Aminotripeptidase, a Cytosol Enzyme from Rabbit Intestinal Mucosa

By CHRISTIANE DOUMENG and SUZANNE MAROUX

Centre de Biochimie et de Biologie Moléculaire, Centre National de la Recherche Scientifique,
31 Chemin Joseph Aiguier, 13274 Marseille Cedex 2, France

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Aminotripeptidase, a cytosol enzyme from rabbit intestinal mucosa, was purified to homogeneity. The pure enzyme is a glycoprotein containing a very small amount of sugar. It is composed of only one subunit of 50000 mol.wt. and possesses 1 zinc atom per molecule. Its specificity is primarily directed towards tripeptides with an *N*-terminal proline residue. However, the enzyme is also able to hydrolyse other tripeptides, except those with either a charged amino acid in the *N*-terminal position or a proline residue in the second position. The purified aminotripeptidase accounts for almost all the tripeptidase activity of the soluble fraction from intestinal mucosa.

The final products of intraluminal protein digestion by gastric and pancreatic proteinases are free amino acids and peptides. Absorption of free amino acids through the brush-border membrane of the enterocytes is controlled by the now well-characterized Na⁺-dependent mechanism (Schultz & Curran, 1970). On the other hand, absorption of amino acids from peptides is frequently more rapid than that of the equivalent free amino acids (Matthews, 1975; Silk, 1977). In that case, two different systems of uptake have been postulated: either (1) the uptake of intact di- and tri-peptides across the brush-border membrane followed by intracellular hydrolysis within the cytoplasm of the cells (Rubino *et al.*, 1971; Burston *et al.*, 1977), or (2) superficial hydrolysis of tripeptides and longer peptides, mediated by the brush-border aminopeptidase (EC 3.4.11.2), followed by uptake of the released amino acids by a membrane-transport system apparently distinct from that involved in the direct absorption of free amino acids (Maroux *et al.*, 1973; Ugolev, 1972). In either event, it is essential to know precisely the subcellular localization and specificity of the intestinal peptidases that are responsible for the complete hydrolysis of peptides to free amino acids.

In a subcellular fractionation of intestinal mucosa (Maroux *et al.*, 1973; Peters, 1970), dipeptidase and aminopeptidase (arylamidase) activities are very distinctly distributed: the total amount of the aminopeptidase activity originally present is recovered in the brush-border membrane fraction, whereas 90% of the activity with dipeptides as substrate is present in the soluble fraction. Indeed the enzymes responsible for these dipeptidase activities have been localized in the cytosol of the enterocytes and purified (Peters, 1970; Das & Radhakrishnan, 1972; Sjöström & Nören, 1974; Nören *et al.*, 1977). By contrast, sub-

cellular distribution of tripeptidase activity is more ambiguous: it is partitioned between the soluble fraction and the membranous material. The aminopeptidase is responsible for all the tripeptidase activity found in the brush-border membrane (Maroux *et al.*, 1973). On the other hand, some years ago, an aminopeptidase that specifically hydrolysed tripeptides to yield the *N*-terminal residue and the intact dipeptide had been characterized in several tissues (Delange & Smith, 1971). More recently, this enzyme has been purified from pig kidney (Chenoweth *et al.*, 1973a). Also, in a subcellular fractionation of pig intestinal mucosa we had ourselves observed that the activity of the soluble fraction on tripeptides was that of an aminopeptidase (Maroux *et al.*, 1973). The present paper describes the purification of the soluble aminotripeptidase from rabbit intestinal mucosa. This enzyme accounts for almost all the soluble activity of the mucosa on tripeptides. Its molecular and enzymic properties are reported.

Experimental

Materials

DEAE-cellulose (fibrous grade DE 11 and microgranular grade DE 32) was purchased from Whatman, Maidstone, Kent, U.K. Sephadex G-100 and concanavalin A-Sepharose were from Pharmacia Fine Chemicals, Uppsala, Sweden. L-Amino acid oxidase (EC 1.4.3.2) was purchased from Worthington Biochemical Corp., Freehold, NJ, U.S.A. Phenazine methosulphate and methoxytetrazolium were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. L-Alanine *p*-nitroanilide, peptides and their derivatives were from Bachem, CH-4416 Budendorf, Switzerland.

