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Research Article

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Digestion and Assimilation of Proline-containing Peptides by Rat Intestinal Brush Border Membrane Carboxypeptidases

Role of the Combined Action of Angiotensin-converting Enzyme and Carboxypeptidase P

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Abstract

Two intestinal brush border membrane carboxypeptidases were found to participate in the sequential digestion of proline-containing peptides representing a novel mechanism of hydrolysis from the COOH terminus. NH_2 -blocked prolyl tripeptides were rapidly hydrolyzed by either brush border membrane angiotensin converting enzyme (ACE, dipeptidyl carboxypeptidase, E.C. 3.4.15.1) or carboxypeptidase P (E.C.3.4.12-) depending on the position of the proline residue. Furthermore, these two enzymes were found to participate in a concerted manner to sequentially degrade larger proline-containing pentapeptides from the COOH terminus. A brush border membrane associated neutral endopeptidase also participated in the hydrolysis of the prolyl pentapeptides.

During *in vivo* intestinal perfusion, the NH_2 -blocked prolyl peptides were degraded and their constituent amino acids efficiently absorbed by the intestine. Furthermore, hydrolysis and absorption of these peptides could be dramatically suppressed by low concentrations of captopril, a specific inhibitor of ACE.

These studies show that prolyl peptides are efficiently and sequentially hydrolyzed from the COOH terminus by the combined action of ACE and carboxypeptidase P, and that these enzymes may play an important role in the digestion and assimilation of proline-containing peptides.

Introduction

Proteins rich in proline such as casein, gliadin and collagen are important dietary constituents. Although many dietary proteins are efficiently hydrolyzed to small peptides and amino acids by the digestive peptidases of gastric and pancreatic origin, prolyl peptide bonds are generally resistant to the action of these enzymes. Therefore, proline-containing peptides may escape from the action of these peptidases and reach the surface of the intestinal brush border membrane relatively intact.

Dipeptidyl aminopeptidase IV (DAP IV,¹ E.C. 3.4.14-) is a proline-specific peptidase associated with the intestinal brush

border membrane, which preferentially hydrolyzes a postproline peptide-bond to release X-Pro type dipeptides from the NH_2 -terminus. This enzyme has been purified, characterized and its physiological importance in the digestion and assimilation of proline-containing peptides has been established (1-5).

In addition, two carboxypeptidases, a proline-specific carboxypeptidase (carboxypeptidase P, E.C. 3.4.12-) and angiotensin-converting enzyme (ACE, dipeptidyl carboxypeptidase, E.C. 3.4.15.1), have also been recently found to be associated with brush border membrane of human and mammalian intestine (5-10). Carboxypeptidase P preferentially hydrolyzes the COOH-terminal amino acid from peptides containing a proline residue penultimate to the COOH terminus, and it has been suggested that this enzyme may play an important role in hydrolysis of proline-containing peptides (10). ACE has been reported by a number of investigators to have a relatively wide substrate specificity (11), however reports from our laboratory have shown that the intestinal enzyme has some of the highest activities toward peptide substrates containing a proline residue at the COOH terminus (12). The hydrolytic rates observed with these proline-containing peptides were comparable to those for DAP IV and aminopeptidase N, both of which are major enzyme constituents of the intestinal brush border membrane (12). These earlier observations suggested that proline-containing peptides might be effectively degraded from the COOH terminus by the concerted action of these two enzymes. It is well established that many oligopeptides can be hydrolyzed by a variety of NH_2 -terminal specific peptidases present in the brush border membrane. However, corresponding information about the role of brush border membrane-associated carboxypeptidases is not available. The present study therefore, was designed to investigate the role of these carboxypeptidases in the digestion and assimilation of several synthetic NH_2 -blocked peptides and show that they may be of particular importance for the hydrolysis and assimilation of prolyl peptides.

Methods

Animals. Male Wistar rats (Harlan Sprague-Dawley, Indianapolis, IN), weighing ~ 300 g, were maintained on a standard laboratory chow diet (Ralston Purina Co., St. Louis, MO) and used throughout the study after overnight fasting.

