J. Membrane Biol. 75, 115–122 (1983)

The Journal of
Membrane Biology

Association of Renal Dipeptidase with the Triton-Insoluble Fraction of Kidney Microvilli

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Summary. Renal dipeptidase, previously identified as a component of renal microvilli, has been investigated to determine its orientation within this organelle. Digestion of porcine renal microvilli with papain released essentially all of aminopeptidase M, an outer membrane marker enzyme from the microvilli within one hour; whereas less than 10% of renal dipeptidase was released under the same conditions. Antibody to purified renal dipeptidase produced 50% inhibition of the purified enzyme at an antibody/antigen molar ratio of 2:1. Inhibition by the renal dipeptidase-directed antibody was not observed when the enzyme was bound within the microvillar structure. Demembranation of the microvilli with Triton X-100 resulted in a distribution of 68% of renal dipeptidase in the insoluble pellet and 32% in the soluble supernatant. The same detergent treatment released 92% of animopeptidase M into the supernatant. These results indicate that renal dipeptidase is not located at the luminal surface of the microvillus membrane where it would be available for release of papain, inhibition by antibody, or solubilization by detergent. Fractionation of the Triton-insoluble pellet with 2 M NaCl resulted in the release of 64% of the peptidase into a pellicle fraction separated from insoluble pellet and soluble supernatant. Finally extraction of Triton-insoluble pellet with 0.05 mM ATP–0.10 mM $MgCl_2 \cdot 6\,H_2O$ solubilized 57% of the renal dipeptidase.

Key Words renal dipeptidase · microvilli · Triton extraction · antibody inhibition · enzyme solubilization

Introduction

Renal dipeptidase (E.C. 3.4.13.11), purified from porcine kidney cortex, has been shown to exhibit hydrolytic activity against a wide range of L-dipeptides but not against esters, tripeptides or proteins (Campbell, 1970). Physicochemical characterization demonstrated that the dipeptidase is a zinc metalloenzyme with a molecular weight of $94{,}000 \pm 300$ containing 2.04 g-atoms of zinc/mole enzyme (Armstrong, Mukhopadhyay & Campbell, 1974). Investigations into the role of renal dipeptidase within the substructure of the mammalian kidney have indicated that the enzyme is located within the microvillus of the proximal tubular cell (Welch & Campbell, 1980). Disintegration of microvillus structure by the nephrotoxic agent, cephaloridine, resulted in the release of the dipeptidase into the urine (Mullins & Campbell, 1980). In the present investigation, the renal microvillus structure was disrupted by means of enzymic digestion, detergent treatment, and sodium chloride extraction to demonstrate that renal dipeptidase is not located primarily at the surface of the microvillus membrane.

Materials and Methods

Preparation of Porcine Renal Microvilli

The procedure of Booth and Kenny (1974) was employed for the isolation of microvilli from porcine kidney cortex. This method as described previously by Welch and Campbell (1980) utilizes a series of differential centrifugations in hypotonic Tris-HCl buffer containing Mg^{2+}. The microvilli preparations were examined by electron microscopy. Samples for electron microscopy were fixed with 2% glutaraldehyde, 0.05 M sodium cacodylate buffer, pH 7.2, and centrifuged at $1000 \times g$ for 20 min. The pellets were post-fixed with 1% osmium tetroxide in Millonig's fixative, pH 7.3, and followed by dehydration steps with ethanol and propylene oxide. The pellets were embedded in Epoxy 812 resin. Thin sections were cut using an MT-2 Porter-Blum ultramicrotome and double-stained with uranyl acetate saturated with 50% ethanol followed by lead citrate. They were examined under the Phillips 300 electron microscope.