The composition of buffer A was 10mM-KH₂PO₄ adjusted to pH 6.0 with 0.1M-KOH.

Methods

Brush-border membrane. Rabbit intestinal brush-border membranes were isolated by the method described for pig jejunum mucosa by Louvard *et al.* (1973) without any modification.

Determination of total protein and sugar content. Proteins were measured spectrophotometrically at 280 nm by using the coefficient $A_{1\text{cm}}^{1\%} = 11.5$ of the purified aminotripeptidase. Neutral sugars, amino sugars and sialic acids were evaluated respectively by the phenol/ H_2SO_4 method, the Elson-Morgan technique as modified by Montreuil & Spick (1963) and by reaction with thiobarbituric acid (Warren, 1959).

Enzymic activities. Activity on L-alanine *p*-nitroanilide as substrate was measured as previously described (Maroux *et al.*, 1973). Activities on peptides as substrates were determined with the aid of two different assays. (1) The rate of hydrolysis of a 5 mM-Ala-Gly-Gly solution in a 50 mM-potassium phosphate buffer (pH 7.0) was recorded spectrophotometrically at 230 nm and 37°C in a Beckman spectrophotometer Acta C III. Complete hydrolysis of 1 μmol of Ala-Gly-Gly corresponded to a decrease in $A_{1\text{cm}}$ of 0.040. (2) Peptide solutions in 50 mM-potassium phosphate buffer (pH 7.0) were incubated at 37°C with the enzyme; at various time intervals, samples were withdrawn from the mixture and diluted with 1 ml of a 66 mM-trisodium citrate solution adjusted to pH 2.2 with 12 M-HCl, to stop the reaction. The nature and amount of the released amino acid(s) were ascertained with a Beckman analyser model 120 C equipped with an expanded scale. Standard conditions were used for column elution.

One unit of enzyme activity is that amount of enzyme hydrolysing 1 μmol of substrate/min and specific activity is expressed in units/mg of protein.

Gel electrophoresis and subsequent staining. Electrophoresis in the absence of sodium dodecyl sulphate was carried out in a Canalco apparatus by the procedure recommended by the manufacturer. The specific staining methods used for protein, glycoprotein and aminopeptidase have been described in detail elsewhere (Louvard *et al.*, 1975). Specific staining for the activity on Ala-Gly-Gly as substrate was performed as follows: a solution (4 ml) containing 0.8 mg of phenazine methosulphate and 3.2 mg of methoxytetrazolium in 10 mM-potassium phosphate buffer (pH 7.0) was prepared in the dark. This reagent was mixed with 1 ml of a 150 mM-Ala-Gly-Gly solution in the same buffer, 0.8 mg of L-amino acid oxidase and 5 ml of 1.6% (w/v) agarose solution in 10 mM-potassium phosphate buffer (pH 7.0) at 50°C. At the end of the electrophoresis the polyacrylamide gel was immersed in this mixture in a test tube (14 mm $\times$ 140 mm) and the agarose was quickly solidified by cooling the tube in crushed ice. After 15–30

min in the dark, the aminotripeptidase activities appeared as black bands. R_f values were measured by reference to the migration of Bromophenol Blue (in general approx. 13 cm).

Polyacrylamide-gel electrophoresis in 5.6% gels in the presence of sodium dodecyl sulphate was performed by the method of Fairbanks *et al.* (1971) after reduction and carboxymethylation of the protein samples (Crestfield *et al.*, 1963). The following proteins were used as standards (molecular weights in parentheses): bovine serum albumin (67 000); ovalbumin (43 000); pig pepsin (34 000); bovine trypsinogen (24 000). Proteins were stained with Coomassie Brilliant Blue.

