Dipeptidyl Peptidase IV, a Kidney Brush-Border Serine Peptidase

By A. JOHN KENNY, ANDREW G. BOOTH, STEPHEN G. GEORGE,*
JEAN INGRAM, DOUGLAS KERSHAW, EDWARD J. WOOD and A. RHOEN YOUNG
Department of Biochemistry, University of Leeds, 9 Hyde Terrace, Leeds LS2 9LS, U.K.

(Received 28 January 1976)

Dipeptidyl peptidase IV, an enzyme that releases dipeptides from substrates with *N*-terminal sequences of the forms X-Pro-Y or X-Ala-Y, was purified 300-fold from pig kidney cortex. The kidney is the main source of the enzyme, where it is one of the major microvillus-membrane proteins. Several other tissues contained demonstrable activity against the usual assay substrate glycylproline 2-naphthylamide. In the small intestine this activity was greatly enriched in the microvillus fraction. In all tissues examined, the activity was extremely sensitive to inhibition by di-isopropyl phosphorofluoridate (Dip-F), but relatively resistant to inhibition by phenylmethylsulphonyl fluoride. It is a serine proteinase which may be covalently labelled with [³²P]Dip-F, and is the only enzyme of this class in the microvillus membrane. The apparent subunit mol.wt. estimated by sodium dodecylsulphate/polyacrylamide-gel electrophoresis and by titration with [³²P]Dip-F was 130000. Gel-filtration and sedimentation-equilibrium methods gave values in the region of 280000, which is consistent with a dimeric structure, a conclusion supported by electron micrographs of the purified enzyme. Among other well-characterized serine proteinases, this enzyme is unique in its membrane location and its large subunit size. Investigation of the mode of attack of the peptidase on oligopeptides revealed that it could hydrolyse certain *N*-blocked peptides, e.g. Z-Gly-Pro-Leu-Gly-Pro. In this respect it is acting as an endopeptidase and as such may merit reclassification and renaming as microvillus-membrane serine peptidase.

Enzymes that hydrolyse dipeptides from the *N*-terminus of susceptible peptide substrates have been named 'dipeptidyl peptidases' by the Enzyme Commission (1973). McDonald *et al.* (1971), however, refer to them as 'dipeptidyl aminopeptidases': cathepsin C, for example, is 'dipeptidyl aminopeptidase I', and the enzyme described in the present paper is designated 'dipeptidyl aminopeptidase IV'. This latter enzyme, first reported as 'glycylproline naphthylamidase' by Hopsu-Havu & Glenner (1966), liberates glycyl-L-proline from glycyl-L-proline 2-naphthylamide. It is a membrane-bound enzyme that has been purified, after autolysis, from rat liver (Hopsu-Havu & Sarimo, 1967) and pig kidney (Hopsu-Havu *et al.*, 1968). The kidney enzyme is maximally active at pH 7.6–7.8, has a mol.wt. of about 250000 and, on the basis of several studies with potential inhibitors and activators, could not be assigned to any of the main classes of proteinases. Barth *et al.* (1974) have re-investigated the enzyme from pig kidney and have shown it to be sensitive to di-isopropyl phosphorofluoridate and to possess two types of specificity involving substrates of the type X-Pro-Y on the one hand and X-Ala-Y on the other.

The rates for the latter type were reported to be about one-fifth of that for the corresponding substrate containing proline.

Although the enzyme hydrolysing glycylproline 2-naphthylamide is widely distributed among mammalian tissues, the kidney is much the richest source, and the activity has been shown histochemically to be located in the cortex, especially in the proximal convoluted tubules (Hopsu-Havu & Ekfors, 1969; McDonald *et al.*, 1971). We have shown that it is a brush-border enzyme, being enriched to a degree comparable with other markers in both brush-border fractions (S. G. George & A. J. Kenny, unpublished work) and microvillus-membrane preparations (Booth & Kenny, 1974). In this respect the enzyme is one of a battery of microvillus-membrane peptidases, so far known to include aminopeptidases A (EC 3.4.11.7) and M (EC 3.4.11.2), γ -glutamyltransferase (EC 2.3.2.2) and a zinc-containing endopeptidase (EC 3.4.24.-) (George & Kenny, 1973). In the present paper we provide evidence that dipeptidyl peptidase IV is the only serine peptidase present in the kidney brush border. When purified it exists as a dimer, each subunit of mol.wt. 130000 containing one active site. Its specificity is not limited to that of a dipeptidyl-peptide hydrolase, but can, with appropriate substrates, include that of an endopeptidase.

* Present address: Institute of Marine Biochemistry, St. Fittick's Road, Aberdeen AB1 3RA, Scotland, U.K.

Experimental

Materials

Pig kidneys, obtained from a slaughter-house, were placed on ice immediately on removal from the carcass. Within about 2 h, each kidney was decapsulated and the cortical tissue, about 100 g/kidney, was frozen in liquid N₂ and stored at -70°C until required. Dip-F,* phenylmethylsulphonyl fluoride, diethyl *p*-nitrophenyl phosphate (colloquially called 'E 600'), papain (twice recrystallized) and trypsin were obtained from Sigma (London) Chemical Co., Kingston-on-Thames, Surrey KT2 7BH, U.K.

[³²P]Dip-F (code PB1; specific radioactivity initially 80 μCi/μmol) was obtained from The Radiochemical Centre, Amersham, Bucks. HP7 9LL, U.K. DEAE-cellulose, CM-cellulose (Whatman microgranular grades DE 52 and CM 52) and chymotrypsin were obtained from BDH Chemicals, Poole, Dorset BH12 4NN, U.K. Sepharose 6B was obtained from Pharmacia, Box 175, S-75104 Uppsala 1, Sweden. Hydroxyapatite had been prepared by Dr. A. J. Turner (of this Department) by the method of Tiselius *et al.* (1956).

Substrates were obtained as follows: Gly-Pro-2-NNap, Ala-Ala-2-NNap and Z-Gly-Pro-Gly-Gly-Pro-Ala from Feinchemikalien A.G., Kasernenstrasse 43a, 4410 Liestal, Switzerland. Gly-Pro-NPhN₂Ph and Ala-Ala-NPhN₂Ph were kindly given by Professor Alfred Barth, and Z-Gly-Pro-Leu-Gly-Pro was from Sigma (London) Chemical Co.

Markers used in the determination of molecular weight by gel filtration or by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis were obtained as follows: β-galactosidase (*Escherichia coli*), pyruvate kinase (rabbit muscle), catalase, glucose oxidase, and ovalbumin from Sigma (London) Chemical Co.; lactate dehydrogenase (pig muscle) and DNA polymerase I (*Escherichia coli*; grade 1) from Boehringer Corp. (London) Ltd., London W5 2TZ, U.K.; bovine serum albumin and urease (jack-bean meal) from BDH Chemicals Ltd., Poole, Dorset, U.K.; histidase and urocanase (*Pseudomonas testosteroni*) were prepared and kindly given by Dr. H. Hassall (of this Department); kidney neutral endopeptidase (rabbit) was prepared as described by Kerr & Kenny (1974a); actin and myosin (rabbit muscle) were prepared by the methods of Spudich & Watt (1971) and Tonomura *et al.* (1966) respectively; samples of ¹²⁵I-labelled serum albumin and ovalbumin were prepared by using the method described for iodo-insulin B chain (George & Kenny, 1973).

* Abbreviations: Dip-F, di-isopropyl phosphorofluoridate; Gly-Pro-2-NNap, glycyl-L-proline 2-naphthylamide; Ala-Ala-2-NNap, L-alanyl-L-alanine 2-naphthylamide; Gly-Pro-NPhN₂Ph, glycyl-L-proline 4-(phenylazo)phenylamide; Ala-Ala-NPhN₂Ph, L-alanyl-L-alanine 4-(phenylazo)phenylamide; Pipes, piperazine-*NN'*-bis-(2-ethanesulphonic acid).

