Biochimica et Biophysica Acta, 689 (1982) 181–193
Elsevier Biomedical Press

BBA 71248

THE EFFECT ON AMINO ACID TRANSPORT OF TRYPSIN TREATMENT OF RAT RENAL BRUSH BORDER MEMBRANES

BETTY YEE-LI HSU, SUSAN M. CORCORAN, CONSTANCE M. MARSHALL and STANTON SEGAL *

Division of Biochemical Development and Molecular Diseases, Children's Hospital of Philadelphia, and the Departments of Pediatrics and Medicine, School of Medicine of the University of Pennsylvania, Philadelphia, PA 19104 (U.S.A.)

(Received December 23rd, 1981)

Key words: Amino acid transport; Brush border membrane; Trypsin; (Rat kidney)

Trypsin treatment of isolated rat renal brush border membrane vesicles which preferentially releases L-leucine aminopeptidase (EC 3.4.11.2) decreases their ability to take up a variety of amino acids under Na^+-gradient conditions. Such treatment did not alter the osmotic properties of the vesicles nor affect their fragility. A linear correlation could be demonstrated between the L-leucine aminopeptidase activity of the membranes and the initial rate of uptake of L-leucine and L-proline. Velocity of uptake-concentration dependence studies with these substrates indicate that the major effect of trypsinization is to decrease the maximum velocity (V_{max_1}) of the low-K_m high-affinity system with little effect on the V_{max_2} of the high-K_m low-affinity transport process and no effect on the apparent Michaelis constants of either. Although the data indicate that L-leucine aminopeptidase activity and uptake of L-leucine and L-proline are affected in parallel, they should not be construed to imply a role of the enzyme in the transport process, especially in view of the global decrease in the uptake of various amino acids and sugars.

Introduction

The brush border microvillar membrane of the renal proximal tubule cell is a complex organelle whose function is important in the reclamation of amino acids and other solutes from the tubule lumen [1]. Numerous studies have indicated the presence of various enzymes, especially hydrolases and peptidases [2–6], several of which have been highly purified and characterized [7–13]. The enzymatic complexity of the microvillar membrane and the localization of the marker enzyme have been examined by a number of investigators by means of proteolytic digestion and detergent extraction [14–17].

In contrast to an abundance of knowledge about enzymic microvillar proteins such as γ-glutamyltranspeptidase [18] and the various peptidases which constitute about 17% of microvillar protein [19], little is known of the proteins concerned with amino acid transport. Our information about amino acid transport is derived primarily from kinetic studies of uptake by isolated brush border membrane vesicles. The transport characteristics of these vesicles with proline [20,21], alanine [22], cystine [23], glutamine [24] and other amino acids [25–27] have been reported. This situation has given rise to the speculation that microvillar peptidases may play a role in amino acid transport. Meister [18] has proposed that γ-glutamyltranspeptidase may mediate the transport of amino acids and Kenny and co-workers [1,19] have suggested that aminopeptidases may be involved in transport of leucine and other amino acids.

We have previously reported on both the delin-

* To whom correspondence should be addressed.

eation of amino acid-transport systems [20,24, 26,27] and the sensitivity to proteolytic digestion and detergent-induced dissociation of proteins and enzymic activities in isolated rat renal brush border membrane preparations [28]. In this paper we have combined both types of approaches and examined the effects of graded trypsin digestion of brush border membrane vesicles on amino acid transport. We have taken advantage of the fact that in our previous studies, trypsin preferentially released leucine aminopeptidase activity from the membrane while not altering the membrane complement of other marker enzymes such as alkaline phosphatase, γ-glutamyltranspeptidase and maltase [28]. The results reported herein indicate that the diminished ability of trypsin-exposed vesicles to take up leucine and proline correlates with the amount of leucine aminopeptidase stripped from the membrane.

Materials and Methods

Preparation of brush border membrane

Adult male Sprague-Dawley rats weighing 250–300 g and fed ad libitum on Purina rat chow were killed by decapitation. Kidneys were removed, decapsulated and placed in 0.9% NaCl solution at 4°C. Rat cortical tissue was removed by using a Stadie-Riggs microtome. Brush border membranes were isolated by using the $MgCl_2$-precipitating method of Booth and Kenny [29]. The increase in alkaline phosphatase activity in the brush border fraction over that in the cortical homogenate was between 10- and 13-fold. The average alkaline phosphatase specific activity of the membrane preparation was between 12000 and 16000 Sigma units/mg membrane protein [30]. Each final membrane fraction to be treated was suspended in buffer A (0.25 M sucrose, 0.01 M triethanolamine hydrochloride, pH 7.6). The protein concentration of the suspension was determined by the method of Bradford [31] using the Bio-Rad protein assay kit from Bio-Rad, Rockville Center, NY. For membrane fractions suspended in buffer B (2 mM Tris-Hepes buffer, pH 7.4, containing 100 mM mannitol), the protein concentration of the membrane preparation was determined by the method of Lowry et al. [32]. Membrane protein concentration was adjusted between 2.5 and 3.0 mg/ml buffer before proteolytic digestion.

Trypsin solubilization of brush border preparation

Trypsin (EC 3.4.21.4) crystallized from bovine pancreas was obtained from Calbiochem as was trypsin inhibitor which was crystallized three times from soybean. Isolated brush border fractions were treated with 0.01, 0.05, 0.2 or 1.0 mg trypsin/ml membrane preparation in buffer A at 37°C for the designated time period. As a control of trypsin digestion, the volume of trypsin and trypsin inhibitor was replaced by buffer or mixed trypsin and trypsin inhibitor before adding to membrane incubation flasks. At the end of incubation, trypsin inhibitor in buffer A was added with the final concentration being twice that of trypsin (w/w). The incubation mixture was spun immediately at $35000 \times g$ for 20 min at 4°C. The trypsin-solubilized fraction and membrane pellet fraction were separated. The protein and enzyme activities which did not sediment after centrifugation of $35000 \times g$ were called the solubilized fraction of the brush border membrane. The membrane pellets were resuspended in buffer B (6-times the incubation volume) and spun at $35000 \times g$ for 20 min. The supernatant was discarded. The washed pellets were resuspended in buffer B. Protein concentration and enzyme activities in both the solubilized fractions and in the membrane pellets, which were resuspended in desired buffer after treatment, were determined and expressed as the percentage of total (supernatant fraction plus pellet fraction) recovered. Where trypsin digestion was involved, protein concentrations in the fractions were corrected for added trypsin and inhibitor by the use of control samples.

