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# AMINOPEPTIDASE II FROM *BACILLUS STEAROTHERMOPHILUS*

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## Summary

1. The low molecular weight aminopeptidase (aminopeptidase II) from *Bacillus stearothermophilus* cells grown at 50°C was purified to a homogeneous state.

2. Molecular weight determination by sodium dodecyl sulfate gel electrophoresis resulted in a value of 46 000 for the subunits. A molecular weight of 80 000—100 000 has been reported for the native enzyme. We therefore conclude that aminopeptidase II is a dimeric enzyme.

3. The amino-terminal sequence, the amino acid analysis and the subunit molecular weight of aminopeptidase II show no relationship to the corresponding data of aminopeptidase I.

4. Aminopeptidase II binds two $Co^{2+}$ per subunit. The dissociation constants of these ions determined by binding studies and by kinetic analysis agree within experimental error.

## Introduction

*Bacillus stearothermophilus* shows remarkable differences in its enzyme pattern, depending on growth temperature [1,2]. Cells grown at 50°C contain mainly two aminopeptidase types [3] (designated aminopeptidase I and II [4]) of different molecular weights. The high molecular weight aminopeptidase I has been characterised [4—6]. It contains two types of subunits. Although the specificity of the two subunits is different they are closely related, since they have identical molecular weights and their amino terminal sequences are homologous. Therefore we were interested to find out if also the second, low molecular weight aminopeptidase II is related with the high molecular weight enzyme. In this paper we report the purification and characterization of the aminopeptidase II as well as its comparison to aminopeptidase I.

In the course of this investigation we found that aminopeptidase II was

much more stable during the purification procedure in the presence of EDTA, although this enzyme depends on metal ions for catalytic activity [4]. This finding suggests that the apoenzyme of aminopeptidase II is remarkably stable. This is in sharp contrast to aminopeptidase I [7] or other high molecular-weight aminopeptidases such as the bovine eye-lens enzyme [8]. This stability of the apoenzyme of aminopeptidase II facilitates several types of experiments about the function of the metal ion, which should be more difficult to investigate in the case of other metal ion-dependent aminopeptidases.

Recently a paper appeared by Myrin and Hofsten [9] about the metal ion activation of aminopeptidase II. Since our results concerning this problem show some new aspects we also include them in this report.

## Experimental Procedure

Cells of *B. stearothermophilus* NCIB 8924 grown at 50°C as described elsewhere [10] were a gift of Ciba-Geigy AG, Basle, Switzerland.

Amino-acid analyses were done in a Beckman analyser. Tryptophan values were determined by the method of Edelhoch [11] or after acid hydrolysis in the presence of thioglycolic acid [12].

The system of Davis [13] was used for disc gel electrophoresis. Sodium dodecyl sulfate gel electrophoresis was performed according to Weber and Osborn [14]. Suberimidate cross-linking was performed according to Davies and Stark [15].

*Sequence analyses.* The amino terminal sequence of the native aminopeptidase II was determined in a Beckman sequencer (by L.H. Ericsson, Seattle) using a modification of the procedure of Edman and Begg [16] as described by Hermodson et al. [17]. Arg (third residue) was determined in the aqueous phase by chemical spot test. All other residues were identified by gas chromatic analysis of the silylated phenylthiohydantoin amino acid derivative [17].

The same buffer was used for the binding studies and the kinetic determination of the dissociation constant of $Co^{2+}$: 0.05 M imidazole · HCl, pH 7.5, containing 0.1 M NaCl. The buffer was kept over Chelex 100 to avoid metal contamination. Glassware was soaked in 6 M HCl and washed with ion-free water. $^{57}Co^{2+}$ delivered by I.C.N. was used as a tracer for the binding studies, which were performed in a special dialysis apparatus (Diachema AG, Ruschlikon, Zurich) designed by Weder et al. [18]. Both types of experiments were done at room temperature.