Enzyme Assays

Renal dipeptidase was assayed by means of the spectrophotometric method of Rene and Campbell (1969). The rate of enzyme-catalyzed hydrolysis of the unsaturated dipeptide, glycyldehydrophenylalanine, was measured by observing the fall in absorbance at 275 nm of a solution of 5.00×10^{-5} M glycyldehydrophenylalanine. Aminopeptidase M was assayed by the method of Louvard et al. (1973). The substrate was 1 mM L-alanine-*p*-nitroanilide in 50 mM potassium phosphate buffer at pH 7.0. Alkaline phosphatase was determined by the procedure described by Simpson and Vallee (1968) which employed 1 mM *p*-nitrophenylphosphate as substrate in 1.0 M Tris-HCl buffer at pH 8.8. All enzyme assays were carried out at 37° C, and

protein concentrations were determined by the method of Lowry, Rosebrough, Farr and Randall (1951). The specific activities are expressed as micromoles of substrate hydrolyzed per minute per milligram of enzyme. All values are the average of four or more determinations and are reported as the mean ±standard deviation.

Papain Release of Peptidases from Microvilli

Papain was activated prior to use according to the method of Louvard, Maroux, Vannier and Desnuelle (1975). In a typical release assay 0.5 ml of microvilli (6 mg/ml) were mixed and incubated with 0.5 ml of activated papain (0.5 mg/ml) at 37° C. Aliquots were removed at appropriate time intervals up to 90 min, and the reaction was stopped by the addition of 100 µl of the 0.1 M N-ethylmaleimide. Supernatant was separated from pellet by centrifugation at 105,000 × *g* for 1 hr. The pellet was resuspended in 1 ml of 2 mM Tris-HCl at pH 7.6, and both the supernatant and pellet suspension were assayed for renal dipeptidase and aminopeptidase M.

Purification of Renal Dipeptidase and Analytical Methods

Purified renal dipeptidase was isolated from porcine kidney cortex by means of solubilization with 1-butanol, isoelectric precipitation, $(NH_4)_2SO_4$ fractionation, and preparative polyacrylamide gel electrophoresis according to procedures reported by Armstrong et al. (1974). Sedimentation velocity experiments were carried out using the Beckman-Spinco Model E analytical ultracentrifuge and the method of Chervenka (1969). Analytical polyacrylamide gel electrophoresis was performed as described by Ornstein and Davis (1964). The Ouchterlony gel diffusion technique was employed as reported by Elgert, Ross, Campbell and Barrett (1974). The immunodiffusion plates were prepared by using 1.25 g Agarose dissolved in 100 ml of 0.05 M barbital-HCl at pH 8.2. After addition of antibodies and antigen to the wells, the plates were incubated in a moist chamber for 24–48 hr. Immunoelectrophoresis was also performed as reported by Elgert et al. (1974) with the modification that 1% Agarose was substituted for Noble agar in the preparation of the immunoelectrophoresis plates. The periodic acid-Schiff reaction (Kapitany & Zebrowski, 1973) was employed to locate glycoprotein bands in polyacrylamide gels, and the quantitative determination of neutral and amino sugars in renal dipeptidase was performed according to the procedure of Mawhinney, Feather, Barbero and Martinez (1980).

Antibody Inhibition of Renal Dipeptidase

Antiserum was prepared by subcutaneous injections of purified renal dipeptidase into rabbits (100 µg per rabbit at 4 intervals over a period of 3 months). The serum was harvested, and immunoglobulin G was purified by ammonium sulfate fractionation (0 to 50% saturation at pH 7.8) followed by DEAE ion exchange chromatography (Stanworth, 1960). Immunoglobulin G was purified from nonimmunized serum using the same procedures to serve as a control in the antibody inhibition experiments. A typical antibody inhibition reaction mixture contained 2 µg/ml purified renal dipeptidase, IgG ranging from 1 to 72 µg/ml, and 5 mM sodium phosphate at pH 8.0 in a total volume of 0.5 ml. A control mixture contained enzyme and buffer without antibody. The tubes were incubated for $2\frac{1}{2}$ hr at 37° C, and then transferred to an ice bath prior to assay for renal dipeptidase activity using glycyldehydrophenylalanine as substrate. Three aliquots of 0.1 ml each were assayed for each data point. Renal dipeptidase activity in the control mixture was assigned an activity of 100%. IgG prepared from nonimmunized serum was substituted for IgG-anti-renal dipeptidase to control for nonspecific inhibition. Inhibition studies with microvilli were carried out in the same way with microvilli substituted for purified renal dipeptidase. The renal dipeptidase content of the microvilli was estimated to be 3.2% according to Welch and Campbell (1980).

