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Proteins of the kidney microvillar membrane

Purification and properties of the phosphoramidon-insensitive endopeptidase ('endopeptidase-2') from rat kidney

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A second endopeptidase is present in the renal microvillar membrane of rats that can be distinguished from endopeptidase-24.11 by its insensitivity to inhibition by phosphoramidon. The purification of this enzyme, referred to as endopeptidase-2, is described. The enzyme was efficiently released from the membrane by treatment with papain. The subsequent four steps depended on ion-exchange and gel-filtration chromatography. These steps were monitored by the hydrolysis of various substrates: ^{125}I-insulin B chain (the normal assay substrate), benzoyl-L-tyrosyl-*p*-aminobenzoate (Bz-Tyr-pAB), azocasein and benzyloxycarbonyl-L-phenylalanyl-L-arginine 7-amino-4-methylcoumarylamide (Z-Phe-Arg-NMec). All four assays revealed comparable stepwise increases in activity in the main stages of the purification, although it was apparent that the last-named fluorogenic assay depended on traces of aminopeptidase activity present in the preparation. The K_m for ^{125}I-insulin B chain was 16 μM and that for Bz-Tyr-pAB was 4.7 mM. Several experimental approaches confirmed that both peptides were hydrolysed by the same enzyme. The pH optimum was 7.3. Phosphate buffers were inhibitory and shifted the optimum to above pH 9. Zinc was detected in the purified enzyme; EDTA and 1,10-phenanthroline were strongly inhibitory. SDS/polyacrylamide-gel electrophoresis revealed polypeptides of equal staining intensity of M_r 80000 and 74000 in reducing conditions. In non-reducing conditions a single band of apparent M_r 220000 was seen. Gel filtration yielded an M_r of 436000. These results are consistent with an oligomeric structure in which the α and β chains are linked by disulphide bridges. Endopeptidase-2 hydrolysed a number of neuropeptides. Enkephalins resisted attack, only the heptapeptide [Met]enkephalin-Arg^6-Phe^7 being susceptible to slow hydrolysis. Luliberin (luteinizing-hormone-releasing hormone) and bradykinin were rapidly hydrolysed. Neurotensin was shown to be slowly attacked at the Tyr^3–Glu^4 bond. Thus the specificity appears to be limited to the hydrolysis of bonds involving the carboxy group of aromatic residues, provided that this P_1 residue is extended by additional residues, at least to the P_3' position. The relationship of this membrane metalloendopeptidase to mouse meprin and human 'PABA peptidase' is discussed.

INTRODUCTION

The brush border of the proximal tubule of the kidney is extraordinarily rich in peptidases. In rabbit, pig and human there is a single endopeptidase (endopeptidase-24.11, EC 3.4.24.11) complemented by a group of exopeptidases (for review see Kenny, 1986). Endopeptidase-24.11 is characteristically inhibited by phosphoramidon (Kenny, 1977), and in an immunoradiometric survey of pig tissues with a monoclonal antibody to endopeptidase-24.11 there was a good correlation between immunoreactivity and phosphoramidon-sensitive endopeptidase activity (Gee *et al.*, 1985). The hydrolysis of ^{125}I-insulin B chain by microvilli prepared from pig kidneys is wholly inhibited by phosphoramidon. In contrast, the activity in rat kidney microvilli is only partially sensitive to phosphoramidon (Kenny *et al.*, 1981). This residual activity (about 50% of the total) could be resolved from endopeptidase-24.11 by ion-exchange chromatography. The second endopeptidase peak ('endopeptidase-2') contained activity hydrolysing both insulin B chain and azocasein (Kenny *et al.*, 1981). At about the same time, Beynon *et al.* (1981) reported that mouse kidney contained a membrane enzyme that hydrolysed azocasein. These authors purified it after solubilization with toluene/trypsin treatment, and noted that it was inhibited by metal-ion-chelating agents but insensitive to phosphoramidon. In addition to several protein substrates, the hydrolysis of Z-Phe-Arg-NMec was also noted. This enzyme was later shown to be associated with the renal brush border, where it coexisted with endopeptidase-24.11 (Mulligan *et al.*, 1982), and was named 'meprin' (Bond *et al.*, 1983). Some strains of mice are known to be deficient in the expression of this enzyme (Beynon & Bond, 1983; Bond & Beynon 1986) and the *Mep*-1 gene controlling this enzyme in mice has been shown to be linked to the major histocompatibility complex (Bond *et al.*, 1984).

Another brush-border endopeptidase, this one in human intestine, has also been reported (Sterchi *et al.*, 1982, 1983). It has been assayed with benzoyl-L-tyrosyl-*p*-aminobenzoate (Bz-Tyr-pAB) as substrate and has come to be referred to 'PABA-peptide hydrolase'. It has not so far been identified in renal microvilli.

Abbreviations used: Bz-Tyr-pAB, *N*-benzoyl-L-tyrosyl-*p*-aminobenzoic acid; Z-Phe-Arg-NMec, benzyloxycarbonyl-L-phenylalanyl-L-arginine 7-amino-4-methylcoumarylamide; LHRH, luteinizing-hormone-releasing hormone (luliberin).

In the present paper we report the purification and properties of endopeptidase-2. It is a metallopeptidase, containing zinc, with the ability to hydrolyse a variety of assay substrates, including ^{125}I-insulin B chain, azocasein, Bz-Tyr-pAB (PABA-peptide) and Z-Phe-Arg-NMec. It also hydrolysed several neuropeptides, including LHRH, bradykinin, substance P, neurotensin and the angiotensins.

EXPERIMENTAL

Materials

Substrates and peptides were purchased as follows. Azocasein and Bz-Tyr-pAB were from Sigma Chemical Co., Z-Phe-Arg-NMec was from Bachem, and substance P was from Cambridge Research Biochemicals. Luliberin, neurotensin, bradykinin, angiotensins I and II and the enkephalins were from Sigma Chemical Co.

Papain (type III) was from Sigma Chemical Co., and peptide *N*-glycanase (glycopeptide *N*-glucosidase, EC 3.2.2.18) was from Genzyme Corp.

Microvillar membranes from rat kidneys

These were prepared as previously (Booth & Kenny, 1974).

Purification of endopeptidase-2

Batches (80–200 g) of rat kidneys, previously stored at −70 °C, were thawed. The preparation of the microsomal fraction (from whole kidneys) was similar to that described previously (Kerr & Kenny, 1974) except that the sucrose medium used for homogenization contained 10 mM-Tris/HCl buffer, pH 7.5. The membrane pellet was resuspended in 10 mM-Pipes/NaOH buffer, pH 6.8, and treated with papain as described previously (Hedeager-Sorensen & Kenny, 1985). The supernatant after this treatment was then chromatographed as described in the Results section.