*Assay.* The hydrolysis of leucine *p*-nitroanilide was followed at 405 nm. A value of $\epsilon = 9.6 \cdot 10^6$ cm$^2$/mol was used to calculate the specific activities. An expanded-scale recorder (0.1 absorbance full scale) was used for the kinetic experiments. The routine assay mixture contained 1 mM leucine *p*-nitroanilide and 1 mM $CoCl_2$ in 0.05 M imidazole buffer at pH 7.5 (adjusted at room temperature). The test temperature was 40°C.

*Protein determination.* The protein was estimated by the method of Lowry et al. [19].

*Enzyme purification.* 500 g of wet *B. stearothermophilus* cells were routinely used for an enzyme preparation. The disruption of the cells, the $(NH_4)_2SO_4$ precipitation and the Sephadex G-150 filtration are described elsewhere [3].

*DEAE-cellulose.* The protein solution after the Sephadex G-150 step (1.2 l) was applied to a column (3.5 × 35 cm) of DEAE-cellulose (Cellex D, Bio Rad) in 0.05 M Tris buffer, pH 7.2 (all pH values were adjusted at room temperature), containing 0.5 mM EDTA. A linear gradient of 0—0.3 M NaCl was applied in a total volume of 2 l. The most active fractions were pooled and concentrated by ultrafiltration to a volume of 25 ml.

*Sephadex G-150.* The concentrated solution was desalted and further purified by passage through a Sephadex G-150 column (5 × 90 cm) in Tris buffer, pH 7.2, containing 0.5 mM EDTA.

*DEAE-Sephadex A-50.* The desalted solution was applied to a DEAE-Sephadex A-50 column in the same buffer and eluted with a linear gradient of 0.08—0.2 M NaCl. The active fractions were pooled, concentrated by ultrafiltration to 15 ml and passed through a 200-ml Sephadex G-25 column in 5 mM sodium phosphate buffer, pH 6.8, containing 0.5 mM EDTA.

*Hydroxyapatite.* A 2 × 20 cm column of hydroxyapatite (Bio-Gel HTP, Bio Rad) was used for this purification step. A linear gradient of 0.005—0.15 M sodium phosphate, pH 6.8, containing 0.5 mM EDTA was used to elute the enzyme. The total gradient volume was 560 ml.

*Preparative gel electrophoresis.* The final purification of the enzyme was achieved by electrophoresis in a Buchler poly-prep apparatus. According to the manual, the enzyme was applied in 0.05 M Tris buffer, pH 7.2, on a gel of 10.5% acrylamide. The pure fractions were again concentrated and passed through a Sephadex G-25 column which removed some low molecular weight ultraviolet-absorbing material.

*Ultracentrifugation.* The sedimentation coefficient in 0.05 M Tris buffer, pH 7.2, was determined in a MSE analytical ultracentrifuge equipped with an ultraviolet scanning system.

*Immunological studies.* Aminopeptidase I (500 μg) was dissolved in 0.3 ml phosphate-buffered saline and emulsified with 0.3 ml Freunds complete adjuvant (Difco). 0.6 ml of the emulsion was injected subcuntaneously in the rabbit at about 20 different points. After 3 weeks a second portion of 500 μg aminopeptidase I in phosphate-buffered saline was injected without Freunds adjuvant. After 5—6 weeks the antiserum was prepared and the cross-reactivity with aminopeptidase II was determined by the immune diffusion method (Ouchterlony [20], 1% Agar).

## Results

### *Enzyme purification*

Aminopeptidase II has been partially purified by Roncari and Zuber (unpublished) as well as by Myrin and Hofsten [9]. However, since structural investigations require a really pure enzyme we decided to purify aminopeptidase II to homogeneity. Table I shows the purification scheme. The enzyme was purified 585-fold resulting in an aminopeptidase with a specific activity of 234 units under our assay conditions. Fig. 1 shows the disc gels of the pure enzyme at pH 8.9 (A) as well as the gels of the sodium dodecyl sulfate disc electrophoresis at pH 7.0 (B).

