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STUDIES ON THE ORGANIZATION OF THE BRUSH BORDER IN INTESTINAL EPITHELIAL CELLS

IV. AMINOPEPTIDASE ACTIVITY IN MICROVILLUS MEMBRANES OF HAMSTER INTESTINAL BRUSH BORDERS

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SUMMARY

1. Microvillus membranes isolated from hamster intestinal epithelial cells are capable of hydrolyzing 8 di- and tripeptides at appreciable rates. Hydrolysis is most rapid at or near neutral pH. Cobalt and zinc restore activity when it is reduced by treatment of the membrane with EDTA. D-Leucyl-glycine, *N*-Z-leucyl-glycine and several proteins are not hydrolyzed. Enzymatic activity is not removed by deoxycholate treatment.

2. The specific activity of membrane aminopeptidase measured with tripeptide substrates is comparable to that of membrane sucrase.

3. Owing to a location at the luminal surface of the epithelial cell, an association with carbohydrate digestive hydrolases and a relatively high specific activity in isolated membranes, brush border aminopeptidase activity is concluded to play a role in terminal protein digestion at the peptide level.

INTRODUCTION

Enzymatic hydrolysis of protein in the intestine of man and animals has two phases; one luminal, the other cellular. Within the lumen, well-characterized peptidases secreted by the stomach and pancreas hydrolyze protein to peptides[1–3] and a smaller amount of free amino acids[4,5]. However, there are also enzymes associated with the mucosal epithelial cells which hydrolyze peptides to free amino acids as evidenced by the following observations: (1) isolated *in vivo* loops of intestine devoid of gastric and pancreatic secretions carry out appreciable hydrolysis[6–8]; (2) isolated and washed *in vitro* sac preparations hydrolyze peptides[8–10]; and (3) individuals with pancreatic insufficiency hydrolyze peptides in substantial quantity[11]. These

Abrevation: Z, benzoyloseycarbonyl group.

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observations are corroborated by direct studies showing that the epithelial cell contains a number of peptidases. For example, SMITH AND BERGMANN reported[12] finding an aminopeptidase in intestinal mucosa which they attempted to purify[13]. Also, ROBINSON[14] found dipeptidases in fractions of rat mucosal homogenates subjected to differential centrifugation. Activity was distributed among various fractions but the largest proportion was in the high-speed supernatant fluid[14].

With respect to digestion, there are several aspects of cellular peptidase activity which are of especial interest to us. First, among the various enzymes present, the aminopeptidases have an exceptional capability as they exhibit a relatively wide spectrum of specificity for splitting N-terminal residues and dipeptides[15]. Second, it is clear that the epithelial cell brush border serves as a digestive–absorptive surface for carbohydrates[16]. It may also serve as a digestive surface for peptides. Studies along these lines have been made practicable owing to the fact that EICHHOLZ AND CRANE have recently succeeded in disrupting isolated hamster intestinal brush borders and recovering a fraction of pure microvillus membranes by density-gradient centrifugation[17,18]. These membranes contain the total cellular activities of alkaline phosphatase, maltase and sucrase, among others, and are enriched in leucyl-naphthylamidase. It is the purpose of this report to describe some characteristics of the latter.

METHODS

Isolated brush borders were prepared, disrupted by 1.0 M Tris and fractionated on a glycerol density gradient as previously described[17]. The various fractions were removed by aspiration from the top of the gradient and separated from glycerol by dialysis using 18/32 tubing treated to remove some contaminants[19], or by centrifugation and washing with appropriate buffers. In some experiments the membrane fraction was treated with various concentrations of freshly prepared deoxycholate. These concentrations ranged from 0.03 to 3.0% and the ratio of deoxycholate to protein varied from 1:1 to 60:1. Deoxycholate was immediately removed by dialysis or by passage through Sephadex G-25 columns equilibrated and eluted with 0.01–0.05 M buffers (pH 7.0–7.5). High-speed centrifugation was performed in the Beckman Spinco Model L preparative ultracentrifuge. Protein was determined by the method of LOWRY *et al.*[20]. Alkaline phosphatase was determined with *o*-nitrophenylphosphate in buffer containing cobalt, manganese and magnesium[17]. Leucyl-naphthylamide hydrolysis was determined by the method of GOLDBARG AND RUTENBERG[21]. The method of DAHLQVIST was used for disaccharidase determination[22]. The hydrolysis of leucinamide, dipeptides and tripeptides was determined by the ninhydrin method of MATHESON AND TATTRIE[23] using incubation in 0.4-ml volumes of 0.05 M buffers with 0.1 – 0.3 μmole of substrate. Standards were prepared using a mixture of substrate and products in proportions to coincide with 5–30% hydrolysis.