Enzyme assays

Endopeptidase-2 was routinely assayed with ^{125}I-insulin B chain with the use of the same protocol for endopeptidase-24.11 (Kenny, 1977; Fulcher & Kenny, 1983) except that the incubation mixture contained 1 μM-phosphoramidon. The hydrolysis of Bz-Tyr-pAB was determined fluorimetrically by a method designed by Dr. R. W. Lobley (personal communication). The incubation mixture (volume 300 μl) contained, in addition to enzyme sample, 3.3 mM-Bz-Tyr-pAB and 0.1 M-triethanolamine/HCl buffer, pH 7.1. After incubation at 37 °C for 1 h, the reaction was terminated by the addition of 2 ml of dimethyl sulphoxide (AnalaR). Fluorescence was determined at 303 nm excitation and 350 nm emission. Standards contained 33.3 μM-*p*-aminobenzoic acid in the incubation buffer. The calibration curve was linear over the range 0–80 μM. When different substrate concentrations were used, as for the determination of K_m, a quench correction was necessary.

The determination of hydrolysis of azocasein was that described by Beynon & Kay (1978) and Beynon *et al.* (1981). The assay with Z-Phe-Arg-NMec was performed in the same way as the exopeptidase assays with NMec derivatives (Fulcher & Kenny, 1983), except that the substrate concentration was 5 μM.

Units of activity

These are expressed as μmol of substrate hydrolysed/min (hence 1 munit = 1 nmol/min), except for the azocasein assay, where activity is in arbitrary units (Beynon *et al.*, 1981).

Precipitation by antiserum raised to rat kidney microvillar membranes

Endopeptidase-2 (11 μg in 10 μl) was incubated for 18 h at 4 °C with increasing amounts of an IgG fraction of an antiserum raised to rat kidney microvillar membranes. After centrifugation of the immunoprecipitates, the supernatant was assayed with ^{125}I-insulin B chain and Bz-Tyr-pAB as substrates.

Hydrolysis of neuropeptides

These were determined by h.p.l.c. analyses, with a μBondapak C_{18} column eluted by a gradient of acetonitrile as previously described (Matsas *et al.*, 1983).

M_r determinations

A Superose 12 column, controlled by a fast-protein-liquid-chromatography system (FPLC; Pharmacia), was used. The buffer was 20 mM-Tris/HCl buffer, pH 7.9, containing 100 mM-NaCl. The M_r markers were thyroglobulin (669000), ferritin (440000), catalase (232000), fructose-bisphosphate aldolase (158000) and α-chymotrypsinogen A (25000). V_0 was determined by Dextran Blue. The column was pumped at 0.5 ml/min.

Zinc determination

This was performed with an atomic absorption spectrophotometer (Perkin–Elmer 303) essentially as described previously (Fulcher & Kenny, 1983). All solutions were prepared in Milli Q (Millipore) water; acid-washed glassware was used; enzyme samples were exhaustively dialysed against 5 mM-Tris/HCl buffer, pH 7.5, before analysis.

Incubation with *N*-glycanase

Three attempts to deglycosylate the purified enzyme were made by incubation with *N*-glycanase at 37 °C for 3–24 h. The incubation mixture (volume 25 μl) contained 1 μg of endopeptidase-2, 0.1 unit of *N*-glycanase (glycopeptide *N*-glycosidase), 0.25 M-Na_2HPO_4/NaH_2PO_4 buffer, pH 8.6, and 12 mM-1,10-phenanthroline. In one experiment, the *N*-glycanase was preincubated for 1 h with 0.1 mM-di-isopropyl phosphorofluoridate.

Other methods

These were those described previously (Fulcher & Kenny, 1983). Protein in fractions from columns was determined by the method of Bradford (1976) with the reagents supplied by Bio-Rad Laboratories.

RESULTS

Phosphoramidon inhibition of endopeptidase activity in rat kidney microvillar membranes

As reported previously, 50% of the activity of these membranes in hydrolysing ^{125}I-insulin B chain could be titrated by phosphoramidon (Fig. 1). This component is attributable to endopeptidase-24.11, and the residual activity is, by definition, endopeptidase-2. The value of a general endopeptidase substrate such as insulin B chain

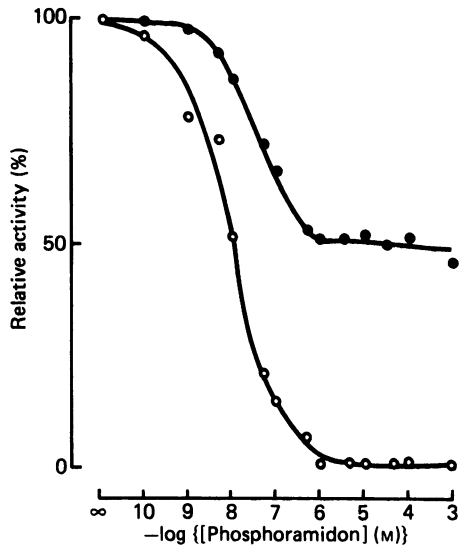

Fig. 1. Titration of peptidase activities in rat kidney microvillar membranes with phosphoramidon

The hydrolysis of ^{125}I-insulin B chain (●) and [D-Ala^2,Leu^5]enkephalin (○) is expressed relative to the uninhibited activities.

Table 1. Endopeptidases in rat kidney microvillar fraction

See the Experimental section for details. ^{125}I-insulin B chain was the substrate in these assays. Values are means for three experiments.

Enzyme	Specific activity (munits/mg of protein)	Enrichment (microvilli/homogenate)
Endopeptidase-2	4.85	11.3
Endopeptidase-24.11	4.57	12.4

in revealing new activities is also shown in Fig. 1 in the titration curve for another peptide substrate [D-Ala^2-Leu^5]enkephalin, the hydrolysis of which is wholly sensitive to phosphoramidon, since this pentapeptide is not a substrate for endopeptidase-2.

Endopeptidase-2 and endopeptidase-24.11 in microvillar-membrane fractions

The specific activities and the enrichment values of the two endopeptidases, assayed with ^{125}I-insulin B chain, were similar in microvillar-membrane fractions prepared from whole rat kidneys (Table 1).

Purification of endopeptidase-2 from rat kidneys

The standard assay depended on the hydrolysis of ^{125}I-insulin B chain, which was also the usual assay substrate for endopeptidase-24.11. The latter enzyme is wholly inhibited by 1 μM-phosphoramidon, and hence endopeptidase-2 was defined as the residual activity observed in the presence of this inhibitor. One purification is shown in Table 2. Unlike endopeptidase-24.11, endopeptidase-2 is readily released from the membrane by treatment with papain, in this case with a yield of 56%. The four subsequent steps, depending on

Table 2. Purification of endopeptidase-2 from rat kidneys

See the Experimental section for details.

