HYDROLYSIS OF THE ISOPEPTIDE BOND OF ε-*N*-L-METHIONYL–L-LYSINE BY INTESTINAL AMINOPEPTIDASE N

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1. Introduction

Amide bonds involving ε-amino groups of lysine and/or ω-carboxyl groups of aspartic and glutamic acid are generally called 'isopeptide bonds' in contrast to the α-peptide bonds which normally link amino acids residues in proteins and peptides. These bonds are known to be responsible for the covalent cross-linking of several proteins [1] and other natural products such as the peptidoglycan network of bacteria cell walls [2]. They may also be formed artifactually by degradative reactions within or between proteins during processing [3] and by chemical grafting of essential amino acids through their α-carboxyl to the ε-amino group of lysine residues in food proteins [4,5]. Here, only latter type of isopeptide bond is considered.

Methionine is known to be a limiting essential amino acid in several food proteins. Its covalent attachment to casein through isopeptide bond formation has been shown to improve the nutritional value of the protein as judged by growth tests on rats [6]. The plasma amino acid patterns were found to be normal in animals fed on the modified casein, suggesting that isopeptide bonds were hydrolyzed during digestion. Evidence obtained with the model isopeptide ε-Met–Lys was consistent with the hypothesis that the intestinal brush border aminopeptidase N may be involved in the process [6]. If confirmed, this finding would be of importance since amino acids ingested as peptides are more efficiently absorbed than in the free form [7].

It will be shown below that, when compared to α-Met–Lys, ε-Met–Lys is actually a good substrate for pure aminopeptidase N from pig and rabbit intestine. The ε-dipeptide was also used to test the properties of the different subsites assumed to be present in the binding site of the enzyme.

Abbreviations: α-Met–Lys and ε-Met–Lys; α-*N*-L-methionyl–L-lysine and ε-*N*-L-methionyl–L-lysine, respectively

2. Materials and methods

The isopeptide ε-Met–Lys was synthesized as in [6]. Its purity was checked by thin-layer chromatography on silica gel plates and by amino acid analysis. The α-dipeptides and the two synthetic substrates, alanine *p*-nitroanilide and glutamic acid *p*-nitroanilide, which are specific for aminopeptidase N and A, respectively, were obtained from Bachem Fine Chemicals. Amino acids were from Sigma Chemical Co. Pig and rabbit intestinal aminopeptidases N and A were purified in this laboratory as in [8–11].

Peptide and isopeptide bond hydrolysis by aminopeptidase was done at 37°C in a 50 mM phosphate buffer (pH 7.0) and followed by determination of the liberated amino acids in a Beckman Model 120C automatic analyzer. Hydrolysis of *p*-nitroanilides was followed spectrophotometrically at 410 nm [8].

Kinetic studies on peptides and alanine *p*-nitroanilide were done at 5 substrate levels from 0.5–20-times K_m. Determinations at each concentration were in triplicate and the kinetic parameters K_m and k_{cat} were derived from double-reciprocal plots of v vs S or computed by the least-squares method. The following kM_r values were used for calculating k_{cat}: 245 for porcine aminopeptidase N and A [8,9]; 125 and 185, respectively, for the corresponding rabbit enzymes [10,11]. Inhibition constants were derived from the $1/S$-axis intercept when inhibition was competitive and from the slope of the reciprocal plot in case of linear mixed-type inhibition [12].

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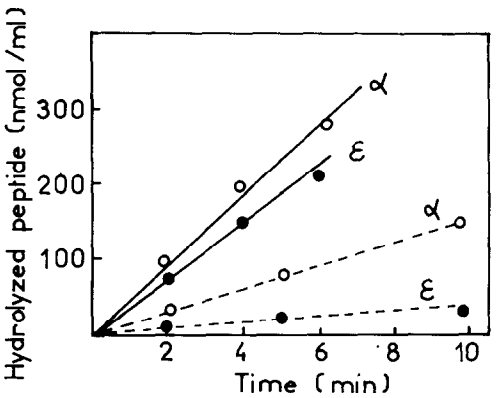

Fig.1. Action of pure aminopeptidase N (solid line) and aminopeptidase A (dotted line) from rabbit intestine on α-Met–Lys (○–○) and ε-Met–Lys (●–●). Substrate was 2 mM in all assays. Enzyme amounts were 57 units/ml for aminopeptidase N and 320 units/ml for aminopeptidase A, using alanine *p*-nitroanilide and glutamic acid *p*-nitroanilide as substrate, respectively. The corresponding enzymes were 30×10^{-15} M and 57×10^{-15} M.

