Co²⁺-mediated time- and temperature-dependent activation of neutral α -D-mannosidase from monkey brain

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Neutral α -D-mannosidase from monkey brain was purified by Co²⁺-chelate affinity chromatography and immunoabsorbent affinity chromatography. The purified enzyme, with subunit M_r 45000, was essentially homogeneous with only traces of two contaminant proteins as revealed by SDS/polyacrylamide-gel electrophoresis and AgNO₃ staining. The purified enzyme, on preincubation with Co²⁺ at 37 °C or 60 °C followed by assay, showed a time-dependent enhancement in activity. The enhanced activity of the enzyme persisted even after removal of the Co²⁺. Bacitracin could partially prevent the activation. An aminopeptidase activity that was stimulated by Co²⁺ both at 37 °C and at 60 °C was present in the purified enzyme. After preincubation of the enzyme with Co²⁺ there was evidence for the release of amino acids, as revealed by t.l.c., but the M_r determined by SDS/polyacrylamide-gel electrophoresis was not appreciably altered. It is suggested that a Co²⁺-stimulated thermostable aminopeptidase, inseparable from the neutral mannosidase, may be involved in the stimulation of neutral mannosidase activity during its preincubation with Co²⁺.

INTRODUCTION

The cytosolic neutral α - and β -D-mannosidases, which exhibit a pH optimum at close to neutrality, are suggested to be genetically, structurally and functionally distinct from the lysosomal acid mannosidases (Shoup & Touster, 1976; Dawson, 1982). Studies by Bischoff & Kornfeld (1983, 1986) have indicated that the rat liver cytosolic neutral mannosidase, which cleaves α -1,2-linked mannoside residues of high-mannose-type oligosaccharides, arises from the endoplasmic-reticulum neutral mannosidase by proteinase action. Neutral α -D-mannosidases of liver and brain, unlike the acid mannosidases or Golgi mannosidases, do not bind to concanavalin A-Sepharose, and they are activated by Co²⁺ (Philips *et al.*, 1976; Shoup & Touster, 1976; Mathur & Balasubramanian, 1981). The activation/stabilization of neutral α -D-mannosidase by Co²⁺ and other bivalent ions such as Mn²⁺ and Fe²⁺ (Philips *et al.*, 1974; Shoup & Touster, 1976; Snaith, 1977; Hirani & Winchester, 1979; Mathur & Balasubramanian, 1981), is unique, because several other glycosidases, acid or neutral (including neutral β -D-mannosidase), are not activated by similar metal ions. Snaith (1977), who studied the metal-ion activation of neutral α -D-mannosidases as a function of pH in different tissue preparations, suggested the existence of multiple forms of the enzyme. Snaith (1977) also observed very high increases in the activity of rat testis neutral mannosidase when it was preincubated with Co²⁺ in comparison with the direct activation by Co²⁺. Stabilization of neutral α -D-mannosidases by Co²⁺ in general and during preincubation has also been reported (Shoup & Touster, 1976; Bischoff & Kornfeld, 1983).

The difficulty with the purification of the neutral α -D-mannosidase, coupled with its instability (Shoup & Touster, 1976), precluded a detailed study of its mechanism of activation by Co²⁺. We have however, reported Co²⁺-chelate affinity chromatography for the purification of monkey brain neutral α -D-mannosidase

(Mathur & Balasubramanian, 1984). In earlier studies (Mathur & Balasubramanian, 1981) on the purification of monkey brain neutral mannosidase we noticed an increased recovery of activity after heat treatment at 60 °C in the presence of Co²⁺. We now present evidence to suggest that an aminopeptidase, inseparable from the neutral α -D-mannosidase, may be involved in the Co²⁺-mediated temperature-dependent conversion of a less active 'precursor' neutral α -D-mannosidase to a more active 'product' neutral mannosidase.

MATERIALS AND METHODS

Assay for neutral α -D-mannosidase

The assay of neutral mannosidase at pH 6.3 with *p*-nitrophenyl α -D-mannoside as the substrate, and the units of enzyme activity, were described previously (Mathur & Balasubramanian, 1984). When 4-methyl-umbelliferyl α -D-mannoside was used as substrate, the assay mixture was the same as above, except that 0.2 μ mol of the fluorescent substrate was used in place of the *p*-nitrophenyl mannoside. The fluorescence was measured (Snaith, 1977) in a Hitachi 204A spectrofluorimeter. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Assay for aminopeptidase

The assay system consisted of 50 mM-potassium phosphate buffer, pH 7.5, either 0.25 mM-leucine β -naphthylamide or 0.25 mM-tyrosine β -naphthylamide as the substrate and 50 μ l of the enzyme (10 μ g of protein) in a final volume of 350 μ l. The tubes were incubated at 37 °C or 60 °C for 30 min to 1 h, the reaction was stopped by keeping the tubes in a boiling-water bath for 1 min, and the released β -naphthylamine was measured fluorimetrically (λ_{exc} . 335 nm, λ_{em} . 410 nm; Hersh & McKelvy, 1981). The aminopeptidase was also measured by using 2 mM-leucine *p*-nitroanilide as a substrate at

pH 7.5, and the released *p*-nitroaniline was measured at 400 nm (Pfleiderer, 1970) in an Ultrospec 4050 spectrophotometer.