Results

Purification of the aminotripeptidase

Enzyme source. Rabbit jejunum and ileum were excised immediately after the death of the animals, thoroughly washed with 10 mM-potassium phosphate buffer (pH 7.4) containing 0.15 M-NaCl and cooled on ice. They were opened longitudinally, and mucosa was scraped off with a microscope slide and stored at -20°C until use.

Extraction. Frozen mucosa (400 g) was cut into pieces and gently stirred in 1200 ml of buffer A for 15 min at room temperature (20–25°C). The suspension thus obtained was filtered through gauze and centrifuged for 15 min at 2000 g to remove the mucus. The supernatant was further centrifuged for 2 h at 37 000 g in a Sorvall centrifuge model RC 2B equipped with a SS34 rotor. Some 80% of the brush-border membrane was recovered in the pellet, as judged by the activity on L-alanine *p*-nitroanilide as substrate (Maroux *et al.*, 1973). The supernatant constituted the aqueous extract of the mucosa and contained the soluble cytoplasmic enzymes.

Electrophoretic migration on polyacrylamide gel and characterization of aminotripeptidase activity. The aqueous extract (20–50 units, Ala-Gly-Gly as substrate) was electrophoresed on polyacrylamide gel. Specific staining for aminotripeptidase activity revealed a very active band characterized by its R_f of 0.57, as well as two weakly active bands. One of these latter bands did not penetrate the gel, but the other one migrated with an R_f of 0.37. Both of them also displayed activity with L-alanine 2-naphthylamide, a specific substrate for the brush-border membrane aminopeptidase. In contrast, the major active band (R_f 0.57) did not hydrolyse this substrate. This result suggests that in the aqueous extract there exists one major enzyme responsible for the hydrolysis of Ala-Gly-Gly that is distinct from a typical aminopeptidase. Table 1 summarizes the main steps for the purification of this enzyme.

Table 1. *Flow sheet for the purification of intestinal aminotripeptidase*
See the Experimental section for details.

Step	Specific activity (units/mg of protein)	Enrichment factor	Yield (%)
Aqueous extract	0.6	1	100
DE 11 DEAE-cellulose chromatography	10	16	50
DE 32 DEAE-cellulose chromatography	95	158	35
DE 32 DEAE-cellulose concentration	100	165	30
Sephadex G-100 filtration	150	250	25
Concanavalin A-Sepharose chromatography	380	630	20

Chromatography on DEAE-cellulose. The aqueous extract (1000–1200 ml) was loaded on a column (9 cm × 40 cm) of DE 11 DEAE-cellulose equilibrated and washed with 5 litres of buffer A. The enzyme was eluted with the same buffer containing 0.15 M-NaCl. The tripeptidase activity emerged from the column as a single peak. The pooled fractions (650 ml) were dialysed overnight against 10 litres of buffer A and further chromatographed on a column (3 cm × 40 cm) of DE 32 DEAE-cellulose equilibrated with the same buffer. The aminotripeptidase activity was eluted with a linear 0.05–0.2 M-NaCl gradient (500 ml) (in buffer A). The fractions of highest specific activity were pooled as indicated in Fig. 1(a) and dialysed against buffer A. The tripeptidase solution (80 ml) was then concentrated on a column (1 cm × 10 cm) of DE 32 DEAE-cellulose equilibrated with buffer A and the enzyme was eluted in a final volume of 7 ml by adding 0.15 M-NaCl to the buffer.

Although at this stage of the preparation the aminotripeptidase had been purified 165-fold compared with the crude homogenate, with an overall yield of 30% (Table 1), it did not seem to represent the major protein of the preparation [see Fig. 1(c), gel 1; band indicated by the arrow (R_F 0.57)].

