Proteins of the kidney microvillar membrane

The amphipathic forms of endopeptidase purified from pig kidneys

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The purification of detergent-solubilized kidney microvillar endopeptidase (EC 3.4.24.11) by immuno-adsorbent chromatography is described. The product (the d-form) was 270-fold purified compared with the homogenate of kidney cortex and was obtained in a yield of 5%. It was free of other peptidase activities and homogeneous by electrophoretic analyses. It contained about 15% carbohydrate and one Zn atom/subunit. Two trypsin-treated forms were also characterized. One (dt-form) was obtained by treatment of the d-form. The other (tt-form) was the result of solubilizing the membrane by treatment with toluene and trypsin. All three forms had apparent subunit M_r values of approx. 89000, but the d-form appeared to be slightly larger than the other two. Estimates of M_r by gel filtration showed that of the tt-form to be 216000 whereas those of the other forms were 320000. An estimate of the detergent (Triton X-100) bound to the d- and dt-forms accounted for this difference. By several criteria, including charge-shift crossed immunoelectrophoresis and hydrophobic chromatography, the d- and dt-forms were shown to be amphipathic molecules. In contrast, the tt-form was hydrophilic in its properties. Differences in ionic properties were also noted, consistent with the loss, in the case of the dt-form, of a positively charged peptide. The results indicate that the native endopeptidase is a dimeric molecule, each subunit being anchored in the membrane by a relatively small region of the polypeptide close to one or other terminus. The d- and dt-forms had similar enzyme activity when assayed by the hydrolysis of ^{125}I-insulin B-chain. Chelating agents and phosphoramidon inhibited the endopeptidase. The kinetic constants were determined by a new two-stage fluorimetric assay using glutarylglycylglycylphenylalanine 2-naphthylamide as substrate and aminopeptidase N (EC 3.4.11.2) to hydrolyse phenylalanine 2-naphthylamide. The K_m was 68 μM and $V_{max.}$ 484 nmol $\cdot$ min^{-1} $\cdot$ (mg of protein)$^{-1}$.

Kidney brush-border neutral peptidase (EC 3.4.24.11) was first purified and characterized in this laboratory from rabbit kidneys (Kerr & Kenny, 1974a,b). An endopeptidase with similar properties

has been found in kidneys of all other species so far examined, including pig, human, rat and mouse, but the two rodent kidneys differ in also containing a second metallo-endopeptidase with different properties (Kenny et al., 1981; Beynon et al., 1981). The endopeptidase is also present in the pig intestinal brush border (Danielsen et al., 1980a) and in membrane fractions from other organs, including brain (Almenoff et al., 1981; Fulcher et al., 1982; Malfroy et al., 1982) and pituitary (Orlowsky & Wilk, 1981). The original purification from rabbit kidneys used toluene/trypsin treatment to release it from the membrane in a hydrophilic form. The purified protein was a monomer of M_r 93000, containing about 15% carbohydrate and an essential Zn atom at the active site. The specificity was

directed towards bonds involving the amino groups of hydrophobic amino acid residues (Kerr & Kenny, 1974a,b). The apparent similarity of the active site to that of thermolysin (EC 3.4.24.4) was strongly supported by its sensitivity to inhibition by phosphoramidon (Kenny, 1977a); indeed it is the only known mammalian example of this class of endopeptidase. Among kidney microvillar hydrolases, the endopeptidase is unusual in resisting release from the membrane by treatment with papain, trypsin or elastase (Kenny, 1977b). Only in combination with toluene was trypsin found to be effective, but the yield of solubilized enzyme was often low. Non-ionic detergents are efficient in solubilizing membrane proteins, but the protein–detergent micelles that are formed are less amenable to fractionation by conventional column chromatography than the hydrophilic proteinase forms. However, only the detergent form can reflect the intact structure of the protein in the membrane. Since there is much current interest in the topology of this group of enzymes (for review, see Kenny & Maroux, 1982), it was important to study the detergent form of the endopeptidase and to compare it with other hydrolases that are readily released by proteinase treatment. In the present paper we report on the purification of the detergent form of the endopeptidase by immunoadsorbent chromatography and have compared its structure with other forms of the enzyme subjected to trypsin treatment. Unlike the rabbit enzyme, all forms purified from the pig were found to be dimeric.

