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PURIFICATION AND METAL ION ACTIVATION OF AN AMINOPEPTIDASE (AMINOPEPTIDASE II) FROM *BACILLUS STEAROTHERMOPHILUS*

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SUMMARY

1. One of the aminopeptidases (aminopeptidase II) of the obligate thermophilic bacterium *Bacillus stearothermophilus* has been purified by gel filtration and chromatography on DEAE-Sephadex and SE-Sephadex. Its molecular weight and isoelectric point are 80 000–100 000 and 4.3, respectively.

2. The enzyme is located in the cytoplasm of the bacterium, which also contains a carboxypeptidase and a D-aminopeptidase.

3. Aminopeptidase II is a metal ion (M^{2+})-dependent enzyme, and no ions other than Co^{2+} or Zn^{2+} were found to give an active ESM^{2+} complex with the substrate L-leucine-*p*-nitroanilide.

4. Kinetic data show that with Co^{2+} the formation of the ESM^{2+} complex is a 2-step process with the substrate molecule binding only to the ECo^{2+} complex. This activation is very fast.

5. In the presence of both Mn^{2+} and Co^{2+}, an inactive $ESMn^{2+}$ complex can be formed. Kinetic experiments indicate separate binding sites for the metal ion and the substrate molecule.

6. A lag in the reaction between apoenzyme and substrate was observed at low concentrations of Co^{2+} and Zn^{2+}. Beside being involved in the hydrolysis of the substrate, these ions probably bring about a structural change in the enzyme protein. Other ions such as Mn^{2+}, Cd^{2+} and Ni^{2+} can also activate the apoenzyme in a similar way without having a catalytic function.

INTRODUCTION

The obligate thermophilic bacterium *Bacillus stearothermophilus* contains at least three different aminopeptidases (α-aminoacylpeptide hydrolases, EC 3.4.11), which can be differentiated on the basis of their molecular weights and substrate specificities. An enzyme of high molecular weight (400 000) called aminopeptidase I which is active against L-leucine-*p*-nitroanilide (LNA) has been studied in detail by

Abbreviations: LNA, L-leucine-*p*-nitroanilide. TNBS, trinitrobenzene sulfonic acid. HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid. NA, p-nitroaniline.

Zuber and co-workers [1, 2]. This enzyme was found to contain both cobalt and zinc and to consist of several subunits [3, 4]. Another aminopeptidase, aminopeptidase II, has a lower molecular weight, and is less heat stable than aminopeptidase I [5]. Aminopeptidase II is also active towards LNA, whereas the third peptidase is inactive against this substrate.

We have purified the aminopeptidase II enzyme and studied its activation by metal ions. Such ions may bring about conformational changes in the enzyme protein which are necessary for its activity, and they may also take part in the catalytic reaction [6]. Our kinetic data show that both these functions are important in activation of the aminopeptidase II enzyme.

MATERIALS AND METHODS

Bacterial strain and cultivation

The bacterium used in this study was *B. stearothermophilus*, NCIB 8924, kindly supplied by Professor H. Zuber, Zürich. This is an obligate thermophilic organism which grows at 55 °C but not at 37 °C. It was grown in a medium of the following composition: 3.5 g NaCl, 1.32 g KH_2PO_4, 3.68 g K_2HPO_4, 1.5 g yeast extract (Difco), 5.0 g peptone (Difco), 1.5 g meat extract (Difco) per l distilled water; pH of the medium was 7.0.

Large batches of bacteria were grown at 55 °C in a 20-l glass bottle containing 15 l of aerated medium and provided with vortex stirring. 500 ml of an actively growing culture was used as inoculum and 1 ml Polyglycol 2000 (Dow) was added to prevent foaming. After 5–7 h, when the culture was at the end of the exponential phase, the bacteria were harvested by centrifugation in the cold. The yield was about 3 g wet cells/l.

Determination of enzyme activities

In the enzyme purification work, hydrolysis of L-leucine-*p*-nitroanilide (Merck) was followed in 0.05 M Tris–HCl buffer, pH 7.5, at a substrate concentration of 0.5 mM in the presence of 1.0 mM $CoCl_2$. The initial increase in absorbance was determined at 410 nm in a Hitachi Model 124 spectrophotometer equipped with temperature control and a recorder.

Kinetic experiments were done by adding 10 μl of enzyme to 3 ml of the assay mixture, and the change in absorbance was converted to concentrations by using a molar absorbance coefficient of 10 000 $M^{-1} \cdot cm^{-1}$ for NA. As shown in Fig. 1, there is a linear relationship between reaction rate and enzyme concentration up to at least 1 μg/ml under these assay conditions, and most experiments were done at a final enzyme concentration of 0.5 μg/ml.