During our purification procedure we realized that the enzyme became in-

TABLE I

PURIFICATION OF AMINOPEPTIDASE II

The protein was estimated by the method of Lowry et al. [19] except for the last step. The final protein concentration was determined by amino acid analysis.

| Fraction | Protein (mg) | Units | Yield | Specific activity |
|---|---|---|---|---|
| Crude extract | 56 753 | 22 800 | 100% | 0.4 |
| $(NH_4)_2SO_4$ fractionation | 16 863 | 20 100 | 88% | 1.2 |
| Sephadex G-150 | 8 004 | 16 800 | 74% | 2.1 |
| DEAE-cellulose | 1 810 | 13 700 | 60% | 7.6 |
| Sephadex G-150 | 798 | 12 500 | 55% | 15.6 |
| DEAE-Sephadex A-50 | 203 | 8 000 | 35% | 39 |
| Hydroxyapatite | 63 | 4 200 | 18.5% | 67 |
| Gel electrophoresis | 12.5 | 2 920 | 13% | 234 |

creasingly unstable unless it was purified in the presence of EDTA. However, the pure enzyme was again practially as stable in $Co^{2+}$-containing buffer as in EDTA. We therefore concluded that this instability was due to a contaminant rather than to autodigestion.

Since aminopeptidase II depends on metal ions for catalytic activity [4,9], it seemed likely that our pure enzyme (purified in the presence of EDTA) was a metal-free apoenzyme. We therefore investigated the pure aminopeptidase II by neutron activation for Zn, Co and Mn, the most common occurring metals in aminopeptidases. Our purified enzyme contained none of these metals. Nevertheless the enzyme was stable for weeks at 4°C at a reasonable high protein concentration (approx. 0.5 mg/ml).

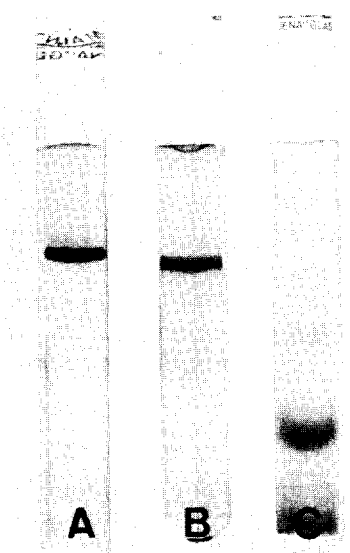


Fig. 1. Polyacrylamide gel electrophoresis of the purified aminopeptidase II. (A) Electrophoresis at pH 8.9 in a 10.5% gel. (B) Electrophoresis in the presence of sodium dodecyl sulfate at pH 7.0 in a 10% gel. (C) Electrophoresis after suberimidate cross-linking in the presence of sodium dodecyl sulfate at pH 7.0 in a 5% gel.

*Molecular weights*

Sodium dodecyl sulphate electrophoresis showed that the subunit(s) of aminopeptidase II had a molecular weight of 46 500. For comparison, the molecular weight of the aminopeptidase I subunits was also determined by this method and a value of 42 000 was obtained. A molecular weight of 80 000—100 000 for the native aminopeptidase II has been estimated by Myrin and Hofsten [9] by Sephadex gel filtration. Ultracentrifugation studies in our laboratory resulted in an *s* value of 6 S, also suggesting a molecular weight around 100 000 for a normal globular protein. We therefore concluded that aminopeptidase II consists of two most probably identical subunits. However, the attempt to confirm this number by cross-linking with suberimidate failed. Fig. 1C shows that under conditions which resulted in four clear bands for rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (not shown) only one major band, corresponding to the monomeric subunit, is visible in the case of aminopeptidase II. The band of the dimer was barely visible. There are several possible explanations of this observation e.g. the absence of appropriately spaced amino groups or a rather unstable dimeric structure which breaks down under the condition of the experiment.