Experimental

Tissues

Kidneys. These were kindly given by ASDA Farm Stores, Lofthousegate, West Yorkshire, U.K.

Chemicals

Substrates. Ala-NMec, α-Glu-NMec, γ-Glu-NMec, Gly-Pro-NMec and glutaryl-Gly-Gly-Phe-NNap were purchased from BACHEM, Hauptstrasse 144, CH-4416 Bubendorf, Switzerland.

Inhibitors. Phosphoramidon, leupeptin, antipain, chymostatin and elastinal were from Protein Research Foundation, Osaka, Japan. Thiorphan was donated by Dr. J. Shaw, ICI Pharmaceuticals, Macclesfield, Cheshire, U.K. Soya-bean trypsin inhibitor was from Sigma (London) Chemical Co.

Chromatography materials. Sepharose 6B, phenyl- and octyl-Sepharose CL-4B, CNBr-activated Sepharose 4B and Sephadex G-25 were from Pharmacia Fine Chemicals. Ultrogels AcA 22 and AcA 34 were from LKB Instruments Ltd. DEAE-cellulose (DE-52) was from Whatman Chemical Separation Ltd.

Proteins and enzymes. Trypsin (Type III), human transferrin, β-galactosidase, ferritin, urease, catalase, glucose oxidase and liver alcohol dehydrogenase, were from Sigma (London) Chemical Co. Anti-(human transferrin) was from DAKO-Immunoglobulins, Copenhagen, Denmark. Calibration markers for M_r determination on polyacrylamide gels (low-M_r range) were from Pharmacia Fine Chemicals.

Detergents. Triton X-100, cetyltrimethylammonium bromide and sodium deoxycholate were from BDH. [*phenyl*-³H(n)]Triton X-100 was from New England Nuclear, Southampton SO2 0AA, U.K. Emulphogene BC 720 was from Sigma (London) Chemical Co.

Enzyme assays. Endopeptidase was routinely assayed with ¹²⁵I-insulin B-chain as substrate (Kenny, 1977b). The unlabelled peptide was prepared as described in that reference, but the iodination method was modified as follows. The mixture (vol. 2.2 ml) contained 14 mg of insulin B-chain in 2 ml of 0.05 M-sodium phosphate, pH 7.5, 4 μmol of KI and 1 mCi of Na¹²⁵I (Amersham International; code IMS 30). Chloramine-T (BDH), 18 mg/ml, was added in six 100 μl portions. After 15 min at 20°C the reaction was quenched by the addition of 100 μl of Na₂S₂O₅ (80 mg/ml) and KI (2.5 mg in 100 μl). The labelled peptide was separated from the other compounds by passage through a column (500 mm × 9 mm diam.) of Sephadex G-25 developed with 20 mM-sodium phosphate (pH 7.5)/100 μM-KI. Labelled fractions were pooled and stored at −20°C.

For the kinetic experiments a fluorimetric assay was used, with glutaryl-Gly-Gly-Phe-NNap as substrate. The incubation mixture (2 ml) contained 0.2 mM-substrate and 0.1 M-Tris/HCl, pH 7.0 (37°C), and the reaction started by the addition of the enzyme. After 15 min incubation at 37°C, 50 μl of 100 μM-phosphoramidon was added, followed by 500 m-units of aminopeptidase N in 150 μl, and the incubation continued while monitoring the hydrolysis fluorimetrically (excitation, 310 nm; emission, 410 nm) until the fluorescence no longer increased (usually within 15 min). At this stage the endopeptidase activity was calculated from the fluorescence of the naphthylamine, or by a colorimetric step (Felgenhauer & Glenner, 1966).

Exopeptidases were assayed with 4-methyl 7-coumarylamide derivatives as substrates (all at 200 μM) and 0.1 M-Tris/HCl as buffer. The substrates and pH values were as follows: Ala-NMec (pH 7.0) for aminopeptidase N (EC 3.4.11.2); α-Glu-NMec (pH 7.0, 1.25 mM-CaCl₂) for aminopeptidase A (EC 3.4.11.7); γ-Glu-NMec (pH 8.5 with 3 mM-glycylglycine as acceptor) for γ-glutamyltransferase (EC 2.3.2.2), and Gly-Pro-NMec (pH 8.0) for dipeptidyl peptidase IV (EC 3.4.14.5). The reaction was started by addition of enzyme to

2 ml of the buffered substrate in a thermostatically controlled cell holder at 37°C in an Aminco–Bowman Fluorimeter, model SPF 125 (excitation, 370 nm; emission, 442 nm). The instrument was calibrated with 20 μM-7-amino-4-methylcoumarin (BACHEM). Alkaline phosphatase was assayed as described previously (George & Kenny, 1973).