Degradation of peptides was assayed by reacting the liberated amino acids with trinitrobenzene sulfonic acid (TNBS) (Sigma) in the presence of Cu^{2+}, which blocks the amino groups of the peptides [7]. 10-μl samples of enzyme were added to 50 μl of a 2 mM solution of each peptide in 0.05 M Tris–HCl, pH 8.0, containing 1 mM Co^{2+}. After 5 min at 55 °C the reaction was interrupted by immersing the tubes in ice water, and 1 ml of 2.7 mM TNBS and 0.5 mM Cu^{2+} in 0.1 M $Na_2B_4O_7$ was added. After incubation at 23 °C for 30 min, the absorbance was measured at 420 nm.

Carboxypeptidase activity was determined with the tricyclohexylamine salt of

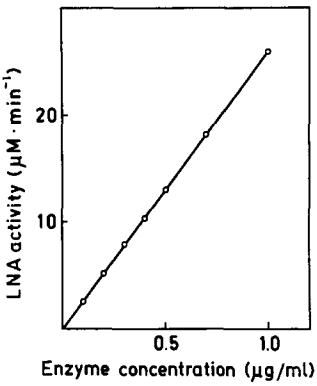


Fig. 1. Effect of aminopeptidase concentration on the initial rate of hydrolysis of LNA measured at 55 °C and a substrate concentration of 0.5 mM in the presence of 1.0 mM Co^{2+}.

Z–L-Ala–L-Trp as substrate. 10 μl of enzyme was added to 100 μl of a 17 mM solution of the substrate in 0.05 M Tris–HCl, pH 8.0, containing 1 mM Co^{2+}. After incubation at 55 °C for 2 h, the mixture was reacted with ninhydrin according to the procedure of Yemm and Cocking [8] and the absorbance read at 570 nm.

Determination of protein and RNA

Protein was determined by the method of Lowry et al. [9] using bovine serum albumin as standard. RNA was determined by the method of Mejbaum [10] using D-ribose as standard.

Separation methods

Chromatography experiments on Sephadex, DEAE-Sephadex A-50 and SE-Sephadex C-50 (Pharmacia Fine Chemicals, Uppsala) were done under conditions described in the legends to the figures.

Purity of the enzyme was tested by polyacrylamide electrophoresis described by Hjertén et al. [11]. The gel had a total concentration of 7% and the degree of cross-linking was 5%. The buffer was 0.025 M Tris–HCl, pH 8.0, and the current density 3 mA/cm^2.

The isoelectric point of the aminopeptidase was determined in a polyacrylamide gel slab according to the method of Vesterberg [12]. Staining was done with Coomassie brilliant blue [13].

Reagents and buffers

All chemicals were of reagent grade, and solutions were tested for metal contamination by 0.01% dithizone in carbon tetrachloride. When necessary, EDTA was added to a final concentration of 1–3 μM to bind contaminating metal ions. All solutions contained 0.02% NaN_3 to prevent microbial growth and they were stored in polyethylene bottles.

N-2-Hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) was obtained from Sigma.

RESULTS

Purification

One of the aminopeptidases of *B. stearothermophilus* (aminopeptidase I) has a high molecular weight, and is associated with the cell membrane [1]. Aminopeptidase II, on the other hand, appears to occur in the cytoplasm since it was readily solubilized by lysing the cells with lysozyme. Table I shows the result of an experiment

TABLE I

EFFECT OF ULTRACENTRIFUGATION ON THE CELL-FREE BACTERIAL EXTRACT

The extract was obtained by treatment with lysozyme as described in Results.

Extract	Activity ($\mu M \cdot min^{-1} \cdot ml^{-1}$)	Protein (mg/ml)	RNA (μg/ml)
After centrifugation 10 000 × *g*, 30 min	1400	3.3	195
After centrifugation 100 000 × *g*, 120 min	1400	1.8	85

in which a suspension containing about 0.1 g cells per ml 0.05 M Tris–HCl buffer, pH 7.2, was treated at 20 °C with 50 μg lysozyme per ml for 30 min. Most cells had then lysed, and the suspension was centrifuged to remove cell debris. Enzyme activity, protein and RNA content of the extract were determined before and after ultracentrifugation. No change in aminopeptidase activity was obtained, whereas both the protein and RNA concentrations decreased to a similar extent indicating sedimentation of the ribosomes, with the enzyme in solution.

A procedure was worked out for obtaining an enzyme preparation which was virtually free from contaminating protein. It involves the following steps which were all performed at about 4 °C using buffer solutions containing 1 mM Co^{2+}. To minimize the effect of proteases present in the bacterial extract, the purification was carried out as rapidly as possible.