Chemicals. L-leucyl-L-alanyl-L-proline (Leu-Ala-Pro) and L-leucyl-glycyl-L-proline (Leu-Gly-Pro) were obtained from Bachem Freinchemikalien AG, Bubendorf, Switzerland). Benzoylglycyl-L-alanyl-L-proline (Bz-Gly-Ala-Pro) and phosphoramidone were purchased from Peninsula laboratories (Belmont, CA). Benzoyloxycarbonylglycylglycyl-L-proline (Z-Gly-Gly-Pro), benzoyloxycarbonylglycyl-L-prolyl-L-leucine (Z-Gly-Pro-Leu), benzoyloxycarbonylglycyl-L-prolyl-L-leucyl-L-alanyl-L-proline (Z-Gly-Pro-Leu-Ala-Pro), benzoyloxycarbonylglycyl-L-prolyl-L-leucylglycyl-L-proline (Z-Gly-

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1. *Abbreviations used in this paper:* ACE, angiotensin converting enzyme; DAP IV, dipeptidyl aminopeptidase IV.

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Pro-Leu-Gly-Pro) and other peptides and amino acids were purchased from Sigma Chemical Co., (St. Louis, MO). Captopril (SQ 14,225) was a gift from E.R. Squibb and Sons, Inc. (Princeton, NJ). [^{14}C]PEG was obtained from Amersham Corp. (Arlington Heights, IL). All other chemicals were of reagent grade quality.

Hydrolysis of NH_2 -blocked peptides by intestinal brush border membrane. The rat small intestine was removed and purified brush border membranes were prepared from mucosal scrapings by the method of Kessler et al. (13). Isolated brush border membranes were incubated with various NH_2 -blocked peptide substrates (5 mM) in 50 mM Hepes–0.15 M NaCl buffer, pH 7.0. After incubation for 30 min at 37°C, the reaction was terminated by the addition of 6% sulfosalicylic acid and precipitated protein was removed by centrifugation. The hydrolytic products were analyzed by amino acid analysis.

When the time course of hydrolysis of Z-Gly-Pro-Leu-Ala-Pro and Z-Gly-Pro-Leu-Gly-Pro was examined, an aliquot of the reaction mixture was removed at various times for periods of up to 3 h.

In vivo perfusion study. A 20-cm long jejunal segment beginning 5 cm distal to the ligament of Treitz was perfused with test solutions containing 2 mM of the same NH_2 -blocked peptides as used in the preliminary in vitro assay. The test solutions were made isoosmotic with NaCl and the pH was adjusted to 7 before perfusion. [^{14}C]PEG was added to the test solutions as a nonabsorbable volume marker. Intestinal perfusion was performed using a steady state perfusion technique described previously (4, 14). After a 30-min equilibration period, the perfusate was collected for three consecutive 10-min periods. Hydrolytic products in perfusates were identified and measured by amino acid analysis. The absorption rate of the constituent amino acids during perfusion was calculated after acid hydrolysis of perfusates in 6 N HCl at 110°C for 22 h.

Amino acid analysis. The amount of free amino acids and peptides in the brush border membrane incubation mixtures and intestinal perfusate were determined by using an automated amino acid analyzer (model 119C; Beckman Instruments Inc., Palo Alto, CA).

Captopril treatment. To investigate the role of brush border membrane ACE in the hydrolysis of NH_2 -blocked peptides, captopril, an active site-specific inhibitor was used to inhibit the enzyme activity. In the in vitro study, purified brush border membrane was preincubated with 10 μM captopril at room temperature for 15 min before assay. During in vivo intestinal perfusion, the test solutions contained 10 μM captopril and was perfused in a separate series of animals under the same conditions described above.

Results

Hydrolysis of NH_2 -blocked peptides by intestinal brush border membrane. The hydrolytic products released from several NH_2 -blocked peptide substrates after incubation with purified brush border membranes and the effect of captopril are shown in Table I. For those tripeptide substrates containing a proline residue at the COOH-terminal position (Bz-Gly-Ala-Pro and Z-Gly-Gly-Pro), the respective COOH-terminal dipeptide (Ala-Pro or Gly-Pro) was the only hydrolytic product detected. No free amino acid was detected in any of the incubation mixtures. When NH_2 -blocked tripeptides containing a proline in the penultimate position from the COOH-terminus such as Z-Gly-Pro-Leu were used, the COOH-terminal amino acid was detected as the only hydrolytic product. In the presence of 10 μM captopril, dipeptide release from the COOH-terminus of Bz-Gly-Ala-Pro and Z-Gly-Gly-Pro was completely inhibited, while release of leucine from Z-Gly-Pro-Leu was not affected. With the two NH_2 -blocked pentapeptides, both the respective COOH-terminal dipeptide and free leucine were the products of hydrolysis. In both cases captopril strongly inhibited dipeptide release while the release of leucine was only partially affected.