Enzyme assays

The two marker enzymes measured were alkaline phosphatase (EC 3.1.3.1) and L-leucine aminopeptidase (EC 3.4.11.2). Alkaline phosphatase determinations were performed according to the Sigma method [30]. The substrate used was disodium *p*-nitrophenol phosphate and activities were expressed as mmol *p*-nitrophenol liberated/mg brush border membrane during 30 min incubation at 37°C. L-Leucine aminopeptidase was assayed according to the method of

Goldmann et al. [33]. L-Leucine *p*-nitroanilide was used as a substrate. The activities were calculated as mmol *p*-nitroaniline released/min per mg membrane proteins. Data are expressed as a percentage of the total activities in both supernatant and pellet fractions of the control vesicles incubated at 4°C for 30 min.

Protein concentrations in the supernatant fractions were determined by the method of Bradford [31], using the Bio-Rad protein assay kit from Bio-Rad, Rockville Center, NY. The washed pellet fraction was assayed for protein utilizing the procedures of Lowry et al. [32]. Data are presented as the percentage of the difference in the amount of protein of pellets at 4 and 37°C as compared to the sum of the protein amount in both supernatant and pellet fraction at 4°C.

Uptake of amino acids and sugars

The resulting membrane vesicles after treatment with trypsin were separated from the solubilized fraction and washed as described in the section for trypsin solubilization of brush border preparation. The final protein concentration of the washed membrane after these manipulations was adjusted to 0.20–0.25 mg/ml buffer B as determined by the method of Lowry et al. [32]. The resulting vesicle suspension was allowed to equilibrate at 22°C for at least 30 min before the uptake studies. The uptake of amino acids at 22°C was determined in the presence of a 100 mM inward Na^+ gradient by using filtration techniques as described by McNamara et al. [20]. Results of amino acid-uptake studies were obtained as uptake of ^{14}C-labeled amino acids in excess of the diffusion component as measured simultaneously by the entrance of 3-*O*-[*methyl*-^{3}H]methyl-D-glucose or L-[1-^{3}H]glucose into the vesicle. These sugars are thought to enter the brush border by diffusion [20,24].

To determine the effect of trypsin solubilization of the brush border membrane on the uptake of amino acids and sugars, 0.5 ml of the prepared membrane vesicles in buffer B, pH 7.4, at 22°C was added at the starting time to a disposable 10 × 75 mm test tube containing 0.1 μCi ^{14}C-labeled amino acid, 0.1 μCi 3-*O*-[*methyl*-^{3}H]methyl-D-glucose or 0.4 μCi L-[1-^{3}H]glucose, 50 μmol NaCl and unlabelled amino acid to bring the incubation mixture to the desired final concentration of substrates. Uptake of the amino acids were calculated as nmol amino acid taken up by the vesicle containing 1 mg membrane protein during the initial 0.5 min of incubation at 22°C. Data were expressed as the percentage of total uptake of the same amino acid in the absence of trypsin.

To study the effects of osmolarity of the medium, various volumes of 2 M sucrose in buffer were added to replace the regular buffer in order to reach the desired osmolarity of the incubation mixture. The final volume of the mixture was 0.565 ml. The samples were vortex mixed during the first 3 s of incubation. After a total incubation time ranging from 6 s to 20 min, the incubation mixture was transferred by Pasteur pipette to a Millipore filter apparatus. Uptake was stopped by rapid filtration of the mixture through a Millipore filter (HAWP, 0.45 μm) which was washed once with 5 ml of solution containing 0.9% NaCl in buffer B, pH 7.4. The filter was air dried and counted in a Packard Tricarb scintillation spectrometer in 4 ml diluted Concentrol (Yorktown Research, Miami, FL). All vesicle preparations used for transport studies were also assayed for L-leucine aminopeptidase and alkaline phosphatase activity and the degree of solubilization of these enzymes by the treatments used was determined.

Test of possible friability of the vesicle after trypsin treatment

Brush border membrane preparations were treated with 0.1% trypsin at 37°C for 25 min as described above. After treatment, the washed membrane pellets were resuspended in buffer B and the final membrane protein concentration was adjusted to about 0.2 mg/ml buffer for transport studies. 0.5 ml membrane vesicle suspension in the presence of a 100 mM inward Na^+ gradient was vortex mixed and incubated at 22°C for 0.25 min. The mixture was filtered through Millipore filter (HAWP, 0.45 μM). Filtrate was collected for alkaline phosphatase assay. Duplicate samples without filtration were also assayed for alkaline phosphatase activity.

Materials

L-[U-^{14}C]Glutamine, L-[U-^{14}C]proline, L-[U-^{14}C]leucine, D-[U-^{14}C]glucose, L-[U-^{14}C]lysine,

[U-^{14}C]glycine, L-[U-^{14}C]methionine, L-[1-^{3}H]glucose and 3-*O*-[*methyl*-^{3}H]methyl-D-glucose were purchased from New England Nuclear Corp. α-[U-^{14}C]Methyl-D-glucoside was obtained from Rosechem. Products (Los Angeles, CA). γ-Glutamyl-*p*-nitroanilide, Hepes, trypsin and trypsin inhibitor were purchased from Calbiochem. All other chemicals were of the highest purity available.

Results

The effect of trypsin concentration and incubation time

The effect of incubating brush border membrane vesicles with various concentrations of trypsin on removal of protein and enzyme activity is shown in Table I. Incubation for 30 min at 37°C in buffer alone affected the release of 5.8% of both L-leucine aminopeptidase and alkaline phosphatase activities together with 13.7% of membrane protein. Essentially, the same results were obtained when the vesicles were incubated at 4°C for 30 min (12.9% protein released and 3.1% of the enzyme activity), thus indicating that exposure of the vesicles to ionic buffer and their manipulation during centrifugation, resuspension and washings only slightly affect the membrane. Treatment of the membrane with 50 μg trypsin/ml resulted in further removal of half of the L-leucine aminopeptidase, little alkaline phosphatase and no detectable protein. Increasing the trypsin concentration to 200 μg/ml released an additional 21% of L-leucine aminopeptidase and 17% of membrane protein while 1 mg/ml caused the liberation of 15% more protein without L-leucine aminopeptidase or alkaline phosphatase activity.