Methods

Kidney microvilli. These were prepared from pig kidneys as previously described (Booth & Kenny, 1974).

[³²P]Dip-labelled microvilli. Microvilli were labelled by incubating 4.8 ml of a suspension of microvilli (2.3 mg of protein/ml) in 10 mM-mannitol/2 mM-Tris/HCl, pH 7.10 (at 4°C), with 0.2 ml of [³²P]Dip-F (2.5 μmol, 200 μCi) for 1 h at 37°C, by which time no hydrolysis of Gly-Pro-NNap was demonstrable. Unbound radioactivity was removed either by dialysis against five 1-litre portions of the same buffer over a period of 96 h or, more quickly, by twice centrifuging the suspension of microvilli through a cushion of 30 ml of 0.3 M-sucrose in a Beckman SW25 rotor at 53000 g for 1 h.

Papain digestion of [³²P]Dip-labelled microvilli. 2-Mercaptoethanol (0.1 ml; 25 mM), 1.25 mg of papain and 0.35 ml of 0.1 M-Pipes/NaOH buffer, pH 6.8, were mixed and incubated at 37°C for 30 min. A sample of microvillus preparation (4 ml, containing 3–4 mg of protein/ml) was then added, and the mixture was incubated at 37°C. The papain was inactivated after 30 min incubation by the addition of 0.6 mg of iodoacetic acid. A portion [10 μl of the incubation volume (4.5 ml)] was removed for assay of radioactivity. The remainder was centrifuged at 100000 g for 1 h in a Beckman type 50 rotor. The supernatant was carefully removed, and a portion (0.1 ml) was counted. This treatment solubilized 75% of the radioactivity. The supernatant was then mixed with 1.2 units of purified dipeptidyl peptidase IV in 30 ml of 5 mM-Tris/HCl buffer, pH 7.9, and applied to a column (9 mm × 50 mm) of DEAE-cellulose equilibrated with the same buffer. Fractions (2 ml) were eluted at 4°C with 100 ml of a 0–150 mM-NaCl gradient, buffered with 5 mM-Tris/HCl, pH 7.0. Enzyme activity and radioactivity were assayed in each fraction.

Sodium dodecyl sulphate/polyacryl-amide-gel electrophoresis. Samples of membrane (100–150 μg of protein) or purified proteins (10 μg) were prepared for electrophoresis by boiling for 5 min in electrophoresis buffer (0.1 M-boric acid/0.1 M-sodium acetate, brought to pH 8.5 with NaOH) containing 2% (w/v) sodium dodecyl sulphate and 1% (v/v) 2-mercaptoethanol. After cooling, 1 drop of glycerol and 2 drops of tracker dye (0.1%, w/v, Pyronin Y) were added and mixed. The samples were applied directly to tubes (10 cm × 0.5 cm) containing 7.7% (w/v) polyacrylamide prepared as described by Weber & Osborn (1969), except that half the amount of cross-linker was used. The samples were electrophoresed into the gel at a current of 1 mA/tube for 1 h, after which time the current was increased to 7 mA/tube. Under these conditions, the tracker dye moved through the gels in about 4 h.

The gels were stained with Coomassie Brilliant Blue,

0.02% (w/v) in 10% (v/v) acetic acid/25% (v/v) propan-2-ol overnight and then 0.004% stain in 10% (v/v) acetic acid/10% (v/v) propan-2-ol for 3–24 h. Destaining was performed in 10% (v/v) acetic acid/5% (v/v) propan-2-ol. Gels were stained for carbohydrate with basic Fuchsin after periodic acid oxidation by the method of Glossmann & Neville (1971). Apparent molecular weights were obtained by comparing the electrophoretic mobility of the sample with standards run in parallel. Standards (with subunit mol.wts.) were: myosin (heavy-chain, 200000), β -galactosidase (130000), DNA polymerase I (108000), neutral endopeptidase (93000) (Kerr & Kenny, 1974b), bovine serum albumin (68000), histidase (50000), actin (42000) and lactate dehydrogenase (36000).

Disc electrophoresis. This was performed in polyacrylamide gels by using the Tris/glycine buffer system as described by Davis (1964).

Radioautography of polyacrylamide gels. After de-staining, gels were cut longitudinally, and the cut surfaces were gently pressed down on to Whatman no. 1 filter paper. The filter paper was sandwiched between polythene sheets and a weight of about 50 g/gel was placed on a Perspex plate over the gels. After drying at 20°C *in vacuo* overnight, the gels retained their overall dimensions but dried down flat to the filter paper. Radioautography of the dried gels was achieved by using Kodak Kodirex X-ray film, which was developed with Kodak DX 80 and fixed with Kodak Unifix.

Assay of dipeptidyl peptidase IV. Except where noted otherwise, Gly-Pro-2-NNap was the assay substrate. This and Ala-Ala-2-NNap were used at a concentration of 0.2 mM in 0.1 M-Tris/HCl, pH 8.0 (at 37°C). The incubation volume was 2.0 ml and the initial velocity was determined by the release of 2-naphthylamine, monitored fluorimetrically (excitation 310 nm, emission 410 nm). The fluorimeter, an Aminco-Bowman SPF 125 fitted with a thermo-

constant cell compartment and recorder, had been calibrated with 20 μ M-2-naphthylamine as a primary, and quinine sulphate as a secondary standard. The emission was corrected for quenching attributable to the 2-naphthylamide substrate. Although the fluorimetric assay was used as a routine, activities were sometimes determined by the colorimetric method (Felgenhauer & Glenner, 1966) in the same buffer but at a substrate concentration of 3 mM. This method was used when checking the specific activities of purified preparations or when assays were performed in the presence of substances exerting a strong quenching effect.

The assay using Gly-Pro-NPhN₂Ph and Ala-Ala-NPhN₂Ph as substrate was based on that described by Barth *et al.* (1974) but with the substrates at a concentration of 0.05 mM in either 0.1 M-Na₂HPO₄ buffer, pH 7.0, or in 0.1 M-Tris/HCl, pH 8.0 (at 37°C), incubated for 30–60 min. Enzyme units are expressed as μ mol of substrate hydrolysed/min at 37°C for each assay system.

Enzyme purification. This is summarized in Scheme 1. The frozen cortex tissue (2000 g) was allowed to thaw at 4°C overnight and was homogenized in 2 vol. of water for 1 min in a Kenwood blender (model A956A). After centrifugation for 15 min at 7500 g, the pellets were re-homogenized in 0.5 vol. of water, re-centrifuged, and the two supernatants combined and adjusted to pH 3.8 with 3.2 M-H₂SO₄. The extract was then stirred at 37°C for 18 h. The autolysed extract was centrifuged for 15 min at 11000 g and the supernatant stirred for 2 h with CM-cellulose (0.5 g/4 g of tissue) previously equilibrated in 10 mM-sodium acetate/acetic acid buffer, pH 4.65. The suspension was filtered on a Buchner funnel and the filtrate stirred for 2 h with the same amount of CM-cellulose as before, filtered, and, after washing, the CM-cellulose was packed into a column (50 mm diam.). The column was washed with 5 bed vol. of the equilibration buffer and then eluted with a linear gradient of sodium

Scheme 1. Summary of enzyme purification

acetate/acetic acid buffers, 1000ml each of 100mM, pH4.3, and 200mM, pH5.8. The active fractions were pooled, dialysed against 5mM-Tris/HCl buffer, pH7.9 (at 5°C), and applied to a column (15mm×200mm) of DEAE-cellulose equilibrated in the same buffer. The column was eluted by 1000ml of a linear NaCl gradient (0–100mM) in the same buffer. The active fractions were pooled, diluted fivefold in water and concentrated on another DEAE-cellulose column (9mm×30mm) similarly equilibrated. The enzyme activity was eluted in a volume of 5–8ml by 100mM-NaCl in 5mM-Tris/HCl, pH7.9 (at 5°C). The concentrated fraction was applied to a column (25mm×900mm) of Sepharose 6B and developed with 5mM-Tris/HCl, pH7.9 (at 5°C). The active fractions were pooled and dialysed against 1mM-NaH₂PO₄/Na₂HPO₄ buffer, pH7.0. The sample was applied to a column (15mm×200mm) of hydroxyapatite equilibrated with the same phosphate buffer. Elution was achieved by 500ml of a linear phosphate gradient from 50–150mM. The active fractions were dialysed against 5mM-Tris/HCl, pH7.9 (at 5°C), and concentrated as before on a small column of DEAE-cellulose.