Fraction	Protein (mg)	Substrate... ^{125}I-insulin B chain				Bz-Tyr-pBA			Azocasein	Z-Phe-Arg-NMec
		Total activity (munits)	Yield (%)	Sp. activity (munits/mg)	Enrichment	Total activity (munits)	Yield (%)	Sp. activity (munits/mg)	Enrichment	Enrichment
Homogenate	35100	20800	100	0.59	1	52400	100	1.5	1	1
Microsomal fraction	6000	14100	68	2.4	4	120000	230	20	1.7	0.5
Papain supernatant	145	7830	38	5.4	9	59500	110	41	6.9	2.2
DEAE-cellulose fraction	212	4340	21	21	35	47000	90	222	51	11
Ultrogel AcA 22 fraction	104	3690	18	36	60	39900	76	383	40	14
Hydroxyapatite fraction	42	2350	11	56	94	28600	55	680	24	38
Mono Q fraction	5.5	475	2.3	87	146	5200	9.9	949	82	35

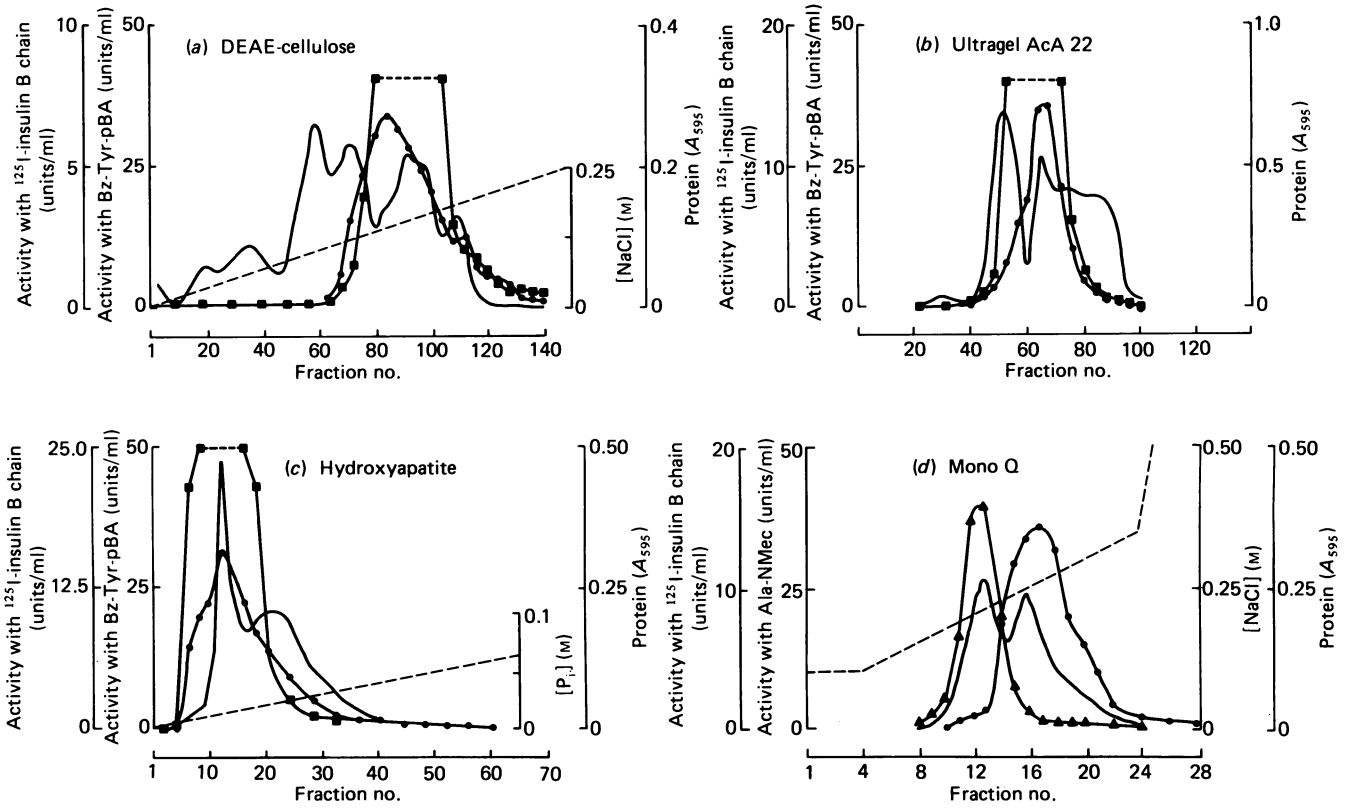

Fig. 2. Column-chromatographic steps in the purification of endopeptidase-2 from rat kidney

See the Experimental section for details. (*a*) DEAE-cellulose (DE52; Whatman). Column volume 50 ml; gradient, 1500 ml total, 0–240 mM-NaCl in 5 mM-Tris/HCl buffer, pH 7.9, at 4 °C; 10 ml fractions. (*b*) Ultrogel AcA 22. Column volume, 490 ml; buffer, 100 mM-NaCl/10 mM-Tris/HCl buffer, pH 7.9, at 4 °C; 5 ml fractions. (*c*) Hydroxyapatite (Ultrogel HA; LKB). Column volume, 25 ml; gradient, 200 ml total, 1–100 mM-Na_2HPO_4/NaH_2PO_4 buffer, pH 6.8–7.2; 5 ml fractions. (*d*) Mono Q (Pharmacia FPLC) anion-exchange chromatography. Column volume, 1 ml; gradient, volume 20 ml, 100–350 mM-NaCl in 20 mM-Tris/HCl buffer, pH 7.9; 1 ml fractions. ——, A_{595} (protein); – – – –, gradient; ●, activity with ^{125}I-insulin B chain; ■, activity with Bz-Tyr-pBA; ▲, activity with Ala-NMec.

ion-exchange chromatography and gel filtration, are shown in Figs. 2(*a*)–2(*d*). In the first three steps, DEAE-cellulose, Ultrogel AcA 22 and hydroxyapatite, the column fractions were also assayed with Bz-Tyr-pAB. The two activities coincided in each step. The fourth step was chromatography on a Mono Q column with a fast-protein-liquid-chromatography system. At each step, the pooled fractions were re-assayed with insulin B chain, Bz-Tyr-pAB, azocasein and Z-Phe-Arg-NMec as substrates. The enrichment values from start to finish for the four substrates showed wide variation, in the range 35–635-fold (Table 2). This may be partly due to the presence of other peptidase activities (capable of hydrolysing some but not all substrates) and/or other substances affecting the assays in the early stages. If the enrichments are recalculated from the papain solubilization step they show much greater agreement: 16-fold (insulin B chain), 23-fold (Bz-Tyr-pAB), 12-fold (azocasein) and 16-fold (Z-Phe-Arg-NMec). This would support the view that all four substrates are hydrolysed by the same enzyme.