RESULTS

As a control on enzyme location, leucyl-naphthylamidase activity of the luminal contents of fed hamsters was determined. The excised intestine was washed with normal saline and the washings were centrifuged at 500 $\times$ g for 10 min to

remove cells and large debris. The specific activity of leucyl-naphthylamidase in the supernatant fluid was less than 4% that of a preparation of isolated brush borders.

HOLT AND MILLER reported that similarly to alkaline phosphatase and sucrase, 75% of the total cellular leucyl-naphthylamidase was recovered with isolated brush borders[24]. We have obtained approximately the same result and further found that the microvillus membranes have a specific activity for hydrolysis of leucinamide, alanyl-glycine and leucyl-glycyl-glycine greatly increased over the isolated brush borders. To be compared with the above is the fact that only 6–8% of the total cellular peptidase activity for leucyl-tyrosine, leucyl-leucine or leucyl-leucyl-leucine is recovered with isolated brush borders.

Metal ions do not appreciably influence the ability of microvillus membranes to hydrolyze leucyl-naphthylamide unless the membranes have been exposed to metal-binding agents. Dialysis of the membrane fraction against 0.01 M EDTA (pH 7.4) at 4° for 20 h, and then against water at 4° for 20 h, reduces hydrolysis of leucyl-naphthylamide and leucyl-glycine to less than 10% of the original. Activity is fully restored by incubation at room temperature with 1 mM cobalt, 90–100% restored with 1 mM zinc and 60–70% restored with 1 mM manganese. Magnesium, 1 mM, is without substantial effect. These results are similar to those reported on a 2650-fold purified amino acid naphthylamidase from human liver[25], and a less purified enzyme from human intestinal extracts[26].

Hydrolysis of leucyl-naphthylamide by membrane preparations that have not been treated with EDTA is maximal at pH 7.0 with a rather broad range from pH 6 to 8. Hydrolysis of leucinamide is maximal at pH 7.5–7.6 with 80–84% of the maximal activity still present at pH 8. Hydrolysis of leucyl-naphthylamide in Tris buffer at pH 7.5 is about 60% of the maximal activity in phosphate buffer of the same pH and molarity.

The rates of hydrolysis of leucyl-naphthylamide, leucyl-leucine, leucyl-leucyl-

TABLE I

DISTRIBUTION OF DIPETIDASE ACTIVITY IN GLYCEROL GRADIENT OF DISRUPTED BRUSH BORDERS

2.8 mg brush borders from the mucosal scrapings of 4 hamsters (2.0 g wet wt.) were disrupted in 3.0 ml of 1.0 M Tris (pH 7.4) as previously described[16]. This is less than optimum protein to Tris ratio for obtaining maximal yields of membrane fraction. Bands A through D correspond to previously reported patterns obtained by transillumination from below[16]. Band C contains microvillus membranes and the highest activities of disaccharidases. Aliquots were removed from the top and dialyzed against 10–50 mM phosphate buffer (pH 7.4).

Bands	*Fraction No.*	*Fraction volume (ml)*	LOWRY *protein (per cent of total)*	*Per cent distribution*				
				Leucyl-naphthyl-amide	*Leucyl-glycine*	*Alanyl-glycine*	*Glycyl-glycine*	*Leucine-amide*
	1	2.1	20	1	1	1	2	2
A	2	4.0	21	6	7	8	7	8
B	3	5.2	10	12	13	14	10	15
C	4	10.6	17	46	40	36	37	36
	5	1.5	4	7	7	8	9	8
C′	6	3.2	7	9	10	10	9	9
	7	3.5	6	7	8	7	12	8
D	8	ppt.	15	12	15	17	13	14

leucine, and glycyl-leucyl-tyrosine are linear with time in 0.05 M phosphate buffer (pH 7.4) without added metals; the rates of hydrolysis of leucinamide and leucyl-glycine increase with time.