Step 1: Extraction of bacteria. 50–100 ml of bacteria (0.5 g wet weight/ml) suspended in 0.05 M Tris–HCl buffer, pH 7.2, were sonicated for 5 min in a Branson Sonifier (Model B12) at 100 W. After centrifugation, the sediment was resuspended in buffer and sonicated once more. The pooled supernatant solutions were concentrated to about 100 ml in a Diaflo cell containing a PM-10 membrane.

Step 2: Gel filtration on Sephadex G-100. The crude bacterial extract was chromatographed on a column of Sephadex G-100 in 0.05 M Tris–HCl, pH 7.2. The elution pattern of one such experiment is illustrated in Fig. 2, which shows that the bacteria contain several peptidases. The high molecular weight aminopeptidase (aminopeptidase I) was eluted with the void volume material, whereas the aminopeptidase II enzyme was more retarded. A broad peak of carboxypeptidase activity was obtained, and an enzyme capable of splitting D-Leu–Gly was also detected. Fractions 54–66 were pooled and concentrated to 40 ml in a Diaflo cell.

Step 3: Chromatography on DEAE-Sephadex A-50. Solid NaCl was added to the pooled solution from Step 2 to give a final concentration of 0.1 M, and the

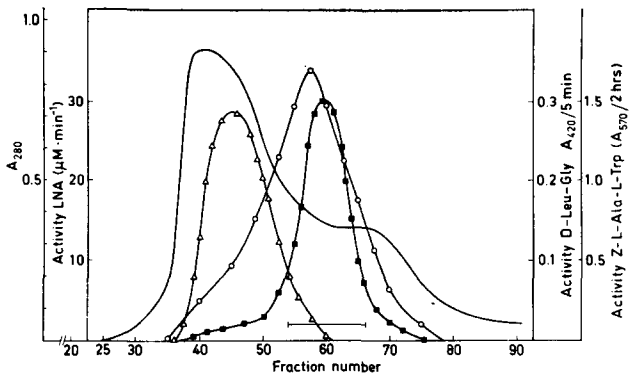


Fig. 2. Fractionation of the crude bacterial extract on Sephadex G-100. The column (7 cm × 60 cm) was equilibrated and eluted with 0.05 M Tris–HCl, pH 7.2, 1 mM Co^{2+}. Sample: 100 ml bacterial extract (2.1 g protein). Fraction volume: 15 ml. Flow rate: 20 ml/h. ———, $A_{280\ nm}$ (Uvicord (LKB) 3 mm cell); ■—■, activity towards LNA expressed as μM·min^{-1}; ○—○, activity towards D-Leu–Gly measured at 420 nm; △—△, carboxypeptidase activity measured at 570 nm.

material chromatographed on DEAE-Sephadex A-50 equilibrated with 0.1 M NaCl in 0.05 M Tris–HCl, pH 7.2. The column was eluted with a linear gradient of 0.1–0.8 M NaCl in the same buffer (Fig. 3) and Fractions 139–146 pooled.

Step 4: Chromatography on SE-Sephadex C-50. The material from Step 3 was dialyzed against 0.05 M sodium acetate buffer, pH 4.7, and chromatographed on SE-Sephadex C-50 equilibrated with the same buffer. The column was eluted with a linear gradient of NaCl up to 0.5 M (Fig. 4). Fractions 97–100 were pooled and dialyzed against 0.05 M Tris–HCl, pH 7.2.

Some properties of the purified enzyme

All subsequent experiments were done with holoenzyme purified by the procedure described above and unless otherwise stated, it contained cobalt as metal.

It was possible to store the enzyme at about 4 °C for a year with only 10%

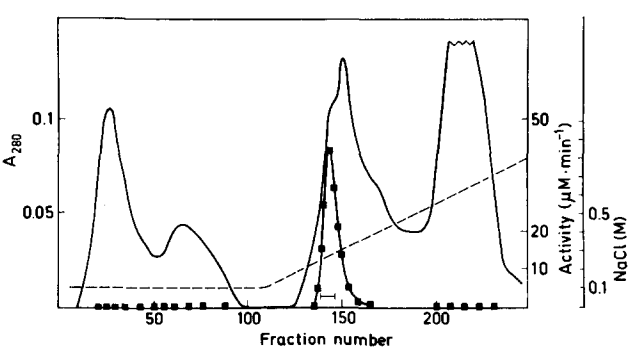


Fig. 3. DEAE-Sephadex chromatography of the aminopeptidase from Step 2. The column (3 cm × 12 cm) was equilibrated with 0.05 M Tris–HCl, pH 7.2, 0.1 M NaCl, 1 mM Co^{2+}, and eluted with a linear NaCl gradient, 0.1–0.8 M. Sample: 40 ml (0.33 g protein). Fraction volume: 5 ml. Flow rate: 20 ml/h. ———, $A_{280\ nm}$ (Uvicord); ■—■, activity towards LNA expressed as μM·min^{-1}; — — —, NaCl concentration.