Assay for carboxypeptidase

Carboxypeptidases A and B were determined spectrophotometrically with 1 mM-hippurylphenylalanine and hippurylarginine respectively as substrate and 100 μ l of the enzyme in 200 μ l of 50 mM-potassium phosphate buffer, pH 7.5 (Petra, 1970; Folk, 1970).

Preincubation experiments

In some of the studies the enzyme was purified up to the end of the Co^{2+} -chelate affinity chromatography, and in others the final purified enzyme was used for the preincubation experiments. Preincubation experiments were conducted by maintaining the enzyme (10–15 μ g of protein in 50 μ l of 20 mM-potassium phosphate buffer, pH 7.5), with any additions as indicated, under different experimental conditions, in a volume of 140 μ l at the given temperature for the desired time intervals. At the end of preincubation, the tubes were chilled on ice and then assayed for the neutral mannosidase activity at pH 6.3 by adding the required components for assay.

T.l.c.

The final purified enzyme (75–150 μ g of protein in 20 mM-potassium phosphate buffer, pH 7.5) was preincubated with different metal ions (1.4 mM- CoCl_2 , - MgCl_2 or - ZnSO_4) at 60 °C for 1 min. The control tubes did not contain any metal ions. The reactions were stopped by keeping the tubes in a boiling-water bath for 2 min, after which time metal salt (1.4 mM) was added to the respective control tubes. After the tubes had cooled, 4 vol. of chilled acetone was added to all the tubes, and they were then centrifuged at 12000 *g* for 30 min. The supernatants were collected and the acetone was evaporated by passing a stream of N_2 . The aqueous phase was freeze-dried and the residue was dissolved in 40 μ l of 10% (v/v) propan-2-ol in 0.1 M-HCl. T.l.c. was carried out on glass plates coated with silica gel G with the solvent system butan-1-ol/formic acid/water (15:3:2, by vol.), and protein was detected by either 0.4% (w/v) ninhydrin or by the Cu^{2+} /ninhydrin spray reagent (Ganapathy *et al.*, 1981).

SDS/polyacrylamide-gel electrophoresis

Electrophoresis (Laemmli, 1970) of the samples prepared in 2% (w/v) SDS, with or without 5% (v/v) 2-mercaptoethanol, was carried out in 10% (w/v) polyacrylamide slab gel in 50 mM-glycine/Tris buffer, pH 8.6, containing 0.1% (w/v) SDS at 40 mA until the tracking dye, Bromphenol Blue, emerged from the gel. The gel was first stained with 0.1% (w/v) Coomassie Brilliant Blue R in methanol/acetic acid/water (5:1:4 by vol.). After destaining in the same solution without the dye, the gel was subjected to the highly sensitive AgNO_3 staining method (Merril *et al.*, 1983). Standard marker proteins used for M_r determination were human IgG (M_r 150 000), bovine serum albumin (M_r 68 000), ovalbumin (M_r 45 000) and myoglobin (M_r 17 800).

Purification of the neutral mannosidase

All the steps were carried out at 4 °C. The neutral mannosidase was purified from frozen monkey brain (250 g) by $(\text{NH}_4)_2\text{SO}_4$ fractionation, concanavalin A-

Sepharose chromatography and finally subjecting the enzyme in the flow-through fractions from concanavalin A-Sepharose to Co^{2+} -chelate affinity chromatography as described previously (Mathur & Balasubramanian, 1984). Further purification of the enzyme was carried out as follows. Concentration of the enzyme during the entire procedure was done by ultrafiltration on Amicon CF-25 Centriflo cones.

Preparation of antibodies against the contaminants. The enzyme obtained after Co^{2+} -chelate affinity chromatography was concentrated to about 5 ml and subjected to a preparative electrophoresis on 7% (w/v) polyacrylamide gel, polymerized in a glass tube (1.1 cm $\times$ 10.5 cm) in 50 mM-glycine/Tris buffer, pH 8.9 (Davis, 1964), at 25 mA until Bromophenol Blue was excluded from the gel. The gel was cut into 1.5 mm-thick slices and extracted in 20 mM-potassium phosphate buffer, pH 7.5. The neutral mannosidase, which was localized in three slices, was separated, and the extracts from the remaining slices were pooled, dialysed against 5 mM-potassium phosphate buffer, pH 7.5, concentrated, and then freeze-dried. The isolated contaminant proteins (2.5 mg) dissolved in 1 ml of 0.15 M-NaCl/5 mM-potassium phosphate buffer, pH 7.5, and mixed with 1 ml of Freund's complete adjuvant were injected intradermally at multiple sites into a rabbit (Vaitukaitis *et al.*, 1971). A booster injection (2.5 mg of protein) was given in Freund's incomplete adjuvant after 3 weeks. At 1 week after the booster dose the rabbit was bled and the total immunoglobulin fraction was prepared from the pooled serum by 50% saturation with $(\text{NH}_4)_2\text{SO}_4$ (Livingston, 1974). Part of it was passed through a 5 ml column of Cibacron Blue F3G-Sepharose in 0.5 M-NaCl/50 mM-Tris/HCl buffer, pH 8.0, to separate the albumin from the immunoglobulins, which were recovered in the run-through fractions (Travis *et al.*, 1976). The fractions were pooled, concentrated and dialysed against distilled water. The IgG fraction from the total immunoglobulin was purified by DEAE-cellulose chromatography at pH 6.8 (Livingston, 1974), concentrated and dialysed against distilled water.