A unit of enzyme activity is defined as 1 μmol of substrate transformed/min at 37°C under the assay conditions specified.

Protein determinations

Protein was assayed by the Lowry method with the addition of 100 μl of 10% (w/v) SDS to 1 ml of samples that contained Triton X-100. In the experiments designed to estimate Triton X-100 binding to the enzyme, protein was assayed by the method of Bradford (1976) after precipitation by an equal volume of 25% (w/v) trichloroacetic acid. The precipitate was washed twice with ethanol/diethyl ether (1:1, v/v) before assay. The calibration had been established with purified endopeptidase (see below). For estimating precise specific activities of the purified endopeptidase and for samples intended for carbohydrate analyses, protein was determined from the amino acid composition after hydrolysis for 36 h by 6 M-HCl/0.3% phenol, at 110°C, with a Rank–Hilger Chromospek J180 amino acid analyser.

Preparation of the immunoadsorbent column

Partial purification of endopeptidases. The starting material was a microsomal fraction in 300 ml of 0.1 M-Hepes/NaOH, pH 6.8, prepared from 400 g of kidney cortex (stored frozen at −70°C) as previously described (Kerr & Kenny, 1974a). Papain (250 mg in 20 ml), activated for 15 min at 37°C in 25 mM-2-mercaptoethanol in the same buffer, was added and the mixture incubated with gentle shaking for 2 h. After the addition of 500 mg of iodoacetamide, the suspension was centrifuged at 31 000 g for 1 h, and the pellet was resuspended in 250 ml of 50 mM-Tris/HCl, pH 8.0. The centrifugation was repeated and the pellet suspended in 10 mM-Tris/HCl, pH 7.8. Emulphogene BC 720 was added to 5% (w/v) and the mixture stirred at 20°C for 1 h. It was re-centrifuged as described above for 2 h and the supernatant incubated for 1 h at 37°C with 200 mg of trypsin (added in four batches during the incubation). Soya-bean trypsin inhibitor (150 mg) was then added and the mixture loaded on to a column containing about 100 ml of DEAE-cellulose equilibrated with 10 mM-Tris/HCl (pH 7.4)/1% (w/v) Emulphogene BC 720. After washing with 200 ml of this buffer, the column was eluted by a linear gradient (0–200 mM-NaCl) in 1 litre of the same buffer. Endopeptidase-containing fractions were pooled, the volume decreased to 20 ml by ultra-

filtration before loading on a column (900 mm × 50 mm) of Sepharose 6B, equilibrated with the same buffer containing detergent. The active fractions were pooled and rechromatographed on a column (vol. 20 ml) of DEAE-cellulose, and eluted as described above. The active fractions were concentrated to 4 ml and chromatographed on a column of Ultrogel AcA 34 (900 mm × 22 mm) developed with 5 mM-Tris/HCl (pH 7.4)/0.1% Emulphogene. The active fractions were pooled and concentrated on a small column (vol. 1 ml) of DEAE-cellulose, the activity being recovered in 5 ml on elution with 200 mM-NaCl in the same buffer.

Line electrophoresis. The partially pure endopeptidase (2 mg of protein in 1 ml) was cast into a slot 80 mm × 20 mm of the agarose gel and run into gel containing an IgG fraction (50 mg of protein) from an antiserum raised to kidney microvilli, as previously described (Danielsen et al., 1980b). The main immunoprecipitate, identified histochemically as that of endopeptidase (Kenny et al., 1981), was excised and stored at −70°C. Pooled material from four runs was used as the antigen.

Specific antiserum. This was raised in a rabbit by an initial injection of the immunoprecipitate emulsified in Freund's complete adjuvant, followed by three injections at 14-day intervals in incomplete adjuvant. Doses were given intracutaneously in multiple sites on the back and the animal bled (45 ml) 7 days after the last injection. Subsequent bleedings were obtained after two injections, one with incomplete adjuvant, followed 14 days later by an intravenous injection of pure endopeptidase (50 μg in 0.5 ml of 0.15 M-NaCl). An IgG fraction was prepared from the serum and coupled to CNBr-Sepharose as described by Danielsen et al. (1980b).