[³²P]Dip-labelled enzymes. Samples of dipeptidyl peptidase IV (about 2mg), trypsin and chymotrypsin (about 5mg each) were each dissolved in 5ml of 10mM-Tris/HCl, pH7.9 (at 37°C). [³²P]Dip-F solution (0.1 ml, containing 1.25 μmol and about 100 μCi) was added and the mixtures were incubated at 37°C until no enzyme activity could be detected; this took 1 h for dipeptidyl peptidase IV and about 6 h for trypsin and chymotrypsin. The activities of these two enzymes were monitored by a sensitive assay using [¹²⁵I]iodo-insulin B chain (George & Kenny, 1973). Their initial specific activities were also checked by the standard assay procedures using benzoylarginine ethyl ester and *N*-acetyltyrosine ethyl ester (Walsh & Wilcox, 1970) and were within 10% of the suppliers' stated activities.

The incubation mixtures were dialysed against 10mM-Tris/HCl, pH7.9, with three changes of medium to remove most of the unbound radioactivity. The buffer for the dipeptidyl peptidase sample was changed to 10mM-sodium acetate/acetic acid, pH4.5, for the last two dialyses. Small columns (9mm×20mm) of CM-cellulose were equilibrated at the appropriate pH, and the enzyme samples loaded and washed with the same buffer until the effluent radioactivity was decreased to a low value. Unlabelled Dip-F and NaH₂PO₄ were added to the buffer to aid removal of unbound radioactivity. The [³²P]Dip-labelled enzymes were finally eluted with 200mM-sodium acetate/acetic acid buffer, pH5.5 (dipeptidyl peptidase), or 1M-NaCl in 10mM-Tris/HCl, pH9.0, for the pancreatic proteinases. Each radioactively labelled protein, in about 5ml of buffer, was assayed for radioactivity and protein.

Molecular-weight determinations on Sepharose 6B. Samples of the purified enzyme and various markers were applied in a minimal volume (1.0–2.5ml) to columns (25mm×900mm) of Sepharose 6B previously equilibrated with 5mM-Tris/HCl, pH7.9, at 5°C. Fractions (3–5ml) were collected at a flow rate of 20ml/h. In some experiments the buffer contained 100mM- or 1M-NaCl.

Calibration markers were as follows: urease (mol.wt. 483000) from jack-bean meal, assayed with 10mM-urea as substrate, NH₃ release being detected with Nessler's reagent; catalase (250000), assayed in 0.1% H₂O₂, monitored by *E*₂₄₀; pyruvate kinase from rabbit muscle (237000), assayed in 0.2mM-NADH, phosphoenolpyruvate and ADP, with 1 unit of lactate dehydrogenase/ml, monitored by *E*₃₄₀; glucose oxidase (154000), assayed with 0.5% glucose solution containing peroxidase (0.5mg/100ml) and *o*-dianisidine (5mg/100ml) (the colour reaction was followed at 445nm); urocanase (110000), assayed with urocanate as substrate in the presence of dichlorophenol-indophenol, the decrease in *E*₆₀₀ being monitored; kidney neutral endopeptidase (93000), assayed as described by Kerr & Kenny (1974a); bovine serum albumin (68000), labelled with ¹²⁵I; [¹²⁵I]ovalbumin (45000). *V*₀ values for the columns were determined with Blue Dextran.

Electron microscopy. The enzyme was made visible by negative staining with 2% uranyl acetate on fresh carbon and Formvar-coated grids. A Philips EM300 electron microscope was used.

Analytical ultracentrifugation. Sedimentation-velocity and sedimentation-equilibrium experiments were performed in a Beckman model E analytical ultracentrifuge equipped with Schlieren and interference optical systems and a rotor-temperature indicating and control system. Sedimentation-velocity experiments were performed in cells of 12mm, or more often 30mm light-path, because of the low protein concentration involved. The plates were measured in a projectorscope (Precision Grinding Ltd., Precision Works, Mitcham Junction, Surrey, U.K.), and the sedimentation coefficients calculated from the rate of movement of the peak maxima in the usual way.

Sedimentation-equilibrium experiments were performed by the high-speed or meniscus-depletion method (Yphantis, 1964). At equilibrium, several exposures were taken by using the interference optical system. Usually some Schlieren pictures were also taken. From the fringe displacement in the interference pictures, the logarithm of the fringe shift [$\ln(Y_r - Y_0)$] at equilibrium was calculated and plotted against the square of the radial distance, *r*². The mol.wt., *M*_w, was calculated from such plots by using the relationship

$$\bar{M}_{w,app} = \frac{2RT}{(1 - \bar{v}\rho)\omega^2} \cdot \frac{d \ln(Y_r - Y_0)}{dr^2}$$

The data from the Schlieren pictures at equilibrium were treated by the Lamm method [log(Schlieren plot), see Chervenka, 1969] to obtain values for M_z (Z -average molecular weight).

Assay of radioactivity. Samples containing ^{32}P were counted in the ^3H channel of a Beckman LS230 liquid-scintillation counter by means of its Čerenkov radiation in water. ^{125}I -labelled proteins were counted in a NaI crystal γ -scintillation counter.

Determination of protein. Protein was determined by the method of Lowry *et al.* (1951) as previously described (George & Kenny, 1973) or by measuring the E_{280} , assuming a value for $E_{280}^{1\%}$ of 10.0, unless values for individual proteins were known.

Buffers. In each case the molarity stated refers to the weak acid or base used.

Hydrolysis of peptides by dipeptidyl peptidase. Oligopeptides were incubated at 37°C with enzyme in a volume of 50–200 μl containing 25 mM-Tris/HCl buffer, pH 7.9. Controls contained, in addition, 2.5 mM-Dip-F. At the end of incubation the mixtures were applied to Whatman 3MM paper and subjected to high-voltage electrophoresis in formic acid/acetic acid buffer, pH 1.85, for 1 h at 125 V/cm (Atfield & Morris, 1961). After drying, a strip was cut and stained with ninhydrin. Areas corresponding to ninhydrin-positive regions were excised and eluted with 1% acetic acid. Portions of the eluate were dansylated before and after hydrolysis with 6M-HCl, and dansyl-amino acids identified on polyamide thin-layer plates (Hartley, 1970).

Results and Discussion

Purification of dipeptidyl peptidase IV from pig kidney cortex

The key step in the purification of any membrane-bound enzyme is that of solubilization. Although papain releases a substantial proportion of dipeptidyl peptidase IV from microvillus fractions, it proved both inefficient and expensive as a means of treating crude kidney extracts on a large scale. However, autolysis at pH 3.8 as used by Hopsu-Havu *et al.* (1968), based on that of de la Haba *et al.* (1959), proved to be very effective in solubilizing about 60% of the enzyme. The enzyme was found to be exceptionally stable during all stages of purification; even during autolysis, the loss at pH 3.8 at 37°C for 18 h was usually less than 15%. Below pH 4.65 the enzyme is cationic and binds well to CM-cellulose, but at pH values above 5 it is readily desorbed. CM-cellulose was therefore a useful means of extracting it from the large volume of the acidic supernatant after autolysis. At neutral pH values the enzyme is anionic and could be chromatographed successfully on DEAE-cellulose. Gel filtration on a column of Sepharose 6B further enriched the preparation. Chromatography on hydroxyapatite was also useful, though desorption of the enzyme at 4°C was erratic, and yields were sometimes poor. These difficulties were not encountered at 20°C . The results from one purification are shown in Table 1. The fluorimetric assay using Gly-Pro-2-NNap as substrate is con-

Table 1. *Purification of dipeptidyl peptidase IV*

In this preparation 2 kg of cortical tissue was used. See under 'Methods' for details of each step. Enzyme units are μmol of substrate hydrolysed/min. The ratio AA/GP is the ratio of specific activities obtained with Ala-Ala-2-NNap and Gly-Pro-2-NNap as substrates.