The results of the effect of various incubation times at 37°C in the presence of 200 μg trypsin/ml membrane preparation upon the net removal of marker enzymes L-leucine aminopeptidase and alkaline phosphatase together with the membrane proteins are shown in Table II. At 15 min, 70% of L-leucine aminopeptidase was removed, very little alkaline phosphatase and 18% of protein was removed above that observed during incubation with buffer alone (4.56% L-leucine aminopeptidase and 11.31% protein).

By increasing the incubation time from 15 to 30 min at 37°C, about 8% more of the membrane L-leucine aminopeptidase together with an additional 3% of the membrane protein were removed with still little alteration in alkaline phosphatase. By increasing the incubation time to 45 min, there was no further effect upon the membrane proteins and L-leucine aminopeptidase removal as compared to that of 30 min incubation. However, there was an additional 7.24% alkaline phosphatase removal as compared to that of 30 min incubation. Since membrane alkaline phosphatase is generally considered as part of the membrane matrix and not a surface protein [28], it therefore appears that prolonged incubation to 45 min at 37°C in the presence of 0.02% trypsin resulted in a disturbance of the membrane matrix. Hence, the data from Tables I and II suggest that the optimal trypsinization condition for membrane L-leucine aminopeptidase would be 0.02% trypsin incubated

TABLE I

EFFECT OF TRYPSIN CONCENTRATION UPON THE REMOVAL OF MEMBRANE PROTEIN, L-LEUCINE AMINOPEPTIDASE AND ALKALINE PHOSPHATASE ACTIVITIES FROM BRUSH BORDER MEMBRANE

Brush border membranes were incubated at 37°C for 30 min. Each value is the mean±S.E. for four determinations.

Trypsin concentration		% of L-leucine aminopeptidase stripped from membrane	% of alkaline phosphatase stripped from membrane	% of protein stripped from membrane
μg/ml membrane	μg/mg membrane protein			
0	0	5.84±0.0	5.82±0.2	13.67±1.1
50	51.0– 58.0	60.13±2.0	6.92±0.1	12.60±1.8
200	204.0– 232.0	81.92±0.6	7.04±0.1	30.35±1.7
1000	1020.0–1161.0	83.64±0.3	6.58±0.1	45.25±1.2

TABLE II

EFFECT OF THE LENGTH OF INCUBATION AT 37°C BY TRYPSIN TREATMENT UPON THE REMOVAL OF MEMBRANE PROTEINS, L-LEUCINE AMINOPEPTIDASE AND ALKALINE PHOSPHATASE ACTIVITIES OF THE BRUSH BORDER MEMBRANE

Each value is the difference from that observed for vesicles incubated for the same length of time in buffer without trypsin.

Time of incubation at 37°C (min)	Trypsin concentration		% of L-leucine aminopeptidase stripped from membrane	% of alkaline phosphatase stripped from membrane	% of protein stripped from membrane
	μg/ml membrane	μg/mg membrane protein			
15	200	146.4–149.6	70.27±0.8	0.62±0.1	18.54±0.4
30	200	147.1–154.2	78.21±0.5	0.44±0.1	21.59±0.6 [a]
45	200	141.5–143.7	78.99±0.3	7.24±0.1	20.73±1.0 [b]

[a] $P<0.01$.

[b] $P>0.05$ for differences from control.

with the renal brush border membrane preparation in buffer A at 37°C for 15 min which results in the maximum amount of membrane L-leucine aminopeptidase removed with least effect upon the alkaline phosphatase activity and the matrix for the membrane vesicles.

Effect of trypsinization on the responses of the membrane vesicles to osmotic perturbations

In order to clarify whether the uptake of substrate by trypsinized vesicles respresents transport into an intravesicular space, the relation of intravesicular volume to steady-state equilibration of substrates was examined. As shown in Fig. 1, steady-state accumulation of both L-leucine and L-glucose by control and trypsinized vesicles, which had been treated with 0.02% trypsin at 37°C for 15 min, were found to be inversely proportional to medium osmolarity and thus, directly related to intravesicular space. Extrapolation to infinite medium osmolarity, i.e., zero intravesicular volume, resulted in no uptake, also indicating no binding to the membrane. Kenny and O'Halloran [19] have shown that vesicles, in the presence of L-leucine, are osmotically active and results indicate a transport of amino acid rather than binding to the membrane surface. No difference is apparent in the osmotic reactivity of the trypsinized vesicles and control vesicles which were also incubated at 37°C and washed in parallel with the trypsinized vesicles.

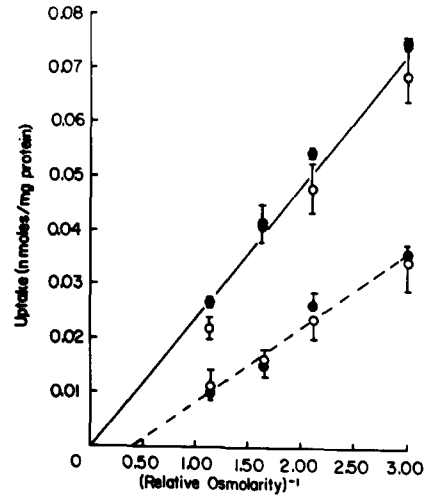

Fig. 1. Effect of medium osmolarity on the uptake of L-leucine (———) and L-glucose (— — —) by control and trypsinized vesicles. Brush border vesicles were incubated at 37°C for 15 min in the absence or presence of 200 μg trypsin/ml membrane preparations in buffer A. Control (●) and trypsinized (○) vesicles were obtained as described in the text. Vesicles were allowed to equilibrate at 22°C for 30 min in buffer B containing 100 mM NaCl and various concentrations of sucrose in order to reach the desired osmolarity of the incubation medium. Since NaCl was equilibrated with the vesicles before uptake measurement, the concentration of NaCl was not counted in relative osmolarity. 0.02 mM L-[^{14}C]leucine (0.1 μCi) and L-[^{3}H]glucose (0.4 μCi) tracer mix were then added and incubated with the vesicles for an additional 24 min, after which the mixture was filtered and radioactivities were determined as described in the text. Results shown are the mean of four determinations at each relative osmolarity. Standard errors are indicated by vertical bars. Relative osmolarity is defined as 300 mosM = 1 = isomolar and 100 mosM = 0.33. The lines were drawn by the least-squares method of a Monroe 1775 programmable calculator.