Distribution of peptidase activity among the fractions of Tris-disrupted brush borders is shown in Table I. Each fraction was dialyzed against 0.01 M sodium phosphate buffer at 4° for 24 h before being tested for activity which leucyl-naphthylamide, leucyl-glycine, alanyl-glycine, glycyl-glycine, and leucinamide as substrates. The distribution of all tested activities is essentially identical; 36–46% is contained in the membrane fraction which contains only 17% of the total protein. These various dipeptidase activities are clearly associated with the microvillus membrane.

The relative specific activities of the microvillus membrane with several peptides is summarized in Table II. Compatible with aminopeptidase activity, there was no hydrolysis of leucyl-glycine. *N*-Z-leucyl-glycine, prolyl-glycine or β-alanylhistidine (Carnosine) under conditions where 0.001 μmole/min per mg could be detected. There was also no significant increase in the ninhydrin color when 10–40 μg (LOWRY *et al.*) of the membrane were incubated with 1 mg/ml of egg albumin, bovine fibrinogen, cytochrome *c* or ribonuclease using leucine as the standard.

As shown in Table II the specific activity of sucrase in the membrane is about 3.0 μmoles/min per mg. Inasmuch as sucrase is an essential digestive enzyme for carbohydrates, the fact that the specific activity for leucyl-leucine is almost one half the sucrase value suggests that peptide hydrolysis is, like glycoside hydrolysis, a significant function of the microvillus membrane.

The possibility that the peptidase activity of the membrane fraction reflected non-specific adsorption during its preparation of enzymes from other cellular locations was excluded by treatment with deoxycholate as described in the METHODS section. Recoveries from the Sephadex G-25 column were in the range of 75–95% for both protein and enzymatic activity. The distribution of protein and disaccharidase and peptidase activity coincided in the elution pattern and were identical with the elution pattern of Dextran Blue 2000. Aliquots from the enzyme fraction were diluted to 9 ml with 0.01 M phosphate buffer and centrifuged at 120 000 × *g* for 1.5–4 h. Samples were aspirated from the top of the tube and the precipitate was

TABLE II

HYDROLYSIS OF VARIOUS SUBSTRATES BY MEMBRANE FRACTION

All determinations (except with sucrose) were carried out at 37° in 50 mM phosphate buffer (pH 7.4) for 10–200 min using 1 to 40 μg of the membrane fraction. Sucrase activity was assayed as noted under METHODS.

Substrate	*Spec. activity (μmoles/min per mg)*
Gly-Gly	0.04
Ala-Gly	0.36
Leu-Gly	0.39
Leu-NH_2	0.50
Gly-Leu-Tyr	0.50
Leu-Leu	1.00
Leu-Gly-Gly	1.05
Leu-Leu-Leu	1.40
Sucrose	3.0

taken up in buffer. The distribution of protein and various enzymatic activities in these fractions is shown in Table III. The distribution ratios of protein, alkaline phosphatase, sucrase, leucyl-naphthylamidase and leucyl-glycinase coincide in all fractions. In other experiments, the distributions of leucyl-leucine and alanyl-glycine hydrolysis were similar. In the experiment of Table III, the largest percentage of

TABLE III

ENZYME ACTIVITY IN ALIQUOTS FROM ULTRACENTRIFUGATION OF THE DEOXYCHOLATE-TREATED MEMBRANE FRACTION

1.1 mg of membrane fraction were incubated at 25° for 10–15 min with 33 mg deoxycholate (30:1). The deoxycholate was freshly prepared prior to each experiment. The sample was eluted from a Sephadex G-25 column at room temperature. The enzyme fraction was taken up to 9.0 ml with 10 mM phosphate buffer (pH 7.4) and centrifuged at 120 000 × *g* for 3 h. Aliquots were removed from the top by aspiration.

Fraction volume (ml)	*Protein (% of total)*	*Per cent distribution of activity*			
		Alkaline phosphatase	*Maltase*	*Leucyl-naphthyl-amidase*	*Leucyl-glycinase*
2.0	11	12	13	12	13
2.0	16	12	13	12	13
2.0	11	12	12	12	13
2.0	12	12	12	12	13
1.0	31	35	38	39	33
ppt.	17	17	13	13	15
Recovery from tube	97%	84%	70%	87%	97%

the activity and protein migrated to the lower 1.0 ml in the centrifuge tube, as might be expected were there micelle formation of the deoxycholate. This possibility was excluded by diluting the active Sephadex G-25 fraction 45-fold, and again centrifuging. The distribution of leucyl-naphthylamidase and maltase activities were determined and found to remain similarly distributed with most of the activity in the lower 1.0 ml of the centrifuge tube. Since the peptidase activities behaved under all these conditions in a closely similar way to enzymes (sucrase, *etc.*) known to be membrane components, it may be concluded that these peptidase activities are also membrane components. It may also be noted that considerable washing of the membrane occurs during its preparation and that leucyl-naphthylamidase activity is removed only after vigorous treatment with papain[27].

