mannosidase from *P. vulgaris* is composed of two identical subunits and has two zinc atoms and two essential tryptophans per molecule [1,3]. In view of these and the present results, it is tempting to assume one active site per subunit, each possessing one tryptophan residue and two carboxyl groups participating in the catalytic function. Zn^{2+} could be bound to the pK 5.7 carboxyl group.

Acknowledgements

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