Fraction	Volume (ml)	Protein (mg)	Units (total)	Recovery (%)	Fluorimetric assays with 0.2mM-Gly-Pro-2-NNap			Colorimetric assays with Gly-Pro-2-NNap	
					Specific activity (units/mg of protein)	Purification (fold)	Ratio (AA/GP)	Specific activity (units/mg of protein)	
								0.2mM	3mM
Homogenate	6000	247000	8610	100	0.035	1	0.19	0.073	0.17
7500g supernatant	5700	182000	6540	76	0.036	1.03	0.18	0.069	0.21
Autolysed extract	5750	111000	5304	62	0.048	1.4	0.13	0.12	0.30
Supernatant (soluble) extract	4915	31700	3690	43	0.117	3.3	0.11	0.21	0.77
CM-cellulose	240	914	2513	29	2.75	79	0.034	3.83	12.6
DEAE-cellulose	433	368	2350	27	6.4	183	0.018	7.6	27.7
Sepharose 6B	80	164	1660	19	10.1	289	0.022	11.2	35.9
Hydroxyapatite	500	150	1575	18	10.5	300	0.019	11.4	46
DEAE-cellulose (concentrated enzyme)	20	96	1017	12	10.6	303	0.04	12.8	56.8

venient, quick and economical in use of substrate. It is therefore ideal for monitoring column effluents during purification, but it suffers two disadvantages: the kinetics are not zero-order, and the fluorescence may be subject to quenching by substrate and other substances. Where enzyme activities need to be expressed in absolute terms, the colorimetric assay may be preferable. Values for both types of assay are shown in Table 1.

The two methods gave comparable values when the same concentration of substrate (0.2 mM) was used. At a higher concentration (3 mM) the rates approach $V_{\max}$ values and specific activities are almost four-fold higher. Gly-Pro-NPhN₂Ph used at a lower concentration (0.05 mM) gave values that were about an order of magnitude lower than those for the fluorimetric assay (homogenate, 0.0022 μ mol hydrolysed/min; concentrated enzyme, 0.78 μ mol hydrolysed/min). Barth *et al.* (1974) used Ala-Ala-NPhN₂Ph for their assays: the specific activity of their purified enzyme is comparable with that of ours, with the same substrate (0.02 unit/mg). The ratio of activities for the two types of peptide substrate, Ala-Ala-X/Gly-Pro-X, are also shown in Table 1. The ratio is about 0.2 in the crudest fractions and is decreased after the first-column fractionation to values in the range 0.02–0.04 as purification proceeds. The ratios obtained for the column fractions when colorimetric assays, including those with the 4-(phenylazo)phenylamide substrates are used, were slightly higher, in the range 0.025–0.11.

Fig. 1. Gel filtration on Sepharose 6B of dipeptidyl peptidase IV and [32 P]Dip-labelled enzyme

See under 'Methods' for details. $\circ$, Radioactivity; $\bullet$, enzyme activity; fraction volume, 10 ml.

During the course of one purification, a sample amounting to 8% of the solubilized extract was treated with [32 P]Dip-F until no dipeptidyl peptidase IV activity could be detected, and the portion added back to the rest of the preparation. The subsequent steps in the purification were monitored for both enzyme activity and radioactivity. The results for the Sepharose 6B column are shown in Fig. 1. The two activities are coincident in this elution pattern (as they were in the earlier chromatographic steps), confirming that the enzyme is the only major protein in the solubilized extract to be labelled by the inhibitor.

Distribution of enzyme activity hydrolysing Gly-Pro-2-NNap in rabbit tissues

The survey of rat tissues made by Hopsu-Havu & Ekfors (1969) showed that glycylproline naphthylamidase is predominantly located in the kidney. Our results, on rabbit tissues, are in agreement and also demonstrate that the activity is sensitive to Dip-F, though not to 0.5 mM-phenylmethylsulphonyl fluoride, in each of the tissues examined (Table 2). The substantial enrichment factor for microvillus fractions prepared from intestine (10.3) suggests that in this tissue, as in the kidney, glycylproline naphthylamidase is a brush-border enzyme. The specific activity in the intestine is, however, only one-tenth of that in the kidney. Bury & Pennington (1975) were unable to detect the hydrolysis of Gly-Pro-2-NNap by human muscle tissue. In rat muscle the activity, though weak, is readily detectable.

Evidence that dipeptidyl peptidase IV is the only major enzyme in kidney microvilli reacting with Dip-F

Microvillus fractions prepared from pig kidney are equally susceptible to inhibition by Dip-F as those from rabbit kidneys. When incubated with [32 P]Dip-F all dipeptidyl peptidase IV activity was lost within 60 min (see under 'Methods' for details). The [32 P]Dip-labelled microvillus fraction so obtained was investigated in two ways. First, the preparation was treated with 2-mercaptoethanol and sodium dodecyl sulphate and subjected to polyacrylamide-gel electrophoresis. The pattern of bands seen after staining by Coomassie Blue is typical of kidney microvillus preparations (Plate 1a). One gel run in parallel and stained in the same way was bisected longitudinally, dried flat and the radioactivity localized by radioautography. A single strong radioactive band was demonstrated, the mobility of which is consistent with that of a polypeptide of mol.wt. 130000 (Plate 2a). The mobilities in sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the labelled protein in the microvillus membrane and the purified enzyme were identical when run in parallel and could not be resolved when the two samples were mixed and run on the same gel.

Table 2. *Dipeptidyl peptidase IV activity in various tissues*

Rabbit tissues were used except for the sample of muscle from rat. Gly-Pro-2-NNap was used as substrate. Microvilli were prepared as described under 'Methods'. Homogenates were prepared in 0.33M-sucrose. The microsomal pellet was obtained by centrifugation at 25000g for 2h after removing the 8000g-15min pellet. Pms-F, phenylmethylsulphonyl fluoride.

Tissue	Fraction	10 ³ × Specific activity (units/mg)	Preincubation time (min) ...	Inhibition (%)					
				0.5mM-Dip-F			0.5mM-Pms-F		
				30	60	90	30	60	90
Kidney (cortex)	Homogenate	37							
	Microvilli	533		98	99	100	10	20	24
	Pure enzyme (pig)	10700		98	100	100	5	16	21
Small intestine	Homogenate	4.4							
	Microvilli	46		97	98	99	2	16	22
Liver	Homogenate	0.35							
	Microsomal fraction	0.5		99	100	100	20	27	26
Lung	Homogenate	0.4							
	Microsomal fraction	1.7		100	100	100	10	16	24
Spleen	Homogenate	0.2							
	Microsomal fraction	0.15		100	100	100	16	26	25
Muscle (rat)	Homogenate	0.042							
	Microsomal fraction	0.27		100	100	100	49	52	52

Fig. 2. *Chromatography on DEAE-cellulose of papain-solubilized [³²P]Dip-labelled microvillus preparation and purified dipeptidyl peptidase IV*

See under 'Methods' for details. ○, Radioactivity; ●, enzyme activity; ····, NaCl gradient; fraction volume, 2ml.