Preparation of total Ig-Sepharose and IgG-Sepharose. Portions (200 mg each) of total immunoglobulin fraction and IgG fraction were coupled separately to 20 ml of Sepharose 4B after CNBr (50 mg/ml of gel) activation of the gel (March *et al.*, 1974). The resultant gels were found to have approx. 10 mg of protein coupled per ml of gel. They were packed in columns (5 cm $\times$ 2.2 cm) and equilibrated with 20 mM-potassium phosphate buffer, pH 7.5.

Chromatography of the neutral mannosidase on the immunoabsorbents. The enzyme obtained after Co^{2+} -chelate chromatography was concentrated (to 5–7 ml), dialysed against 20 mM-potassium phosphate buffer, pH 7.5, and applied to the total Ig-Sepharose. After it had been allowed to remain in the column for 6–8 h, fractions were collected. The column was washed with the same buffer. The run-through fractions, which contained mannosidase activity, were pooled, concentrated and passed through the IgG-Sepharose in exactly the same way. The unbound fractions, which contained neutral mannosidase activity, were pooled and concentrated.

Fig. 1. SDS/polyacrylamide-gel electrophoresis and AgNO_3 staining of neutral α -D-mannosidase eluted from the Co^{2+} -chelate column (A and C) and purified by passage through the immunoabsorbent columns (B and D)

The contaminant proteins eluted from the Ig-Sepharose (E) and IgG-Sepharose (F) are also shown. Samples A and B were without 2-mercaptoethanol, and samples C-F with mercaptoethanol. Electrophoresis was conducted with 10–12 μg of samples as described in the text.

Other methods

Metal ion-chelate gels were prepared by the procedure of Porath *et al.* (1975), mannosamine-Sepharose by the procedure of Mathur & Balasubramanian (1981), and Cibacron Blue F3G-Sepharose as described previously (George & Balasubramanian, 1983). The dansylation procedure for *N*-terminal amino acid analysis, followed by hydrolysis and subsequent two-dimensional t.l.c. on polyamide sheets in the solvents 1.5% (v/v) formic acid in the first dimension and benzene/acetic acid (9:1, v/v) in the second dimension, was as described by Niederwieser (1972). A partially purified preparation (200 μg) of monkey brain neutral mannosidase (Mathur & Balasubramanian, 1981) was used to raise an anti-(neutral mannosidase) antibody in a rabbit. Immunoprecipitation was carried out at 4 °C for 4 h in the presence of 4% (w/v) poly(ethylene glycol) 6000, followed by centrifugation at 12000 *g* for 30 min. Proteinase inhibitors, marker proteins and other chemicals were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. $^{57}\text{CoCl}_2$ was from the Bhabha Atomic Research Centre, Trombay, India.

RESULTS

Purification of the neutral α -D-mannosidase

Of several methods employed for purification of the neutral mannosidase after the Co^{2+} -chelate affinity chromatography (such as chromatography on other metal ion-chelate gels, DEAE-Sephadex, ECTEOLA-cellulose, Sephadex G-200, mannosamine-Sepharose,

phenyl-Sepharose and Cibacron Blue F3G-Sepharose), only a preparative gel electrophoresis could remove the impurities to the maximum extent, although not completely. An affinity column with antibody raised against purified mannosidase as ligand did not bind and permit elution of the enzyme efficiently, for reasons that are not clear. Therefore we purified the enzyme by using a different approach, with the antibodies raised against the impurities. After passage through the immunoabsorbents, the enzyme was free of most of the contaminants, as observed by SDS/polyacrylamide-gel electrophoresis. A single prominent protein band was seen when the gel was stained with Coomassie Brilliant Blue, but the highly sensitive AgNO_3 staining method showed trace amounts of two additional protein bands (Fig. 1). The subunit M_r of the enzyme was found to be 45000. The subunit M_r values of rat liver cytosolic neutral mannosidase and a rat brain neutral mannosidase are reported to be 107000–110000 (Shoup & Touster, 1976; Bischoff & Kornfeld, 1986) and 62000 (Zanetta *et al.*, 1982) respectively. The use of both the immunoabsorbents was found to be necessary to remove the maximum amount of impurities. The proteins eluted from the immunoabsorbent columns with 6 M-urea corresponded in electrophoretic mobility to the contaminant proteins found in the enzyme fraction eluted from the Co^{2+} -chelate column (Fig. 1). Recovery of the enzyme from the total Ig Sepharose was 50–60%, and that from the IgG-Sepharose was 60–70%. Because of the faster loss of activity during purification, there was only a marginal increase in the specific activity of the purified enzyme (3.4 μmol of *p*-nitrophenol liberated/h per mg of protein).