Table I. Hydrolysis of N -blocked Peptides by Intestinal Brush Border Membrane

Substrate*	Hydrolytic products†	Inhibition by captopril‡
		%
Bz-Gly-Ala-Pro	Ala-Pro	100
Z-Gly-Gly-Pro	Gly-Pro	99
Z-Gly-Pro-Leu	Leu	0
Z-Gly-Pro-Leu-Ala-Pro	Ala-Pro	96
	Leu	31
Z-Gly-Pro-Leu-Gly-Pro	Gly-Pro	97
	Leu	66

* NH_2 -blocked prolyl peptides (5 mM) were incubated with purified brush border membrane at 37°C for 30 min.

† Hydrolytic products were determined by the use of an automated amino acid analyzer.

‡ Brush border membranes were preincubated with captopril (10 μM) at room temperature for 15 min before assay.

Z-Gly-Pro-Leu-Ala-Pro or Z-Gly-Pro-Leu-Gly-Pro were incubated with brush border membrane for various periods of time, and the appearance of hydrolytic products was monitored (Fig. 1 A and B). The COOH-terminal dipeptides, Ala-Pro or Gly-Pro, were rapidly released in a linear fashion during the first 15 min of the incubation period. The initial velocity of hydrolysis was approximately two times faster for the Ala-Pro peptide when compared with Gly-Pro. After incubation of these pentapeptides with brush border membrane for periods of up to 180 min, the concentration of COOH-terminal dipeptide in the incubation mixtures reached ~ 5 mM, indicating that almost all of the peptide substrate was hydrolyzed. Free leucine was also detected in these incubation mixtures and the amount of leucine increased in a linear fashion. However, the rate of leucine release was lower than that observed for the COOH-terminal dipeptide. Trace amounts of free alanine and proline or free glycine and proline were detected with each pentapeptide after the first 30 min of incubation.

In the presence of 10 μM captopril, the rate of dipeptide release from the COOH terminus of both pentapeptides was strongly inhibited, however COOH-terminal dipeptide did appear at 3–4% of the rate observed in the absence of inhibitor (Fig. 2). In addition, free leucine was also released and the rate of its appearance was approximately equal to that observed for the respective COOH-terminal dipeptide. An increase in captopril concentration to 50 μM did not alter the appearance of hydrolytic products. When bestatin, an inhibitor of aminopeptidase N (15), was included in the incubation mixture with captopril, Leu-Ala-Pro and Leu-Gly-Pro were the only hydrolytic products. Their appearance paralleled that of leucine and COOH-terminal dipeptide observed in the presence of captopril only. The appearance of tripeptide could be completely inhibited by phosphoramidone, a specific neutral metalloendopeptidase inhibitor (16).

In vivo perfusion study. The luminal appearance rate of hydrolytic products during in vivo perfusion of Bz-Gly-Ala-Pro or Z-Gly-Gly-Pro is shown in Fig. 3 A. The main hydrolytic products detected in the perfusates were the COOH-terminal dipeptides (Ala-Pro or Gly-Pro) and two constituent amino acids (alanine and proline, or glycine and proline). The rate of