Purification of endopeptidase

Frozen pig kidney cortex (40 g) was thawed and a microvillus fraction prepared (Booth & Kenny, 1974). It was resuspended in 20 ml of 50 mM-Tris/HCl, pH 7.4, to which 2.5 ml of 20% (w/v) Triton X-100 was added. After 1 h at 20°C, the mixture was centrifuged at 31 000 g for 1 h and the supernatant retained and stored at −20°C. The immuno-absorbent column was equilibrated with 50 mM-Tris/HCl/500 mM-NaCl/0.1% (w/v) Triton X-100, pH 8.0. A batch of solubilized enzyme (150 m-units) was diluted 4-fold with the equilibration buffer and pumped at 5 ml/h on to the column. It was then washed with 250 ml of the same buffer before elution with 0.1 M-NaHCO₃/0.5 M-NaCl/0.1% Triton X-100, pH 10.6. The active fractions were pooled and dialysed immediately against 5 mM-Tris/HCl/0.1% Triton, pH 7.4, and concentrated by binding and elution (200 mM-NaCl

in same buffer) from a small column (100μl) of DEAE-cellulose. The activity was obtained in 1 ml of the elution buffer. After use, the immunoabsorbent column was washed with 100 ml of 0.2 M-glycine/HCl/0.5 M-NaCl/0.1% Triton X-100, pH 2.3, and re-equilibrated with the starting buffer. Between uses it was stored with 0.02% NaN$_3$ in the buffer. All operations were at 4°C.

The toluene/trypsin(tt-form)-solubilized enzyme was prepared as described previously (Kerr & Kenny, 1974a) up to the stage at which the soluble fraction was obtained. In place of (NH$_4$)$_2$SO$_4$ precipitation, the fraction was dialysed against 10 mM-Tris/HCl, pH 7.4, and chromatographed on DEAE-cellulose with a gradient of 0–200 mM-NaCl in the same buffer. The pooled fractions were purified by the immunoadsorbent column and also passed through an anti-impurity column containing antibodies to microvillar proteins.

Trypsin treatment

The dt-form was produced by incubation of the d-form with trypsin (d-form/trypsin, 10:1, w/w) at 37°C for 2 h in 50 mM-Tris/HCl, pH 8.0. In some previous experiments, incubation at 4°C for 18 h was used, with essentially the same effect. The mixture was dialysed against 5 mM-Tris/HCl, pH 7.4, applied to a column (vol. 1 ml) of DEAE-cellulose and eluted batchwise with 200 mM-NaCl. All buffers contained 0.1% Triton X-100.

Immunoelectrophoresis

Crossed immunoelectrophoresis was performed as previously described (Danielsen et al., 1980b). Crossed charge-shift immunoelectrophoresis was performed as described by Helenius & Simons (1977).

Determination of Triton X-100 binding to purified endopeptidase

The method was based on that of Clark (1975), except that the sucrose gradient was 5–30% (w/v); 50μCi of [^{3}H]Triton X-100 was present in each gradient; centrifugation at 100000g for 8 h was in a Kontron TST-54 rotor. The endopeptidase (100μg) was layered on top.

Determination of zinc

A sample (300μg) of the purified dt-form was exhaustively dialysed against deionized (Millipure) water and zinc determined by an Instrumentation Laboratory model 157 atomic-emission spectrophotometer.

Determination of carbohydrate

The method of Chaplin (1982) was used.

Gel electrophoresis

SDS/polyacrylamide gels were run by using the Laemmli (1970) system with a 7–17% poly-acrylamide gradient. Isoelectric focusing was performed in 0.5 mm-thick agarose gels in the pH range 4–6 by using the LKB Multiphor System, at 10°C, as detailed by the manufacturers. The pH was determined by a surface electrode.

Ornstein–Davis polyacrylamide-disc-gel electro-phoresis (Hames, 1981) was performed with a polyacrylamide gradient (7–17%) in the presence of 1% Triton X-100 and 0.02% Pyronin Y (marker dye; Sigma). Endopeptidase was located histo-chemically (Kenny et al., 1981).