DISCUSSION

The work of CRANE and his associates, confirmed by others using a different technique[28] has demonstrated that the brush border membrane of the intestinal epithelial cell plays a dominant role in terminal hydrolysis of carbohydrates at the disaccharide level; maltase, sucrase, isomaltase and lactase are located therein. Also, alkaline phosphatase[29,30] and leucyl-naphthylamidase[31,32] have been histochemically localized to the brush border. The importance to digestion of the brush border organelle implied by these findings has been amply confirmed in recent years by clinical

reports of individuals with disaccharidase deficiencies and transport defects[33]. The results of the present study appear to extend the potential of the microvillus membrane to include di- and tripeptide hydrolysis at levels of activity consistent with digestive function.

Those of our findings that are applicable are in agreement with the report of ROBINSON[14] who found 7–11% of the total glycyl-glycine and leucyl-glycine hydrolytic capacity from rat intestinal homogenates to be present in the 'microsomal' fraction in association with 56–67% of the total alkaline phosphatase. Since brush borders fragment and are primarily found in such microsomal fractions when special conditions for their preparations as intact organelles are not used, this result is expected. ROBINSON also found 80–90% of the total hydrolytic capacity for these two dipeptides to remain in the high-speed supernatant which is comparable to the 92–94% in the supernatant from our brush border preparations.

The association of aminopeptidase and leucyl-naphthylamidase activities in the same subcellular fraction; *i.e.*, the microvillus membrane, would appear to be an unusual finding. Previous investigators, working with serum, tissue extracts and high-speed supernates from tissue homogenates have found that leucyl-naphthylamide hydrolysis is readily separable from leucinamide or leucyl-glycine hydrolysis. In preparations from intestine also, both human[26] and mouse[34], these activities were separable. Most of the intestinal leucyl-naphthylamidase is associated with particulate fractions. Most leucinamidase is not. For example, although quantitative distributions were not reported, the specific activity of a high-speed precipitate for leucyl-naphthylamidase was found to be the same as that of the high-speed supernate from the mouse[34]. Also most of the leucyl-naphthylamidase of guinea pig and rabbit intestinal homogenates remains with particles[35], although more vigorous disruption procedures may have yielded additional activity in the supernate[26,34]. Recently, the presence of aminotripeptidase and dipeptidase has been reported in rat brush borders separated by density gradient centrifugation; the brush border fraction contained 22% of the total aminotripeptidase and 35% of the aminopeptidase. However, the predominant particulate associated proteolytic activity was apparently not a brush border enzyme[36]. The special characteristics of microvillus membrane peptidase activity which we have seen above were undoubtedly obscured in these various studies because the activity is such a small proportion of the total cellular peptidase activity.

It is worth noting that a portion of leucyl-naphthylamidase of other tissues appears to be particulate. In ascites tumor cells, 40% of the leucyl-naphthylamidase is present in a fraction which is either membranes or microsomes[36], whereas 85–100% of leucinamide hydrolysis is associated with the high-speed supernatant. Leucinamide hydrolase of rat liver[37,38] is also a predominantly soluble enzyme.

An answer to the question of whether digestion occurs at the membrane or at an intracellular locus clearly depends on which peptide is meant.

NEWEY AND SMYTH concluded from a series of investigations with intestinal sac preparations that the hydrolysis of glycyl-glycine occurred intracellularly rather than at the membrane surface[39]. The relatively slow rate of glycyl-glycine hydrolysis by the microvillus membrane is consistent with this conclusion. Our investigations do not bear on the possibility of transport of glycyl-glycine into the cell where hydrolysis could then occur. However, as seen from Table III, peptides other than

glycyl-glycine are hydrolyzed at a rapid rate by the membrane. For example, glycyl-leucyl-tyrosine is hydrolyzed 12 times more rapidly than glycyl-glycine.

Conclusions drawn primarily from studies with glycyl-glycine are not generally applicable to overall digestive hydrolysis.

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