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Subunit Structure of the Thermophilic Aminopeptidase I from *Bacillus stearothermophilus*†

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ABSTRACT: The subunit structure of the high molecular weight aminopeptidase I from *Bacillus stearothermophilus* was investigated. The enzyme contains two different subunit types in the ratio 2:1. The amino-terminal sequences of the subunits were determined. The predominant component has the sequence H-Ala-Lys-Leu-Asp-Glu-Thr-Leu-Thr-Met-Leu-Lys-Ala-Leu-Thr-Asp-Ala-. The minor component has the sequence H-Met-Asn-Glu-Glu-Thr-Leu-Gln-. The partially purified aminopeptidase preparation also contains two minor components, both of which have the same two subunit types as the major component but in different relative amounts, *i.e.*, 1:1 and most probably 5:1. Only the predominant type of subunit is necessary for aminopeptidase activity since a fully active aminopeptidase containing just this subunit can be prepared. The function of the other type of subunit is not known.

Recently we have described the purification of an aminopeptidase from *Bacillus stearothermophilus* with an apparent molecular weight of 400,000 (Zuber and Roncari, 1967; Roncari and Zuber, 1969; Moser *et al.*, 1970). This enzyme was designated aminopeptidase I as *B. stearothermophilus* also contains two low molecular weight aminopeptidases. The high molecular weight aminopeptidase I is thermostable up to 80° and has a specific quaternary structure. This enzyme could be split into subunits, which showed a molecular weight of 36,000 and appeared to be homogeneous on disc electrophoresis at alkaline pH in the presence of urea, as well as in the analytical ultracentrifuge (Moser *et al.*, 1970). Amino-terminal sequence analyses however showed two different peptide chains and we have therefore reinvestigated the subunit structure of this enzyme. These studies clearly show that aminopeptidase I consists of two different types of subunit which can combine in different ratios.

Experimental Section

Materials. B. stearothermophilus cells (strain NCIB 8924) were a gift from Ciba-Geigy AG, Basle, Switzerland. Cellogel electrophoresis strips were obtained from Chemetron, Milan, Italy. Leucine-*p*-nitroanilide was purchased from Serva, Heidelberg, Germany, and glycyl-L-leucyl-L-tyrosine from Fluka AG, Buchs, Switzerland. All other chemicals were highly purified commercial products.

Enzyme Assay. Aminopeptidase I activity was measured spectrophotometrically by following the hydrolysis of a 1 mM leucine-*p*-nitroanilide solution at 405 nm. The usual assay conditions were: 0.05 M imidazole hydrochloride buffer, containing 1 mM cobalt(II) chloride. The temperature was 55°; the pH was previously adjusted to 7.4 at room temperature. At a concentration of 1 mM leucine-*p*-nitroanilide does not saturate the enzyme. The Michaelis constant in imidazole hydrochloride buffer is 1.3 and 7 mM in Tris buffer. However at leucine-*p*-nitroanilide concentrations above 1 mM substrate inhibition becomes quite severe.

Enzyme Purification. Cells (500 g) were routinely suspended in 1.5 l. of 0.05 M Tris-HCl buffer (pH 7.2) (adjusted at room temperature) containing 1 mM cobalt(II) chloride (Tris–cobalt buffer). The cells were disrupted in a Manton–Gaulin press and centrifuged at 25,000*g* for 30 min. The supernatant was saved. Solid ammonium sulfate up to 47.5% saturation was added (295 g/l.). The pellet was discarded after centrifugation and the supernatant was brought to 75% saturation with solid ammonium sulfate (192 g/l.). The suspension was centrifuged and the supernatant discarded. The pellet was dissolved in 250 ml of Tris–cobalt buffer. The enzyme was then purified to a homogeneous state by Sephadex G-150 filtration, heat treatment, DEAE-Sephadex A-50 chromatography and preparative polyacrylamide gel electrophoresis