3. Results

3.1. *Hydrolysis of α- and ε-Met–Lys by intestinal aminopeptidases*

As shown in fig.1, ε-Met–Lys was hydrolyzed by pure rabbit aminopeptidase N almost as well as the normal α-dipeptide. Both peptides were also hydrolyzed by aminopeptidase A but more slowly. These preliminary data were confirmed and markedly extended by determination of the kinetic parameters of the reactions (table 1). As expected from the curves reproduced in fig.1, the results obtained with the rabbit enzymes showed no wide variations in affinity and maximal hydrolysis rates from the normal dipeptide to the corresponding isopeptide. The pig enzymes were slightly more efficient towards the α-dipeptide (lower K_m and higher k_{cat}).

Our results on both the dipeptide and the isopeptide are comparable to those found here with different peptide substrates [13] and to those reported on other aminopeptidases [14–16]. However, both dipeptides could now be shown to be much better substrates for the N enzyme compared to its A analog. Bulky and hydrophobic N-terminal neutral residues are known to be preferentially cleaved by aminopeptidase N [14].

Table 1
Hydrolysis of a lysine dipeptide and its related isodipeptide by intestinal aminopeptidases N and A

Enzyme	α-*N*-L-Met–L-Lys		ε-*N*-L-Met–L-Lys	
	K_m (mM)	k_{cat} (s^{-1})	K_m (mM)	k_{cat} (s^{-1})
Rabbit aminopeptidase N	0.35	45	0.17	22
Pig aminopeptidase N	0.05	70	0.17	31
Rabbit aminopeptidase A	0.58	5.9	0.70	1.5
Pig aminopeptidase A	0.33	8.5	2.20	2.6

3.2. *Inhibitory effect of α- and ε-dipeptides*

The respective affinity of α- and ε-Met–Lys for pig aminopeptidase N was further investigated by a study of their inhibitory effect on the hydrolysis of the tripeptide Leu–Gly–Gly and alanine *p*-nitroanilide catalyzed by the enzyme. As shown in table 2 and fig.2, both compounds were inhibitory irrespective of the nature of the substrate. The affinity of the α-peptide was somewhat higher, as expected from the above K_m-values. But, the difference was again small. It would presumably be still smaller or even non-existent with the rabbit enzymes.

The inhibition constants listed in table 2 are similar to those reported for various dipeptides and free amino acids [13]. Free methionine, methionyl and leucyl dipeptides were powerful inhibitors for reasons given earlier. By contrast, free lysine was a poor

Table 2
Inhibition constants for peptides and amino acids of porcine aminopeptidase N

Inhibitor	Substrate	Inhibition type	K_i (mM)
α-*N*-L-Met–L-Lys	Leu–Gly–Gly	Competitive	0.02
ε-*N*-L-Met–L-Lys	Leu–Gly–Gly	Competitive	0.07
α-*N*-L-Met–L-Lys	AAN	NCM	0.03
ε-*N*-L-Met–L-Lys	AAN	NCM	0.10
Met–Gly	AAN	NCM	0.12
Leu–Met	AAN	Competitive	0.04
Ala–Gly	AAN	Competitive	5.50
Met	AAN	NCM	0.10
Lys	AAN	NCM	13

AAN, Alanine *p*-nitroanilide; NCM, non-competitive mixed inhibition

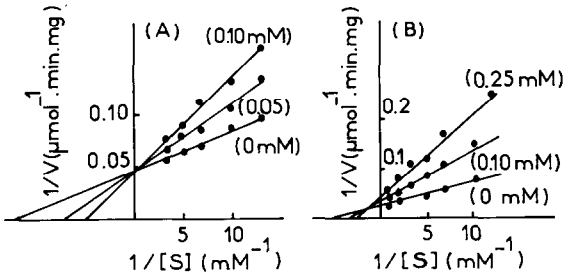

Fig.2. Double-reciprocal plot of the inhibition induced by various concentrations of ε-Met–Lys on the hydrolysis of Leu–Gly–Gly (A) and alanine *p*-nitroanilide (B) by pig aminopeptidase N. Figures in parentheses indicate the inhibitor concentrations.

