The Function of the Two Subunits of Thermophilic Aminopeptidase I
(peptide degradation/specificity/homology/multienzyme complex)

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ABSTRACT The thermophilic high-molecular-weight aminopeptidase I (EC 3.4.11.1) from Bacillus stearothermophilus is composed of 12 subunits of two different types (α,β) which can combine in various ratios. Only one type of subunit (α) is needed for the degradation of neutral peptides, but dipeptides having amino-terminal aspartic or glutamic acid are substantially hydrolyzed only by enzyme containing the other subunit (β) as well. Asp-Gly inhibits the enzymatic hydrolysis of glutamic acid 1-(4-nitroanilide) very strongly but hardly affects the hydrolysis of leucine p-nitroanilide. These results indicate that both types of subunit have hydrolytic activity but different specificity. The two subunits have identical molecular weights and their amino-terminal regions are homologous, suggesting that the two chains originate from a single ancestral gene by gene duplication and independent mutation.

Recently we described the subunit structure of the high-molecular-weight aminopeptidase I (EC 3.4.11.1) from Bacillus stearothermophilus (1). This enzyme contains two different subunits which combine in three different ratios, resulting in the three hybrid aminopeptidases IA, IB, and IC. In addition, we were able to reactivate an aminopeptidase consisting of only one type of subunit, which we designated the α subunit. However, we never succeeded in reactivating an enzyme containing only the other subunit, referred as the β type, a result that would not be surprising for a highly complex enzyme system (2), but is unexpected in the present case. The three isolated aminopeptidases and the reactivated α-subunit enzyme have the same molecular weight. We concluded from the known data for the molecular weights of the subunits as well as of the native aminopeptidase I (3), that the subunit structure of the three hybrids is ααββ for aminopeptidase IA, ααββ for aminopeptidase IB, and ααββ for aminopeptidase IC. The reactivated α-subunit enzyme has the structure αα. However, the function of the β subunit remained obscure since the αα enzyme is fully active in all our routine assays: It hydrolyzes peptides such as Gly-Leu-Tyr or Leu-Gly, splits leucine p-nitroanilide, and it also deformsylates slowly certain N-formyl methionylpeptides (1, 4). In contrast the β subunit does not hydrolyze leucine p-nitroanilide or does so only at a very slow rate (1). We have now investigated the specificity of the isolated hybrids and of the αα enzyme towards different peptide substrates and found that dipeptides containing an aminoterminal aspartic or glutamic acid are only very slowly hydrolyzed by the αα enzyme but are good substrates for the hybrids containing β subunits. Aminoterminal-sequence analyses revealed that the α and β chains are homologous in that region.

EXPERIMENTAL PROCEDURE

Cells of B. stearothermophilus NCIB 8924 grown at 50° as described (5) were a gift of Ciba-Geigy Ltd., Basle, Switzerland.

L-Leucine p-nitroanilide was purchased from Serva, Heidelberg, Germany, and L-glutamic acid 1-(4-nitroanilide) from Merck, Darmstadt, Germany. α-L-Aspartyl-glycine was a product of Bachem. Liestal, Switzerland, and α-L-glutamyl-L-tyrosine of Cyclo Chemical, Los Angeles, Calif. The different aminopeptidase hybrids were purified as described (1). Aminoterminal-sequence analyses of each subunit were done in a Beckman sequencer (6).

Assays. The hydrolysis of the p-nitroanilides was followed spectrophotometrically at 405 nm (7) in an expanded-scale recorder (0.1-absorbance full scale). A value of ε = 9.6 × 10² cm²/mol was used to calculate the specific activities. The assays were done in 50 mM Tris-HCL or in imidazole-HCL buffer at pH 7.5 (adjusted at room temperature) containing 0.1 mM cobalt(II) chloride at 40°.

Peptide substrates were hydrolyzed in 50 mM Tris-HCL, pH 7.5, in the presence of 1 mM cobalt(II) chloride at 40°. The reaction was terminated by addition of 0.2 N sodium citrate buffer, pH 2.2. The released amino acids were quantitatively determined with the amino-acid analyzer. Care was taken to avoid more than 10% degradation of the substrates.

Specific activity determinations of the hydrolysis of leucine p-nitroanilide by the three aminopeptidases IA, IB, and IC showed that within experimental error the β chain was inactive. Hence, enzyme solutions containing αα, ααββ, ααββ, and ααββ were titrated to identical activity against leucine p-nitroanilide before being used to degrade different peptides. The resulting activities were plotted against the ratio β/α.

RESULTS AND DISCUSSION

Preliminary experiments with the αα enzyme and aminopeptidase IC (ααββ) showed that both enzymes degraded peptides containing lipophilic amino acids, but that only the latter showed significant activity against dipeptides containing an aminoterminal aspartic or glutamic acid. Fig. 1 shows the result after the incubation of a 30 mM Asp-Gly solution with the aminopeptidases IA, IB, and IC, the αα enzyme, and a blank. No amino acids appear in the sample that was incubated together with the αα enzyme, but an increasing amount of aspartic acid and glycine can be de-
activity towards cine. acid and I. am.opeptidase 3782 Biochemistry: a12 of experiment, but peptidases IA, and in the detected was determined with enzymes against 1.0, peptide solutions plotted against at 400.