Extraction Methods

Triton X-100 extraction of microvilli was carried out as reported by Kramers and Robinson (1979). A typical demembranation mixture consisted of 27 mg microvilli protein, 0.5 M sucrose, 1% Triton X-100, and 10 mM Tris-HCl at pH 7.4 in a total volume of 12 ml. The mixture was stirred gently for 15 min at 4° C and centrifuged at 105,000 × *g* for 90 min. The Triton-insoluble residue was recovered as a pellet which is designated demembranated microvilli. The pellet was resuspended in 2 mM Tris-HCl at pH 7.6, and both supernatant and pellet were assayed for enzyme activity immediately. Samples containing Triton X-100 were precipitated with trichloroacetic acid as described by Koepsell (1978) to avoid interference by detergent with color development in the Lowry protein method.

The Triton-insoluble fraction was extracted with sodium chloride solution as described by Kramers and Robinson (1979). In this method 44 mg of Triton-insoluble protein were resuspended in 4 ml of a sodium chloride extraction medium in a Teflon glass homogenizer operated by hand (5 strokes). The sodium chloride extraction medium consisted of 2 M NaCl, 0.5 M sucrose, and 10 mM Tris-HCl at pH 7.4. After homogenization, the mixture was incubated for 15 min in an ice bath and centrifuged at 105,000 × *g* for 90 min at 4° C. Three fractions were produced within the centrifuge tube: the pellet at the bottom of the tube, the supernatant, and a white pellicle layer which floated on the top of the supernatant. The pellet and pellicle fractions were resuspended separately in 10 mM Tris-HCl at pH 7.4. All three fractions were assayed for protein, renal dipeptidase, and alkaline phosphatase. The microvillar Triton-insoluble fraction was also extracted with an ATP-Mg^{2+} solution as reported by Matsudaira and Burgess (1979). The pellet and supernatant fraction from these extractions were assayed for protein and renal dipeptidase.

Materials

Glycyldehydrophenylalanine was synthesized by methods previously described (Campbell, Lin & Bird, 1963). Papain was purchased from ICN Pharmaceuticals, Inc. All other reagents were obtained from commercial sources and were of the best quality available.

Results

Electron micrographs of the microvilli showed them to be vesicular and polydisperse in size, ranging from 0.2 to 0.8 µm in diameter. The vesicles were formed by a trilaminar lipid bilayer which enclosed discrete dots which have previously been identified as cross sections of actin microfilaments (Booth & Kenny, 1976). The microvilli appeared to be relatively free of other cell constituents and the vesicles were predominantly closed. A comparison of the specific activity of renal dipeptidase in

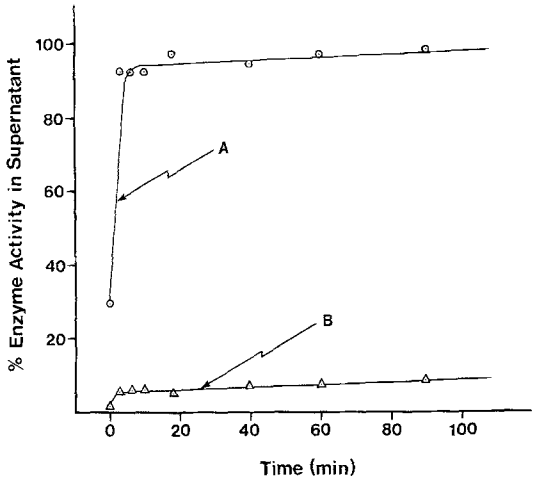

Fig. 1. Papain release of peptidases from microvilli. A series of samples containing equal volumes of microvilli and activated papain were prepared and incubated at 37° C as described in Materials and Methods. The reactions were stopped at appropriate time intervals by adding N-ethylmaleimide and centrifuged at 105,000 × *g* for 1 hr. Both supernatant and pellet were assayed for renal dipeptidase and aminopeptidase M. Curve *A* represents release of aminopeptidase M and curve *B* represents release of renal dipeptidase.