*Amino-acid analyses and amino-terminal sequence*

Since aminopeptidase II contains several amino acids in rather low quantities, a minimal molecular weight could be calculated by amino-acid analyses. The best fit from five determinations resulted in a molecular weight of 45 000 which agrees favorably with the sodium dodecyl sulfate electrophoresis value of 46 500 for the subunit of aminopeptidase II. We therefore used a value of 46 000 for the further investigations.

Table II shows the amino acid analyses for the aminopeptidase II subunit as well as for the $\alpha$-subunit of aminopeptidase I (the $\alpha$-subunit of aminopeptidase I has a similar specificity as aminopeptidase II, in contrast to the $\beta$-subunit). As can be seen from Table II real differences exist between the two enzymes especially for aspartic acid, glycine, methionine, serine, tyrosine and tryptophan. The protein factor for 1 absorbance unit at 280 nm was determined for aminopeptidase II from the amino-acid analyses and is 0.65 mg/ml.

Table III shows the amino terminal sequence of the native aminopeptidase II. 10 mg of protein were subjected to sequenator analyses from two different preparations. Only one amino-acid residue was found per degradation step which demonstrates that the two subunits of aminopeptidase II are identical. No homology exists in the amino-terminal region between aminopeptidase II and the published amino-terminal sequences of the $\alpha$- and $\beta$-subunits of aminopeptidase I [5].

*Immunological studies*

Antiserum prepared against aminopeptidase I was examined for cross-reactivity with aminopeptidase II by immunodiffusion. Aminopeptidase I was used as a positive control. Dilution of the antiserum by a factor of 100 still allowed the detection of the precipitation line in the control. But aminopeptidase II at the same concentration as aminopeptidase I did not show any precipitation with undiluted serum.

TABLE II

AMINO ACID ANALYSES

| | Aminopeptidase II (subunit) | | | | Aminopeptidase I (α-subunit) | | | |
|---|---|---|---|---|---|---|---|---|
| | Determined number | | | Nearest integer | Determined number | | | Nearest integer |
| | 24 h | 48 h | 72 h | | 24 h | 48 h | 72 h | |
| Lysine | 20.4 | 20.7 | 21.1 | 21 | 26.3 | 25.6 | 25.8 | 26 |
| Histidine | 11.3 | 11.1 | 11.2 | 11 | 9.6 | 9.9 | 9.9 | 10 |
| Arginine | 20.4 | 20.2 | 20.1 | 20 | 16.9 | 17.3 | 16.9 | 17 |
| Aspartic acid | 56.3 | 55.2 | 54.2 | 55 | 35.6 | 35.7 | 36.1 | 36 |
| Threonine | 20.7 | 19.8 | 18.7 | 22 [a] | 23.3 | 22.1 | 21.0 | 25 [a] |
| Serine | 14.3 | 12.6 | 11.1 | 16 [a] | 9.3 | 8.2 | 7.3 | 10 [a] |
| Glutamic acid | 43.8 | 43.8 | 43.6 | 44 | 38.7 | 39.0 | 39.3 | 39 |
| Proline | 19.7 | 19.4 | 20.2 | 20 | 19.8 | 20.0 | 18.0 | 20 |
| Glycine | 25.4 | 25.3 | 25.7 | 26 | 38.6 | 38.8 | 39.0 | 39 |
| Alanine | 45.7 | 45.3 | 45.3 | 45 | 36.1 | 36.5 | 37.0 | 37 |
| Valine | 27.4 | 28.0 | 28.8 | 29 | 33.9 | 35.5 | 35.6 | 36 |
| Methionine | 6.0 | 5.9 | 6.1 | 6 | 11.9 | 12.3 | 12.1 | 12 |
| Isoleucine | 19.8 | 20.0 | 20.0 | 20 | 24.8 | 26.1 | 26.3 | 26 |
| Leucine | 35.8 | 35.8 | 35.2 | 36 | 29.4 | 30.6 | 30.0 | 30 |
| Tyrosine | 13.0 | 12.9 | 12.7 | 13 | 8.5 | 8.3 | 8.1 | 8 |
| Phenylalanine | 15.1 | 15.5 | 14.9 | 15 | 11.8 | 12.1 | 11.8 | 12 |
| Cysteic acid | 2.1 | | | 2 | | | | |
| Tryptophan | 6.2 [b], 6.9 [c] | | | 7 | 3.0 [b] | | | 3 |
| Total | | | | 408 | | | | 386 |
| Molecular weight of the subunits | | | | 45 500 | | | | 42 000 |