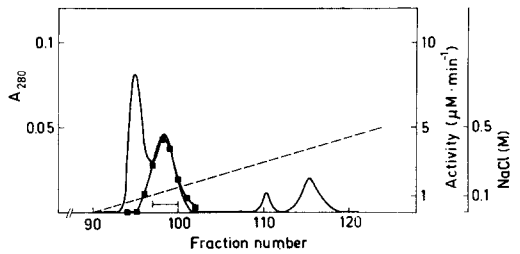


Fig. 4. SE-Sephadex chromatography of aminopeptidase from Step 3. The column (3 cm × 12 cm) was equilibrated with 0.05 M sodium acetate buffer, pH 4.7, 1 mM Co^{2+}, and eluted with a linear gradient of NaCl up to 0.5 M. No protein and LNA activity were found up to Fraction 90. Sample: 10 ml (18 mg protein). Fraction volume: 6 ml. Flow rate: 17 ml/h. Symbols as in Fig. 3.

loss in activity. Polyacrylamide electrophoresis showed one strong and two weak bands after staining with Coomassie brilliant blue, but attempts at removing these small amounts of impurities by preparative gel electrophoresis resulted in less stable preparations.

The molecular weight of the enzyme was estimated by gel filtration on calibrated columns of Sephadex G-100 and G-200 and found to be 80 000–100 000. Its isoelectric point was found to be 4.3.

Determination of the apparent pK_{HS} value for LNA

It was important to know the pK_{HS} value for LNA in order to estimate the degree of dissociation of the substrate at the pH used in the kinetic experiments. 20 ml of a solution of 0.8 mM LNA in 0.1 M NaCl was therefore titrated with 0.05 M NaOH in the pH range 7–9 at 23 °C. A Radiometer 26 pH Meter with expanded scale was used to read the pH after addition of 10-μl portions of the NaOH. From the titration curve a pK_{HS} value of 7.8 was obtained. The kinetic experiments were done at pH 7.0, and it can be estimated that about 40% of the substrate is in the unprotonated form under these conditions. As the enzyme reaction was found to be independent of the ionic strength, it is reasonable to assume that only the unprotonated form of the substrate is bound to the enzyme.

Estimation of the apparent dissociation constant of Co^{2+}–LNA

The degree of interaction between LNA and Co^{2+} may be estimated by determination of the dissociation constant:

$$K_{MS} = \frac{[M^{2+}][S]}{[MS^{2+}]}$$

Because of the low solubility of LNA (maximal solubility 1 mM) it was, however, difficult to obtain an exact value for K_{MS}, and an indirect method for its determination was therefore used.

10 ml of 0.8 mM LNA in 0.1 M NaCl was titrated with 10-μl portions of 1 M Co^{2+}. The reaction was started at pH 7.8, and an almost linear decrease in pH occurred down to about pH 7.7. The ΔpH value after addition of 50 μmoles of Co^{2+} was found to be 0.05. The corresponding ΔpH value for L-leucineamide ($pK_{HS} = 7.8$;

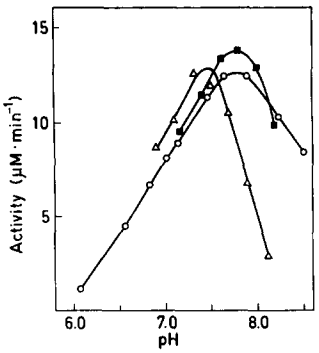


Fig. 5. pH optimum curves for the aminopeptidase in different buffers. The pH of the buffer solutions was measured at 23 °C and the assays carried out at 55 °C at a substrate concentration of 0.5 mM in the presence of 1.0 mM Co^{2+}. △—△, 0.05 M HEPES adjusted with HCl; ■—■, 0.05 M Tris adjusted with HCl; ○—○, 0.025 M imidazole and 0.025 M Tris adjusted with HCl.

K_{MS} = 10 mM) [14] was determined to be about 0.15. From these data the apparent K_{MS} for Co^{2+}–LNA was determined to be about 20 mM, assuming a 1:1 complex formation for the compounds. At higher temperatures this value will increase, and at 50 °C the constant is approx. 40 mM.