The purification illustrated in Table 2 was selected because it contained information from four different assays. In all, eight batches of endopeptidase-2 have been purified, all after solubilization by papain. The protocols varied slightly with respect to the order in which the steps were placed. The mean specific activity (^{125}I-insulin B chain assay) for the preparations not subjected to chromatography on a column of Mono Q was 44±7 munits/mg of protein (mean±S.E.M.) and the mean enrichment was 96-fold. The two preparations in which the final column was a Mono Q gave a mean specific activity of 77.5 munits/mg of protein and an enrichment of 195-fold, indicating the need for a final step with high resolution.

The release of the endopeptidase in a hydrophilic form by papain was important to the success of the purification. In two attempts to isolate a detergent-solubilized form by the same protocol, a specific activity of 4 munits/mg of protein and an enrichment of 11-fold were all that could be achieved. Papain was remarkably efficient: over all the preparations, it released 73% of the membrane activity.

The pooled fractions from the Mono Q step were not wholly free of aminopeptidase N activity (the specific activity with Ala-NMec as substrate was 0.11 unit/mg of protein). On the assumption that rat aminopeptidase N has a similar activity to that purified from pig kidneys (19.4 units/mg), this would indicate that 0.6% of the protein was aminopeptidase N. The SDS/polyacrylamide-gel electrophoretogram (Fig. 3) shows the pattern of bands during the steps of purification. The

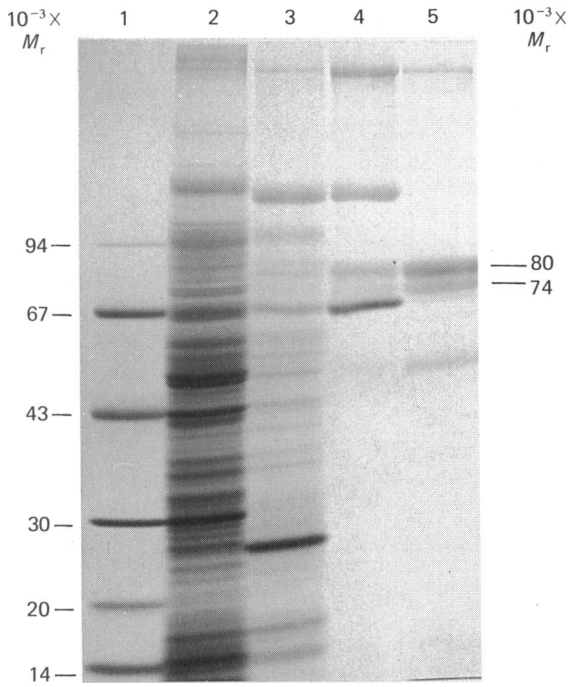

Fig. 3. SDS/polyacrylamide-gel electrophoresis of endopeptidase-2 during purification

See the text and Table 2 for details. A gradient gel of 7–17% polyacrylamide was used. Track 1, marker proteins ($M_r \times 10^{-3}$); track 2, microsomal pellet; track 3, papain supernatant; track 4, pooled fractions from hydroxyapatite; track 5, pooled fractions from Mono Q. Apparent subunit M_r values ($\times 10^{-3}$) are indicated.

Mono Q step yielded a preparation showing two heavily staining bands, polypeptides of M_r 80000 and 74000 (track 5). Some aggregated protein is also visible, and some faint bands of M_r 120000 and 50000, typical of papain-released aminopeptidase N. The preparation was further purified by passage through an immunoaffinity column containing an IgG fraction raised to rat kidney microvillar proteins. This preparation now contained only 0.006 unit of aminopeptidase N/mg of protein. Other peptidases were also depleted, the residual activities being (unit/mg of protein): aminopeptidase A, 0.10; dipeptidyl peptidase IV, 0.023; γ-glutamyl transferase, 0.005. No endopeptidase-24.11 activity was detected. The 80000-M_r and 74000-M_r bands have been observed in all the purified preparations, usually of about equal staining intensity, but sometimes the larger was slightly dominant, as in this case. These bands are discernible in the papain supernatant fraction (track 3) and are enriched as the purification proceeded.

On present evidence it is probable that the 80000-M_r and 74000-M_r bands are both attributable to endopeptidase-2, but it remains to be established whether they represent dissimilar subunits or whether they are generated by limited proteolysis in the course of the papain treatment. An experiment in which the purified enzyme was incubated with papain is shown in Fig. 4. Both bands were slowly degraded over the 6 h incubation. There was no evidence that the 80000-M_r band was converted into the 74000-M_r polypeptide, an observation supporting the existence of dissimilar subunits. Incubation in these conditions, with or without papain, caused some aggregation, seen as a band of very high M_r at the top of the gel (arrow).

Enzyme kinetics

K_m values from Lineweaver–Burk plots were obtained in experiments in which insulin B chain was assayed over the range 1–20 μM). For ^{125}I-insulin B chain, K_m was 16.4 μM and $V_{max.}$ 0.25 μmol/min per mg (mean for two experiments). For Bz-Tyr-pAB, K_m was 4.7 mM and $V_{max.}$ 3.2 μmol/min per mg. Values for $k_{cat.}/K_m$, calculated assuming a subunit of M_r 80000, were 1.2 for insulin B chain and 54 for Bz-Tyr-pAB, but it should be borne in mind that an undetermined number of peptide bonds of insulin B chain are hydrolysed, but only a single bond in the synthetic substrate. Bz-Tyr-pAB was a competitive inhibitor in the insulin B chain assay, giving a K_i of 9.2 mM.

Further evidence that the hydrolysis of ^{125}I-insulin B chain and Bz-Tyr-pAB is by the same peptidase

The enzyme activity was lost rapidly on incubation at 60 °C. The inactivation revealed by both assays approximated to first-order decay curves, the $t_{\frac{1}{2}}$ values being 3.2 min for the hydrolysis of Bz-Tyr-pAB and 3.7 min for ^{125}I-insulin B chain.

An antiserum raised in a rabbit to rat microvillar membranes was also employed to precipitate the enzyme. The titration curves for the two activities were superimposable (Fig. 5).