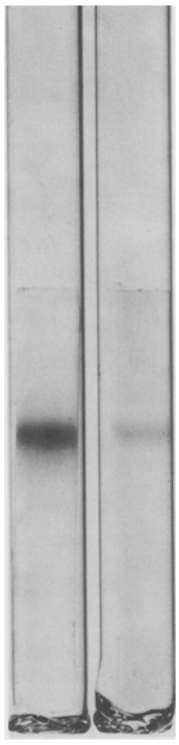

Fig. 2. Polyacrylamide gel electrophoresis of purified renal dipeptidase. The polyacrylamide gel consisted of a 2.5% stacking gel and an 8% separating gel. Electrophoresis was carried on at pH 8.3 for 35 min at 4 mamp per gel. The gel on the left was stained for protein using 0.5% Buffalo Black NBR and destained in 7.5% acetic acid. The gel on the right was stained by the periodic acid-Schiff reaction as described in Materials and Methods.

the renal cortex homogenate to that in the microvilli gave an average relative specific activity of 20.9 for 4 preparations.

Those enzymes that appear to be fixed at the outer surface of the microvillus membrane and to protrude from it can sometimes be released from the membrane by digestion with papain. One such enzyme investigated in the intestinal microvilli was aminopeptidase M (Louvard et al., 1975). To determine if such was the case with renal dipeptidase, the renal microvilli vesicles were treated with papain, and the release of aminopeptidase M was compared with the release of renal dipeptidase. The results shown in Fig. 1 demonstrate that over 90% of aminopeptidase M was released from the microvilli into the supernatant by papin within 5 min. An additional 5% of aminopeptidase M was released slowly over 1 hr. However, less than 10% of renal dipeptidase was released during 90 min of papain digestion under the same conditions. There was no measureable loss of the total activity of aminopeptidase M or renal dipeptidase during the course of these experiments.

The purification of renal dipeptidase from 469 g (protein concentration) of renal cortex homogenate resulted in the isolation of 16.2 mg of purified enzyme with a specific activity of 6.94 micromoles substrate/min/mg protein using the standard glycyldehydrophenylalanine assay at a substrate concentration of 5.00×10^{-5} M. The Schlieren photographs of the sedimentation velocity analysis of this preparation exhibited one symmetrical peak after 60 min at 59,780 rpm. Analytical polyacrylamide gel electrophoresis at pH 8.3 resulted in the appearance of only one protein band which also gave a positive pink stain in the periodic acid-Schiff reaction (Fig. 2). The carbohydrate content of the purified renal dipeptidase was $6.04 \pm 0.02\%$ (wt/wt). The results of the analyses for the individual monosaccharides are reported in Table 1. The molar ratios indicate that 31 monosaccharide molecules are attached per molecule of renal dipeptidase (molecular weight from Armstrong et al., 1974). Other monosaccharides analyzed for but not detected by the gas liquid chromatographic technique are given in Table 1. Sulfate ester, bound lipid, and phosphate were also analyzed for and not detected.

Immunization of rabbits with purified renal dipeptidase followed by collection of antiserum and isolation of IgG resulted in an immunoglobulin fraction that proved to be over 97% pure when examined in the analytical ultracentrifugation. Minor contamination (<3%) was evidenced by a

Table 1. Carbohydrate analyses of purified renal dipeptidase

	Carbohydrate (μg)	Content (nmol)	Sugar Enzyme molar ratio
Renal dipeptidase	293	3.08	
N-acetylglucosamine	4.81±0.01	21.75	7.1
fucose	2.07±0.01	12.61	4.1
mannose	5.54±0.01	30.75	10.0
galactose	5.27±0.01	29.25	9.5
arabinose	ND[a]	TOTAL	30.7
xylose	ND		
glucose	ND		
N-acetylgalactosamine	ND		
sialic acid	ND		
glucuronic acid	ND		
galacturonic acid	ND		
sulfate ester	ND		
bound lipid	ND		
2-deoxyribose	ND		
ribose	ND		
phosphate	ND		

[a] not detected. The level of detection for these compounds using the procedure of Mawhinney et al. (1980) is 0.01%.