Filtration through Sephadex G-100. At this step, the solutions resulting from three preparations were pooled and filtered through a column (2.5 cm × 300 cm) of Sephadex G-100, equilibrated and eluted with buffer A containing 0.15 M-NaCl. The aminotripeptidase emerged from this column as a symmetrical peak corresponding to approx. 1.5 void vol. Fractions with constant specific activity were pooled as indicated in Fig. 1(b). The patterns obtained for this material on polyacrylamide gels are shown in Fig. 1(c). As indicated by the arrows, two very close but distinct bands were now revealed by the chromogenic substrate reaction, the slower-migrating one corresponding to the unique band (R_F 0.57) already characterized in the previous steps of the purification. The faster-migrating band was found in all preparations, but the relative proportions of the two species varied from one preparation to the other. This new active material was never detected before

the Sephadex G-100 step, except in only one preparation: these observations suggest that this form had been generated from the native enzyme. The transformation did not seem to alter the activity, since the specific activity of the final product was not dependent on the ratio of the two forms of the enzyme.

Chromatography on concanavalin A-Sepharose. The two glycoproteins that still contaminated the two active bands after filtration through Sephadex G-100 were eliminated by chromatography on a (20 ml) concanavalin A-Sepharose column (2.5 cm × 5 cm) equilibrated with buffer A containing 0.15 M-NaCl. Under these conditions, the aminotripeptidase activity was unretarded, whereas both glycoproteins were bound to the lectin derivative. The mixture of the two active forms was pure as judged by electrophoresis on polyacrylamide gel: 20–30 mg of enzyme could be obtained starting from 1.2 kg of mucosa, with a 630-fold enrichment factor.

Separation of the two forms of the enzyme has not been attempted because the specific activity of the preparation was not dependent on the relative proportions of the native and modified enzymes, and, as described below, no difference in the molecular properties of the two forms could be observed.

Molecular properties

Determinations of molecular parameters of a protein are often considered as criteria for homogeneity, since the techniques used are very sensitive and can detect very small differences between the molecular properties of two proteins that cannot be separated by classical purification procedures. For this purpose, the mixture of the two forms of aminotripeptidase has been studied by these methods.

Identification of the N-terminal residues. Two preparations containing different ratios of the two forms were investigated. In both cases, only 1 proline N-terminal residue was characterized by the dansyl technique as modified by Charles *et al.* (1974).

Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. Electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulphate of the reduced and carboxymethylated

Fig. 1. Chromatography and polyacrylamide-gel electrophoresis of aminotripeptidase (a) Chromatography on DE 32 DEAE-cellulose; — represents the NaCl gradient. (b) Filtration through Sephadex G-100 (V_0 , 300ml). In (a) and (b), the volume of the fractions was 13ml. The horizontal bars indicate the pooled fractions. For details see the Results section. (c) Polyacrylamide-gel electrophoresis of aminotripeptidase: (1) pooled fractions from DE 32 DEAE-cellulose; (2) and (3), pooled fractions from Sephadex G-100; (4) aminotripeptidase after purification on concanavalin A-Sepharose (see the text). In gels (1), (2) and (4) proteins were stained with Aniline Blue Black. In (3) glycoproteins were stained with Schiff's reagent (the shaded area represents a broad band). The arrows point towards the bands specifically stained for aminotripeptidase activity with Ala-Gly-Gly as substrate. Abbreviations and symbols: O, origin; F, Bromophenol Blue band; $\circ$, A_{280} ; $\bullet$, activity (units/ml).

aminotripeptidase preparation gave a single band corresponding to a mol.wt. of 57000.

Ultracentrifugation assays. All assays were performed with the aid of a Beckman Spinco model E analytical ultracentrifuge. For all experiments the protein samples were dialysed for 24h before use, against buffer A containing 0.15M-NaCl.

After centrifugation at 20°C and 60000 rev./min, the enzyme solution (0.6%, w/v) behaved as a homogeneous protein with a sedimentation coefficient, $s_{20,w}$, equal to 4.07 S.

No difference in the molecular weight of the two forms could be detected by the polyacrylamide-gel electrophoresis method in the presence of sodium dodecyl sulphate. By the equilibrium method of Yphantis (1963) with a sample containing 1mg of protein/ml, and centrifuging at 26000 rev./min for 28h at 20°C, the plot $\log \Delta y$ against r^2 yielded a straight line, which led to an apparent mol.wt. of

49900. The partial specific volume (0.698 ml/g) was measured with the aid of a microdensitometer (model 120C, A; Parr, Graz, Austria) in the same buffer as that used for centrifugation assays.