Influence of pH and buffer ions

The pH–activity curve for the hydrolysis of ^{125}I-insulin B chain is shown in Fig. 6. The optimum pH with a variety of buffers was 7.3. Phosphate buffers were strongly inhibitory, especially so at pH values below 8, and no maximum could be determined over the range pH 5–10. At pH 7, in 10 mM-Tris/HCl, the addition of NaCl stimulated activity slightly, an increase of 15%, plateauing at 50–200 mM.

Effect of chelators and inhibitors

Endopeptidase-2 was sensitive to EDTA and 1,10-phenanthroline. The enzyme was preincubated for 30 min at 37 °C with the inhibitors over the range 1 μM–1 mM before assay. EDTA was the more effective: with insulin B chain as substrate IC_{50} was 25 μM, and with Bz-Tyr-pAB IC_{50} was 13 μM. For 1,10-phenanthroline the IC_{50} values were 180 μM and 130 μM. Inhibitors of aminopeptidases, namely 1 mM-amastatin and 100 μM-bestatin, were tested. No effect on the hydrolysis of ^{125}I-insulin B chain was observed alone or in combination. The hydrolysis of Z-Phe-Arg-NMec was inhibited 30% by amastatin but unaffected by bestatin. The hydrolysis of Arg-NMec by traces of aminopeptidase N in our preparation was inhibited 61% by amastatin and 90% by bestatin.

An inhibitor of endopeptidase-24.15, *N*-[1-(*RS*)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-*p*-aminobenzoate (kindly given by Dr. Marian Orlowski) was also tested over a concentration range 1–100 μM. No inhibition of activity in either the ^{125}I-insulin B chain or the Bz-Tyr-pAB assay was observed.

Specificity of endopeptidase-2

The hydrolysis of Bz-Tyr-pAB to release *p*-aminobenzoic acid indicated an endopeptidase attack on this

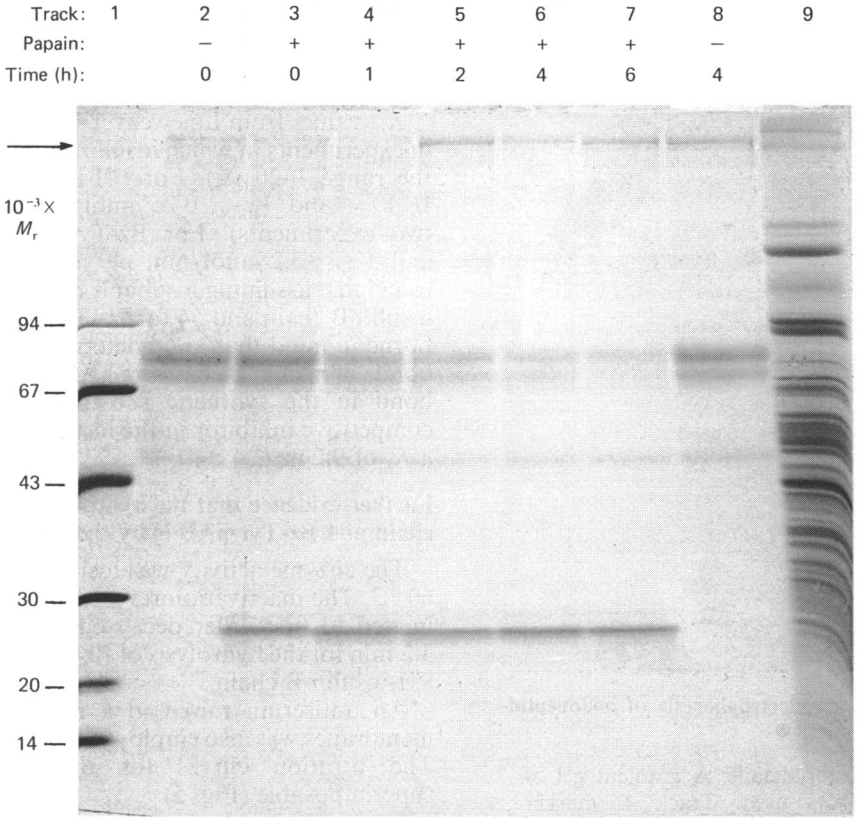

Fig. 4. Effect of papain on endopeptidase-2

Endopeptidase-2 (25 μg) was incubated with 2.5 μg of papain (activated with 2-mercaptoethanol) for various times (0–6 h). The reaction was stopped by the addition of E-64 (L-*trans*-3-carboxy-2,3-epoxypropionyl-leucylamido-(4-guanidino)butane] in a 10-fold molar excess over papain and then subjected to SDS/polyacrylamide-gel electrophoresis after boiling with dissociation buffer. Track 1, marker proteins ($M_r \times 10^{-3}$); track 9, pig kidney microvilli; other tracks, as indicated. The band at M_r 26000 is papain. The arrow indicates the aggregated form generated on incubation, with or without papain.

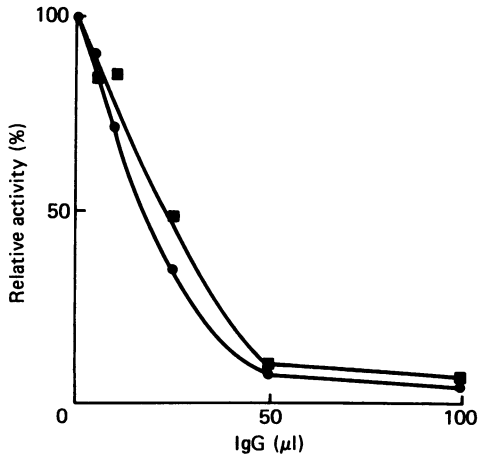

Fig. 5. Precipitation of activities hydrolysing ^{125}I-insulin B chain and Bz-Tyr-pAB from rat kidney microvillar membranes

See the Experimental section for details. After incubation for 18 h at 4 °C, the tubes were centrifuged and the supernatant fractions assayed with each substrate. The activities are plotted relative to the control without addition of IgG (results of two experiments). ■, Activity with ^{125}I-insulin B chain; ●, activity with Bz-Tyr-pAB.