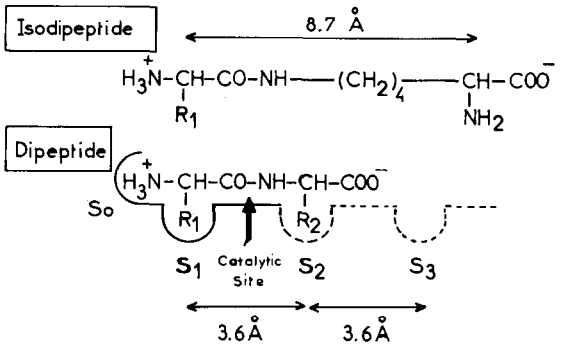

Fig.3. A tentative scheme for the topology of intestinal aminopeptidase subsites. The distances between two adjacent carbons in the α- and ε-dipeptides are calculated assuming that all chains are in a fully extended conformation [20]: S0, subsite recognizing the α-NH_3^+ group of the dipeptide and isopeptide; S1, S2, 0 subsites interacting with other residues in the chain. The minimal role of subsites S2 and following is indicated by a dotted line. R1 and R2 stand for the side chains of methionine and lysine, respectively.

inhibitor even though it enhanced the effect of methionine when at the second position in the dipeptide Met–Lys. The dipeptide Leu–Met with two bulky hydrophobic amino acids was also strongly inhibitory.

As already shown using other derivatives [13], the inhibition induced by α- and ε-Met–Lys was found to be competitive when the substrate was a peptide and generally of the non-competitive mixed type with a *p*-nitroanilide. This finding may reflect a different mode of association of the two substrates with aminopeptidase.

4. Discussion

The major peptidases of the brush border of pig [8,9] and rabbit [10,11] intestine are two aminopeptidases denoted N and A. While the N enzyme displays broad specificity for neutral N-terminal amino acids in proteins and peptides, aminopeptidase A is mostly specific for acidic residues [9,11,13,17,18]. A significant point here is that both enzymes hydrolyzed the isopeptide bond in ε-Met–Lys quite efficiently, even though aminopeptidase N is considerably more active. When the negative effect exerted by Ca^{2+} on the activity of the A enzyme towards neutral substrates [13,17] is taken into account, it then appears that the methionyl isopeptide should be cleaved mostly by aminopeptidase N under the conditions prevailing in vivo during protein digestion. The same conclusion probably applies to grafted methionine and other essential amino acid residues in modified proteins, thus explaining their biological availability in vivo [6].

A few years ago, kinetic studies on the substrate specificity and inhibition of several aminopeptidases by free amino acids and peptides have suggested that the binding site of the enzyme probably includes 3 or 4 subsites which should interact with the side chains of an equal number of amino acids in the substrate [14,19]. If the concepts currently accepted in the field of carboxypeptidase can be applied to aminopeptidases, the most important subsite in aminopeptidase should be the one denoted (S0) in fig.3 which exerts an all or none effect by the specific recognition of the N-terminal α-NH_3^+ group of the substrate. It is noteworthy that the methionine residues linked by isopeptide bonds also possess an α-NH_3^+ group which enables them to comply with the first condition for productive binding. The second subsite (S1) is known to modulate the aminopeptidase activity through a specific recognition of the side chain of the N-terminal residue. As pointed out earlier, the hydrophobic character of methionine in normal and isopeptides appears to guarantee a good fit with this subsite and consequently a high hydrolysis rate in both cases.

The role played by the subsite (S2) in catalysis is attested by the fact that aminopeptidase activity on the first residue is blocked when the second residue is in the D-configuration [13] and also by the lower K_i observed with the dipeptide α-Met–Lys compared to free methionine (table 2). In this latter case, the subsite (S2) appears to recognize the hydrophobic side

chain of lysine well and its terminal positive charge in α-Met–Lys. However, the aminopeptidase activity remains almost unchanged when the substrate is ε-Met–Lys in which, as shown in fig.3, the general arrangement of atoms in the lysine region and the distance separating the two adjacent assymetric carbons are substantially different. This suggests a relatively loose attachment of the substrate to the subsite (S2). No further information could be derived from our assays about the other aminopeptidase subsites.

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