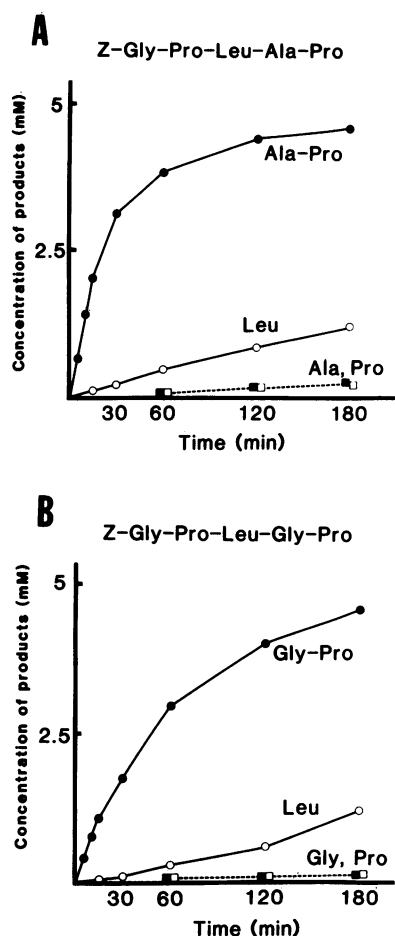


Figure 1. Time course of appearance of hydrolytic products during incubation of brush border membranes with prolyl pentapeptides. (A) Z-Gly-Pro-Leu-Ala-Pro. (B) Z-Gly-Pro-Leu-Gly-Pro. Peptides (5 mM) were incubated with brush border membrane at 37°C. Aliquots were removed from incubation mixtures and the reaction stopped by addition of 6% sulfosalicylic acid. After centrifugation at 3,000 g, samples of the supernatant were analyzed on an amino acid analyzer.

luminal appearance of COOH-terminal dipeptides was approximately four to six times higher when compared with that for the individual amino acids. Addition of captopril to the perfused solution completely inhibited the appearance of all these hydrolytic products (data not shown).

The appearance of hydrolytic products during perfusion of Z-Gly-Pro-Leu-Ala-Pro or Z-Gly-Pro-Leu-Gly-Pro are shown

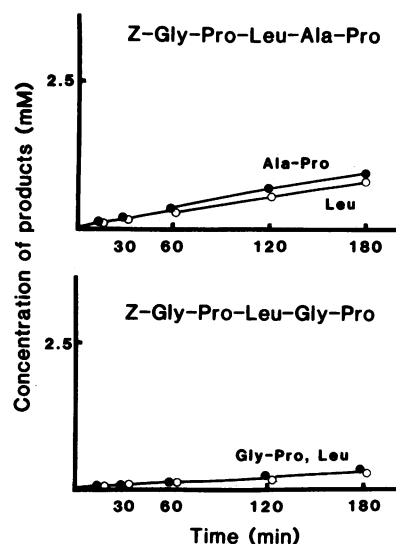


Figure 2. Effect of captopril on prolyl pentapeptide hydrolysis by brush border membrane. Captopril (10 μ M) was included in the incubation mixture described in Fig. 1.

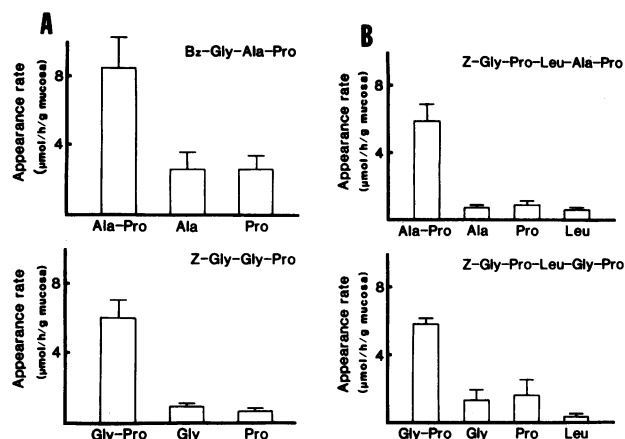


Figure 3. Luminal appearance rates of hydrolytic products during in vivo intestinal perfusion of prolyl peptides. (A) Prolyl tripeptides, (B) prolyl pentapeptides. An isoosmotic solution containing 2 mM of the indicated peptide was perfused through a 20-cm segment of rat jejunum. Samples of perfusates were collected, treated with 6% sulfosalicylic acid, centrifuged and samples of the supernatant analyzed on an amino acid analyzer. Appearance rates were calculated as described (14). Mean of five experiments and SEM are shown.

in Fig. 3 B. In this study, the major hydrolytic products were Ala-Pro and Gly-Pro, respectively. Free alanine and proline or free glycine and proline were also detected in the perfusates as shown. Free leucine was present in both perfusates and its appearance rate was similar to that observed when 2 mM Z-Gly-Pro-Leu was perfused (0.3 ± 0.1 μ mol/h per g mucosa). Analysis of the hydrolytic products appearing during perfusion of Z-Gly-Pro-Leu demonstrated that free leucine was the only hydrolytic product.

The absorption rates of constituent amino acids from NH_2 -blocked tripeptides indicated that alanine and glycine were absorbed from Bz-Gly-Ala-Pro and Z-Gly-Gly-Pro, respectively, at rates similar to those observed for proline from both peptides (Fig. 4 A). No glycine absorption was observed from Bz-Gly-Ala-Pro and amino acid absorption from both peptides was completely inhibited by captopril.