Results

Purification of the endopeptidase

The key step was immuno-adsorbent chromato-graphy on a column in which the IgG fraction from the rabbit immune serum was coupled to CNBr-activated Sepharose 4B. The column showed remarkable consistency in behaviour over many months. The binding capacity remained constant at about 115 m-units of endopeptidase when the amount applied varied from 150 to 540 m-units and the pH of the application buffer varied from 7.0 to 8.0. The yield was also constant in ten runs at 19.6 ± 1.4 (s.d.)%. This low yield needs some qualification: in part it is due to an underestimate by our assay, the results of which are amplified by the presence of aminopeptidases (Kerr & Kenny, 1974a). Hence crude samples, rich in amino-peptidase N, exaggerate the endopeptidase activity, whereas the assay value for the eluted sample approximates to the true activity. The corrected yield is about 25–30%. Nevertheless, the loss of 70% of the activity was not fully explained. Since the capacity of the column did not diminish on repeated use, we can assume that the unrecovered antigen did not bind irreversibly. Probably the polyclonal antibodies exhibited a wide range of affinities such that some of the activity was eluted slowly and in a dilute form during the washing cycles both before and after elution. Several elution buffers were tested, but only two were found to be effective. They were bicarbonate buffer, pH 10.6, and a low-ionic-strength medium of 1 mM-Tris/HCl, pH 8.0. Both gave similar yields, but the elution peak was broader in the latter system. Buffers at low pH or others containing 10% (v/v) dioxan, 50% (v/v) ethylene glycol or 4 M-MgCl$_2$ either did not elute the enzyme or caused irreversible inactivation. Table 1 shows the results of ten purifications, each from 40 g of kidney cortex. The immunoadsorbent column had a low capacity, so that eight applications and elutions would be needed to process the 912 m-units of the previous step. The integrated yield therefore amoun-

Table 1. *Purification of d-form of the endopeptidase*

See the Experimental section for details. The data are for ten preparations, except for the final step, which is based on five preparations for which protein was determined accurately by amino acid analysis. Protein at other steps was determined by the Lowry method. Note that the capacity of the final step was limited to 115 m-units, hence the overall recovery value has been adjusted for the chromatography of 912 units.

Fraction	Protein (mg)	Endopeptidase activity			
		Total (m-units)	Specific activity [m-units · (mg of protein)$^{-1}$]	Recovery (%)	Purification factor
Cortex homogenate	5600	4402	0.79 ± 0.25	100	1
Microvilli	119	918	7.70 ± 0.17	21	9.8
Triton X-100-solubilized	49	912	18.7 ± 0.22	21	24
Immunoadsorbent chromatography	0.13 ± 0.003	22.9 ± 0.5	177 ± 3.0	4	224

ted to about 4% of the homogenate activity. When the activity in the homogenate is corrected for the contribution of aminopeptidase, the specific activity became 0.65 ± 0.21 m-units · (mg of protein)$^{-1}$. Taking this value, the true yield was 5% and the purification factor became 270-fold.

Purity of the endopeptidase

Two criteria of purity were regularly used. In Fig. 1, the immunoprecipitate obtained by crossed immunoelectrophoresis is seen to be a single arc when the purified enzyme was run into an antiserum raised to all microvillar components; this contrasts with the complex pattern when detergent-solubilized membrane was substituted. Since the endopeptidase is a weaker antigen than many of the other enzymes, this analysis is a stringent test of purity. In Fig. 2 the SDS-denatured endopeptidase is seen to migrate as a single band when 15 μg of protein was applied to the track. There was no detectable activity when the preparation was assayed for aminopeptidase N, aminopeptidase A, γ-glutamyltransferase, dipeptidyl peptidase IV (all fluorimetric assays) and alkaline phosphatase. A second immunoadsorbent column was later prepared from an IgG fraction obtained from a second bleeding. This column yielded a preparation that was not quite homogeneous by these criteria. However, passage through a small column containing an IgG fraction from an antiserum raised to kidney microvilli successfully removed these contaminants without significant losses of endopeptidase.