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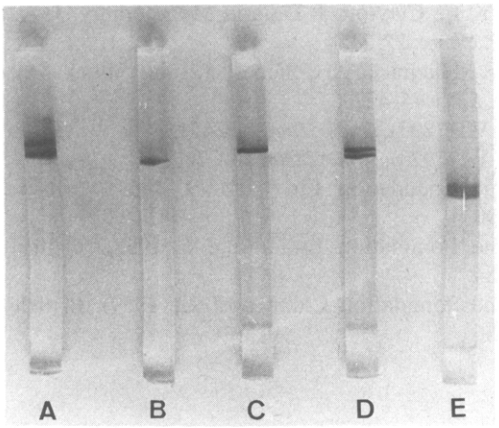

FIGURE 1: (A, B) Polyacrylamide gel electrophoresis at pH 4.5 in 6 M urea, 7.5% gel. (A) Subunits of aminopeptidase I. Disrupted α-subunit enzyme. (C, D, E) Polyacrylamide gel electrophoresis at pH 8.9 in a 5.6% gel. (C) α-Subunit enzyme. (D) Aminopeptidase I plus α-subunit enzyme. Aminopeptidase I represents the band with the higher mobility. (E) IA, IB, plus IC. The mobility increases from IA to IC.

as described by Roncari and Zuber (1969). The pure enzyme was stored in a 80% saturated ammonium sulfate solution.

Subunits of Aminopeptidase I. The precipitated aminopeptidase I was recovered from the ammonium sulfate solution by centrifugation. The pellet was dissolved and dialyzed at 4° against an 8 M urea solution in 0.025 M formate buffer (pH 4.3 at room temperature) containing 0.01 M EDTA and 5 mM cysteine or mercaptoethanol.

Electrophoresis. Polyacrylamide disc electrophoresis at pH 8.9 was performed according to the method of Ornstein (1964) and Davis (1964). For the disc electrophoresis in 6 M urea at pH 4.5 the system of Reisfeld *et al.* (1962) was modified. Urea (3.6 g) was dissolved in the following components: 2.5 ml of buffer (10 ml of 2 M potassium hydroxide, 2 ml of Temed,[1] 10 ml of acetic acid, and water up to 50 ml), 2.5 ml of 30% acrylamide, 0.8% bisacrylamide, and water up to 9.5 ml. Ammonium persulfate solution (0.5 ml; 14 mg/ml) was added to start the polymerization. The electrode buffer consisted of 31.2 g of β-alanine, 10 ml of acetic acid, and 0.875 g of cysteine hydrochloride per l. The gels were freed of persulfate by a prerun.

Electrophoresis on Cellogel strips was made at pH 4.3 (0.25 M sodium acetate, 0.25 M formic acid, 6 M urea, and 5 mM mercaptoethanol or cysteine). Staining was done according to Heil and Zillig (1970).

SE-Sephadex C-50 Chromatography. The subunits of aminopeptidase I were separated by SE-Sephadex chromatography at 4° in 0.02 M sodium formate buffer (pH 4.3, adjusted at room temperature) containing 6 M urea, 0.5 mM EDTA, and 5 mM mercaptoethanol. In a typical experiment the subunit mixture from 5 mg of this enzyme was added to a 30-ml column of SE-Sephadex. A concentration gradient from 0 to 0.5 M sodium chloride in a total volume of 300 ml of buffer was applied to elute the subunits.

Sequence Analysis. The amino-terminal sequence of each subunit was determined in a Beckman sequencer using a modification of the procedure of Edman and Begg (1967) as described by Hermodson *et al.* (1972).

[1] Abbreviation used is: Temed, *N,N,N′,N′*-tetramethylethylenediamine.

Reactivation. The enzyme subunits in urea were recombined by lowering the concentration of the urea by dilution. The urea was removed by dialysis. The enzyme (1 ml) in urea solution was added to 9 ml of 0.05 M Tris buffer (adjusted to pH 9.0 at room temperature) containing 2 mM cobalt(II) chloride and ammonium sulfate to 10% saturation. To aid the renaturation process 20 mg of the good aminopeptidase I substrate glycyl-L-leucyl-L-tyrosine was routinely added (Teipel and Koshland, 1971). The final protein concentration was in the region of 0.1 mg/ml. This mixture was then dialyzed at 4° against 1 l. of 0.05 M Tris-HCl buffer (adjusted to pH 8.0 at room temperature) containing 2 mM cobalt(II) chloride and ammonium sulfate to 10% saturation and finally against 1 l. of Tris–cobalt buffer.