Preincubation of the enzyme at 37 °C

The enzyme purified by Co^{2+} -chelate chromatography showed a minor loss in activity when preincubated at 37 °C for various time intervals up to 30 min (20% loss at 30 min) (Fig. 2a). There was no change in activity when the enzyme was preincubated with 1.4 mM- CoCl_2 at 4 °C for up to 30 min. However, with 1.4 mM- CoCl_2 and preincubation at 37 °C there was a gradual rise in activity, which was 2.5–3.5-fold at 15 min (Fig. 2a) and which then remained approximately constant for up to 60 min. With lower concentrations of CoCl_2 (up to 0.14 mM) the rate of increase was diminished, but higher concentrations (up to 7 mM) did not further increase the rate (results not shown). The use of MnCl_2 (1.4 mM) instead of CoCl_2 also resulted in about 2-fold activation under similar conditions (results not shown). ZnSO_4 and MgCl_2 did not activate the enzyme at any concentration tested (0.14–7 mM). EDTA (2.8 mM) in the presence of 1.4 mM- CoCl_2 during preincubation completely reversed the stimulation of mannosidase activity. Similar results to those described above were obtained when 4-methylumbelliferyl α -D-mannoside was used as substrate for the assay of neutral mannosidase.

Effect of preincubation at 37 °C on the 'product' neutral mannosidase

If the Co^{2+} -mediated increase in neutral mannosidase activity were due to the formation of a new form of neutral mannosidase (henceforth the enzyme preincubated with Co^{2+} and dialysed to remove the metal ion is referred to as 'product' mannosidase and the enzyme before preincubation with Co^{2+} is referred to as the

Fig. 2. Preincubation of the neutral mannosidase (15 μg in 20 mM-phosphate buffer, pH 7.5) at 37 °C or 4 °C for various time intervals with CoCl₂

At the end of preincubation the tubes were chilled on ice and assayed for enzyme activity with 1 mM-CoCl₂ present in all assay tubes. The activity is expressed as μmol of *p*-nitrophenol liberated/h. (a) Effect of preincubation on the 'precursor' mannosidase. O, Enzyme alone, preincubated at 37 °C; x, enzyme with 1.4 mM-CoCl₂, preincubated at 4 °C; Δ, enzyme with 1.4 mM-CoCl₂, preincubated at 37 °C. (b) Effect of preincubation on the 'product' mannosidase (enzyme already subjected to preincubation with 1.4 mM-CoCl₂ at 37 °C and dialysed against 20 mM-phosphate buffer, pH 7.5). O, Enzyme alone, preincubated at 37 °C; x, enzyme with 1.4 mM-CoCl₂, preincubated at 4 °C; Δ, enzyme with 1.4 mM-CoCl₂, preincubated at 37 °C. Details of the experiment are given in the text.

Fig. 3. Preincubation of the 'precursor' (a) and the 'product' (b) neutral mannosidase (15 μg of protein in 20 mM-phosphate buffer, pH 7.5) at 60 °C for various time intervals with the indicated additions

At the end of preincubation the tubes were chilled on ice and assayed for enzyme activity with 1 mM-CoCl₂ present in all assay mixtures. The activity is expressed as percentage of control activity (activity of the enzyme preincubated at 4 °C taken as 100 % in each case). O, Enzyme alone, preincubated; Δ, enzyme preincubated with 1.4 mM-CoCl₂; □, enzyme preincubated with 5 mM-glucosamine; x, enzyme preincubated with 1.4 mM-CoCl₂ and 5 mM-glucosamine.

'precursor' mannosidase), then we reasoned that the enhanced activity of the enzyme should remain even after removal of the Co²⁺. Accordingly, the enzyme that had been subjected to preincubation at 37 °C with 1.4 mM-CoCl₂ for 30 min was dialysed exhaustively against 20 mM-potassium phosphate buffer, pH 7.5, at 4 °C. After dialysis the 'product' enzyme continued to retain 80 % of the enhanced mannosidase activity. When subjected to gel filtration on Sephadex G-200 almost 100 % of the enhanced activity was retained. When this enzyme was further preincubated at 37 °C with 1.4 mM-CoCl₂ for

various time intervals up to 30 min it showed only a slight increase in mannosidase activity, in contrast with observations made with the 'precursor' mannosidase (Fig. 2b).

Preincubation of the enzyme of 60 °C

The effects of preincubation of the 'precursor' and the 'product' enzymes at 60 °C for different time intervals in the presence and in the absence of CoCl₂, and with or without D-glucosamine, are shown in Fig. 3. [D-Glucosamine is a weak competitive inhibitor (*K_i* 2.8 mM) that

Fig. 4. Preincubation of the 'precursor' neutral mannosidase (15 μ g in 20 mM-phosphate buffer, pH 7.5) at 60 °C (○), 65 °C (△) and 70 °C (□) with 1.4 mM- CoCl_2 and 5 mM-glucosamine for various time intervals