Effect of trypsinization upon the friability of the membrane vesicles

An experiment was designed to test the stability of the trypsinized vesicles by assessment of the amount of alkaline phosphatase that could be filtered when the vesicles were applied to the 0.45 μm Millipore filter. Results have shown that a total of 6.56% of the alkaline phosphatase activity passed through the filter when the trypsinized vesicles were filtered as compared to 2.58% of alkaline phosphatase activity of the control vesicles. Thus, if we used alkaline phosphatase retention as a measure of the stability of the membrane matrix, the vesicles' response to mechanical stress was little affected by trypsinization.

Relationship between membrane L-leucine aminopeptidase and amino acid uptake by the membrane vesicles

There is a striking correlation between the amount of the membrane L-leucine aminopeptidase remaining on the trypsinized vesicles and the extent of proline and leucine uptake by these vesicles, as shown in Table III. As increasing activity of L-leucine aminopeptidase is removed from the vesicle, the uptake of amino acids decreases. When brush border membranes were treated with 200 μg/ml trypsin, 70% of L-leucine aminopeptidase was removed from the membranes accompanied by a loss of 81–87% of initial (15 s) proline uptake and about 70% loss of initial (15 s) leucine uptake when compared to control membranes. At the same time, there was little or no removal of protein and alkaline phosphatase as compared to that of control (i.e., zero concentration of trypsin).

Since the total recovery of the membrane proteins, alkaline phosphatase and L-leucine aminopeptidase activities from washed pellets and corresponding supernatant fractions was constant, the decrease in L-leucine aminopeptidase activity associated with the membrane fraction represented solubilization of the enzyme rather than inhibition.

Fig. 2 shows a linear correlation between the activities of L-leucine aminopeptidase on the vesicle membranes and the initial (15 s) amino acid uptake by the vesicles. In Fig. 2A, the initial (15 s) 0.02 and 2.1 mM proline uptake by the trypsinized vesicles is plotted against the activity of membrane L-leucine aminopeptidase and is seen to decrease as the membrane L-leucine aminopeptidase decreased. Extrapolation to zero amount of membrane L-leucine aminopeptidase indicates there would be no uptake of L-proline if it were possible to remove all of the L-leucine aminopeptidase activity on the vesicle membranes. The uptake of 0.02 and 2.1 mM L-leucine by trypsinized vesicles vs. the amount of membrane L-leucine aminopeptidase as shown in Fig. 2B also reveals a decrease as the L-leucine aminopeptidase activity diminishes. The extrapolation in Fig. 2B does not go through zero, indicating that at zero amount of membrane L-leucine aminopeptidase, there is still about 20% of the control L-leucine uptake remaining in the trypsinized vesicles.

The uptake of various substrates by trypsinized vesicles

Since previous reports have demonstrated the existence in vesicles of low- and high-K_m transport systems, we surveyed the alteration in the uptake of various amino acids and sugars at both high and low concentrations. The results are shown in Table IV. Not only was the uptake of several groups of amino acids, including neutral and dibasic types, decreased after trypsin exposure but also that of the sugars, D-glucose and α-methyl-D-glucoside. The data indicate that there is a global depression of transport systems in trypsinized membranes. The effect appeared to extend to both high- and low-affinity processes but for a full comprehension of the transport defect the velocity-concentration dependence studies reported below are required.

Effect of trypsinization of vesicle membrane upon the time course of amino acid transport

The removal of 70% membrane L-leucine aminopeptidase as the result of trypsinization exerts an inhibitory effect on the Na^+-gradient-dependent overshoot phenomenon in the brush border membrane for both proline and leucine. Fig. 3 shows the time course of uptake of 0.02 mM L-[^{14}C]proline by control and trypsinized membrane vesicles. The uptake of L-proline in the presence of 100 mM inward Na^+ gradient was decreased 62–90% in the trypsinized vesicles as compared to that of control vesicles over the first 3 min of incubation. It is during this initial 3 min

TABLE III

EFFECT OF TREATMENT OF VESICLES WITH VARIOUS CONCENTRATIONS OF TRYPSIN ON THE MARKER ENZYMES AND UPTAKE OF AMINO ACIDS

Each value for L-leucine aminopeptidase, alkaline phosphatase and membrane protein removed is the mean ± S.E. for four determinations. Each value for proline and leucine uptake is the mean ± S.E. for eight determinations. $P < 0.001$ for differences from control, when not referred to by a footnote.

Trypsin concentration		% of membrane protein removed	% of alkaline phosphatase removed	% of L-leucine aminopeptidase removed	Proline uptake (%)		Leucine uptake (%)	
μg/ml membrane	%				0.02 mM	2.1 mM	0.02 mM	2.1 mM
0	0	12.01 ± 0.2	13.15 ± 0.2	16.83 ± 0.3	100 ± 4.5	100 ± 5.8	100 ± 1.7	100 ± 4.0
10	0.001	9.51 ± 1.3 [d]	15.16 ± 0.2	33.74 ± 0.3	75.18 ± 2.2	77.08 ± 14.0 [d]	89.67 ± 3.9 [b]	72.16 ± 4.8
50	0.005	14.21 ± 1.9 [b]	15.84 ± 0.1	68.01 ± 0.9	32.08 ± 2.4	32.19 ± 2.7 [a]	53.61 ± 4.0	53.87 ± 4.4
200	0.020	14.10 ± 0.7 [c]	13.96 ± 0.2	87.60 ± 0.7	13.37 ± 2.4	18.86 ± 11.8	30.33 ± 4.5	31.03 ± 10.3

[a] $P < 0.01$ for differences from control.