Ultracentrifugation. Sedimentation coefficients in Tris–cobalt buffer were determined in an analytical ultracentrifuge, Beckman Spinco Model E.

Ratio of the Subunits. The intensity of the spots of the two subunits after Cellogel electrophoresis and Amido Black staining was determined with a Joyce-Loebl Chromoscan.

Sepharose 6B Gel Filtration. A 2.5 × 86 cm column of Sepharose 6B was used in these experiments. It was eluted with Tris–cobalt buffer collecting 5-ml fractions. The first 29 fractions represent the void volume, and fraction 48 the peak of aminopeptidase I. For comparison apoferritin (mol wt 460,000) was eluted in fraction 49 from the same column.

Results

Sequenator Analysis of Aminopeptidase I. Three different preparations of purified aminopeptidase I (4.5, 6, and 10 mg) were subjected to sequenator analysis. In each preparation two amino acid residues were observed in each cycle in approximate molar ratios of 2:1. Thus it was possible to deduce tentative amino-terminal amino acid sequences for the major (α) and the minor (β) polypeptide components (subunits) based on the quantities of the amino acid phenylthiohydantoins released. Since the analysis is only semiquantitative (Hermodson *et al.*, 1972), errors in assignment of residues to the major and minor species were a distinct possibility, especially at such residues as threonine or glutamine. However, sequenator analysis of the separated species completely confirmed the initial assignments and also confirmed Thr_5 and Gln_7 in the sequence of the β subunit which were only tentatively identified in the mixtures. The amino-terminal sequence of the α chain is H-Ala-Lys-Leu-Asp-Glu-Thr-Leu-Thr-Met-Leu-Lys-Ala-Leu-Thr-Asp-Ala-. The β chain showed the sequence H-Met-Asn-Glu-Glu-Thr-Leu-Gln.

Separation of the Subunits. Polyacrylamide gel electrophoresis of the subunits of aminopeptidase I in 6 M urea showed a single band at pH 8.9. However the enzyme subunits behaved differently in the acid pH region. Figure 1A shows the disc gel after a run at pH 4.5 in 6 M urea. Two bands of different intensity can be clearly seen. The band with the higher mobility represents the α subunit, the other the β subunit. Resolution of the two subunits could also be achieved by chromatography on SE-Sephadex. Figure 2 shows the elution pattern from such a column. Two peaks of different size can be seen; the larger has the more positive charge, a pattern that agrees with the pattern of the gel in Figure 1A.

Reactivation of Aminopeptidase I. Aminopeptidase I split into subunits could be reactivated as described in the Experimental Section. The most interesting question was now: are both subunits required for an active enzyme or is it possible to gain an active aminopeptidase consisting only of one type of

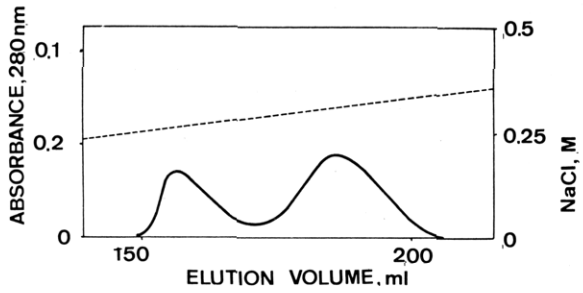

FIGURE 2: Separation of the two subunits by SE-Sephadex chromatography, as described in the Experimental Section.

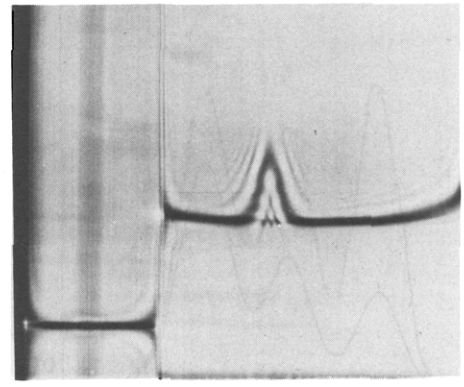

FIGURE 3: Schlieren pattern from the α-subunit enzyme.