At the end of preincubation the tubes were chilled on ice and assayed for enzyme activity. Results are expressed as percentage of control activity (activity of the enzyme preincubated at 4 °C taken as 100%). Inset shows the enzyme activity when preincubated in the absence of CoCl_2 and glucosamine at 60 °C (○) and 70 °C (□).

was found to stabilize the enzyme against thermal denaturation and could only be replaced by D-mannosamine but not by other amino sugars or monosaccharides. Under the usual assay conditions 5 mM-glucosamine inhibited the neutral mannosidase by approx. 15%.] Preincubation of the 'precursor' enzyme for 10 min at 60 °C resulted in the loss of about 55% of the activity, but in the presence of glucosamine this loss did not occur (Fig. 3a). With 1.4 mM- CoCl_2 in the absence of glucosamine there was a 3-fold increase in activity after 1 min of preincubation, but this rise in activity declined to about 1.7-fold at 3 min and about 30% of the original activity had been lost at 10 min. When both 1.4 mM- CoCl_2 and 5 mM-glucosamine were present together, there was a continuous rise in activity, which reached about 3.5-fold at 9 min (Fig. 3a). [Lower concentrations of glucosamine (0.1–1 mM) were less effective, but at 3 mM it was as effective as at 5 mM.]

Similar experiments conducted with the 'product' enzyme are shown in Fig. 3(b). Preincubation at 60 °C of the 'product' enzyme resulted in its rapid inactivation, and within 5 min 95% of the activity was lost. In the

presence of 1.4 mM- CoCl_2 the rate of inactivation was slightly lower up to 3 min, but by 5 min 90% of the activity was lost. This was in contrast with the significant rise in activity at 1 min observed for the 'precursor' enzyme in Fig. 3(a). With 5 mM-glucosamine and 1.4 mM- CoCl_2 the rate of inactivation was lowest, with about 30% loss in activity at 10 min. This again is in contrast with the continuous rise in activity observed with the 'precursor' enzyme in the presence of CoCl_2 and glucosamine shown in Fig. 3(a).

Effects of temperatures of 60 °C and higher during preincubation in the presence of CoCl_2 and glucosamine

The effects of preincubation of the 'precursor' enzyme at 60, 65 and 70 °C in the presence of CoCl_2 and glucosamine for various time intervals are shown in Fig. 4. The maximum increase in activity at 60 °C occurred at 9 min, at a lower rate when compared with that at 65 °C or 70 °C, and there was a decline in activity after 15 min. At 65 °C the peak increase in activity occurred at 2–3 min at a higher rate, followed by a rapid decline. At 70 °C the peak increase occurred at 1 min, followed by

Fig. 5. Preincubation of the purified 'precursor' neutral mannosidase (10 μ g of protein in 20 mM-phosphate buffer, pH 7.5) at 60 °C for various time intervals with the indicated additions

At the end of preincubation the tubes were chilled on ice and assayed for the enzyme activity by the standard assay system with the respective metal ions (1 mM) present in all assay mixtures. The activity is expressed as percentage of control activity (activity of the enzyme preincubated at 4 °C taken as 100% in each case). ○, Enzyme alone, preincubated; ● and △, enzyme preincubated without (●) or with (△) 5 mM-glucosamine in the presence of 1.4 mM- CoCl_2 (a), 1.4 mM- MnCl_2 (b), 1.4 mM- MgCl_2 (c) or 1.4 mM- ZnSO_4 (d).

a rapid decline in activity. In the absence of CoCl_2 and glucosamine the enzyme rapidly lost its activity during preincubation at 60 °C and at 70 °C (Fig. 4 inset). These results suggested that the CoCl_2 -mediated stimulation of mannosidase occurred at a higher rate at higher temperatures, but with increasing temperature and time the 'product' mannosidase lost its activity.

Preincubation studies with the purified enzyme

When the preincubation experiments with CoCl_2 were done with the neutral mannosidase purified by immuno-adsorbent affinity chromatography, very similar results were obtained. Preincubation at 37 °C for 15 min resulted in a 6-fold activation with 1.4 mM- CoCl_2 and a 2.3-fold activation with 1.4 mM- MnCl_2 , but no activation occurred with either 1.4 mM- MgCl_2 or 1.4 mM- ZnSO_4 (results not shown). Fig. 5 shows the effect of preincubation with various metal ions with or without glucosamine on the purified enzyme at 60 °C. The purified enzyme in the absence of metal ions and glucosamine lost 90% activity in 10 min at 60 °C (Fig. 5a). With 1.4 mM- CoCl_2 , there was a 1.8-fold activation at 1 min, followed by a rapid inactivation. With both 5 mM-glucosamine and 1.4 mM- CoCl_2 there was a continuous increase in activity that reached a maximum of over 3-fold at 5–10 min. Similarly with 1.4 mM- MnCl_2 (Fig. 5b) a 1.7-fold activation at 1 min, followed by a rapid loss of activity, was observed. With both 5 mM-glucosamine and 1.4 mM- MnCl_2 there was a small activation, although to a lesser extent (1.5-fold at 5 min) compared with the activation by CoCl_2 . With 1.4 mM- MgCl_2 or 1.4 mM- ZnSO_4 (Figs. 5c and 5d) there was no activation either in the presence or in the absence of glucosamine. On the contrary, the enzyme was inactivated, but the stabilizing effect of glucosamine was evident at all time periods.