Effect of pH on enzyme activity

The hydrolysis of LNA was determined in three different kinds of buffers as illustrated in Fig. 5. An apparent pH optimum of 7.8 was obtained in 0.05 M Tris–HCl, and the same value was obtained in imidazole + Tris. Above pH 8, a coloured complex was formed between Co^{2+} and Tris which interfered with the assay. A slightly lower pH optimum, 7.4, was observed in 0.05 M HEPES buffer. These values are too high because the buffers were adjusted at room temperature and the assays carried out at 55 °C. With corrections for the temperature coefficients of Tris and HEPES (0.028 and 0.014, respectively), the true pH optimum of the enzyme is 7.0 in these buffer systems.

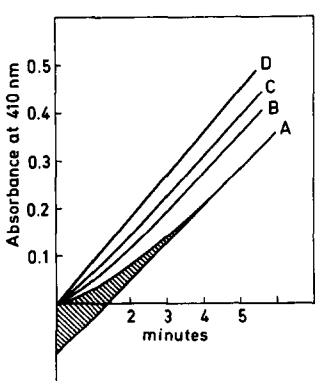


Fig. 6. Effect of preincubation of the aminopeptidase with Zn^{2+}. 10-μl samples of enzyme (150 μg/ml) dialyzed against EDTA were assayed at 55 °C without preincubation (A) and after incubation with 0.01 mM Zn^{2+} at 0 °C for 15 min (B), 30 min (C) and 60 min (D). Substrate concentration: 0.8 mM LNA in the presence of 0.15 mM Co^{2+}. The lag was estimated as the area indicated in the figure.

TABLE II

ACTIVITY OF THE AMINOPEPTIDASE II WITH DIFFERENT METAL IONS

A preparation of the apoenzyme was obtained by dialyzing 1 ml of the purified holoenzyme for 24 h against 800 ml 5 mM EDTA in 0.05 M Tris–HCl, pH 7.5, followed by dialysis against the same buffer without EDTA. The substrate concentration and the temperature of the assays were 0.5 mM and 55 °C, respectively, and the enzyme was not preincubated with the metal ions. The activity values are expressed as percentage of the activity of the holoenzyme containing Co^{2+}.

Metal ion	Concentration (mM)	Activity (%)
Co^{2+}	1.0	95
Zn^{2+}	1.0	3
Zn^{2+}	0.1	9
Mn^{2+}	1.0	0
Mn^{2+}	0.1	0
Ni^{2+}	1.0	0
Ni^{2+}	0.1	0
Ca^{2+}	1.0	0
Mg^{2+}	1.0	0
None		0

Initial reaction kinetics of the apoenzyme

When the apoenzyme, obtained after dialysis against 5 mM EDTA for 16 h, was assayed at 55 °C with 0.8 mM LNA and 0.15 mM Co^{2+}, there was a distinct lag period before a constant reaction rate was obtained. The extent of this lag was determined as the area indicated in Fig. 6. It was possible to reduce the lag by pre-incubating the enzyme at 0 °C with Zn^{2+} or Co^{2+}. Fig. 6 shows that the activation process with 0.01 mM Zn^{2+} is relatively slow, and similar curves were obtained when

TABLE III

THE EFFECTS OF METAL IONS ON THE LAG PHASE OF THE AMINOPEPTIDASE REACTION

The apoenzyme was assayed with 0.8 mM LNA and 0.15 mm Co^{2+} at 55 °C. The different metal ions were included in the assay medium. For estimation of the lag see Fig. 6.

Metal ion	Concentration (mM)	Reduction of the lag (%)
Ni^{2+}	0.1	50*
Ni^{2+}	0.01	0
Zn^{2+}	0.1	100*
Zn^{2+}	0.01	60*
Cd^{2+}	0.01	100*
Cd^{2+}	0.001	80*
Ca^{2+}	1	0
Mg^{2+}	1	0
Mn^{2+}	1	50
Co^{2+}	1	60

* Inhibition of the reaction rate also occurred.

1 mM Co^{2+} was used instead of Zn^{2+}. If a higher Zn^{2+} concentration, 0.1 mM, was included in the assay medium no lag could be detected, indicating that the activation was rapid. Table III shows that the lag period was also reduced by other metal ions, but no similar effect was observed by increasing the ionic strength of the assay medium or by adding L-leucine. 1010 mM.