[b] $P < 0.02$.

[c] $P < 0.05$.

[d] $P > 0.1$ for no significant differences from control.

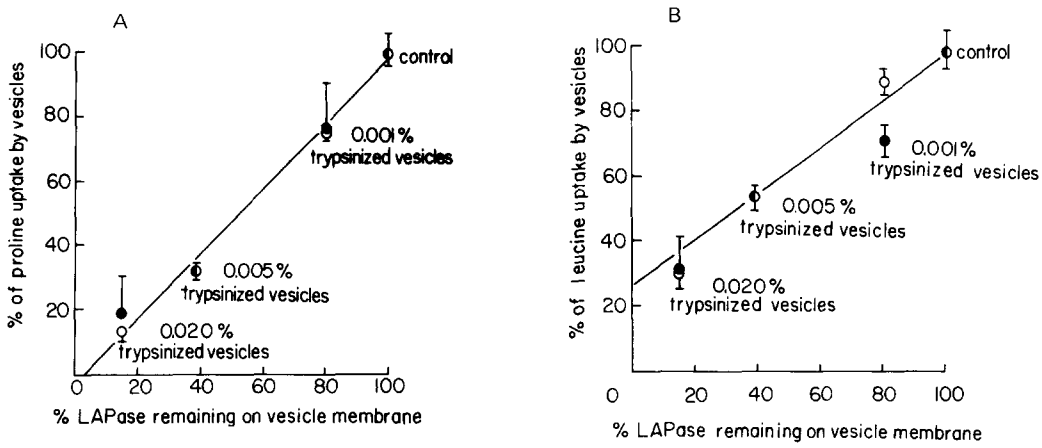

Fig. 2. Relation of membrane L-leucine aminopeptidase to the uptake of (A) L-proline and (B) L-leucine for 0.02 mM and 2.1 mM substrate concentrations. Percent L-leucine aminopeptidase (LAPase) remaining on vesicle membrane after trypsinization with the designated trypsin concentration is data taken and normalized from Table IV. The membrane L-leucine aminopeptidase activity remaining on the control vesicles is taken as 100% (i.e., when treated with no trypsin present). Uptake of 0.02 mM (○) and 2.1 mM (●) substrate concentration was measured after 0.5 min incubation at 22°C as described in the legend to Table IV under 100 mM Na^+-gradient condition. All points are expressed relative to uptake in the absence of trypsin which was taken as 100%. Values given are the mean for eight determinations for each data point. Standard errors are indicated by vertical bars. The lines were drawn by the least-squares method.

that the Na^+-gradient-dependent 'overshoot' phenomenon is observed. Fig. 4 shows the time courses of uptake of 0.02 mM L-[^{14}C]leucine by control and trypsinized membrane vesicles. The uptake of L-leucine by trypsinized vesicles was 45–76% less than that of the control vesicles over the first 3 min of incubation. As in the time curves of L-proline uptake, the trypsinization effect upon L-leucine was evident during the first 3 min of uptake. At the end of 20 min of incubation, when the Na^+ gradient is dissipated, there is no significant difference in the accumulation of L-leucine and L-

TABLE IV

EFFECT OF TRYPSINIZATION UPON THE UPTAKE OF VARIOUS AMINO ACIDS AND SUGARS BY THE MEMBRANE VESICLES

Uptake of the amino acids and sugars was measured at the end of 0.5 min incubation at 22°C. The number of determinations is indicated in the parentheses behind the mean value±S.E.

^{14}C-labeled substrate	% of initial rate of uptake of control vesicles			
	0.02 mM substrate in medium		2.1 mM substrate in medium	
	Control	Trypsinized	Control	Trypsinized
D-Glucose	100±5.7 (12)	25.20±1.7 (12)	100±3.0 (10)	41.90±3.5 (12)
α-Methyl-D-glucoside	100±1.7 (10)	36.09±1.8 (10)	100±2.1 (11)	36.96±4.5 (12)
L-Leucine	100±3.7 (13)	36.62±1.9 (14)	100±2.5 (13)	46.34±2.3 (15)
L-Proline	100±1.7 (16)	22.79±2.5 (16)	100±3.9 (11)	40.64±6.5 (12)
L-Glycine	100±6.1 (15)	41.54±5.3 (14)	100±3.4 (12)	31.54±4.7 (13)
L-Methionine	100±5.3 (10)	24.44±2.3 (12)	100±3.5 (10)	50.07±5.3 (9)
L-Glutamine	100±2.4 (15)	25.00±4.4 (14)	100±3.9 (15)	35.44±3.3 (14)
L-Lysine	100±3.7 (12)	32.00±3.8 (11)	100±7.7 (6)	32.73±10.1 (6)

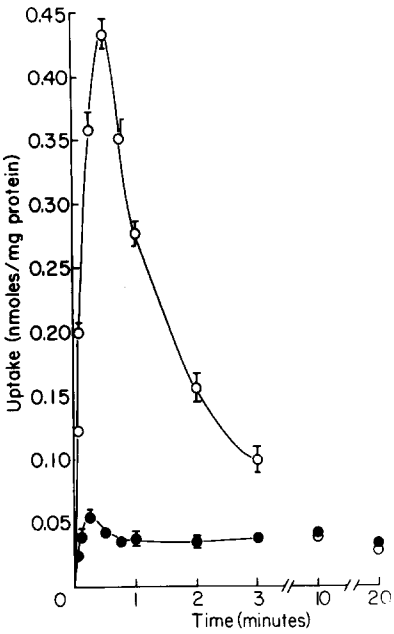

Fig. 3. Effect of trypsinization of vesicle membranes on the time course of uptake of 0.02 mM L-proline by control and trypsinized vesicles. Brush border vesicles were incubated at 37°C for 15 min in the absence or presence of 200 μg trypsin/ml membrane preparations in buffer A. Control (○) and trypsinized (●) vesicles were obtained as described in the text. The time course of uptake by membrane in buffer B incubated with 0.02 mM L-[^{14}C]proline was measured under the conditions of a 100 mM Na^+ gradient at 22°C as described in Materials and Methods. Values shown are the means ± S.E. for eight determinations. Data points without vertical bars indicate that the errors of the data points are included within the size of each point.