subunit? Therefore α and β subunits were purified on SE-Sephadex and individually reactivated. It was indeed possible to regain an active aminopeptidase consisting only of α subunits. Polyacrylamide gel electrophoresis of the subunits of the reactivated enzyme in 6 M urea at pH 4.5 showed no trace of the β subunit (Figure 1B). The schlieren pattern from this α-subunit enzyme in the analytical ultracentrifuge was a symmetrical peak (Figure 3). The s_{20} value was determined and compared to the s_{20} value of the native enzyme. Table I shows the results from three runs. The s_{20} values of both enzymes agree very well, suggesting an identical molecular weight. In addition the reactivated α enzyme had a mobility in the polyacrylamide gel nearly identical with that of the native aminopeptidase I (see Figure 1C,D). On the other hand, we were not able to regain an active enzyme consisting only of β subunits. The purified β subunits precipitated during the reactivation procedure and no activity was regained.

Different Aminopeptidase I Enzymes. The reactivation experiments showed that it is possible to replace the β subunits of aminopeptidase I by α subunits. It seemed therefore possible that also *in vivo* different α:β ratios in this enzyme might exist. This idea was strengthened by the observation that during the normal purification of the enzyme by DEAE-Sephadex chromatography two shoulders could be seen on the aminopeptidase I peak (see Figure 4). Therefore the main peak (fractions 28–31) was saved and the two shoulders (fractions 24–27 and 32–38) were pooled. Figure 1E shows a polyacrylamide disc electrophoresis of these pooled fractions. There appear to be three bands on the gel with nearly identical mobilities. The pooled fractions were again chromatographed on DEAE-Sephadex and the elution pattern in Figure 5 was obtained. Three distinct protein peaks appeared and all three showed aminopeptidase activity. The three aminopeptidases were designated from left to right as IA, IB, and IC (order of elution). Aminopeptidases IA and IC were homogeneous at this stage, as judged by polyacrylamide gel electrophoresis. Aminopeptidase IB showed still two minor slow moving contaminations and was therefore completely purified by preparative polyacrylamide gel electrophoresis. All three peaks were now rechromatographed individually on DEAE-Sephadex to remove the overlaps of the different aminopeptidases. Figure 6 shows the electrophoretic subunit pattern of the three aminopeptidases on Cellogel. All three contain the two types of subunits but the ratio of the subunits is different. The intensities of the subunit spots after Amido Black staining were measured at 620 nm. To check that the intensity of the spots varied in a linear fashion with the amount of protein, a calibration run with different amounts of aminopeptidase IB was made and measurements were only taken in the region where linearity existed. The ratio of the intensities of the two subunits from two independent determinations of all three aminopeptidases can be seen in Table II. Since the color value for the reaction with Amido Black for each of the monomers may differ, the α to β ratio after Amido Black staining should not be interpreted *a priori* as the true α to β relationship. However the α to β ratio values from Table II for the three aminopeptidases can be directly compared.

TABLE I: Sedimentation Coefficients of the Reactivated α-Subunit Enzyme and the Native Aminopeptidase I.

Enzyme	Protein Concn (mg/ml)	s_{20}, Tris–Cobalt (S)
Reactivated α-subunit enzyme	1.4	15.95
	1.0	16.1
Aminopeptidase I	1.1	16.0
Aminopeptidase I	0	16.5[a]

[a] $s_{20,w}$ value, determined by Moser *et al.* (1970).

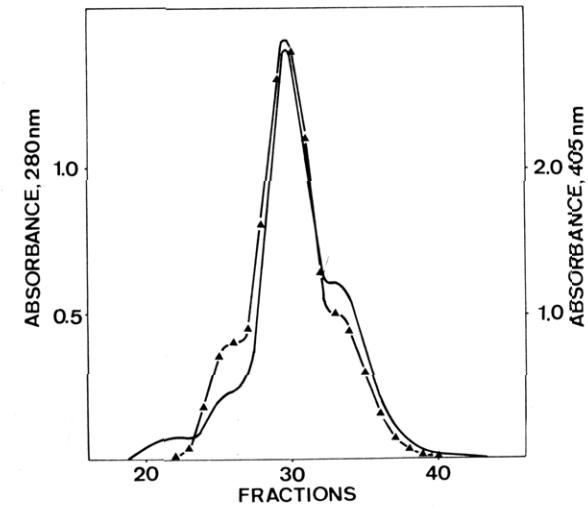

FIGURE 4: Elution pattern of the chromatography of an aminopeptidase I batch on a 2.5 × 25 column of DEAE-Sephadex A-50. Gradient: 0.1–0.5 M NaCl in Tris–cobalt buffer. Total volume: 1.2 l. Fractions of 20 ml were collected. (——) Protein curve (280 nm); (▲) activity curve (405 nm).