Mechanism of formation of an ESM^{2+} complex

An active ESM^{2+} complex can be formed by three binary reaction pathways as shown below:

$$E + S \overset{K_1}{\rightleftarrows} ES \tag{1a}$$

$$ES + M^{2+} \overset{K_2}{\rightleftarrows} ESM^{2+} \tag{1b}$$

$$E + M^{2+} \overset{K_3}{\rightleftarrows} EM^{2+} \tag{2a}$$

$$EM^{2+} + S \overset{K_4}{\rightleftarrows} ESM^{2+} \tag{2b}$$

$$S + M^{2} \overset{K_5}{\rightleftarrows} SM^{2+} \tag{3a}$$

$$SM^{2+} + E \overset{\rightleftarrows}{K_6} ESM^{2+} \tag{3b}$$

Under equilibrium conditions, which are assumed to exist, the concentration of ESM^{2+} is independent of the pathways, leading to the following relationship for the dissociation constants:

$$K_1 \cdot K_2 = K_3 \cdot K_4 = K_5 \cdot K_6 \tag{4}$$

A series of experiments was done at 50 °C in which the initial reaction rates were determined at varied LNA and Co^{2+} concentrations. As the holoenzyme containing Co^{2+} does not give a lag in the reaction, this form of the aminopeptidase was used rather than the apoenzyme. The latter would give a lag which makes the interpretation of kinetic data difficult. The assay mixture also contained 3 μM EDTA to remove any contaminating metal ions. The results are presented as Lineweaver–Burk plots in Fig. 7.

The dissociation constant of the Co^{2+}–LNA complex is relatively large (approx. 40 mM at 50 °C), which gives a very low concentration of SM^{2+}. Under the experimental conditions only 1–2% of the substrate is therefore in complex form. Further evidence for a low concentration of SM^{2+} is that no straight lines would be obtained in Fig. 7 if the concentration of the Co^{2+}–LNA complex were not negligible.

Effect of Mn^{2+} on the reaction

Preliminary experiments showed that Mn^{2+} interfered with the binding of Co^{2+} to the enzyme, although Mn^{2+} cannot form an active ESM^{2+} complex (Table II). To investigate this reaction more closely, the experiments presented in Fig. 7 were repeated in the presence of 0.16 mM Mn^{2+}. The results are shown in Fig. 8.