Absorption rates of alanine and proline from Z-Gly-Pro-Leu-Ala-Pro (Fig. 4 B) were comparable to those observed from Bz-Gly-Ala-Pro (Fig. 4 A). However, glycine and proline were absorbed from Z-Gly-Pro-Leu-Gly-Pro (Fig. 4 B) at significantly lower rates when compared with those from Z-Gly-Gly-Pro. The other constituent amino acid, leucine, was absorbed at rates similar to those observed when Z-Gly-Pro-Leu was perfused (6.0 ± 0.7 μ mol/h per g mucosa), although the absorption rate of leucine from Z-Gly-Pro-Leu-Gly-Pro was about one-third of that from Z-Gly-Pro-Leu-Ala-Pro. No glycine was absorbed during Z-Gly-Pro-Leu-Ala-Pro perfusion. As expected, captopril significantly decreased the absorption rates of all amino acids, but equal rates of absorption of the three COOH-terminal amino acids was observed (Fig. 5).

Discussion

The digestion of proline-containing peptides by intestinal brush border membrane peptidases is nutritionally important

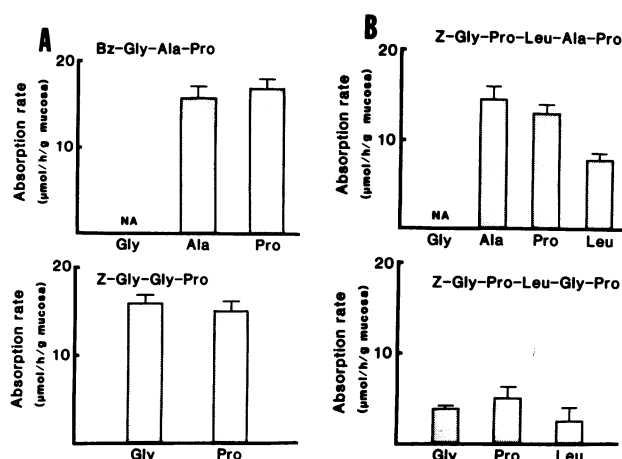


Figure 4. Absorption rates of constituent amino acids during in vivo intestinal perfusion of prolyl peptides. (A) Prolyl tripeptides. (B) Prolyl pentapeptides. Perfusates from Fig. 3 were subjected to acid hydrolysis (6 N HCl, 110°C, 22 h) and analyzed for amino acids. Absorption rates were calculated as described (14). NA, not absorbed. Means of five experiments and SEM are shown.

because prolyl peptide bonds are not readily hydrolyzed by most digestive proteases of gastric and pancreatic origin. In a previous report from our laboratory, hydrolysis of proline-containing peptides by brush border membrane DAP IV was shown to be an important step in the assimilation of these peptides (4). However, DAP IV and most of the brush border membrane-associated peptidases that have been studied to date hydrolyze peptide bonds from the NH₂-terminal end of oligopeptides (17). Recent observations have suggested that the intestinal brush border membrane contains at least two types of carboxypeptidases, ACE and carboxypeptidase P, which may be involved in the digestion of prolylpeptides (10, 12). This indicated that degradation of proline-containing peptides by COOH-terminal specific brush border membrane peptidases might be an important mechanism for their assimilation.

ACE has been purified and characterized from a variety of mammalian tissues and its role in the regulation of vasoactive

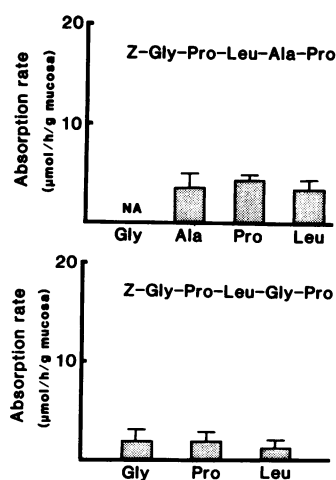


Figure 5. Effect of captopril on the absorption rates of constituent amino acids during in vivo intestinal perfusion of prolyl pentapeptides. Perfusion solutions contained 10 μM captopril in addition to the indicated pentapeptide (2 mM). Perfusates were collected, treated and analyzed as described in Figs. 3 and 4.