A study of the dependence of activation on the preincubation pH was carried out at six different pH values (from pH 5.5 to pH 8.0) in 20 mM-phosphate buffer in the presence of Co^{2+} . After 30 min of pre-

incubation the enzymes were dialysed at 4 °C against 20 mM-phosphate buffer, pH 7.5, followed by assay for mannosidase. A set of control enzymes preincubated in the absence of Co^{2+} were also treated similarly and the increase in mannosidase activity was plotted as a function of preincubation pH. A maximum activation at pH 7.5 and sharp falls in activation at pH 5.5 and at pH 6.0 were seen.

Direct effect of CoCl_2 at different pH values on the activity of the 'precursor' and the 'product' mannosidases

In order to determine the direct effect of CoCl_2 , if any (as opposed to the effect during preincubation), on the neutral mannosidase activity, we carried out pH-activity experiments with or without Co^{2+} both in non-chelating buffers and in citrate/phosphate buffer. As shown in Fig. 6 both the 'precursor' and the 'product' enzymes were activated by 1 mM- CoCl_2 (included in the assay mixture) significantly at pH 5.0 and at pH 5.5. Direct activation by Co^{2+} was relatively insignificant above pH 5.5. Similar results were obtained when the experiments were carried out in citrate/phosphate buffer (results not shown).

Direct effects of Co^{2+} at various concentrations and on the time course of activity

With different concentrations of Co^{2+} (from 0 to 5.7 mM) in the standard assay mixture the maximum activity of 155% by the precursor mannosidase was seen at approx. 0.56 mM, above which concentration there was a continuous fall in activity to 125% at 2.8 mM (results not shown). This suggested that Co^{2+} did not bind to a saturable site that affects the activity of the enzyme. Time-course studies with both 'precursor' and 'product' mannosidases in the presence and in the absence of Co^{2+} (1 mM) in the standard assay mixture showed a linear increase in the concentration of *p*-nitrophenol liberated up to 60 min of incubation (results not shown), suggesting that Co^{2+} did not remove an

endogenous inhibitor of mannosidase in a time-dependent fashion. The linear increase also suggested that in the presence of the substrate *p*-nitrophenyl mannoside the preincubation effect of Co^{2+} did not occur.

Effect of preincubation with CoCl_2 in the presence of various proteinase inhibitors

In order to explore the possibility that a metal-ion-dependent proteinase was responsible for the time- and temperature-dependent activation of the neutral mannosidase during preincubation, we tested the effect of several known proteinase inhibitors [bacitracin, puromycin, phenylmethanesulphonyl fluoride (each 1.4 mM), bestatin, pepstatin (each 0.1 mM), aprotinin (200 units) and trypsin inhibitors (10 μg)] during preincubation of the enzyme with 1.4 mM- CoCl_2 at 37 °C up to 30 min. Except for bacitracin, none of the inhibitors could prevent the Co^{2+} -mediated increase in the activity. The proteinase inhibitors themselves had no significant effect on mannosidase activity when added at the time of assay. Inhibition by bacitracin at 1.4 mM during preincubation with CoCl_2 at 37 °C and at 60 °C was found to be variable and ranged from 45 to 80 % depending upon the purity of the enzyme. At a lower (0.7 mM) or a higher (2.7 mM) concentration the extent of inhibition was very similar. The bacitracin inhibition could not be overcome by an excess of CoCl_2 (7 mM) during preincubation. Moreover, bacitracin had no inhibitory effect on the direct activation by Co^{2+} of 'precursor' mannosidase at pH 5–5.5.

Aminopeptidase activity in the enzyme purified by immunoabsorbent chromatography

With leucine β -naphthylamide, tyrosine β -naphthylamide or leucine *p*-nitroanilide as substrate we were able to detect an aminopeptidase activity in the purified enzyme when it was incubated at 37 °C or 60 °C. The plot of pH versus activity studied at six different pH values (from pH 5.5 to pH 8.0) in phosphate buffer with leucine β -naphthylamide as substrate gave a bell-shaped curve with an optimum pH of 7.0 for the aminopeptidase. Product formation was linear with time up to 60 min both at 37 °C and at 60 °C in the presence and in the absence of 1 mM- CoCl_2 . When the β -naphthylamides were used as substrates the aminopeptidase was activated by 1 mM- CoCl_2 2-fold at 37 °C and 6-fold at 60 °C. With leucine *p*-nitroanilide as the substrate it was activated by 1 mM- CoCl_2 about 2-fold at 37 °C and 4-fold at 60 °C. The specific activity for the hydrolysis of leucine *p*-nitroanilide was 1.45 $\mu\text{mol/h}$ per mg of protein at 60 °C and 0.3 $\mu\text{mol/h}$ per mg of protein at 37 °C in the presence of 1 mM- CoCl_2 , indicating that the aminopeptidase could withstand high temperatures of incubation. The aminopeptidase activity with leucine β -naphthylamide as substrate was not activated significantly by 1 mM- MnCl_2 and was inhibited by 1 mM- ZnSO_4 . It was not inhibited by 1 mM-puromycin, 1 mM-phenylmethanesulphonyl fluoride, 0.25 mM-bestatin or 0.25 mM-pepstatin, but bacitracin at 1 mM caused 15 % inhibition.