The absorption coefficient ($A_{1\text{cm}}^{1\%}$, 280) was determined by using interferential optics on a solution of aminotripeptidase containing 2mg of protein/ml and was found equal to 11.5.

Determination of sugar content. Positive staining of the two forms of the enzyme with Schiff's reagent suggested that it was a glycoprotein. Neither amino sugars nor sialic acids could be detected. Only 1.4 mol of neutral sugars/mol of the mixture of the two forms was measured, if a mol.wt. of 50000 is assumed for the enzyme. Microheterogeneity of the sugar moiety is often responsible for the fractionation of glycoproteins by polyacrylamide-gel electrophoresis. However, the method used for the sugar-content determination was not sensitive enough to assess if

the fractional value found indicated a different amount of sugars in the two forms.

Zinc content. Many aminopeptidases are zinc metalloenzymes (Delange & Smith, 1971). The zinc content of the aminotripeptidase was thus measured by atomic-absorption analysis in a Perkin-Elmer 300 spectrophotometer. Enzyme solutions (1.3 and 0.6 mg/ml) were dialysed for 24 h against 50 mM-potassium phosphate buffer (pH 6.0) in twice-distilled water containing 1% glycerol; 1.1 g-atom of zinc/mol of aminotripeptidase was found, by assuming a mol.wt. of 50000.

Inhibition by EDTA. As with other metalloenzymes, aminotripeptidase was inhibited by EDTA. The enzyme solution (10 µg/ml) was dialysed for 24 h against a 50 mM-potassium phosphate buffer containing 1 mM-EDTA either at pH 6.0 or at pH 8.0 and assayed with Pro-Gly-Gly (5 mM) as substrate at pH 7.0 by the amino acid-analyser method. Losses of activity of 40 and 80% were observed at pH 6.0 and 8.0 respectively. It may be pointed out that inhibition of the brush-border aminopeptidase by EDTA is higher at pH 6.0 than at pH 8.0 (H. M. Ferracci, unpublished work).

Enzymic properties

Specificity. A number of peptides and derivatives have been tested as substrates for the aminopeptidase.

The dipeptides Ala-Gly, Ala-Ala and Leu-Gly, the tetrapeptides Ala-Gly-Gly-Gly and Gly-Gly-Gly-Gly, alanylalanine amide, Ala-Ala-Ala-COCH₃ and *N*-acetyl-Ala-Ala-Ala were not hydrolysed at all by the enzyme. With the exception of *N*-acetyl-Ala-Ala-Ala, which has not been tested, all these peptides, as well as the free amino acids alanine and leucine, completely inhibited the hydrolysis of a solution of 5 mM-Ala-Gly-Gly when used at 25 mM concentration.

With the exception of Ala-Pro-Gly, all the tripeptides (Tables 2 and 3) having a neutral *N*-terminal

residue were rapidly hydrolysed: only the *N*-terminal residue was released and an L-configuration for the first two amino acids was required. Tripeptides with a charged *N*-terminal residue such as lysine or glutamate were poor substrates. The kinetic constants for the hydrolysis at 37°C and pH 7.0 of some tripeptides are given in Table 2.

Although the number of peptides tested was probably not sufficient to permit generalization, the results strongly suggest that the specificity of the aminotripeptidase for the *N*-terminal residue is very broad. The more surprising observation deals with the hydrolysis of the peptide Pro-Gly-Gly. Since the preparation that has been used for these studies contained the two forms of the enzyme, it was important to investigate whether or not the same active site was responsible for the hydrolysis of Ala-Gly-Gly and Pro-Gly-Gly. Therefore the inhibition of Pro-Gly-Gly hydrolysis by Ala-Gly-Gly was studied. As depicted in Fig. 2, the inhibition was competitive. The inhibition constant ($K_i = 1.4$ mM) was found equal to the Michaelis constant for the hydrolysis of this peptide by the enzyme (Table 2).