Secondly, the labelled microvillus fraction was treated with papain, an enzyme capable of solubilizing, to different extents, several microvillus enzymes (George & Kenny, 1973), including dipeptidyl peptidase IV. In this experiment, 73 % of the radioactivity was released in 30min. When a sample of purified active dipeptidyl peptidase IV was added to the soluble extract and the mixture applied to a DEAE-cellulose column, the peaks of radioactivity and

enzyme activity were found to be coincident (Fig. 2).

These experiments show that the only major [³²P]-Dip-labelled protein in the kidney microvillus is indistinguishable in sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and (when solubilized) on DEAE-cellulose chromatography from the purified dipeptidyl peptidase IV.

The specific radioactivities of the [³²P]Dip-labelled membrane and purified enzyme permit the calculation

of the content of the enzyme in the membrane. The values for a sample of enzyme and microvillus membrane, labelled at the same time with the same batch of [32 P]Dip-F, were 303 158 c.p.m./mg of protein and 11 823 c.p.m./mg of protein respectively. This indicates that dipeptidyl peptidase comprised 3.9% of the microvillus protein. A similar calculation may be made using specific enzyme activities: the mean of three batches of the purified pig enzyme was 10.7 units/mg of protein and for pig microvillus preparations, the mean was 0.53 unit/mg of protein, indicating that the enzyme contributes about 5% of the membrane protein.

Molecular-weight determinations

The molecular weight of a membrane protein, particularly one containing carbohydrate, is a relatively complex concept in comparison with that of simple proteins. Dipeptidyl peptidase IV is isolated after a step involving proteolysis by papain or by cathepsins active during autolysis. In either case, a fragment of the native protein is likely to have been shed. In addition, the occurrence of oligomers in the purified preparation may be an artifact and may not reflect the native organization in the membrane. The presence of carbohydrate in a membrane glycoprotein may affect the determination of the molecular weight by most of the conventional methods. We have attempted to resolve some of the uncertainties with respect to the molecular weight by using several different methods: gel filtration, ultracentrifugation and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. In the last instance it has been possible to examine both the native (i.e. membrane-bound) enzyme and the purified preparations. A purely chemical method based on the stoichiometric reaction of the active-site serine with [32 P]Dip-F has also yielded results for the size of the active-site subunit.

Apparent subunit molecular weight estimated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

Samples of the purified enzyme, which had been boiled with 2% (w/v) sodium dodecyl sulphate, migrated on polyacrylamide gels as a single band of mobility (relative to Pyronin Y) of 0.21 (Plate 1c). The band stained with both Coomassie Blue and basic Fuchsin after periodic acid oxidation. The mobility was unchanged when the enzyme was reduced with 2-mercaptoethanol (Plate 1b). However, thiol treatment did reveal some faint, diffusely staining material of greater mobility, possibly produced as a result of the autolytic step in the purification. The [32 P]Dip-labelled enzyme migrated as a single band of identical mobility exactly corresponding to the radioactive band demonstrated by radioautography (Plate 2b and 2c). Marker proteins (listed under 'Methods') spanned the molecular-weight range 37 000–200 000. The relationship between log(molecular weight) and

relative mobility was linear over this range. By interpolation, the mol.wt. of dipeptidyl peptidase IV was estimated to be 130 000. β -Galactosidase from *E. coli* has a subunit molecular weight of 130 000 (Ullmann *et al.*, 1968). This enzyme had the same relative mobility (0.21), and only one band was visible when it was mixed with dipeptidyl peptidase IV. We conclude that the subunit mol.wt. in the presence or absence of thiol is 130 000. Within the limits of error of the method (about 5%) the purified and the labelled membrane-bound enzymes had identical mobilities. This indicates that any residual polypeptide fragment remaining after solubilization of the active enzyme by proteolysis is unlikely to be greater than 6500 daltons.

Subunit molecular weight estimated by reaction with [32 P]Dip-F

The stoichiometric reaction of the active-site serine with Dip-F has enabled us to determine the molecular weight of the subunit by active-site titration. The principle is simple enough; if the specific radioactivity of [32 P]Dip-F is known, and the specific radioactivity of the purified [32 P]Dip-labelled enzyme can be determined, then the equivalent weight of the enzyme can be calculated. At first this approach gave unexpectedly high values until we realized that the batch of [32 P]Dip-F contained radioactive impurities. These were revealed when a sample of [32 P]Dip-F was examined by high-voltage electrophoresis at pH 6.5 in pyridine/acetic acid buffer at 50 V/cm for 50 min. Some 60% of the radioactivity remained at the starting line (as would be expected for the uncharged Dip-F) and 40% migrated as two anionic species 8 cm and 11 cm towards the anode. It was therefore possible to correct the apparent specific radioactivity of [32 P]Dip-F by a factor of 0.6. To confirm the validity of this assumption two 'standard' serine proteinases, trypsin and chymotrypsin, were also treated with [32 P]Dip-F, and the radioactivity covalently bound to 1 nmol of each enzyme was determined. By this approach it was possible to calculate the weight of dipeptidyl peptidase IV binding the same amount of radioactivity, a calculation involving no assumptions about the radiochemical purity of the sample of [32 P]Dip-F. However, other assumptions are necessary: that the standard enzymes were pure and fully active before incubation with the inhibitor; that all the enzyme was converted into Dip-labelled enzyme; that the label was not lost during purification of the protein; that the purified [32 P]Dip-labelled proteins were homogeneous and free of non-covalently bound radioactivity. Some of the assumptions could be tested. Thus the samples of trypsin and chymotrypsin used were found on assay to have the enzyme activities expected for the pure enzymes. The degree of inhibition of all enzymes was shown to be 98% complete by the end of the incubation.

EXPLANATION OF PLATE I

Polyacrylamide-gel electrophoresis of microvillus membrane and purified dipeptidyl peptidase IV

The gels are stained with Coomassie Brilliant Blue. See under 'Methods' for details. (a) Sodium dodecyl sulphate-treated microvillus membrane (100 μ g of protein). The pattern includes a major band with a mobility similar to that of a polypeptide of mol. wt. 130000. (b) Purified dipeptidyl peptidase IV (10 μ g of protein) treated with 2-mercaptoethanol and sodium dodecyl sulphate. (c) As (b), but with mercaptoethanol omitted. The mobility of the purified enzyme in both gels corresponds to that of a polypeptide of mol. wt. 130000. (d) Disc electrophoresis of purified dipeptidyl peptidase IV (10 μ g of protein) previously reduced with 1% mercaptoethanol. (e) As (d), but with mercaptoethanol omitted.

EXPLANATION OF PLATE 2

Polyacrylamide-gel electrophoresis of microvillus membrane and purified dipeptidyl peptidase IV

The left of each pair is the appearance of a dried gel that has been stained for protein; that on the right is the radioautograph obtained by exposure of the dried gel. See under 'Methods' for details. (a) [32 P]Dip-labelled microvillus preparation (100 μ g of protein) (sodium dodecyl sulphate/polyacrylamide-gel electrophoresis). The strong radioactive band has the mobility of a polypeptide of mol.wt. 130 000. (b) [32 P]Dip-labelled enzyme (10 μ g of protein). The labelling was performed early in the course of a purification. All the radioactivity is associated with the stained band. (c) As (b), but run in a disc-electrophoresis system.

EXPLANATION OF PLATE 3

Electron micrograph of dipeptidyl peptidase IV made visible by uranyl acetate negative stain

The typical appearance is that of a dimeric protein; the mean diameter of each subunit is 7.1 nm. Many of the subunits appear to be dimpled, so that each resembles a doughnut, and each dimer a numeral 8.