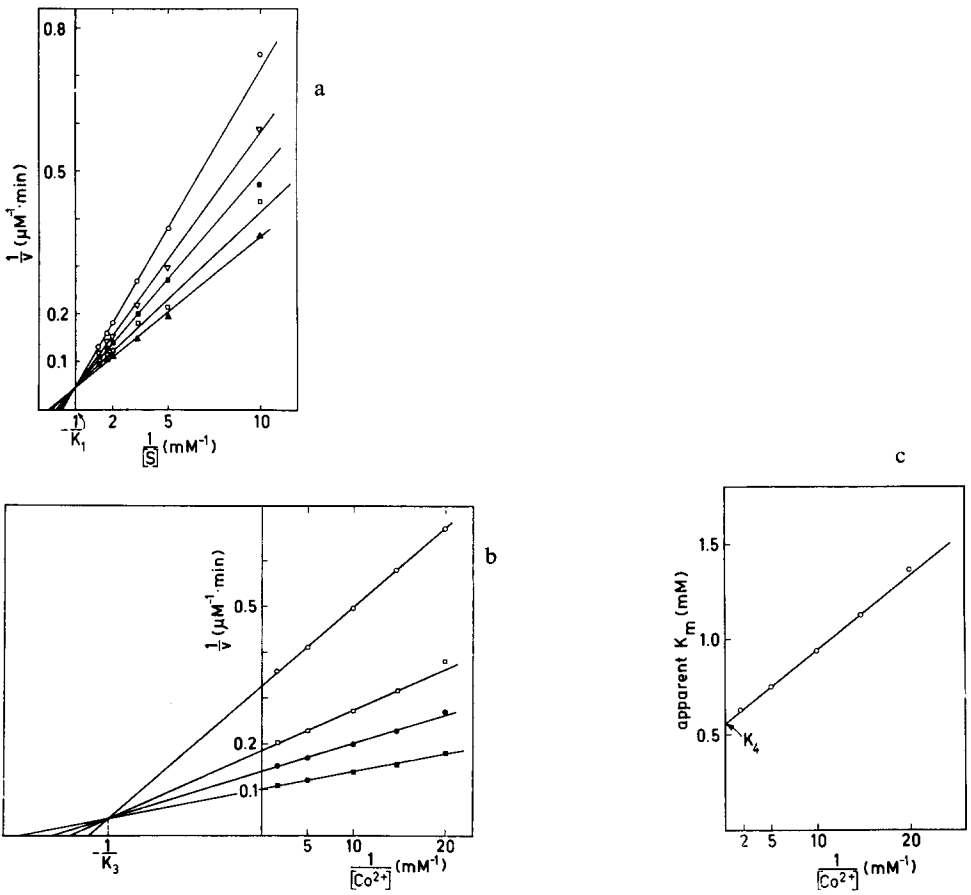


Fig. 7. Kinetics of the aminopeptidase reaction at different concentrations of LNA and Co^{2+}. 10-μl samples of enzyme (1.5 μg) were assayed at 50 °C. (a) Reciprocal rates plotted against reciprocal LNA concentrations at different Co^{2+} concentrations (mM): ○—○, 0.05; ▽—▽, 0.068; ■—■, 0.1; □—□, 0.2; ▲—▲, 0.6. (b) Reciprocal rates plotted against reciprocal Co^{2+} concentrations at different LNA concentrations (mM): ○—○, 0.1; □—□, 0.2; ●—●, 0.3; ■—■, 0.5. (c) Apparent K_m values obtained from Fig. 7a plotted against the reciprocal Co^{2+} concentration.

Determination of the dissociation constants

K_1 values were obtained from Figs 7a and 8a where the intersection of the lines gives $-1/K_1$ when projected down on the abscissa.

For determination of K_3 the data in Figs 7a and 8a were replotted in the form $1/v$ vs $1/[Co^{2+}]$ at different concentrations of S. Figs 7b and 8b show these curves, and the values for $-1/K_3$ were obtained as the intersection of the lines projected down on the abscissa.

From Figs 7a and 7b it follows that K_2 must be zero in the absence of Mn^{2+} because K_1 is ∞. The determination of K_2 in the presence of Mn^{2+} was made as indicated in Fig. 8c. The V values from Fig. 8a were replotted as $1/V$ vs $1/[Co^{2+}]$, giving $-1/K_2$ as intercept on the abscissa (see, for example ref. 15).

In the absence of Mn^{2+} the constant K_4 can be determined by plotting the

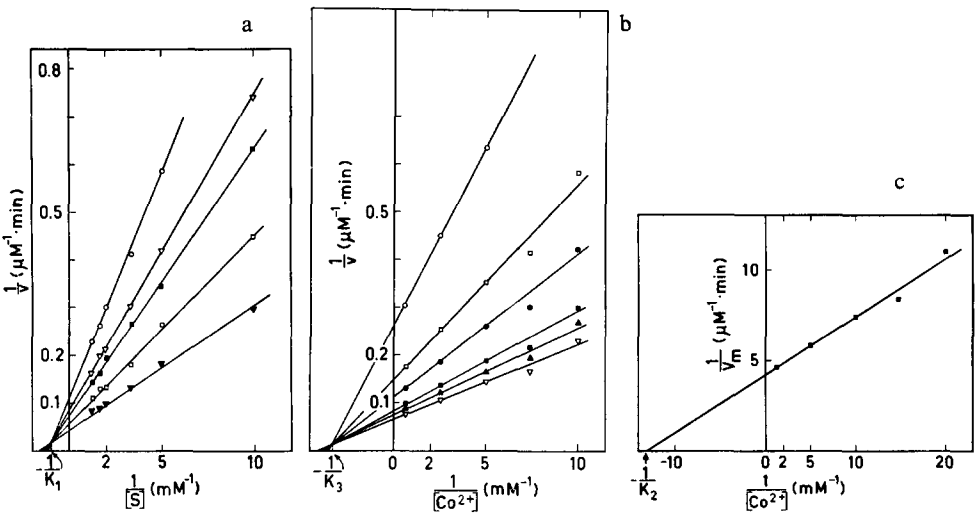


Fig. 8. Effect of 0.16 mM Mn^{2+} on the aminopeptidase reaction at different concentrations of LNA and Co^{2+}. Assay conditions are the same as described in Fig. 7. (a) Reciprocal rates plotted against reciprocal LNA concentrations at different Co^{2+} concentrations (mM): ○—○, 0.05; ▽—▽, 0.068; ■—■, 0.1; □—□, 0.2; ▼—▼, 0.6. (b) Reciprocal rates plotted against reciprocal Co^{2+} concentrations at different LNA concentrations (mM): ○—○, 0.1; □—□, 0.2; ●—●, 0.3; ■—■, 0.5; ▲—▲, 0.6; ▽—▽, 0.8. (c) Reciprocal V values in Fig. 8a plotted against the reciprocal Co^{2+} concentrations.

apparent K_m values obtained from Fig. 7a versus the reciprocal concentration of Co^{2+} (Fig. 7c). K_4 is the ordinate intercept in this figure. K_4 in the presence of Mn^{2+} was obtained from Eqn 4. The various constants are listed in Table IV. K_1 and K_4 have been corrected by multiplication with a factor of 0.4, as 40% of the substrate is in the enzymatically active form. No corrections have been made for the interaction between Co^{2+} and Tris, but this is so slight that it does not affect our conclusions.