[a] Extrapolated to zero time.
[b] Thioglycolic acid method.
[c] Edelhoch method.

*Cobalt activation*

Oligomeric enzymes often tend to dissociate in very dilute solution (e.g. [21—23]) and changes in the quaternary structure can affect the kinetic constants. These effects have been carefully investigated in the case of hexokinase [24]. Such a situation complicates all types of experiments which follow the binding of a ligand. We were therefore interested to check whether a similar problem exists with aminopeptidase II. For this the dissociation constant of $Co^{2+}$ from aminopeptidase II was determined both by binding studies and by initial velocity kinetics, since Myrin and Hofsten's experiments suggested that $Co^{2+}$ and leucine *p*-nitroanilide in the uncomplexed form are the cofactor and the substrate for aminopeptidase II [9]. The difference in protein concentration required by these two methods was of the order of $10^4$. Preliminary ex-

TABLE III

AMINO-TERMINAL SEQUENCE OF NATIVE AMINOPEPTIDASE II

| | | | | 5 | | | | | | 10 | | | | | | 15 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| H — | Met — | Asn — | Arg — | Trp — | Glu — | Lys — | Glu — | Leu — | Asp — | Lys — | Tyr — | Ala — | Glu — | Leu — | Ala — | | |

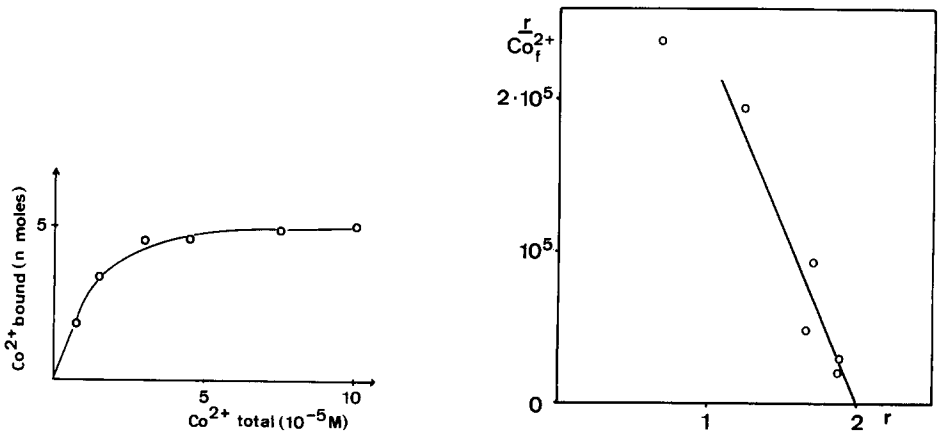


Fig. 2. Binding of $Co^{2+}$ to aminopeptidase II as studied by equilibrium dialysis. $1.34 \cdot 10^{-9}$ mol of enzyme ($2.68 \cdot 10^{-9}$ mol of subunits) were used for each determination.