Table 3. *Estimate of active-site subunit molecular weight of kidney dipeptidyl peptidase IV*

See under 'Methods' for details. The purified enzyme (DPP IV) was fully inhibited by incubation with [32 P]Dip-F. 'Standard' serine proteinases, chymotrypsin (CT) and trypsin (T), were similarly treated. After removal of non-protein radioactivity the specific radioactivities were assayed, and the results used to calculate the weight of dipeptidyl peptidase IV protein binding 1 nmol of di-isopropyl phosphate. The mean of all estimates was 138000.

Experiment	Specific radioactivities				Estimates of active-site molecular weight	
	[32 P]Dip-F (c.p.m./nmol)	[32 P]Dip-CT (c.p.m./nmol)	[32 P]Dip-T (c.p.m./nmol)	[32 P]Dip-DPP IV (c.p.m./mg)	Relative to Dip-F	Relative to trypsin and chymotrypsin
1	10735	13270 (Mean 13084)	12898	79876 (E_{280})	134000	164000
2	7727	10704 (Mean 10965)	11226	80420 (Lowry)	133000	163000
				70307 (E_{280})	109000	156000
				76039 (Lowry)	102000	144000

Table 4. *Molecular weight of dipeptidyl peptidase IV determined by gel filtration on Sepharose 6B*

See under 'Methods' for details (V_e , elution volume; V_0 , void volume).

Elution medium	V_e/V_0	log(Mol.wt.)	Mol.wt.
5 mM-Tris/HCl, pH 7.9	1.4	5.48	302000
5 mM-Tris/HCl, pH 7.9, 100 mM-NaCl	1.85, 1.87	5.46, 5.48	288400, 302000
10 mM-Tris/HCl, pH 7.9, 1 M-NaCl	1.94	5.47	295100

Stability of the label was checked by re-chromatography of the Dip-labelled enzymes on CM-cellulose, over 95% of the radioactivity being eluted with the protein. Before this step, each enzyme had been subjected to ion-exchange chromatography and eluted only when the column had been washed free of radioactivity attributable to the unbound inhibitor. The purified Dip-labelled protein was shown to be homogeneous by polyacrylamide-disc-gel electrophoresis as well as by electrophoresis in the presence of sodium dodecyl sulphate (Plate 1). The results are given in Table 3. The specific activities of the proteins are, of course, subject to the errors inherent in the determination of protein. For chymotrypsin and trypsin, the published values for $E_{280}^{1\%}$ were used. For dipeptidyl peptidase, protein was determined by two methods. The value for $E_{280}^{1\%}$ for a pure sample of enzyme checked by dry weight was 11.5. The values obtained by this method agreed well with those obtained by using the method of Lowry *et al.* (1951).

The estimates of the size of the active-site subunit of dipeptidyl peptidase were found to be in the range 102000–134000 (mean 119500) relative to Dip-F, and 144000–164000 (mean 156750) relative to the other Dip-labelled enzymes. These results (overall mean 138125) indicate that there is only one site reacting with Dip-F in each monomer of mol.wt. 130000.

Molecular weight determined by gel filtration

Columns containing Sepharose 6B were calibrated, as described under 'Methods' with three different media: 5 mM-Tris/HCl, pH 7.9, the same buffer containing 100 mM-NaCl and 10 mM-Tris/HCl, pH 7.9, 1 M-NaCl. The ratio V_e/V_0 for dipeptidyl peptidase was affected by the presence of salt in the buffer as were the values for the marker proteins. It was therefore necessary to prepare calibration curves for each of the media used. Otherwise the higher V_e/V_0 values observed in the presence of salt might erroneously suggest dissociation of the enzyme into monomers. In fact, the molecular weights, shown in Table 4 (mean 297000), indicate that the enzyme exists as a dimer in each of the media studied. No evidence of dissociation was found.

Molecular weight by analytical ultracentrifugation

Purified dipeptidyl peptidase IV dissolved in 0.1 M-Tris/HCl buffer, pH 7.9, sedimented as a single symmetrical boundary in sedimentation-velocity experiments. Over the concentration range 0–0.5 mg/ml, linear extrapolation of the sedimentation coefficient to zero protein concentration (Fig. 3) gave a value for $s_{0,w}^{20}$ of 10.12 ± 0.12 S. In sedimentation equilibrium (Fig. 4), the plot of $\ln(\Delta Y)$ against r^2 (calculated from the interference photographs) was linear, indicating that the material was homogeneous. The apparent

Fig. 3. Dependence of sedimentation coefficient of dipeptidyl peptidase IV on protein concentration

The enzyme was dissolved in 5 mM-Tris/HCl, pH 7.3, containing 0.1 M-NaCl. s Values were corrected to $s_{20,w}^0$ in the usual way. Value for $s_{20,w}^0$, 10.12 ± 0.12 S.

Fig. 4. High-speed sedimentation-equilibrium experiment with dipeptidyl peptidase IV

Protein concentration was initially 0.42 mg/ml in 5 mM-Tris/HCl buffer, pH 7.9, containing 0.1 M-NaCl. Conditions of run: speed, 14656 rev./min; temperature, 25°C; interference photograph taken after 65 h. Mol.wt. ($\bar{M}_{w,app.}$), $239\,700 \pm 6700$.

molecular weight, $\bar{M}_{w,app.}$, calculated from the slope of the plot, was $239\,700 \pm 6700$. An apparent Z -average molecular weight $\bar{M}_{z,app.}$ of $268\,000 \pm 12\,500$ was calculated from the slope of the log(Schlieren plot).

As is normally found with such plots, there was much more scatter than is found with interference measurements, and the experimental error was considerably larger. Therefore not too much reliance should be put on this value, except to note that it agrees reasonably well with the weight-average molecular weight, but could indicate the presence of some heavier material. In these calculations of molecular weights, a value for the partial specific volume, $\bar{v}$, of 0.73 ml/g was calculated from the amino acid composition.

One batch of the enzyme that had lost most of its enzymic activity was found to have a much lower sedimentation coefficient than the active purified material. Uncorrected sedimentation coefficients of 6.0–6.6 S were measured at protein concentrations in the range 0–0.5 mg/ml. A sedimentation-equilibrium experiment with this material yielded a mol.wt. ($\bar{M}_{w,app.}$) of $149\,100 \pm 9500$. It seems likely that this is equivalent to one-half of an active molecule, and such a finding would be in line with the results of sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, and active-site labelling experiments, which indicate a subunit mol.wt. of 130 000.

In an attempt to see if the active enzyme molecule dissociated in the presence of 1 M-NaCl, the sedimentation coefficient was determined in 5 mM-Tris/HCl buffer, pH 7.9, containing 1 M-NaCl. An uncorrected value of 7.82 S was found. Correction of this to $s_{20,w}$ would yield a value of about 9.3 S, indicating that the molecule was still in the 'dimeric' form under these conditions.

Electron microscopy

The conclusion that the purified enzyme is a dimer comprising two active subunits of 130 000 mol.wt. is supported by electron microscopy. The negatively stained preparation shown in Plate 3 reveals that nearly all the forms are dimers. The diameter of a monomer is 7.1 nm (mean of 50 measurements). The centre of each subunit appears dimpled, a concavity or hole retaining some stain so that the dimer often resembles the numeral 8. If each subunit is assumed to be spherical, and the value of v taken as 0.73, the subunit mol.wt. may be calculated as 154 000. In fact, the subunits appear to be ellipsoids, so that this value is not inconsistent with other estimates.