peptides (11, 18) and other peptide hormones (18) is well known. In addition, its substrate specificity has been extensively studied and a number of active site specific inhibitors such as captopril (19, 20) have been synthesized. Observations by our group (12) and others (8, 9) have indicated that the intestinal brush border membrane contains high levels of ACE and recent studies from our laboratory have shown that it functions as a digestive peptidase as does carboxypeptidase P (12). Though ACE can hydrolyze a wide variety of substrates, both it and carboxypeptidase P exhibit high hydrolytic rates with peptides containing proline in either the COOH-terminal or penultimate position, respectively (10, 12). Therefore the presence of these two enzymes in the brush border membrane of small intestine suggested that they may be important components in the sequential degradation of prolyl peptides from the COOH-terminal end.

To investigate this possibility, purified brush border membrane from rat intestine were incubated with NH₂-blocked prolyl peptides of defined composition. We initially used several tripeptide substrates and found that either a free amino acid or dipeptide was removed from the COOH terminus, depending on the position of the proline residue. When proline was present at the COOH terminus, the COOH-terminal dipeptide was removed by ACE. This activity could be completely inhibited by captopril. When proline was in the penultimate position, the COOH-terminal amino acid was released by carboxypeptidase P (10) and was not affected by captopril.

We next chose two commercially available pentapeptide substrates containing proline in defined positions as potential candidates to examine the sequential action of ACE and carboxypeptidase P in the hydrolysis of proline peptides. When Z-Gly-Pro-Leu-Ala-Pro and Z-Gly-Pro-Leu-Gly-Pro were incubated with brush border membranes there was a rapid release of the respective COOH-terminal dipeptide and a slower, continuous release of leucine. In addition, captopril inhibited the appearance of all hydrolytic products. These observations indicated that ACE is responsible for removing the COOH-terminal dipeptide from these two substrates, thereby initiating their hydrolysis. The Z-Gly-Pro-Leu peptide which is also a product, is then further hydrolyzed by carboxypeptidase P, releasing free leucine.

Interestingly, both pentapeptides were hydrolyzed to a small extent under conditions where ACE activity was completely inhibited by captopril (Fig. 2). This suggested the possibility that another enzymatic mechanism might be involved in the degradation of these pentapeptides. Indeed, when bestatin, an inhibitor of brush border membrane aminopeptidase N (15), was added to incubation mixtures along with captopril, Leu-Ala-Pro and Leu-Gly-Pro were found to be the only hydrolytic products. The concentration of the tripeptide products was equal to those of leucine and the COOH-terminal dipeptides originally observed in the presence of captopril only. It was subsequently found that the release of these two tripeptides could be completely inhibited by low concentrations of phosphoramidone, a known specific inhibitor of neutral metalloendopeptidases (16). These results indicate that a phosphoramidone-sensitive metalloendopeptidase in the intestinal brush border membrane is also involved in hydrolysis of these two pentapeptides, releasing the COOH-terminal tripeptide. These released tripeptides are then degraded to free leucine and proline dipeptide by the action of brush border membrane

aminopeptidase N. A phosphoramidone-sensitive brush border membrane metalloendopeptidase has been studied by our laboratory and its physiological role in the digestion of dietary protein examined (21). Thus, the present study confirms our initial observation that neutral endopeptidases may play a significant role in protein digestion.

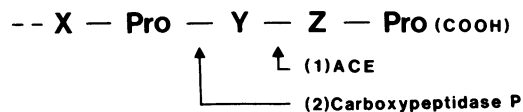
To briefly summarize, the results of the *in vitro* studies indicate that the mechanism of hydrolysis of the NH_2 -blocked prolyl pentapeptides proceeds as follows; (a) initially, the COOH-terminal dipeptides are removed by brush border membrane ACE; (b) the newly exposed COOH-terminal leucine residue is next released by the action of carboxypeptidase P; (c) these pentapeptide substrates are also hydrolyzed by a neutral endopeptidase, releasing the COOH-terminal tripeptide that is rapidly degraded by brush border membrane aminopeptidase N to a free amino acid and prolyl dipeptide.