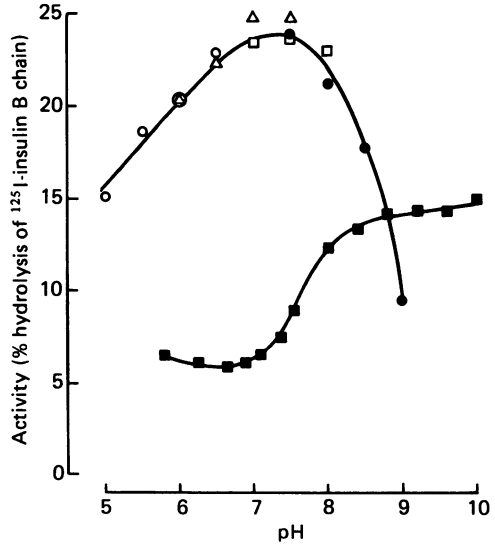

Fig. 6. pH-activity curves for endopeptidase-2

The substrate was ^{125}I-insulin B chain; the incubation mixture contained 100 mM-buffer and 100 mM-NaCl. ○, Mes/NaOH; △, Pipes/NaOH; □, Hepes/NaOH; ●, Tris/HCl; ■, NaH_2PO_4/Na_2HPO_4.

Table 3. Hydrolysis of neuropeptides

Endopeptidase-2 (25 ng) was incubated with 0.5 mM-peptide for 30–120 min at 37 °C. Hydrolysis was assessed by diminution of the area of the substrates peak determined by h.p.l.c. analysis, except for neurotensin, where the products were identified and quantified.

Peptide	Time (h)	Hydrolysis (%/h)
[Leu^5]enkephalin	2	0
[Met^5]enkephalin	0.5	0
[D-Ala^2,Leu^5]enkephalin	2	0
[D-Ala^2,Leu^5]enkephalinamide	1	0
[Leu^5]enkephalinamide	2	0
[Leu^5]enkephalin-Arg^6	2	0
[Met^5]enkephalin-Arg^6-Phe^7	2	3.5
Bradykinin	0.5	16
LHRH (luliberin)	1	30
Substance P	1	19
Angiotensin I	6	4
Angiotensin II	6	1.2
Neurotensin	6	0.6

substrate. The ability of the enzyme to hydrolyse a number of neuropeptides was studied. First, a series of enkephalins and their analogues was examined. The lack of any phosphoramidon-insensitive hydrolysis of [D-Ala^2-Leu^5]enkephalin by microvilli (Fig. 1) had indicated that the enzyme was unlikely to attack these pentapeptides. This was confirmed (Table 3) for both [Leu]enkephalin and [Met]enkephalin as well as the [D-Ala^2] analogue. Further, the amidated forms of [Leu]enkephalin and [D-Ala^2,Leu]enkephalin also resisted attack. So, too, did [Leu]enkephalin-Arg^6. But, when the heptapeptide [Met]enkephalin-Arg^6-Phe^7 was tested, slow hydrolysis was detected. A number of other neuropeptides were also susceptible to hydrolysis: these included the angiotensins, neurotensin, substance P, bradykinin and LHRH, with the last three named being hydrolysed fairly rapidly. Since LHRH is a relatively poor substrate for endopeptidase-24.11, the rates of hydrolysis of three peptides by the two endopeptidases were compared (Fig. 7). For endopeptidase-24.11, the preferred order was substance P, [Met]enkephalin-Arg^6-Phe^7, then LHRH, with the last-named being attacked extremely slowly. For endopeptidase-2, LHRH and substance P were hydrolysed at similar rates and the extended enkephalin was only slowly attacked.

The bond-specificity was established for neurotensin. This substrate was hydrolysed at only one peptide bond in these conditions, giving rise to two products (Fig. 8, peaks 1 and 2). The bond hydrolysed was identified by amino acid analyses as Tyr^3–Glu^4. [Met]enkephalin-Arg-Phe was also hydrolysed to give two products, but there was insufficient material in the collected fractions to quantify the amino acid composition. Peak 2 contained tyrosine, glycine and phenylalanine, consistent with hydrolysis at Phe^4–Met^5. These experiments, together

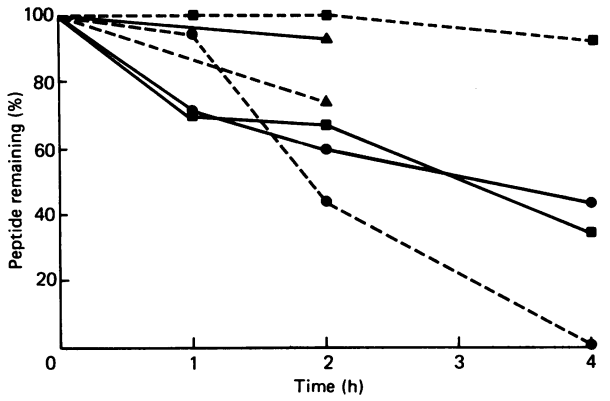

Fig. 7. Rates of hydrolysis of substance P and LHRH by rat endopeptidase-2 and pig endopeptidase-24.11

Equal amounts (25 ng) of each enzyme were incubated with 0.5 mM-peptide for periods up to 4 h in the presence of 10 μM-amastatin (total volume 100 μl). The incubation was stopped by heating at 100 °C for 4 min, and the extent of hydrolysis was assessed by disappearance of the substrate peak after h.p.l.c. analysis. ——, Endopeptidase-2; – – – –, endopeptidase-24.11. ■, LHRH; ●, substance P; ▲, [Met]enkephalin-Arg^6-Phe^7.