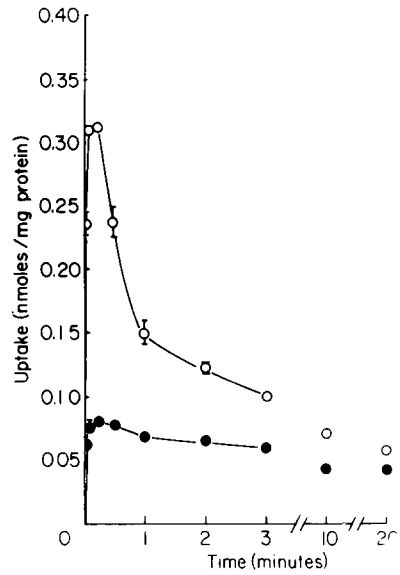

Fig. 4. Effect of trypsinization of vesicle membranes on the time course of uptake of 0.02 mM L-leucine by control and trypsinized vesicles in the presence of an Na^+ gradient. Brush border vesicles were incubated with or without trypsin as described in the legend to Fig. 3. The time courses of uptake of 0.02 mM L-[^{14}C]leucine by control (○) and trypsinized (●) vesicles were measured under the conditions of a 100 mM Na^+ gradient at 22°C as described in the text. Values shown are the means ± S.E. for eight determinations. Data points without vertical bars indicate that the errors of the data points are included within the size of each point.

proline by the control and trypsinized vesicles ($P > 0.05$, not significant).

Effect of trypsinization of vesicle membranes upon the kinetic parameters of amino acid-transport systems

We have previously reported that the kinetics of proline transport by brush border membrane vesicles indicate the presence of two saturable systems for uptake [20,27]. The initial rate of uptake of proline by brush border membrane vesicles at 22°C, after the membrane vesicle had been incubated at 37°C for 15 min in the presence of 0.02% trypsin, was still concentration dependent and saturable, as shown in Fig. 5. The Hofstee plot for the concentration dependence of proline uptake at 15 s shows a two-limbed curve that indicates the presence of multiple transport systems for the vesicles in the absence of trypsinization. The transport parameters determined from the Hofstee plot were: K_{m_1}, 0.095 ± 0.007 mM; V_{max_1}, 2.38 ± 0.17 nmol/15 s per mg; K_{m_2}, 2.21 ± 0.39 mM; V_{max_2}, 11.32 ± 1.06 nmol/15 s per mg. With the Hofstee plot parameters as initial estimates, we calculated kinetic parameters from Eqn. 1 to give the best fit to the observed total uptake by using a Digital PDP 12 computer:

$$V_{total} = \frac{V_{max_1}[S]}{[S] + K_{m_1}} + \frac{V_{max_2}[S]}{[S] + K_{m_2}} \quad (1)$$

The calculated values were: K_{m_1}, 0.095 mM; V_{max_1}, 1.87 nmol/15 s per mg; K_{m_2}, 7.50 mM; V_{max_2}, 16.5 nmol/15 s per mg. The Hofstee plot values for proline uptake by trypsinized membrane vesicles

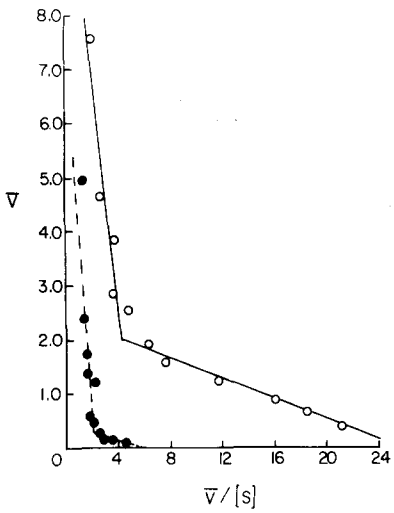

Fig. 5. Concentration-dependent L-proline uptake by control and trypsinized vesicles. Control (○) and trypsinized (●) vesicles were prepared as described in the legend to Fig. 3. Vesicles were incubated for 15 s in buffer B, pH 7.4, with varying L-proline concentrations (0.018–3.91 mM) under the condition of a 100 mM Na^+ gradient at 22°C. Uptake was measured as described in Materials and Methods. v is the velocity in nmol/15 s per mg membrane protein. [S] is the substrate concentration in mM. Each data point is the average of 20 determinations.

at 22°C were K_{m_1}, 0.071 ± 0.016 mM; V_{max_1}, 0.40 ± 0.092 nmol/15 s per mg; K_{m_2}, 2.99 ± 0.76 mM; V_{max_2}, 7.29 ± 1.88 nmol/15 s per mg. The calculated values were K_{m_1}, 0.095 mM; V_{max_1}, 0.10 nmol/15 s per mg; K_{m_2}, 6.19 mM; V_{max_2}, 12.50 nmol/15 s per mg. The V_{max_1} values for L-proline between the control and trypsinized vesicles transport are significantly different when compared with Student's t-test ($P < 0.001$). The K_{m_1} values for L-proline by control and trypsinized vesicles are not statistically different when compared with Student's t-test ($P > 0.2$). Thus, the removal of 70% membrane L-leucine aminopeptidase as the result of trypsinization of the membrane vesicle appeared to lower the V_{max_1} of proline entry on the low-K_m system to 10% of that of the control vesicles. In addition, the V_{max_2} of proline entry on the high-K_m system was lowered by 24% compared to that of the control vesicles ($P < 0.02$). Trypsinization of the membrane vesicle does not seem to alter the substrate affinities in either of the two transport components ($P > 0.6$ for K_{m_2}).

The velocity measurements of L-leucine transport at various substrate concentrations by control vesicles incubated at 37°C indicate the presence of two saturable systems for uptake as shown in Fig. 6. The transport parameters determined from the Hofstee plot were K_{m_1}, 0.47 ± 0.04 mM; V_{max_1}, 7.43 ± 0.53 nmol/15 s per mg; K_{m_2}, 1.83 ± 0.27 mM; V_{max_2}, 17.87 ± 1.65 nmol/15 s per mg. With the Hofstee plot parameters as initial estimates, the calculated values were: K_{m_1}, 0.54 mM; V_{max_1}, 6.25 nmol/15 s per mg; K_{m_2}, 8.35 mM; V_{max_2}, 22.15 nmol/15 s per mg.