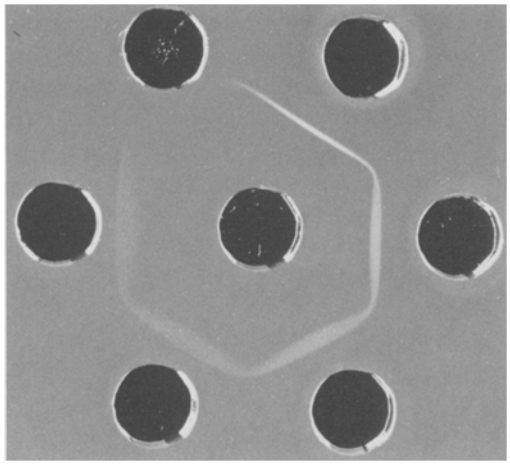

Fig. 3. Immunodiffusion analysis of renal dipeptidase. The Ouchterlony double-diffusion test was carried out as described in Materials and Methods. The center well contained 25 μl of purified enzyme at a concentration 0.732 mg/ml. The outer wells starting at the upper right-hand corner contain 25-μl aliquots of IgG at concentrations of 5, 4, 3, 2, 1, 0.5 mg/ml IgG

small peak with an observed sedimentation coefficient of 22.75. The specificity of the antibody preparation for renal dipeptidase was examined using the Ouchterlony double-diffusion test. The results shown in Fig. 3 demonstrate the formation of a single precipitin line between antibody and enzyme. The antibody preparation was employed in inhibition assays of purified renal dipeptidase and

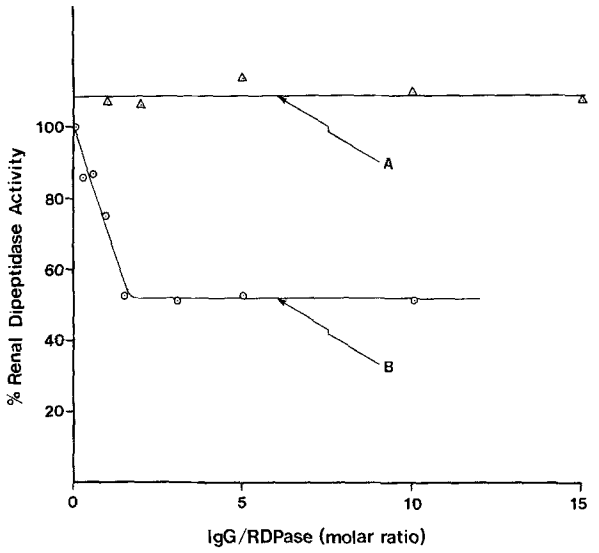

Fig. 4. Antibody inhibition of renal dipeptidase. The reaction mixtures contained increasing amounts of IgG added to a constant amount of enzyme (purified renal dipeptidase or microvilli) and the volumes were brought to 0.5 ml with 5 mM sodium phosphate buffer at pH 8.0. The details of the experimental conditions are described in Materials and Methods. (*A*) represents activity of microvilli and (*B*) represents activity of purified renal dipeptidase

the renal dipeptidase activity expressed by the renal microvilli. The results are presented in Fig. 4. These data show that the antibody produces approximately 50% inhibition of the purified enzyme at an antibody/antigen molar ratio of 2:1. However, the antibody did not inhibit the peptidase at all when the enzyme was bound within the microvilli. It was also observed that the antibody fraction prepared from nonimmunized rabbit serum did not inhibit the purified renal dipeptidase nor the enzyme bound to the microvilli.

Demembranation of the renal microvilli by treatment with the nonionic detergent, Triton X-100, resulted in the separation of two fractions, the supernatant and the insoluble pellet. Evidence that the membranes had been removed from the cytoskeleton was obtained by electron microscopic examination of the demembranated microvilli. The electron micrographs are shown in Fig. 5 *A* and *B*. Enzymic analyses of microvilli before and after demembranation are reported in Table 2. Examination of the Triton supernatant fraction by immunoelectrophoresis against renal dipeptidase-specific antiserum revealed only one precipitin arc identified as that formed by renal dipeptidase with its antibody.