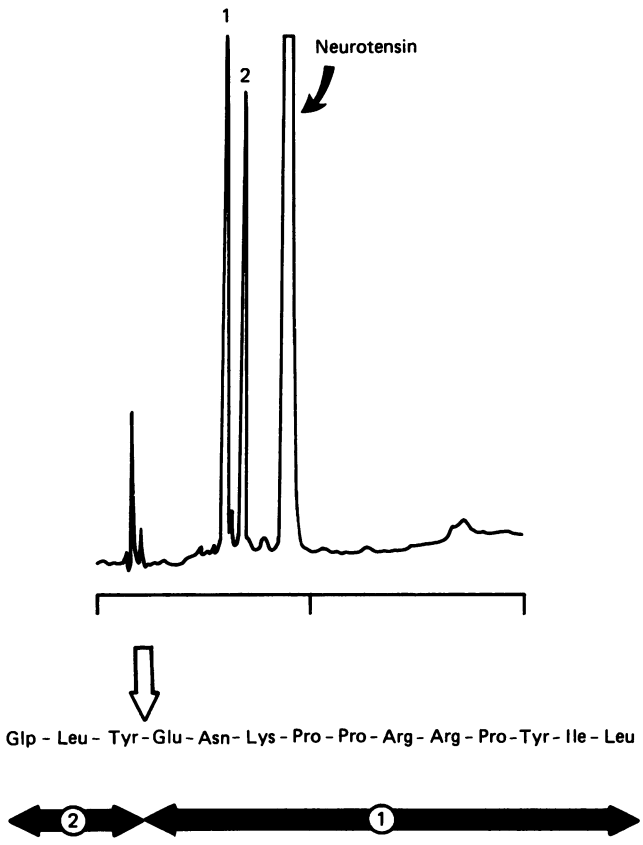

Fig. 8. Mode of hydrolysis of neurotensin

Peptide (0.5 mM) was incubated with 100 ng of endopeptidase-2 in 0.1 M-Tris/HCl buffer, pH 7, for 6 h. The reaction mixture contained phosphoramidon, captopril and amastatin (all 5 μM) and 0.1 mM-di-isopropyl phosphorofluoridate. The reaction was terminated by heating at 100 °C for 4 min, and, after removal of any precipitate, the products were analysed by h.p.l.c. The product peaks were subjected to hydrolysis and amino acid analysis (values relative to Leu). Peak 1: Leu, 1.00; Asp, 1.14; Glu, 1.04; Ile, 0.97; Tyr, 1.11; Lys, 1.09; Pro, 2.32; (Arg, not resolved from NH_3 peak). Peak 2: Leu, 1.00; Glu, 1.20 Tyr, 1.04.

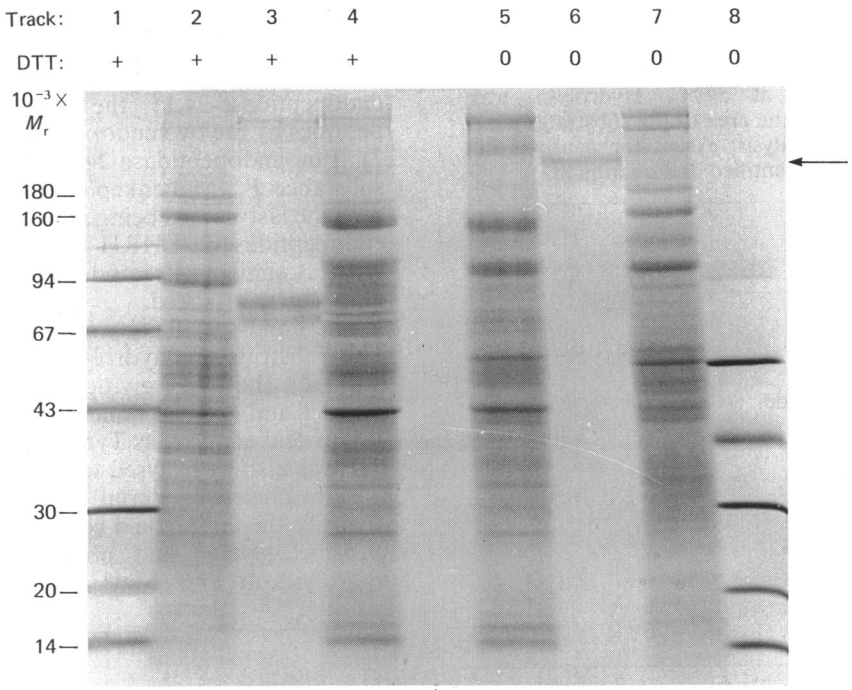

Fig. 9. SDS/polyacrylamide-gel electrophoresis of endopeptidase-2 in the presence and in the absence of dithiothreitol

See the Experimental section for details. A gradient gel of 7–17% polyacrylamide was used. Tracks 1–4 contain samples treated with 80 mM-dithiothreitol (DTT) in the dissociation buffer. Tracks 5–8 contained non-reduced samples. Tracks 1 and 8, markers; tracks 2 and 7, pig kidney microvilli; tracks 3 and 6, purified endopeptidase-2; tracks 4 and 5, rat kidney microvilli. Apparent subunit M_r values ($\times 10^{-3}$), 180 and 160, are assessed from bands in track 2 of pig kidney microvilli. The arrow indicates non-reduced endopeptidase-2.

with the hydrolysis of Bz-Tyr-pAB, show that endopeptidase-2 hydrolysed peptide bonds in which tyrosine and phenylalanine contribute the carbonyl group.

Molecular properties of endopeptidase-2

Oligomeric structure. The M_r of the native enzyme was investigated by gel filtration with the use of a Superose 12 column and a Pharmacia FPLC control system. The standards were Blue Dextran, thyroglobulin, ferritin, catalase, aldolase and chymotrypsinogen A. Two calibrations were made (r values 0.957 and 0.989); in the second run the buffer contained 0.1% Emulphogen. The addition of detergent did not significantly affect the elution volumes of the standards. The M_r of endopeptidase-2 was calculated to be 403000 and 470000 in the two experiments (mean 436000).

When fully reduced by dithiothreitol, the apparent M_r values in SDS/polyacrylamide-gel electrophoresis for the two polypeptide bands were consistently 80000 and 74000 (Fig. 3, track 5; Fig. 9, track 3). However, in non-reduced samples the apparent M_r value, now as a single band, was 220000 (Fig. 9, track 6), indicating that the dissimilar subunits are cross-linked by disulphide bridges.

Treatment with peptide *N*-glycanase. Under conditions in which other microvillar enzymes, such as endopeptidase-24.11, were apparently successfully deglycosylated by peptide *N*-glycanase, endopeptidase-2 suffered progressive proteolysis, which was not prevented by 12 mM-1,10-phenanthroline or, in one experiment, by also preincubating the glycanase with 0.1 mM-di-isopropyl phosphorofluoridate. Even over shorter periods of treatment (3–6 h) no subunits of lower M_r could be detected on SDS/polyacrylamide-gel electrophoresis. Thus evidence of glycosylation is lacking in these experiments.

Zinc analyses. Two preparations of purified endopeptidase were analysed for zinc by atomic absorption spectrophotometry. Assuming a subunit of M_r 77000, values of 0.49 and 0.26 mol of Zn atoms/mol were obtained.

DISCUSSION

Endopeptidase-2 is a brush-border endopeptidase

Our operational definition of endopeptidase-2 is the activity in microvillar membranes hydrolysing ^{125}I-insulin B chain that is insensitive to 1 μM-phosphoramidon. This criterion distinguishes it from endopeptidase-24.11. Both endopeptidases are located in the renal brush border of the rat, being enriched to comparable degrees, but only endopeptidase-2 is released from the membrane by treatment with papain. This single step therefore serves to separate the two activities and avoids some of the difficulties associated with resolving them after detergent solubilization. It was used as the starting point in our purification. The activity was enriched about 200-fold in the most highly purified preparations. Comparison of the specific activity of the pure enzyme with that of a microvillar-membrane fraction indicates that it constitutes about 6% of the microvillar-membrane protein, and

is therefore a major constituent of the membrane. One may compare this with similarly calculated estimates for other peptidases in pig kidney microvilli: endopeptidase-24.11 and dipeptidyl peptidase IV are about 4%, aminopeptidase N about 8% and aminopeptidase A and W and carboxypeptidase P about 1% of the membrane.