Fig. 3. Scatchard plot for the $Co^{2+}$ binding of aminopeptidase II. The experimental degree of binding $r$ is defined as: concentration of bound ligand divided by the aminopeptidase II concentration. $Co^{2+}_f$ represents the concentration of free $Co^{2+}$. A concentration of $1.34 \cdot 10^{-5}$ M of aminopeptidase II subunits was used for the experiment.

periments showed that the dissociation constant for $Co^{2+}$ is below $10^{-5}$ M. We therefore decided to do our experiments in imidazole buffer, which is a weak complexing agent for $Co^{2+}$ [25] and allows the saturation kinetics of $Co^{2+}$ to be followed at somewhat higher metal ion concentrations compared to the concentration required in a non-complexing solution. This seemed necessary since the leucine *p*-nitroanilide test is rather insensitive and a final concentration of leucine (which is a good complexing agent for $Co^{2+}$ [25] of $10^{-5}$ M is required for a change in absorbance of 0.1. However, since we followed the hydrolyses on an expnaded scale recorder which made it possible to work with absorbance changes in the range of 0.02 we still obtained reasonably good kinetics with metal ion concentrations as low as $0.8 \cdot 10^{-5}$ M. Lineweaver-Burke plots for the $Co^{2+}$ activation yielded an apparent dissociation constant for $Co^{2+}$ of $0.59 \cdot 10^{-5}$ M. The apparent dissociation constant determined by binding studies in the same buffer solution was $0.44 \cdot 10^{-5}$ M. Fig. 2 shows the binding curve obtained and Fig. 3 the corresponding Scatchard plot. The molecular weight of the subunit (46 000) was used for the calculation of the experimental points, resulting in a maximal binding number of two $Co^{2+}$ per subunit or four for the dimeric enzyme aminopeptidase II. A repetition of the binding experiment with a 4-fold higher protein concentration resulted in an apparent dissociation constant of $0.4 \cdot 10^{-5}$ M and 2.2 bound $Co^{2+}$ per subunit. Since, as already mentioned above, the kinetic determination of the apparent dissociation constant cannot be very accurate, we think that the agreement is quite satisfactory.

## Discussion

Aminopeptidase II was at first described by Zuber and Roncari [4]. The enzyme was identified as an aminopeptidase primarily due to its high hydrolytic

activity towards leucine *p*-nitroanilide and towards small peptides. Kinetic investigations with oligopeptides as substrate are still missing. However, inhibition studies [26] and investigations on the $Co^{2+}$ activation [9] of this enzyme have been reported. In this paper we compared the purified aminopeptidase II with the well characterised aminopeptidase I [4—6]. No close relationship seems to exist, however. The molecular weights of the subunits are definitely different. Since both enzymes contain no carbohydrates the values determined by sodium dodecyl sulfate gel electrophoresis should be reliable. The amino-acid analyses showed real differences as well. No homology is detectable in the amino-terminal region. In addition, immunological investigations showed no cross-reactivity. However, a remote relationship is still possible. In this respect, a recent paper of Li et al. [27] is interesting. These authors detected an amino-terminal homology in anthranilate synthetases from different sources, enzymes which differ in their molecular weights up to a factor of 3 and in addition also show differences in their catalytic capabilities.

On the other hand, aminopeptidase II should be suited for a further investigation of metal activation. In this respect it offers several advantages compared with the bovine eye-lens enzyme, which is the best investigated aminopeptidase in this respect to-date [28]. These advatages are: (1) The quaternary structure is comparatively simple. The enzyme is a dimer and the subunits are most probably identical since they have identical molecular weights and only one amino-terminal sequence was obtained. (2) The activating metal ion can easily be removed and replaced by others, e.g. metal ions which are suitable for spectroscopic investigations. (3) Since kinetic measurements and binding studies at different protein concentrations resulted in similar apparent dissociation constants, we conclude that the $Co^{2+}$ binding constant for the second $Co^{2+}$ is independent of protein concentration.

Myrin and Hofsten [9] concluded from pure kinetic studies that two different types of metal ions are bound by aminopeptidase II. This result agrees with our binding studies. It is interesting that this bacterial, low molecular weight aminopeptidase binds two metal ions per subunit, the same number that was determined for the bovine lens enzyme [28] and the thermophilic aminopeptidase I [29].

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