The pellet, supernatant, and pellicle fractions obtained by 2 M sodium chloride extraction of the Triton-insoluble fraction were assayed for protein,

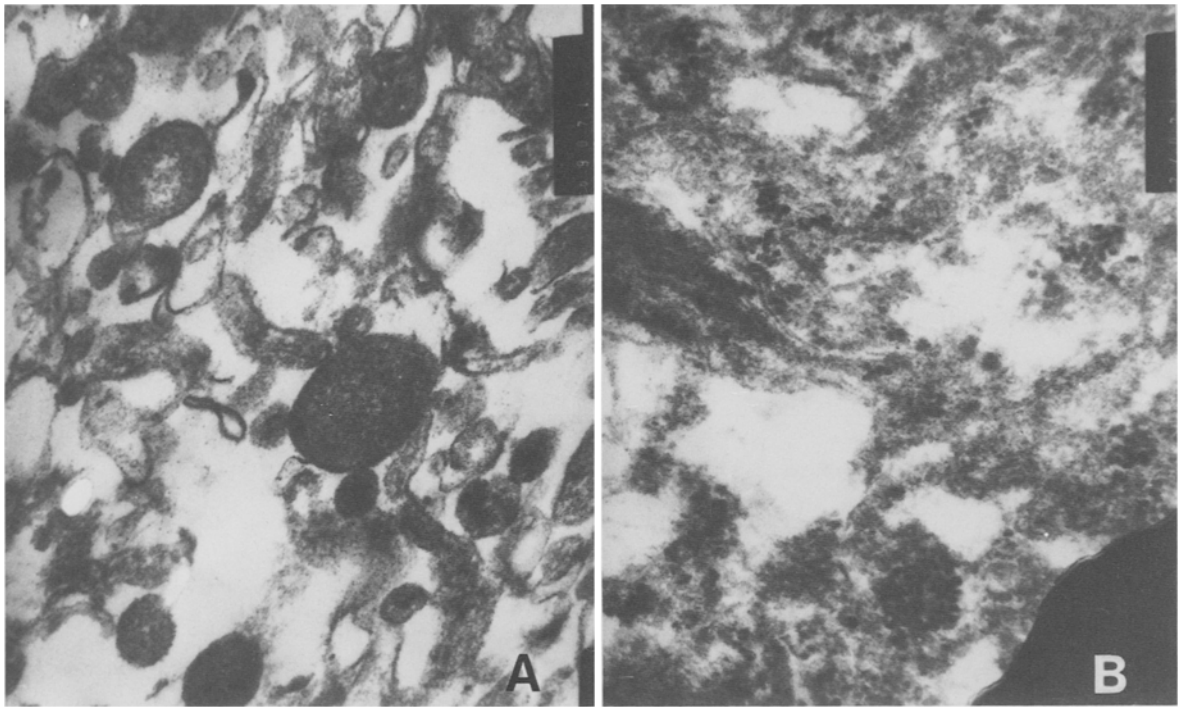

Fig. 5. Electron micrographs of microvilli before and after treatment with Triton X-100. (*A*) renal microvilli prepared as described in Materials and Methods. (*B*) Triton-insoluble preparation obtained by treating microvilli with Triton X-100 as described in Materials and Methods. The preparations were double-stained with uranyl acetate saturated with 50% ethanol followed by lead citrate stain. A magnification of 84,000 × was employed

Table 2. Demembranation of microvilli with Triton X-100

		Microvilli	Supernatant	Pellet	Recovery (%)
Protein	Total (mg)	26.29 ± 1.58	17.00 ± 0.73	1.51 ± 0.76	104
	Dist. (%)		61.80	38.20	
Renal Dipeptidase	Total (unit)[a]	1.97 ± 0.00	0.65 ± 0.06	1.37 ± 0.07	102
	Dist. (%)[b]		32.18	67.82	
	S.A. (10^3)[c]	74.93 ± 1.67	38.24 ± 1.00	130.35 ± 2.19	
	RSA[d]	1.00	0.51	1.74	
Alkaline Phosphatase	Total (unit)	12.59 ± 0.93	4.14 ± 0.26	6.55 ± 0.57	85
	Dist. (%)		38.72	61.27	
	S.A. (10^3)	478.89 ± 35.60	242.80 ± 14.91	626.49 ± 59.72	
	RSA	1.00	0.51	1.31	
Aminopeptidase M	Total (unit)	18.82 ± 0.35	17.20 ± 0.34	1.55 ± 0.09	100
	Dist. (%)		91.74	8.26	
	S.A. (10^3)	715.86 ± 13.71	1015.10 ± 18.95	159.07 ± 9.37	