The discovery of 'PABA-peptide hydrolase' in human intestinal brush border (Sterchi *et al.*, 1982, 1983) led us to check whether our partially purified enzyme hydrolysed Bz-Tyr-pAB. It did so with great efficiency and, following this finding, a number of purifications were also monitored by assays with this substrate. The discovery of meprin in mouse kidneys (Beynon *et al.*, 1981) led us to do the same with the two substrates these authors had employed, azocasein and Z-Phe-Arg-NMec. All four activities were enriched by our purification procedure, but overall the enrichment values showed a rather wide variation, from 36- to 635-fold. The total activity assayed with Bz-Tyr-pAB more than doubled between the homogenate value and that of the papain supernatant, suggesting that the activity was grossly underestimated in the starting material. When the enrichments were recalculated with the use of the papain supernatant fraction as the reference point, the wide discrepancies in the four assays largely disappeared, the range of values now being from 12- to 23-fold. The Bz-Tyr-pAB-hydrolysing activity was further observed to coincide with the peaks of endopeptidase-2 in the first three chromatographic steps. Both activities were similarly affected by EDTA, 1,10-phenanthroline, heat treatment and immunoprecipitation. In addition, the synthetic peptide was a competitive inhibitor of insulin B chain hydrolysis. For these reasons we can be confident that insulin B chain and Bz-Tyr-pAB were hydrolysed by a single peptidase. We have not examined the remaining substrates in the same detail, but believe that a similar conclusion may tentatively be reached. However, Z-Phe-Arg-NMec does pose a mechanistic question, since by analogy with Bz-Tyr-pAB one would expect hydrolysis to occur at the Phe–Arg bond and the release of 7-amino-4-methylcoumarin would require the intervention of an aminopeptidase. Traces of aminopeptidase N were present in our preparation and the fluorimetric assay with Z-Phe-Arg-NMec was partially inhibited by amastatin.

Specificity

Although Bz-Tyr-pAB was hydrolysed by endopeptidase-2, it was a very poor substrate (K_m 4.7 mM) compared with insulin B chain (K_m 16 μM). this suggests that chain length is important in binding to the enzyme, and indeed such subsite interactions are a feature of many peptidases [e.g. endopeptidase-24.11 (Orlowski & Wilk, 1981; Almenoff & Orlowski, 1983; Matsas *et al.*, 1984)]. The enkephalin peptides also illuminate this feature. Neither the pentapeptides nor the *C*-terminally amidated enkephalins nor [Leu]enkephalin-Arg^6 were substrates. Only the heptapeptide [Met]enkephalin-Arg^6-Phe^7 was hydrolysed, and this at a very low rate, probably at the Phe^4–Met^5 bond, suggesting that subsite interactions of P_1', P_2' and P_3' are important. In the case of neurotensin, hydrolysis occurred at Tyr^3–Glu^4 but not at Tyr^{11}–Ile^{12}, again indicating a requirement for the S_3' site to be occupied. This may explain why the 'PABA peptide', Bz-Tyr-pAB, has such a low affinity (K_m 4.7 mM). It is clear that endopeptidase-2 hydrolyses bonds involving the carboxy groups of two aromatic residues, but we cannot at present affirm that the specificity is strictly limited in this way and that bonds involving other hydrophobic residues are necessarily resistant to attack. Meprin has a similar specificity; it has been reported to hydrolyse the Tyr^4–Ile^5 bond of angiotensin II (Bond *et al.*, 1986), though in bradykinin the Gly^4–Phe^5 bond was attacked (Butler *et al.*, 1987).

Molecular properties

Among other microvillar peptidases, endopeptidase-2 is unusual in its oligomeric structure. There appear to be two subunits, of M_r 80000 and 74000, linked by disulphide bonds to form (most probably) a tetramer $(\alpha-\beta)_2$ in undenaturing conditions. This interpretation is based on the non-reduced polypeptide on SDS/polyacrylamide-gel electrophoresis giving a single band of M_r 222000 and the gel-filtration experiments an undenatured M_r of 436000. The apparent M_r values obtained in the presence of SDS would also be compatible with other oligomeric structures, e.g. $(\alpha_2-\beta)_2$ or $(\alpha_2-\beta_2)_2$, if the non-reduced form migrated anomalously faster, by virtue of its more compact structure. The latter model has the attraction of being comparable with that reported for meprin. None of the peptidases, including endopeptidase-24.11, in pig and rabbit microvilli possess covalently linked subunits (for reviews see Kenny & Maroux, 1982; Semenza, 1986). However, in mouse kidneys meprin exhibits similar molecular properties: M_r 85000 on SDS/polyacrylamide-gel electrophoresis in reducing conditions but remaining tetrameric in non-reducing conditions (Bond *et al.*, 1986; Butler *et al.*, 1987). The Zn analysis yielded a value of less than 1 atom/subunit, consistent with either 1 or 2 atoms of zinc bound/tetramer, though it is also possible that the metal is less tightly bound than in other microvillar metalloenzymes. Endopeptidase-2 exhibited a tendency to aggregation when incubated for several hours. It was also unusually sensitive to proteolytic digestion by papain and by proteinases in the *N*-glycanase preparation. Apart from these unusual oligomeric structural properties, endopeptidase-2 is typical of many other microvillar peptidases. It is an ectoenzyme, releasable by papain, and contains a metal essential for activity.

Proposed nomenclature for rat endopeptidase-2 and its relationship to pig endopeptidase-24.11, mouse meprin and human 'PABA peptide hydrolase'

Endopeptidase-2 and endopeptidase-24.11 are undoubtedly distinct enzymes, with different properties in respect of inhibitors, specificity, topology and oligomeric structure. In rat microvilli both enzymes coexist but can be readily separated. On the other hand, there are obvious overlapping properties of rat endopeptidase-2, mouse meprin and human 'PABA peptidase', and there is a need for some direct comparisons to be made to establish whether these three enzymes deserve to be classed as a single type of endopeptidase or whether they should retain separate identities and names. The name 'endopeptidase-2' should be regarded as a temporary expedient, pending the allocation of an EC number. It should then be renamed in line with the convention established for endopeptidase-24.11 (Matsas *et al.*, 1983) and endopeptidase-24.15 (Chu & Orlowski, 1985). Perhaps the enzyme recently characterized by Checler

et al. (1986) might be endopeptidase-24.16, and the peptidase reported here endopeptidase-24.17.

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