The Hofstee plot for the concentration dependence of uptake at 15 s of leucine by trypsinized vesicles is also presented in Fig. 6. The transport parameters observed were K_{m_1}, 0.36 ± 0.07 mM; V_{max_1}, 3.16 ± 0.56 nmol/15 s per mg; K_{m_2}, 4.03 ± 1.16 mM; V_{max_2}, 22.64 ± 3.90 nmol/15 s per mg. The calculated values were K_{m_1}, 0.54 mM; V_{max_1}, 2.75 nmol/15 s per mg; K_{m_2}, 8.35 mM; V_{max_2}, 23.50 nmol/15 s per mg. The removal of 70% membrane L-leucine aminopeptidase after the trypsinization of the membrane vesicles resulted in

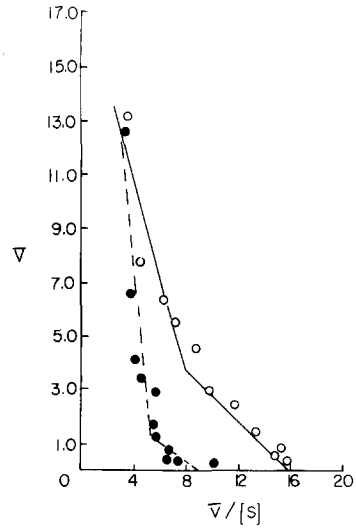

Fig. 6. Concentration-dependent L-leucine uptake by control and trypsinized vesicles. Control (○) and trypsinized (●) vesicles were prepared as described in the legend to Fig. 3. Various L-leucine concentrations (0.018–3.19 mM) were incubated with vesicles under the condition of a 100 mM Na^+ gradient at 22°C. Initial rate of uptake by the vesicles was measured as described in the text. v is the velocity in nmol/15 s per mg membrane protein. [S] is the substrate concentration in mM. Each data point is the average of four determinations.

TABLE V

THE RELATIVE CONTRIBUTION TO TOTAL UPTAKE OF THE LOW-K_m (HIGH-AFFINITY) SYSTEM AND THE HIGH-K_m (LOW-AFFINITY) SYSTEM CALCULATED FOR PROLINE AND LEUCINE UPTAKE

Brush border membranes were incubated at 37°C for 15 min. Initial rate (0.25 min) of uptake was measured at 22°C.

Substrate concentration (mM)		Type of vesicle	Calculated contribution to each component (%)	
			Low-K_m system	High-K_m system
L-Proline	0.018	Control	88.4	11.6
		Trypsinized	30.5	69.5
	3.91	Control	24.7	75.3
		Trypsinized	1.98	98.9
L-Leucine	0.018	Control	80.9	19.1
		Trypsinized	63.7	36.3
	3.91	Control	43.7	56.3
		Trypsinized	24.4	75.6

lowering of the V_{max_1} ($P < 0.01$) of leucine entry through the low-K_m system. But K_{m_1} was unaffected ($P > 0.2$) compared to control vesicles. Both K_{m_2} and V_{max_2} of leucine entry through the high-K_m system were increased slightly but not significantly as the results of trypsinization ($P > 0.1$ for K_{m_2} and $P > 0.3$ for V_{max_2}). Table V shows the relative contributions for either the low-K_m or high-K_m system to the total uptake of L-proline and L-leucine at two designated concentrations, 0.018 and 3.91 mM, by control and trypsinized brush border vesicles. By substituting the calculated values of the kinetic parameters into Eqn. 1, the uptake theoretically mediated by each of two transport components was determined by the substrate concentration desired. The contributions indicate that trypsin treatment with the removal of 70% membrane L-leucine aminopeptidase from the vesicles lowered the relative contributions from the low-K_m (high-affinity) system to the total uptake of L-proline and L-leucine by 57.9 and 17.2%, respectively, in the presence of 0.018 mM substrate concentrations. At the substrate concentration of 3.91 mM, the uptake of proline by the trypsinized vesicles was mediated almost solely by the high-K_m system; and 75.6% of the total uptake of L-leucine by the trypsinized vesicles was contributed from the high-K_m system.

Discussion

The treatment of renal brush border membrane vesicles with trypsin causes the release from the membrane of leucine aminopeptidase activity and a decrease in the ability to take up various amino acids. The ability of the vesicles to take up leucine and proline correlates with the residual aminopeptidase and not with the amount of protein released. Our data lend some support to the suggestion of Kenny and O'Halloran [19] that peptidases may be involved in Na^+-dependent leucine transport. There appears, however, to be a lack of specificity of the trypsin treatment. Not only is the transport of several groups of amino acids impaired but that of sugars as well. Our findings do not imply that leucine aminopeptidase acts as a transport protein. It may well be that when trypsin releases leucine aminopeptidase, it also removes other proteins associated with amino acid and sugar transport that have a similar topographical relationship in the membrane [28].

From the point of view of transport kinetics, the exposure to trypsin had quite specific effects on leucine and proline uptake. The low-K_m transport systems appear to be dramatically altered, leaving the high-K_m low-affinity component only slightly affected. In addition, the maximum velocity of the leucine and proline high-affinity system was affected with no change in the K_m. This may be construed to mean that the number or efficiency of saturable binding sites was reduced but that the sites remaining possessed an unchanged affinity. The fact that trypsin treatment resulted in dramatically decreased uptake via the low-K_m sys-

tem while leaving the high-K_m system functioning almost normally is consistent with the separate nature of these transport components.

The result of trypsin digestion of the membrane vesicle is a decrease in the Na^+-dependent uptake of leucine and proline. Previous analysis of the low-K_m transport system for proline revealed two effects of Na^+ on the system, one being on the apparent affinity of the binding site for the amino acid and the other where the Na^+ gradient established a membrane potential which is used as a driving force for concentrative uptake of the amino acid [27]. Our present studies show no alteration in the K_m or affinity for proline but only of the V_{max}. It is, therefore, possible that trypsin incubation caused a change in the ability of the membrane vesicles to dissipate the Na^+ gradient or establish a membrane potential. This possibility remains to be determined by measurements of Na^+ flux and membrane potential. An effect on Na^+ flux or generation of membrane potential would explain the rather global effect of trypsin on amino acid and sugar transport.