[a] Enzyme unit = μmol glycyldehydrophenylalanine/min.
[b] % distribution is based on the sum of recovered activity in the supernatant and pellet.
[c] S.A. = Specific Activity = units/mg.
[d] RSA = Relative Specific Activity = Ratio of specific activity of the enzyme in the fraction to the specific activity of the enzyme in the microvilli.

alkaline phosphatase, and renal dipeptidase and the results are presented in Table 3. Assays of the Triton-insoluble fraction for protein and renal dipeptidase before and after extraction with ATP-Mg^{2+} are reported in Table 4.

Discussion

The microvillus preparation employed in these investigations has previously been characterized by marker enzyme assay and electron microscopic ex-

Table 3. Sodium chloride extraction of Triton-insoluble fraction of microvilli

		Triton-insoluble fraction	Pellicle	Supernatant	Pellet	Recovery (%)
Protein	Total (mg)	10.51± 0.77	1.89± 0.54	3.67± 0.09	3.90±0.24	90
	Dist. (%)		19.98	39.00	41.23	
Renal Dipeptidase	Total (unit)[a]	1.37± 0.07	0.70± 0.09	0.09± 0.03	0.35± 0.05	88
	Dist. (%)[b]		61.40	7.89	30.70	
	S.A. (10^3)[c]	130.35± 2.19	370.37± 49.09	24.52± 3.40	89.74± 7.91	
	RSA[d]	1.00	2.84	0.19	0.67	
Alkaline Phosphatase	Total (unit)	6.55± 0.57	4.09± 0.28	0.24± 0.05	2.47± 0.19	103
	Dist. (%)		60.03	3.54	36.43	
	S.A. (10^3)	626.49±59.72	21.53±176.52	65.40±10.29	633.33±64.94	
	RSA	1.00	3.44	0.10	1.01	

[a] Enzyme unit = μmol glycyldehydrophenylalanine/min.
[b] % distribution is based on the sum of the recovered activity in the pellicle, supernatant and pellet.
[c] S.A. = Specific Activity = units/mg.
[d] RSA = Relative Specific Activity = Ratio of specific activity of the enzyme in the fraction to the specific activity of the enzyme in the Triton-insoluble fraction.

Table 4. ATP-Mg extraction of Triton-insoluble fraction of microvilli

		Triton-insoluble fraction	Supernatant	Pellet	Recovery (%)
Protein	Total (mg)	3.46±0.22	0.99±0.07	2.50±0.04	100
	Dist. (%)		28.37	71.64	
Renal Dipeptidase	Total (unit)[a]	0.17±0.01	0.10±0.01	0.07±0.00	100
	Dist. (%)[b]		56.89	43.11	
	S.A. (10^3)[c]	49.88±3.07	95.57±7.11	28.62±0.27	
	RSA[d]	1.00	1.92	0.59	

[a] Enzyme unit = μmol glycyldehydrophenylalanine/min.
[b] % distribution is based on the sum of recovered activity in the supernatant and pellet.
[c] S.A. = Specific Activity = μmol/min/mg.
[d] RSA = Relative Specific Activity = Ratio of sepcific activity of the enzyme in the fraction to the specific activity of the enzyme in the Triton-insoluble fraction of microvilli.

amination and has been reported to be in a substantially homogeneous state relatively free of other subcellular components such as endoplasmic reticulum, mitochondria, lysozymes, and basolateral membranes (Welch & Campbell, 1980). The specific activity of renal dipeptidase in the microvilli is 20.9 times greater than that of kidney cortex homogenate which compares with a value of 20.4 reported for previous preparations (Welch & Campbell, 1980). Haase, Schafer, Murer and Kinne (1978) and Vannier, Louvard, Maroux and Desnuelle (1976) have shown by electron microscopic, enzymatic, and immunological techniques that the renal microvillus vesicles, isolated by the method of Booth and Kenny (1974), are oriented "luminal side out;" that is, in the same direction as their *in vivo* state.