Fig. 2. Inhibition of Pro-Gly-Gly hydrolysis by Ala-Gly-Gly. The initial rate of hydrolysis, v (µmol hydrolysed/min per mg of aminotripeptidase), of Pro-Gly-Gly at different concentration(s) was determined at 37°C and pH 7.0 by using the amino acid-analyser technique (see under 'Methods'). $1/v$ was plotted against $1/[S]$ in the absence of Ala-Gly-Gly (●) and in the presence of 2.5 mM- (Δ) and 5 mM-Ala-Gly-Gly (□).

Table 2. Kinetic constants for the hydrolysis of some tripeptides by the aminotripeptidase at pH 7.0 and 37°C

	k_{cat} ,* (s ⁻¹)	K_m (mM)
L-Pro-Gly-Gly	385	1, 3
L-Gly-Gly-Gly	88	7, 5
L-Leu-Gly-Gly	190	0, 55
L-Ala-Gly-Gly	365	1, 4
L-Ala-L-Ala-L-Ala	298	0, 52
L-Ala-L-Ala-D-Ala	240	1, 4
L-Ala-D-Ala-L-Ala	0	
D-Ala-L-Ala-L-Ala	0	
L-Ala-L-Pro-Gly	0	

* Calculated assuming a mol.wt. of 50000.

pH-dependence of aminotripeptidase. Pro-Gly-Gly is one of the best (Table 2) and (for reasons discussed below) the most specific substrate of the enzyme. It

Fig. 3. *pH-dependence of aminotripeptidase* Activity was assayed at 37°C on a 5 mM-Pro-Gly-Gly solution in 50 mM-potassium phosphate buffer adjusted to various pH values with NaOH.

was chosen to evaluate the influence of the pH for the enzyme activity at 37°C, and its hydrolysis was measured by the amino acid-analyser technique. Fig. 3 shows that the activity reached its maximum value at pH 7.5.

Relative activities of the purified aminotripeptidase, the aqueous extract of the mucosa and the brush-border membrane. With the activity on Pro-Gly-Gly as substrate as a reference, the activity of the aqueous extract and that of the purified enzyme on some tripeptides listed in Table 3 were compared.

The results summarized in Table 3 show that the purified aminotripeptidase accounts for all of the soluble activity on those tripeptides that have been routinely used to characterize a 'tripeptidase' activity. However, the result obtained with Lys-Ala-Ala and Glu-Ala-Ala could suggest the presence in the aqueous extract of soluble enzymes specific for the hydrolysis of these tripeptides. The purified brush-border membrane from rabbit intestine had a high activity on all the tripeptides tested, except Pro-Gly-Gly.

Discussion

The enzyme responsible for almost all the aminotripeptidase activity of the soluble fraction from the rabbit intestinal mucosa has been purified. The purified enzyme is an authentic aminotripeptidase that does not hydrolyse dipeptides or peptides larger than tripeptides.

In the purification procedure a degraded form of

Table 3. *Relative activities of the purified aminotripeptidase, the aqueous extract from the mucosa and the brush-border membrane in hydrolysing various tripeptides* relative to the activities with Pro-Gly-Gly as substrate*

	Aqueous extract	Purified aminotripeptidase	Brush-border membrane
Ala-Ala-Ala			
Pro-Gly-Gly	0.57	0.50	52
Ala-Gly-Gly			
Pro-Gly-Gly	0.85	0.97	36
Leu-Gly-Gly			
Pro-Gly-Gly	0.72	0.72	10
Tyr-Gly-Gly			
Pro-Gly-Gly	0.24	0.23	25
Gly-Gly-Gly			
Pro-Gly-Gly	0.27	0.23	65
Lys-Ala-Ala			
Pro-Gly-Gly	0.13	0.08	11.8
Glu-Ala-Ala			
Pro-Gly-Gly	0.19	0.065	12.4
Alanine <i>p</i> -nitroanilide			
Pro-Gly-Gly	0.004–0.02	0	8

* The concentration of all peptides was 5 mM, except for Gly-Gly-Gly, which was at a concentration of 20 mM.

the enzyme, revealed by electrophoresis on polyacrylamide gels, has been generated from the native protein. The transformation, which probably modified some charged group(s) of the molecule, did not alter its molecular parameters or its enzymic properties. For these reasons, no attempt has been made to separate the two forms.