Specificity of dipeptidyl peptidase IV

The hydrolysis of a bond adjacent to a proline residue is only one aspect of the specificity of dipeptidyl peptidase IV. The enzyme cleaves this bond in the assay substrates, Gly-Pro-2-NNap and Gly-Pro-NPhN₂Ph as well as in a number of tripeptides. In each case the N -terminal dipeptide is one of the products. The other well-characterized type of specificity is directed towards alanine peptides. Ala-Ala-2-

NNap and Ala-Ala-NPhN₂Ph are hydrolysed; so, too, are simple alanine peptides. Barth *et al.* (1974) have shown the longer alanine peptides are degraded by a step-by-step mechanism liberating dipeptides sequentially. However, alanyl peptides are also excellent substrates for another kidney brush-border peptidase, aminopeptidase M. Crude preparations of the enzyme containing traces of aminopeptidase M may give unexpected results, e.g. the formation of free alanine from tetra-alanine. The occurrence of (Ala)₃-NPhN₂Ph as a product from (Ala)₄-NPhN₂Ph (Barth *et al.*, 1974) could also be explained on this basis. The apparent relative rates for the two types of substrates may likewise be affected by the presence of aminopeptidase M in impure preparations. This is the most likely explanation for the decreasing ratios (Ala-Ala-2-NNap/Gly-Pro-2-NNap, Table 1) obtained as purification proceeded. Indeed each batch of purified enzyme assayed with both substrates has given a ratio in the range 0.02–0.04. When the values for $V_{\max}$ were compared, the ratio was 0.04. The use of the colorimetric assay with either 2-

naphthylamide or 4-(phenylazo)phenylamide substrates gave values slightly outside this range, but never approaching the value of 0.2 reported by Barth *et al.* (1974). We have no explanation of this discrepancy, but the possibility must exist that the higher ratio was due to traces of aminopeptidase M.

The two types of specificity exhibited by purified preparations might be attributed to the presence of two distinct dipeptidyl peptidases. However, careful monitoring with both substrates throughout one purification failed to show any suggestion of resolution of the two activities. If two enzymes do exist, their physical properties are indistinguishable by any of the techniques so far used.

The probability that both types of substrate are hydrolysed at the same active site of dipeptidyl peptidase IV is increased when considering the results obtained by another experiment. When Ala-Ala-2-NNap and Gly-Pro-2-NNap were used in the colorimetric assay at concentrations corresponding to their K_m values (0.24 mM and 0.21 mM respectively), the rates (means of three experiments) for each substrate

Fig. 5. Effect of inhibitors on dipeptidyl peptidase IV

(a) Purified enzyme; (b) microvillus membrane; ●, Dip-F, enzyme assayed with Gly-Pro-2-NNap; ○, Dip-F, enzyme assayed with Ala-Ala-2-NNap; in both cases the values are the means of six experiments. ■, Diethyl *p*-nitrophenyl phosphate; ▲, phenylmethylsulphonyl fluoride, in both cases assayed with Gly-Pro-2-NNap. ····, Dip-F, Gly-Pro-NPhN₂Ph as substrate; ----, Dip-F, Ala-Ala-NPhN₂Ph as substrate.

separately were 0.77 and 22.5 $\mu\text{mol}/\text{min}$ per mg of enzyme, and when both were present in the assay the rate was 17.3 $\mu\text{mol}/\text{min}$ per mg, i.e. 74% of the sum of the separate rates. Dixon & Webb (1964) have pointed out that a simple summation of rates would be predicted if two enzymes are responsible for the two specificities. On the other hand, the theoretical value for a single enzyme is 67% of the sum. Our experimental result is consistent with the latter interpretation. For a dimeric enzyme it is also an argument against the occurrence of dissimilar subunits in each dimer.

Effect of serine inhibitors

The effects of a 1 h incubation with Dip-F, diethyl *p*-nitrophenyl phosphate and phenylmethylsulphonyl fluoride are shown in Figs. 5(a) and 5(b). The activity is strikingly sensitive to Dip-F, 50% inhibition being achieved at less than 10 μM , and the sensitivity is similar for both the purified enzyme and the microvillus preparation. In comparison diethyl *p*-nitrophenyl phosphate is about 100-fold less effective for both the pure enzyme and the microvillus preparation and phenylmethylsulphonyl fluoride is a feeble inhibitor of the pure enzyme and almost ineffective with the microvillus preparation.

Barth *et al.* (1974) have reported a curious phenomenon in that the inhibition curves obtained with Dip-F were different with the two substrates they used, namely Gly-Pro-NPhN₂Ph and Ala-Ala-

NPhN₂Ph, the latter showing greater sensitivity (about sixfold) to the inhibitor. The Dip-F curves in Fig. 5(a) are the means of six experiments and they confirm this report, although the magnitude is much less (<twofold concentration difference at 50% inhibition). Our experiments were performed with the 2-naphthylamide substrates, but the results were not significantly altered by repeating the experiment with the 4-(phenylazo)phenylamide substrates (·····, ----, Fig. 5a). Nor was the different sensitivity attributable to an effect of the unbound inhibitor, since assays repeated after dialysing the partially inhibited samples of enzyme failed to abolish the difference. The existence of two curves is at first sight surprising if the same enzyme is hydrolysing the Ala-X and Pro-X bonds. There is only one active-site serine in each subunit, and the irreversible conversion of one-half of the active-sites into the Dip form might be expected to result in 50% inhibition, irrespective of the substrate used. The fact that this simple expectation is not fulfilled in practice deserves comment, if not explanation.

The simplest explanation, that two enzymes, or a dimer comprising dissimilar subunits, are responsible for the two specificities has been dismissed by the arguments above. Another possibility is that some form of interaction occurs between subunits such that di-isopropyl phosphorylation of one active site affects the catalytic activity of the other subunit. We know that the reaction of Dip-F with the enzyme does

Table 5. *Hydrolysis of oligopeptides by dipeptidyl peptidase IV*

See under 'Methods' for details. Control incubations, containing 2.5 mM-Dip-F or enzyme preincubated with 0.5 mM-Dip-F, were run for each peptide; in no case could any ninhydrin-positive products be demonstrated with the inhibited enzyme. Appropriate markers for the peptides and amino acids identified were used, except for the peptides released from the Z-substituted substrates; their identification relies on the dansyl end-group and the amino acid composition after hydrolysis of the eluted peptide. Since these were not quantitative, the second glycine residue is an assumed component.

Peptide sequence	Incubation conditions			Ninhydrin-positive products
	Peptide (μg)	Enzyme (μg)	Incubation time (h)	
Gly-Pro-Ala	50	2	0.5	Gly-Pro, Ala
Ala-Pro-Gly	50	2	0.5	Ala-Pro, Gly
Gly-Ala-Ala	50	2	0.5	Gly-Ala, Ala
Val-Ala-Ala-Phe	50	2	2.5	Val-Ala, Ala-Phe
Ala-Ala	50	2	4	None detected
Ala-Ala-Ala	50	2	0.5	Ala-Ala, Ala
Ala-Ala-Ala-Ala	50	2	0.25	(Ala) ₂ [with traces of (Ala) ₃ and Ala after 2.5 h incubation]
Ala-Ala-Ala-Ala-Ala	50	2	0.25	(Ala) ₃ , (Ala) ₂ [with traces of (Ala) ₄ and Ala after 2.5 h incubation]
Gly-Gly-Pro	50	2	4	None detected
Gly-Pro-Pro	50	2	4	None detected
Z-Gly-Pro-Leu-Gly-Pro	880	87	5	Leu-Gly-Pro
Z-Gly-Pro-Gly-Gly-Pro-Ala	500	156	4	Gly(-Gly)-Pro, Gly(-Gly)-Pro-Ala, Ala
Z-Pro-Ala	50	2	4	None detected
Z-Ala-Pro-Gly	50	2	4	None detected

not bring about dissociation of the dimer, since the labelled enzyme co-elutes with the active form on Sepharose 6B; hence an allosteric or even a simple steric effect of the Dip-group is a possibility. However, partial inhibition of the enzyme had only a slight effect on the K_m values. For Ala-Ala-2-NNap it was 0.22 mM (uninhibited, 0.24 mM) and for Gly-Pro-2-NNap it was 0.18 mM (uninhibited 0.21 mM). These are the means of two experiments, and the differences may well be within experimental error.