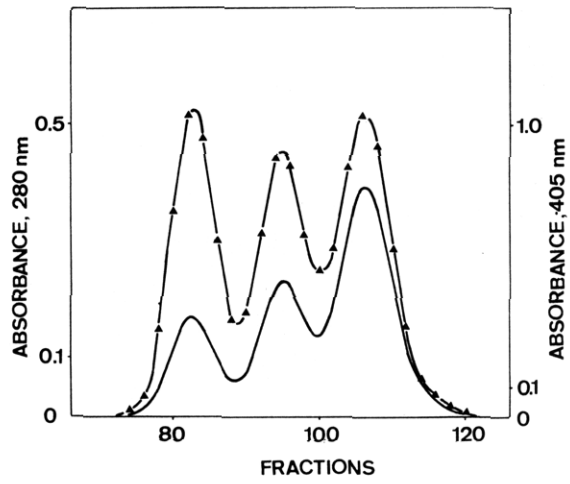

FIGURE 5: Rechromatography of the minor fractions of Figure 4 on an identical column and gradient. Fractions of 6 ml were collected. (——) Protein curve (280 nm); (▲) activity curve (405 nm).

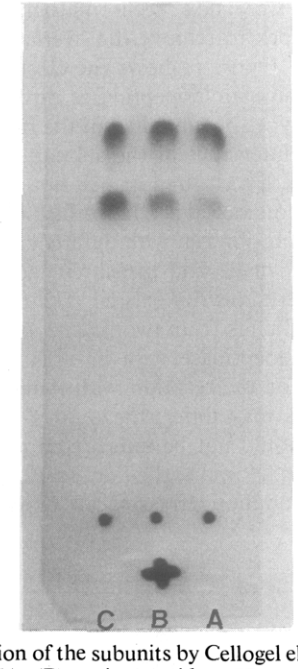

FIGURE 6: Separation of the subunits by Cellogel electrophoresis. (A) Aminopeptidase IA, (B) aminopeptidase IB, and (C) aminopeptidase IC.

The calculated values for aminopeptidases IA:IB:IC is then 5.0:1.9:1 for the first determination and 5.4:2.0:1 for the second determination. These results show that the three enzymes represent stochiometric complexes.

We also considered if these unusual different aminopeptidase I forms could be due to the heat step during the purification procedure. This seems not to be the case, since we were able to get multiple aminopeptidase I peaks from DEAE Sephadex columns after omitting the heat step.[2]

It was previously known from the sequence analyses data that the α to β relationship is in the range of 1:2 to 1:3. The Amido Black values are in favor of a ratio of 1:2. To obtain a third, independent value we determined the α to β relationship of aminopeptidase IB after SE-Sephadex chromatography. The resulting two peaks were measured at 280 nm and integrated. These ratios also agree with the Amido Black values and are shown in Table II.

TABLE II: Subunit Ratio of the Different Aminopeptidase I Enzymes.

Aminopeptidase	Method	α Subunit: β Subunit
IA	Amido Black	5.4
	Amido Black	6.3
IB	Amido Black	2.0
	Amido Black	2.3
	280 nm	1.75
	280 nm	2.1
IC	Amido Black	1.07
	Amido Black	1.17

Sepharose Gel Filtration. When a mixture of aminopeptidase IC and the α-subunit enzyme was subjected to Sepharose 6B gel filtration as described in the Experimental Section the elution pattern showed only one protein peak, which contained all the hydrolytic activity. The fractions were further investigated by disc electrophoresis, since the two enzymes may easily be separated by this technique. The results showed that the two enzymes had cochromatographed exactly. The first fraction that showed activity contained both enzymes, which were identified by comparison to authentic material. This experiment confirms that the β subunit is a real subunit and not a contaminant. If it were a contaminant one of the two possible results should have been observed. Either a separation of aminopeptidase IC (which contains 50% of the β subunit) from the α-subunit enzyme or the contaminant would also have been bound to the α-subunit enzyme and additional bands due to the formation of aminopeptidases IA and IB would have been seen.