Fig. 1. Hydrolysis of Asp-Gly by the different hybrids of aminopeptidase I. The electrophoretic separation of aspartic acid and glycine was done on cellulose plates in water–pyridine–acetic acid 900:100:4. a = aspartic acid; d = dipeptide; g = glycine. The aminopeptidases were titrated to identical hydrolytic activity towards leucine p-nitroanilide. From right to left: blank, α12 enzyme, aminopeptidase IA, IB, and IC.

tected in the samples that were incubated with the aminopeptidases IA, IB, and IC. Fig. 2 shows basically the same experiment, but this time the absolute value of aspartic acid was determined as a function of the ratio β/α, which is zero for the α12 enzyme, 0.2 for aminopeptidase IA, and 0.5 and 1.0, respectively, for the aminopeptidases IB and IC. There is obviously a linear relationship between the activity of the enzymes against Asp-Gly and the amount of β chain. No hydrolysis of this peptide could be detected after incubation with the α12 enzyme. A similar experiment with a different peptide substrate, Glu-Tyr, is also included in Fig. 2. Glu-Tyr is also hydrolyzed by the α12 enzyme. Under the conditions used, the rate is about 5% of that for the aminopeptidase IC. The values of Glu-Tyr hydrolysis by the three aminopeptidases IA, IB, and IC fall on a straight line, but the value for the α12 enzyme is definitely too high. The fact that all four values are on a straight line means that the two subunit types are absolutely independent of each other—and this is obviously not true for the hydrolysis of Glu-Tyr.

Since the analysis of the peptide substrates after aminopeptidase degradation is rather laborious, we also used glutamic acid 1-(4-nitroanilide) to test the activity of the β chain. This substrate was degraded by all the aminopeptidase hybrids containing the β-subunit, but the activity of the α12 enzyme is less than 1% of that of aminopeptidase IC. Fig. 3 shows a Lineweaver–Burk plot for the hydrolysis of

Fig. 2. Hydrolysis rates of Asp-Gly (○) and Glu-Tyr (●) plotted against the ratio of the subunits β/α. 0.25 ml of 8 mM peptide solutions were incubated with the corresponding enzymes at 40°.

Fig. 3. Lineweaver–Burk plot for the hydrolysis of leucine p-nitroanilide and glutamic acid 1-(4-nitroanilide) by aminopeptidase IB. Leucine p-nitroanilide in Tris (●) and in imidazole buffer (×); glutamic acid 1-(4-nitroanilide) in Tris (○) and in imidazole buffer (■). The velocity, ν, is expressed in μmol/min per mg of enzyme. Numbers on abscissa have been multiplied by 10⁻¹.

Fig. 4. Hydrolysis rates of leucine p-nitroanilide (L, 1 mM) and glutamic acid 1-(4-nitroanilide) (G, 0.2 mM) by the different hybrids plotted against the ratio of the subunits β/α.
The hydrolysis of leucine p-nitroanilide by the same enzyme is also included. It can be seen that the former substrate has a much lower Michaelis constant and lower maximal velocity than the latter. The hydrolysis of both substrates is inhibited by Tris buffer in a competitive way compared to imidazole buffer. The maximal velocity in both buffers is the same within experimental error. Table 1 shows the resulting kinetic parameters.

Fig. 4 shows that also in this case an essentially linear relationship exists between subunit content of the aminopeptidases and the hydrolytic activity.

The most reasonable interpretation of these results is that aminopeptidase I is a simple multienzyme complex which consists of two subunits with aminopeptidase activity but different specificity. However, another interpretation is still possible: the specificity change could be induced on the α subunit by the β chain. The restriction is that a β subunit affects only one α subunit because of the linear activity relationship (Figs. 2 and 4). To distinguish between these two possibilities we investigated the influence of Asp-Gly on the hydrolysis of leucine p-nitroanilide and of glutamic acid 1-(4-nitroanilide) by the αβI enzyme. Fig. 5 shows that 4 × 10⁻⁴ M Asp-Gly hardly affects the degradation of leucine p-nitroanilide by aminopeptidase I. On the other hand the hydrolysis of glutamic acid 1-(4-nitroanilide) is strongly inhibited by much lower concentrations of Asp-Gly. In fact, we were not able to measure this inhibition in the usual way since the hydrolysis of Asp-Gly is too fast to obtain meaningful inhibition values at low substrate concentrations. Fig. 6 shows that the hydrolysis of 0.1 mM glutamic acid 1-(4-nitroanilide) is inhibited by 4 × 10⁻⁴ M Asp-Gly to at least 80% at the beginning. This inhibition decreases quickly and finally fades. Asp-Gly initially inhibits the hydrolysis of glutamic acid 1-(4-nitroanilide) over 10-fold more strongly than the hydrolysis of leucine p-nitroanilide. This result is evidence that both subunit types of aminopeptidase I have hydrolytic activity but different specificity, resulting in an enzyme with broad specificity.

The molecular weights of the α and β chains are identical (1, 3). We, therefore, extended our aminoterminal-sequence analyses to check if the primary structure of the two chains is also related. Table 2 shows the result. A comparison of the first 30 residues of the two chains demonstrates that 67% are identical or chemically similar (8). A remarkable degree of homology can be seen, suggesting that at least this part of the two chains originates from a single ancestral gene. This result may explain why aminopeptidase I exists in different forms. The three hybrids are probably the remnants of a whole set of hybrid enzymes, a rather uncommon event in bacteria (9).

Table 2. Aminoterminal sequences of α and β subunits of aminopeptidase I

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* Residues that are identical in α and β subunits are underlined. Residues that are chemically similar (8) or identical in α and β subunits are blocked.
However, in the case of aminopeptidase I, mutation resulted in structural and functional differences of the two chains, leading to an enzyme structure that, to our knowledge, is unique.

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