There was no detectable carboxypeptidase activity in the purified enzyme preparation when assayed at 37 °C and at 60 °C in the presence and in the absence of Co^{2+} .

In one experiment we heated the purified enzyme at 60 °C for 15 min in the absence of glucosamine, and found that the heated enzyme had completely lost the mannosidase activity but retained the aminopeptidase

Fig. 6. pH-activity profiles of the 'precursor' neutral mannosidase (—) and 'product' neutral mannosidase (---) assayed in the presence (●) and in the absence (○) of 1 mM- CoCl_2 .

Buffers (100 mM) under standard assay conditions were used. Activity is expressed as μmol of *p*-nitrophenol liberated/h. Sodium acetate/acetic acid buffer (pH 5.0–6.0), Mes/NaOH buffer (pH 6.0–7.0) and Hepes/NaOH buffer (pH 7.0–7.5) were used. The same amounts of 'precursor' and 'product' mannosidase protein were used in each assay.

activity. When this heated enzyme was added to the 'precursor' mannosidase during preincubation with Co^{2+} there was an additional increase (from 200 to 235 %) in the stimulation of mannosidase activity.

T.l.c. and *N*-terminal amino acid analysis

T.l.c. was carried out as described in the Materials and methods section on the enzyme purified by immunoabsorbent chromatography. The results consistently showed that when the enzyme was preincubated with CoCl_2 at 60 °C for 1 min at least three purple spots were visible on the t.l.c. plates after spraying with ninhydrin. One of the spots had a mobility similar to that of glutamic acid, glutamine or alanine (R_F 0.49) and the other had a mobility similar to that of leucine or isoleucine (R_F 0.63). The third spot (R_F 0.26) did not correspond with any of the 15 standard amino acids used for comparison. The control samples did not show these spots. No ninhydrin-positive spot on t.l.c. plates was seen when the enzyme was preincubated with MgCl_2 or ZnSO_4 . When the plates were sprayed with Cu^{2+} /ninhydrin reagent to differentiate between peptides and amino acids (Ganapathy *et al.*, 1981) there was no appearance of bright yellow spots, suggesting the absence of peptides.

N-Terminal amino acid analysis of both 'precursor' and 'product' enzymes, revealed a major fluorescent spot on the polyamide sheet under u.v. light corresponding to dansylalanine. Traces of other fluorescent spots were also seen, possibly arising from the contaminating proteins in the purified enzyme.

Other characteristics of the 'precursor' and the 'product' mannosidases

Both the 'precursor' mannosidase and the 'product' mannosidase obtained after preincubation with Co^{2+} at 37 °C as well as at 60 °C gave similar elution profiles on Sephadex G-200 gel filtration and showed similar rates of migration on SDS/polyacrylamide-gel electrophoresis, supporting the view that no significant change in the M_r value of the 'product' enzyme had occurred. Moreover, the 'product' mannosidase did not show any new protein bands other than those seen with the 'precursor' mannosidase on SDS/polyacrylamide-gel electrophoresis. The 'product' enzyme had approximately the same K_m value (0.1 mM) as the 'precursor' enzyme, but its $V_{\max}$ value was about 3.4-fold higher than that of the 'precursor' enzyme when assayed with Co^{2+} . It also showed similar immunoprecipitation characteristics to those of the 'precursor' enzyme when the antibody raised against a partially purified neutral mannosidase was used at different concentrations.

Experiments with $^{57}\text{CoCl}_2$

The 'precursor' mannosidase was preincubated with $^{57}\text{CoCl}_2$ (25 μCi) under the same conditions as were required for the activation of the enzyme. It was subsequently subjected to Sephadex G-75 gel filtration (25 cm $\times$ 1.5 cm column, equilibrated and eluted with 0.05 M-NaCl/20 mM-phosphate buffer, pH 7.5, at 4 °C) or SDS/polyacrylamide-gel electrophoresis. The fractions from the gel-filtration column as well as the gel slices after electrophoresis were monitored for radioactivity. There was no evidence for any radioactivity bound to the enzyme.

Evidence to discount the involvement of a Co^{2+} -dependent phosphatase

The possibility of the involvement of a Co^{2+} -dependent phosphatase in the activation of the neutral mannosidase was ruled out because the presence of inhibitors of alkaline phosphatase and phosphoprotein phosphatase (Goodno, 1979; Li *et al.*, 1979), such as inorganic phosphate (20 mM), sodium pyrophosphate, NaF or vanadate (each 1 mM), during preincubation with Co^{2+} failed to prevent the activation.