Hydrolysis of oligopeptides by dipeptidyl peptidase IV

Table 5 shows that a number of simple peptides containing a proline or alanine residue in the second position were readily hydrolysed in a predictable fashion, except for Gly-Pro-Pro, which apparently resisted attack. Tetra- and penta-alanine were similarly attacked in short incubations, but longer periods yielded traces of products inconsistent with a step-by-step dipeptidyl attack. Two peptides with blocked α -amino groups were also hydrolysed by the enzyme, albeit with higher enzyme/substrate ratios and with longer incubation times. All the positive results in Table 5 were attributed to dipeptidyl peptidase IV, since the hydrolyses were sensitive to inhibition with Dip-F in every case. Although none of these results is quantitative, it appears that the dipeptidyl peptidase activity of the enzyme is only one aspect, though perhaps the preferred one, of its catalytic repertoire. In particular, a free α -amino group is not an absolute requirement, at least for prolyl bonds in two *N*-blocked oligopeptides.

Dipeptidyl peptidase IV deserves to be reclassified as a serine endopeptidase, (EC 3.4.21.-) in which case it might be renamed 'microvillus-membrane serine peptidase'. This mode of attack explains reports of the anomalous behaviour of commercial preparations of aminopeptidase M used as a tool in elucidating amino acid sequences. Some results have suggested the presence of one or more endopeptidases sensitive to Dip-F (Deftos & Potts, 1969). Plummer (1969) identified tyrosylproline among the products in his investigation, which strongly suggests the presence of dipeptidyl peptidase IV in the preparation. In defining the specificity of the Zn^{2+} -activated neutral endopeptidase of rabbit kidney (Kerr & Kenny, 1974a) with insulin B chain as substrate, the identification of lysylalanine after prolonged incubation is also consistent with the hydrolysis by traces of dipeptidyl peptidase IV of the Pro²⁸-Lys²⁹ bond.

The other unusual attributes of this enzyme are its molecular properties and location in the microvillus membrane. No other well-characterized serine proteinase is known to be an intrinsic membrane protein and none has a subunit mol.wt. as large as 130000, a characteristic probably related to its insertion into the membrane. Cathepsin A, a lysosomal serine peptidase, has been described as existing in two

forms with mol.wts. 500000 and 100000 (Kawamura *et al.*, 1975), but it is not clear whether the latter represents the subunit size. The dimeric form of dipeptidyl peptidase IV in the isolated state is well established, but it is unknown if this is the form of the enzyme in the microvillus membrane.

Dipeptidyl peptidase IV is one of a group of peptidases located in the microvillus membrane. Between them they cover a range of specificities capable of degrading oligopeptides but not proteins. Their physiological role is obscure: in particular the load of susceptible substrates in the tubular lumen seems unlikely to warrant this concentration of enzymes. Two other roles are conceivable: the microvillus membrane might be consumed during pinocytosis of proteins and peptides, and contribute to pinocytic vesicles an internal surface rich in hydrolytic activity, which could later reinforce the lysosomal proteinases. However, in so far as apical (pinocytic) vesicles have been isolated (Bode *et al.*, 1974), they have been reported to be devoid of microvillus enzymes. The other possibility is a role in transport of amino acids, perhaps peptides, across the membrane. An analogy in a related membrane is the postulated role of the sucrase-isomaltase complex in sugar transport across the intestinal microvilli. Here, a dimeric hydrolase, similar to dipeptidyl peptidase IV in its position in the membrane and molecular weight (Cogoli *et al.*, 1973; Mosimann *et al.*, 1973), purified and reconstituted into lipid vesicles, has been shown to mediate sugar transport (Storelli *et al.*, 1972). At present, the possible roles of the kidney microvillus hydrolases in hydrolysis, pinocytosis or transport have not been investigated.

We thank the Medical Research Council for a grant, and for a studentship to A. R. Y.

References

- Atfield, G. N. & Morris, C. J. O. R. (1961) *Biochem. J.* **81**, 606-614
- Barth, A., Schulz, H. & Neubert, K. (1974) *Acta Biol. Med. Germ.* **32**, 157-174
- Bode, F., Pockrandt-Hemstedt, H., Baumann, K. & Kinne, R. (1974) *J. Cell Biol.* **63**, 998-1008
- Booth, A. G. & Kenny, A. J. (1974) *Biochem. J.* **142**, 575-581
- Bury, A. F. & Pennington, R. J. (1975) *Biochem. J.* **145**, 413-416
- Chervenkova, C. H. (1969) *A Manual of Methods for the Analytical Ultracentrifuge*, pp. 1-61, Spinco Division of Beckman Instruments Inc., Palo Alto
- Cogoli, A., Eberle, A., Sigrist, H., Joss, C., Robinson, E., Mosimann, H. & Semenza, G. (1973) *Eur. J. Biochem.* **33**, 40-48
- Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404-427
- Deftos, L. J. & Potts, J. T., Jr. (1969) *Biochim. Biophys. Acta* **171**, 121-127

- de la Haba, G., Cammarata, P. S. & Timasheff, S. N. (1959) *J. Biol. Chem.* **234**, 316–319
- Dixon, M. & Webb, E. C. (1964) *Enzymes*, 2nd edn., pp. 84–87, Longmans, Green & Co. Ltd., London
- Enzyme Commission (1973) *Enzyme Nomenclature*, p. 237, Elsevier, Amsterdam
- Felgenhauer, K. & Glenner, G. G. (1966) *J. Histochem. Cytochem.* **14**, 401–413
- George, S. G. & Kenny, A. J. (1973) *Biochem. J.* **134**, 43–57
- Glossmann, H. & Neville, D. M. (1971) *J. Biol. Chem.* **246**, 6339–6346
- Hartley, B. S. (1970) *Biochem. J.* **119**, 805–822
- Hopsu-Havu, V. K. & Ekfors, T. O. (1969) *Histochemie* **17**, 30–38
- Hopsu-Havu, V. K. & Glenner, G. G. (1966) *Histochemie* **7**, 197–201
- Hopsu-Havu, V. K. & Sarimo, S. R. (1967) *Hoppe Seyler's Z. Physiol. Chem.* **348**, 1540–1550
- Hopsu-Havu, V. K., Rintola, P. & Glenner, G. G. (1968) *Acta Chem. Scand.* **22**, 299–308
- Kawamura, Y., Matoba, T., Hata, T. & Doi, E. (1975) *J. Biochem. (Tokyo)* **77**, 729–737
- Kerr, M. A. & Kenny, A. J. (1974a) *Biochem. J.* **137**, 477–488
- Kerr, M. A. & Kenny, A. J. (1974b) *Biochem. J.* **137**, 489–495
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- McDonald, J. K., Callahan, P. X., Ellis, S. & Smith, R. E. (1971) *Tissue Proteinases* (Barrett, A. J. & Dingle, J. T., eds.), pp. 69–107, North Holland Publishing Co., Amsterdam and London
- Mosimann, H., Semenza, G. & Sund, H. (1973) *Eur. J. Biochem.* **36**, 489–494
- Plummer, T. H., Jr. (1969) *J. Biol. Chem.* **244**, 5246–5253
- Spudich, J. A. & Watt, S. (1971) *J. Biol. Chem.* **246**, 4866–4871
- Storelli, C., Vögeli, H. & Semenza, G. (1972) *FEBS Lett.* **24**, 287–292
- Tiselius, A., Hjertén, S. & Levin, Ö. (1956) *Arch. Biochem. Biophys.* **65**, 132–155
- Tonomura, Y., Appel, P. & Morales, M. F. (1966) *Biochemistry* **5**, 515–521
- Ullmann, A., Goldberg, M. E., Perrin, D. & Monod, J. (1968) *Biochemistry* **7**, 261–265
- Walsh, K. A. & Wilcox, P. E. (1970) *Methods Enzymol.* **19**, 31–41
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412
- Yphantis, D. A. (1964) *Biochemistry* **3**, 297–317