Our ultrastructural studies on the effects of trypsin indicated that surface protein was stripped from the microvillar membrane, leaving exposed so-called 'lollipop' structures [28,34]. The treatment apparently leaves the vesicular membrane intact, since we find no alkaline phosphatase released; the vesicles reveal normal osmotic responses and the friability during filtration procedures is essentially unaltered. The intactness of the membrane appears to coincide with inaccessibility of about 20% of the leucine aminopeptidase to trypsin activity. This activity appears to be associated with or buried in the lipid matrix, since it is extracted by detergents [3]. By using the monovalent Fab fragment of the brush border membrane aminopeptidase antibody, Louvard et al. [35] found that 20% of brush border membrane aminopeptidase enzyme cannot be inhibited even in the presence of substantial antibody excess. This is consistent with the hydrophobic anchors of the enzyme interacting with the lipid bilayer [3]. It is interesting to note that the cleavage of enzyme from the membrane results in no loss of enzyme activity in that the activity released and that residual in the membrane are about equal to the total enzyme activity present before trypsin action. However, the existence of the small portion of the inverted vesicles cannot be excluded [1].

Along with showing a positive correlation of vesicle transport with leucine aminopeptidase, our results show a negative one with γ-glutamyltranspeptidase activity. Trypsin treatment does not solubilize the transpeptidase activity [28] yet alters the transport of amino acids. If, as Meister [18] predicts, this enzyme functions as an amino acid carrier, one would expect that transport would be unchanged if the vesicle possessed essentially all of its γ-glutamyltranspeptidase activity. This appears to be additional evidence that the transpeptidase does not act as an amino acid transporter although one could postulate that trypsin alters the carrier function of the enzyme but leaves the transpeptidase activity in its native state.

Acknowledgement

This work was supported by Grant AM 10894 from the National Institutes of Health, Bethesda, MD, U.S.A.

References

1 Kenny, A.J. and Booth, A.G. (1978) Essays Biochem. 14, 1–14
2 Brinkley, F., King, N., Milikin, E., Wright, R.K., O'Neal, C.H. and Waldman, I.J. (1968) Science 162, 1009–1011
3 Thomas, I. and Kinne, R. (1972) Biochim. Biophys. Acta 255, 114–125
4 Glossman, H. and Neville, D.M. (1972) FEBS Lett. 19, 340–344
5 George, S.G. and Kenny, A.J. (1973) Biochem. J. 134, 43–57
6 Kenny, A.J. and Booth, A.G. (1980) Int. J. Biochem. 2, 215–217
7 Kerr, M.A. and Kenny, A.J. (1974) Biochem. J. 137, 477–488
8 Kerr, M.A. and Kenny, A.J. (1974) Biochem. J. 137, 489–495
9 Maroux, S., Louvard, D. and Baratti, I. (1973) Biochim. Biophys. Acta 321, 282–295
10 Kenny, A.J., Booth, A.G., George, S.G., Ingram, J., Kershaw, D., Wood, E.J. and Young, A.R. (1976) Biochem. J. 155, 169–182
11 David, R., MacNair, C. and Kenny, A.J. (1979) Biochem. J. 179, 379–395
12 Booth, A.G., Hubbard, L.M.L. and Kenny, A.J. (1979) Biochem. J. 179, 397–405
13 Danielson, E.M., Vyas, J.P. and Kenny, A.J. (1980) Biochem. J. 191, 645–648
14 Triplett, R.B. and Carraway, K.L. (1972) Biochemistry 11, 2897–2903

15 Armstrong, C.M., Bezanilla, F. and Rojas, E. (1973) J. Gen. Physiol. 62, 375–391
16 Louvard, D., Maroux, S., Vannier, C. and Desnuelle, P. (1975) Biochim. Biophys. Acta 375, 236–248
17 Preston, R.L. (1979) Life Sci. 24, 633–638
18 Meister, A. (1973) Science 180, 33–39
19 Kenny, A.J. and O'Halloran, D.M. (1979) FEBS Lett. 101, 407–410
20 McNamara, P.D., Ozegovic, B., Pepe, L.M. and Segal, S. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 4521–4525
21 Hammerman, M. and Sacktor, B. (1977) J. Biol. Chem. 252, 591–595
22 Fass, S.J., Hammerman, M.R. and Sacktor, B. (1977) J. Biol. Chem. 252, 583–590
23 McNamara, P.D., Pepe, L.M. and Segal, S. (1981) Biochem. J. 194, 443–449
24 Hsu, B.Y.-L., Marshall, C.M., McNamara, P.D. and Segal, S. (1980) Biochem. J. 192, 119–126
25 Evers, J., Murer, H. and Kinne, R. (1976) Biochim. Biophys. Acta 426, 598–615
26 Weiss, S.D., McNamara, P.D., Pepe, L.M. and Segal, S. (1978) J. Membrane Biol. 43, 91–105
27 McNamara, P.D., Pepe, L.M. and Segal, S. (1979) Biochim. Biophys. Acta 556, 151–160
28 Hsu, B.Y.-L., McNamara, P.D., Schlesinger, H., Pepe, L.M., Marshall, C.M. and Segal, S. (1980) Enzyme 25, 170–181
29 Booth, A.G. and Kenny, A.J. (1974) Biochem. J. 142, 575–581
30 Sigma Technical Bulletin No. 104 (1963) The Colorimetric Determination of Phosphatase, Sigma, St. Louis, MO
31 Bradford, M. (1963) Anal. Biochem. 72, 248–254
32 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, J. (1957) J. Biol. Chem. 193, 265–272
33 Goldmann, D.R., Schlesinger, H. and Segal, S. (1976) Biochim. Biophys. Acta 419, 251–260
34 Pockrandt-Hemstedt, H., Schmitz, J.E., Kinne-Saffran, E. and Kinne, R. (1972) Pflügers Arch. Ges. Physiol. 333, 297–313
35 Louvard, D., Maroux, S. and Desnuelle, P. (1975) Biochim. Biophys. Acta 389, 389–400