In a further experiment a mixture of all the three aminopeptidase I enzymes was subjected to Sepharose gel filtration and again the enzymes exactly cochromatographed. This shows clearly that the molecular weights of all four enzymes are very similar.

Discussion

Little is known about the subunit structure of high molecular weight aminopeptidases. Swine kidney leucine aminopeptidase has been reported to consist of four subunits (Melins *et al.*, 1970) and the subunit structure of leucine aminopeptidase isolated from bovine eye lenses has been investigated by several groups (Melbye and Carpenter, 1971; Kretschmer, 1967, 1968; Kretschmer and Hanson, 1968; Weber and Osborn, 1969). The results however are a matter of controversy. In this report we describe the quite unusual subunit structure of the thermophilic aminopeptidase I from *B. stearothermophilus*. This enzyme contains two different types of subunits, which can be separated by electrophoresis and chromatography and which also behave differently during our reactivation procedure. Each subunit is a single peptide chain and both chains have a different amino-terminal sequence. The reactivated aminopeptidase I that consists only of α subunits

[2] L. Jung and H. Zuber, unpublished results.

shows the enzymatic activities that are known for the native aminopeptidase I (Zuber, 1970). It hydrolyzes peptides and leucine-*p*-nitroanilide and also slowly deformylates *N*-formyl peptides. Aminopeptidase I is thermostable up to 80° and also in this respect the enzyme without β subunits shows no difference.

It can clearly be seen from Figure 5 that the specific activity toward leucine-*p*-nitroanilide of the three aminopeptidase I enzymes decreases from left to right, while the percentage of β subunit increases (Figure 6 and Table II). The Michaelis constant for leucine-*p*-nitroanilide of each of the three aminopeptidase I enzymes is identical and so it is doubtful that the β subunit hydrolyzes leucine-*p*-nitroanilide. The fact that we have never succeeded in reactivating a high molecular weight enzyme consisting only of β subunits may mean that such a species cannot exist. On the other hand, it is possible that our reactivation conditions which are surely different from the conditions in the living cell favored the precipitation of the β subunits. The observation that if both subunits are present in the ratio $\alpha:\beta = 2:1$ during reactivation, only traces of the β subunit are reincorporated into the enzyme while the β subunit tends to precipitate supports this possibility. Finally, very little is known about the physiological function of the high molecular weight aminopeptidases in microorganisms (Sussman and Gilvarg, 1971; Vogt, 1970) and therefore no information about the possible function of a second subunit can be gained from this direction. Further studies are in progress to obtain an insight into the function of the β subunits.

Ultracentrifugation studies resulted in molecular weights of 400,000 (±45,000) from sedimentation equilibrium runs and 395,000 (±30,000) from the sedimentation and diffusion coefficients. A molecular weight of 36,500 (±4000) was determined for the subunits. It seems therefore that the enzyme consists of 9–13 subunits. Values like 9, 11, and 13, however, seem very unlikely, whereas enzymes with 10 and 12 subunits are well known (Klotz *et al.*, 1970). The ratios in Table II support strongly the existence of 12 subunits in aminopeptidase I. With this number the accurate subunit ratio $\alpha:\beta$ would then be 5:1 for IA, 2:1 for IB, and 1:1 for IC.

An interesting question concerns the subunit arrangement in the native enzyme. Preliminary electron micrographs suggest the possibility of a similar arrangement to that of *Escherichia coli* glutamine synthetase where 12 subunits are assembled in two stacked six-membered rings (Valentine *et al.*, 1968). A similar arrangement for aminopeptidase I would also allow a certain symmetry for the proposed subunit relationships of 5:1 for IA ($\alpha_{10}\beta_2$), 2:1 for IB ($\alpha_8\beta_4$), and 1:1 for IC ($\alpha_6\beta_6$), which could easily be accommodated in six-membered rings. The most important species of the three aminopeptidases is IB, representing about 70% of the total aminopeptidase I protein, whereas only about 10% is IA and 20% IC.

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