The physiological importance of this mechanism of enzyme action was examined by perfusing the NH_2 -blocked proline-containing peptides through segments of rat jejunum *in vivo*. Analysis of the hydrolytic products appearing in the perfusates demonstrated that the *in vivo* hydrolytic pattern is the same as that observed *in vitro*. Absorption rates of the constituent amino acids originating from the COOH-terminal dipeptide were similar for Bz-Gly-Ala-Pro, Z-Gly-Gly-Pro, and Z-Gly-Pro-Leu-Ala-Pro peptides. On the other hand, absorption of glycine and proline from Z-Gly-Pro-Leu-Gly-Pro was lower, a result that is consistent with the lower rate of hydrolysis found for this peptide in the *in vitro* study (Fig. 1 B). Absorption rates for leucine originating from both pentapeptides and Z-Gly-Pro-Leu shows that carboxypeptidase P also plays an important physiological role in the degradation and assimilation of these peptides by the intestinal mucosa. In general, the absorption rates of amino acids derived from the released COOH-terminal dipeptides were equivalent to those observed when substrate for aminopeptidase N, a major constituent of the brush border membrane, was perfused in a similar system (14).

In the presence of captopril, hydrolysis and absorption of constituent amino acids from Bz-Gly-Ala-Pro and Z-Gly-Gly-Pro was completely inhibited. With the two pentapeptides, absorption of constituent amino acids was considerably decreased by captopril but still occurred at measurable rates due to the participation of neutral endopeptidase and aminopeptidase N in their hydrolysis (Fig. 5) as observed in the *in vitro* study.

The results of the *in vivo* perfusion study shows that NH_2 -blocked peptides containing a proline residue at the COOH terminus are hydrolyzed by ACE, which appears to be essential for absorption of their constituent amino acids. The X-Pro type dipeptides released by ACE are not readily hydrolyzed by brush border membrane peptidases and are probably absorbed intact (22, 23). It has been shown that amino acids are more efficiently absorbed in peptide form when compared to free amino acid (17, 24, 25). Therefore, the fact that the COOH-terminal dipeptides are the major hydrolytic products from these proline-containing peptides indicates that they have an advantage in being efficiently absorbed by the intestinal mucosa. In the present study, comparatively high amounts of the respective COOH-terminal proline dipeptide was observed in perfusates during peptide perfusion. However, the appearance rates of these dipeptides are less than, or in one instance ap-

Carboxy Terminus



Amino Terminus

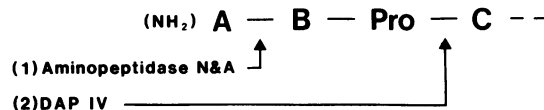


Figure 6. Proposed mechanism of hydrolysis of prolyl peptides by intestinal brush border membrane peptidases. ACE and carboxypeptidase P can act in a concerted manner to sequentially degrade proline peptides from the COOH terminus. Aminopeptidases N and A sequentially remove NH_2 -terminal amino acids from the prolyl peptide but cannot cleave the B-Pro bond. DAP IV specifically removes the NH_2 -terminal B-Pro dipeptide, thereby continuing hydrolysis.

proximately equal to the absorption rates for the constituent amino acids. This indicates that absorption of the proline dipeptides is probably not the rate limiting step in their assimilation by the intestine.

As this study and others from our laboratory have demonstrated, the intestinal brush border membrane contains several different peptidases that are unique in their ability to hydrolyze prolyl peptides (2, 10, 12). This is in contrast to the enzymes of gastric and pancreatic origin that are either unable to cleave peptide bonds involving proline or have low activity towards them. For instance, pancreatic carboxypeptidases A and B can cleave the Pro-X peptide to a limited degree, but they are inactive towards peptides with proline in the COOH-terminal position (26, 27). Thus, the presence of ACE, DAP IV and carboxypeptidase P in the small intestine is complementary to the known substrate specificities of pancreatic enzymes and serve to insure the rapid hydrolysis and assimilation of prolyl peptides as shown in Fig. 6.

In summary, it is clear that proline-containing peptides can be sequentially hydrolyzed by the concerted action of brush border membrane carboxypeptidases and are efficiently assimilated as dipeptides and free amino acids. This COOH-terminal hydrolytic activity in combination with the action of intestinal DAP IV on the amino terminus serves to efficiently hydrolyze prolyl peptides. These results also suggest that COOH-terminal hydrolysis of oligopeptides may not be limited to those containing proline since ACE has a relatively wide substrate specificity (11, 12). From this stand point, further studies are necessary to fully elucidate the mechanisms involved in the intestinal digestion and assimilation of dietary proteins.

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