TABLE IV

EQUILIBRIUM CONSTANTS FOR THE VARIOUS REACTIONS LEADING TO AN ACTIVE ESM^{2+} COMPLEX

The constants have been determined from the data in Figs 7 and 8 and are expressed in terms of millimolar.

Constant designation in Eqns 1 and 2	With Co^{2+} (mM)	With Co^{2+} and Mn^{2+} (mM)
K_1	∞	0.41
K_2	0	0.08
K_3	0.06	0.15
K_4	0.22	0.21

DISCUSSION

The aminopeptidase purified and studied by us has a much lower molecular weight than the aminopeptidase I enzyme studied by Zuber and co-workers [1–4],

and its Michaelis–Menten constant with LNA as substrate is 0.6 mM in comparison with the value of 8 mM reported for aminopeptidase I with the same substrate. We, therefore, conclude that our enzyme is identical with the aminopeptidase II enzyme originally described by Roncari and Zuber [1]. Its relationship with the membrane-bound aminopeptidase I is not known, but it appears to be located in the cytoplasm as it was easily solubilized by lysing the bacteria with lysozyme. Other bacterial aminopeptidases have also been reported to occur in the cytoplasm [16–18], whereas a basic aminopeptidase was reported by Matheson [19] to be associated with the ribosomes of *Escherichia coli*. Aminopeptidase II of *B. stearothermophilus* has many properties in common with "SAP I", an aminopeptidase from *Bacillus subtilis*, studied by Matsumura et al. [20]. The molecular weights, isoelectric points and metal ion requirements of the two enzymes are almost identical, but no detailed kinetic study of the metal activation of the *B. subtilis* enzyme appears to have been made.

The occurrence of a lag period in the activation of an apoenzyme by metal ions has been interpreted as the result of a conformational change induced in the protein molecule [21, 22]. We found that this process was rather slow, but the rate was increased by raising the temperature and the metal ion concentration. We suggest that metal ions have a stabilizing effect on the enzyme conformation and that this function is important in regulating aminopeptidase activity in the cell.

The results of the kinetic experiments presented in Fig. 7 can be interpreted to mean that an active ESM^{2+} complex is formed either by binding of a preformed SM^{2+} complex to the enzyme or by binding of the substrate to an EM^{2+} complex. To distinguish between these two mechanisms it is necessary to determine the constant K_6 in Eqn 3b, but direct measurement of this constant is difficult because of the low affinity of the metal ion for the substrate. From Eqn 4, K_6 can be calculated to be about 0.001 mM, which indicates that binding of SM^{2+} to the enzyme cannot be excluded. However, as mentioned earlier, the concentration of free SM^{2+} is very low compared with that of S. The contribution of SM^{2+} to the formation of the active enzyme complex must be very small. We, therefore, conclude that the main pathway is that given by Eqns 2a and 2b. Further evidence for this pathway can be seen in Fig. 8 which shows the kinetics when both Co^{2+} and Mn^{2+} are present in the assay medium. The constants given in Table IV show that there is a binding of substrate to enzyme in the presence of Mn^{2+} (K_1), and K_4 has the same value irrespective of the presence of Mn^{2+}. However, K_2 and K_3 are increased, indicating that Mn^{2+} competes with Co^{2+} at the binding site. As seen in Table II, the substrate molecule cannot be hydrolyzed when Mn^{2+} is the sole metal ion. Co^{2+} is therefore essential for hydrolysis although Mn^{2+} can form an EM^{2+} complex to which the substrate can bind.

The exact function of metal ions in the aminopeptidase is difficult to assess solely from kinetic experiments, but the following functions must be involved. Firstly, there is a relatively slow reaction between the apoenzyme and metal ions:

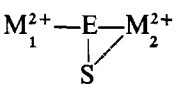
$$E + M_1^{2+} \rightleftarrows EM_1^{2+}$$

This slow activation can be accomplished by several metal ions such as Co^{2+}, Mn^{2+}, Zn^{2+}, Cd^{2+} and Ni^{2+} (Table III). Secondly, there is a rapid activation making it possible for the substrate molecule to bind to the enzyme:

$$EM_1^{2+} + M_2^{2+} \rightleftarrows EM_1^{2+}\ M_2^{2+}$$

$$EM_1^{2+}\ M_2^{2+} + S \rightleftarrows EM_1^{2+}\ M_2^{2+}\ S$$

The activated enzyme complex can be schematically represented as follows:



The binding sites for M_1^{2+} and M_2^{2+} must be different because S cannot bind to EM_1^{2+} (see K_1 in Table IV).

Our experiments do not enable us to state whether there is a direct or indirect connection between the metal ion and the substrate. Further work is necessary to settle the question.

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