The slow release of renal dipeptidase from microvilli by papain as compared to the rapid release of aminopeptidase M under the same conditions (Fig. 1) suggests that renal dipeptidase does not exist primarily at the luminal surface of the membrane at a site available for papain digestion. Physicochemical analyses of the purified renal dipeptidase gave the same results as those reported for earlier preparations (Armstrong et al., 1974; Ferren, Ward & Campbell, 1975) attesting to the homogeneity of the preparation. Demonstration of the glycoprotein nature of renal dipeptidase is provided for the first time by the positive periodic acid-Schiff test shown in Fig. 2 and the individual analyses of the monosaccharide components. A significant carbohydrate component has been shown to be characteristic of other hydrolases known to be present in renal microvilli by Booth and Kenny (1976). The single precipitin line formed between renal dipeptidase and its antibody in the Ouchterlony analysis (Fig. 3) confirms the homogeneity of the antigen and demonstrates specific interaction between the peptidase and anti-

body. The effect of this interaction is to produce the inhibition of the purified enzyme shown in Fig. 4. The total lack of antibody inhibition of microvillus renal dipeptidase also presented in Fig. 4 further suggests that the enzyme is not located at the luminal surface of the microvillus membrane where it would be available for antibody binding and inhibition.

Electron microscopic examination of the microvilli before and after extraction with Triton X-100 (Fig. 5) revealed that the outer membranes of the vesicles formed by the lipid bilayers have been removed by the detergent. Such demembranation should concentrate enzymes located within the outer membrane in the supernatant and those not primarily associated with the outer membrane in the Triton-insoluble pellet. The data reported in Table 2 indicate that 94.5% of aminopeptidase M is found in the Triton supernatant. Aminopeptidase M has previously been established as a component of the outer membrane of renal microvilli (Haase et al., 1978). The analysis of the microvillus fractions for alkaline phosphatase gave values of 38.8% in the supernatant and 61.7% in the Triton-insoluble pellet. Previous investigations have reported that alkaline phosphatase in renal microvilli was almost completely resistant to papain treatment and that only 20% was released from the microvilli by extraction with 1% Triton X-100 (Kramers & Robinson, 1979). On the basis of these data alkaline phosphatase has been referred to as a core enzyme (George & Kenny, 1973; Kramers & Robinson, 1979). Analyses of renal dipeptidase reported in Table 3 (31.0% supernatant; 69.0% pellet) are closely similar to those found for alkaline phosphatase. The single precipitin line obtained by immunoelectrophoresis of the Triton supernatant against renal dipeptidase antiserum confirms the presence of some enzyme (31%) in the supernatant and suggests that possibly the enzyme may be involved in a linkage between the cytoskeleton and the membrane bilayer.

Studies of the renal microvillus cytoskeleton by Kramers and Robinson (1979) using negative-staining electron microscopy demostrated that the cytoskeleton contained not only microfilamantes but also vesicles of lipid. These workers also showed that these lipid vesicles concentrated into the pellicle fraction when the cytoskeleton was extracted with 2 M NaCl. They suggested that proteins found in these inner vesicles, separated as pellicle fraction, may represent the interconnections between the microfilaments of the cytoskeleton and the outer microvillus membrane. Analysis of the microvilli fractions for alkaline phosphatase and renal dipeptidase presented in Table 3 again show a close similarity in the distribution of the two enzymes. The data show that 64% of renal dipeptidase and 65.8% of alkaline phosphatase are extracted into the pellicle fraction. Matsudaira and Burgess (1979) by treatment with ATP-Mg^{2+} selectively extracted between 30 and 80% of the cross bridges between the actin microfilaments and the membrane coat of chicken intestinal cytoskeleton. The results presented in Table 4 show that 56.9% of the renal dipeptidase component of the Triton-insoluble fraction of renal microvilli was extracted by the ATP-Mg^{2+} treatment of Matsudaira and Burgess (1979). The similar solubility characteristics of alkaline phosphatase and renal dipeptidase suggest that they may perform their physiological functions at a similar location within the renal microvillus.

We thank Dr. Thomas Mawhinney for carbohydrate analyses and Dr. Don Thompson for electron microscopic examination of microvilli preparations.

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Received 27 July 1982; revised 23 February 1983