The intestinal aminotripeptidase is a monomeric enzyme of 50000–57000 mol.wt. A 50000 mol.wt. value has been used in all calculations for the determination of the molecular constants of the enzyme. It is a glycoprotein containing a very small amount of sugar (1–2 mol/mol). As with the brush-border aminopeptidase, the aminotripeptidase is an enzyme inhibited by EDTA and containing 1.1 g-atom of zinc/mol. These characteristics are very similar to those of the renal enzyme (Chenoweth *et al.*, 1973a,b).

The main structural requirements for a tripeptide to be hydrolysed by the enzyme are: a free α -amino group, a free α -carboxy group and an L-configuration for the first two amino acid residues if either is other than glycine. The residue located in the second position cannot be a proline residue. As observed for aminotripeptidases from other tissues (Delange & Smith, 1971; Chenoweth *et al.*, 1973a), the specificity for the N-terminal residue of the substrate tripeptides is quite broad.

Except for the free α -carboxy group, the brush-border aminopeptidase requires exactly the same structural properties in a tripeptide for it to be recognized as a substrate. The kinetic constants for the hydrolysis of a given peptide are very similar for both enzymes (H. M. Feracci, unpublished work). The essential difference between the catalytic properties of the two enzymes resides in the specificity of the aminotripeptidase for tripeptides with a proline as N-terminal residue. Table 3 shows that Pro-Gly-Gly is the most specific tripeptide that could be used to monitor the aminotripeptidase in a subcellular fractionation. By contrast, as described previously (Maroux *et al.*, 1973), the *p*-nitroanilide or 2-naphthylamide derivatives are the best markers for the brush-border membrane.

Two dipeptidases have been purified from intestinal mucosa and localized in the cytoplasm of the enterocyte (Nören *et al.*, 1977). The aminotripeptidase displays its maximum activity in the same pH range as these two dipeptidases and closely parallels their aqueous extraction from the mucosa (Peters, 1970). Therefore it is reasonable to assume that the dipeptidases and the aminotripeptidase have the same cytoplasmic localization.

One of the most challenging properties of the aminotripeptidase is its high specificity for tripeptides with a free α -amino group as well as an excellent specificity for peptides containing a proline as N-terminal residue. In general only very specific enzymes are able to split bonds in which a proline

residue is involved (Sjöström & Nören, 1974; Koida & Walter, 1976; Delange & Smith, 1971), whereas proteinases, even those characterized by a broad specificity, cannot hydrolyse these bonds. Noteworthy is the fact that the same unusual double specificity is also a property of another intestinal and renal enzyme, the dipeptidyl peptidase IV, implicated in the degradation of proline-containing peptides (Kenny *et al.*, 1976).

It is important to realize that the final digestion of the regions surrounding proline residues of the dietary proteins requires the action of the intestinal peptidases. Pepsin and the pancreatic proteinases are not capable of splitting such peptide bonds (Smyth, 1967). The smaller peptides (X-Pro-Y) that they generate are indeed substrates for the brush-border dipeptidyl peptidase IV (Kenny *et al.*, 1976), and the product of the reaction, X-Pro, is then hydrolysed by a specific enzyme in the cytosol, the aminocacyl-L-proline hydrolase (Sjöström & Nören, 1974). The tripeptides of the type Pro-X-Y could also be residual peptides from intraluminal digestion if the residue Y is not susceptible to removal by carboxypeptidases. To our knowledge, only the aminotripeptidase described in the present paper has the required specificity to hydrolyse these peptides once they have crossed the brush-border membrane. The dipeptide X-Y released simultaneously with free proline could then be degraded by a cytoplasmic dipeptidase (Sjöström *et al.*, 1973; Das & Radhakrishnan, 1972).

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