DISCUSSION

The present studies suggest, but do not prove, that a less active form of the purified brain neutral mannosidase is converted into a more active form by preincubation with Co^{2+} at 37 °C or at 60 °C. Several lines of evidence suggest the involvement of an exopeptidase, most probably an aminopeptidase, in the activation process, as follows. (1) The enhanced activity of the neutral mannosidase after preincubation with Co^{2+} was permanent and did not disappear on removal of free Co^{2+} by dialysis or gel filtration on Sephadex G-200, thereby suggesting a change in the enzyme molecule as such. (2) The stimulation by Co^{2+} occurred only at 37 °C or higher temperatures, but not at 4 °C. (3) The stimulation was time-dependent. (4) Bacitracin, an aminopeptidase inhibitor, could partly prevent the Co^{2+} -mediated activation. (5) An aminopeptidase activity, which was active at 37 °C and at 60 °C and which could be stimulated several-fold by Co^{2+} , was found to be present in the purified enzyme.

The inability to remove the aminopeptidase activity from the purified mannosidase suggested that they have closely similar physical properties. (6) There was evidence for the release of amino acids from the enzyme after preincubation with Co^{2+} . The apparently similar M_r values of both the 'precursor' and 'product' enzymes, as observed by SDS/polyacrylamide-gel electrophoresis and gel filtration, indicated that any change in M_r caused by the release of amino acid residues was too small to be detected by these procedures. The presence of the same *N*-terminal amino acid (alanine) in the 'precursor' and 'product' enzymes might have been coincidental. It is possible that the alanine in the 'product' enzyme was originally present a few amino acid residues away from the *N*-terminus of the 'precursor' enzyme, and that the specificity of the aminopeptidase action prevented it from removing the alanine in the 'product' enzyme because of an unfavourable amino acid adjacent to it on the *C*-terminus. (7) The possibility of a time- and temperature-dependent irreversible binding of Co^{2+} to the enzyme resulting in its activation was discounted because there was no evidence for the binding of $^{57}\text{CoCl}_2$ to the enzyme. Other experiments excluded the possible involvement of a Co^{2+} -dependent phosphatase in the activation. The possibility of a permanent conformational change in the enzyme resulting in its activation caused by a reversible binding of Co^{2+} cannot be completely excluded.

The aminopeptidase activity present in the neutral mannosidase is activated by Co^{2+} , similarly to observations made with several other aminopeptidases from brain tissue. Some of these aminopeptidases are also known to be inhibited by bacitracin, and their susceptibility to inhibition by puromycin, bestatin and phenylmethanesulphonyl fluoride depends on the source and preparation method of the enzyme (Schnebli *et al.*, 1979; Hersh & McKelvy, 1981; Hersh, 1981; Wagner *et al.*, 1981; Hui *et al.*, 1982; Shimamura *et al.*, 1983; Hersh, 1985). The resistance to inactivation at high temperatures shown by the aminopeptidase activity present in the neutral mannosidase is similar to observations made with the kidney leucine aminopeptidase (stable at 65 °C) (Pfleiderer, 1970) and a brain aminopeptidase (optimum temperature of 40 °C and stable at 45 °C) (Wagner *et al.*, 1981). A liver aminopeptidase has been found to be stimulated only by Co^{2+} maximally at 50 °C (Garner & Behal, 1974).

There were several similarities in the activation process of the 'precursor' mannosidase and the properties of the aminopeptidase, suggesting that the activation process involves the action of the same aminopeptidase. Thus both were stimulated by Co^{2+} but not by Mg^{2+} or Zn^{2+} , both were resistant to high temperatures, and both were inhibited to variable extents by bacitracin but not by other proteinase inhibitors. However, there were some dissimilarities. Mn^{2+} , although it did not stimulate the aminopeptidase activity, was able to activate the neutral mannosidase to a smaller extent in comparison with Co^{2+} . Co^{2+} was an absolute requirement for the activation process, but the aminopeptidase activity was detectable even in the absence of Co^{2+} . The pH optimum of the aminopeptidase was 7.0, whereas the activation occurred maximally at pH 7.5. These differences and also the lesser susceptibility to inhibition by bacitracin of the aminopeptidase, could be attributed to differences in the nature of the substrates involved (Dahlmann *et al.*, 1985).

In comparison with the lysosomal acid glycosidases, knowledge of the processing of non-lysosomal neutral glycosidases is scanty. Bischoff & Kornfeld (1983, 1986) isolated from rat liver endoplasmic reticulum a neutral α -D-mannosidase that was similar to the soluble neutral mannosidase, acted on *p*-nitrophenyl mannoside, had a pH optimum of 6.5, did not bind to concanavalin A and was stabilized by Co^{2+} . They suggested that the endoplasmic-reticulum neutral mannosidase gave rise to the soluble neutral mannosidase by the action of proteinases that were insensitive to many known proteinase inhibitors. Similarly an α -glucosidase II (pH optimum 6.8) from rat liver microsomal fraction has been shown to be converted by controlled proteolysis into a fully active form, and the action of an endogenous proteinase in the cleavage has been implicated (Hino & Rothman, 1985). In conclusion, our studies clearly indicate that the mechanism of Co^{2+} stimulation of neutral α -D-mannosidase is more complex than just a direct activation. This is also reflected in papers by different investigators reporting on stabilization or activation of the enzyme by Co^{2+} (Shoup & Touster, 1976; Snaith, 1977; Bischoff & Kornfeld, 1983).

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