Carbohydrate and zinc content

Total carbohydrate, excluding glucose, was determined on three preparations of the d-form of the enzyme. The mean was 150 ± 16 (s.d.) μg · (mg of protein)$^{-1}$. The zinc content of one sample of the dt-form of the endopeptidase (50 repetitive readings) was 0.93 ± 0.06 g-atoms per protein molecule of M_r 89000.
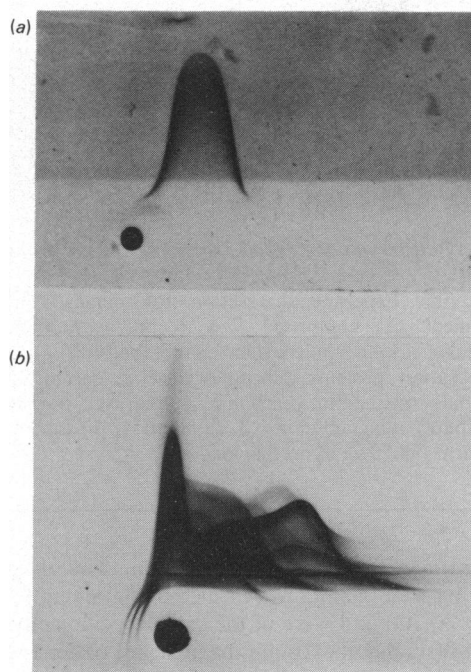

Fig. 1. *Crossed immunoelectrophoresis of purified endopeptidase and microvillar proteins*

See the Experimental section for details. In each case the second dimension contained an IgG fraction from an antiserum raised to microvilli (Booth *et al.*, 1979). (*a*) d-form of endopeptidase (5 μg of protein); (*b*) microvillar membrane (40 μg of Triton X-100-solubilized protein).

Forms of the endopeptidase obtained by treatment with trypsin

Two forms of the enzyme that had been exposed to trypsin were also studied. One (the dt-form) was obtained by treatment of the purified detergent form. The second (the tt-form) was purified after initial release of the enzyme from the membrane by
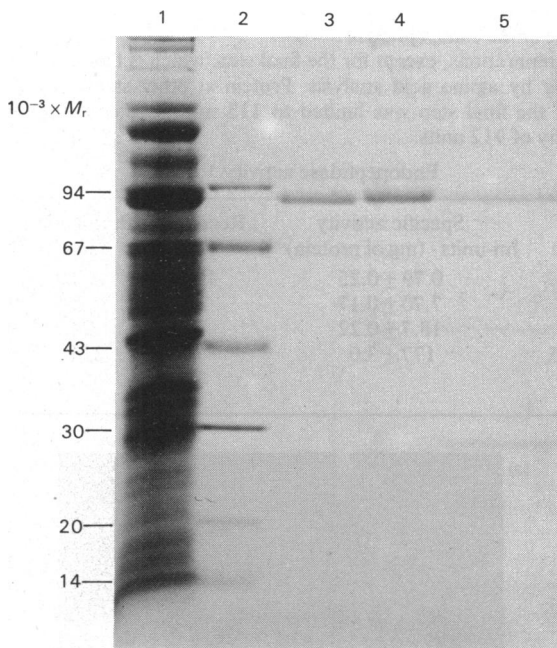
Fig. 2. *Polyacrylamide-gel electrophoresis in the presence of SDS*

See the Experimental section for details. The gradient gel comprised 7–17% polyacrylamide. Tracks: 1, kidney microvillar membrane (100 μg); 2, calibration proteins (phosphorylase *b*, serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, α-lactalbumin); 3, dt-form; 4, d-form; 5, tt-form (all 20 μg of protein loaded).

Fig. 3. *Gel filtration on a column of Ultrogel AcA 22*
Column dimensions, 900 mm × 22 mm; buffer, 10 mM-Tris/HCl, 100 mM-NaCl, pH 7.4, 0.1%-Triton X-100; V_0 (Blue Dextran), 108 ml. The column was calibrated ($M_r \times 10^{-3}$ in parentheses) with β-galactosidase (520), ferritin (483), urease (480), aminopeptidase N (proteinase form, 280), dipeptidyl peptidase IV (proteinase form, 266), catalase (232), IgG (150) and liver alcohol dehydrogenase (80). Each marker was run twice. A linear regression line was calculated; $r^2 = 0.98$. All experiments were performed at room temperature. O, d-form; ▲, dt-form; ■, tt-form of endopeptidase.

treatment with toluene and trypsin. The d- and dt-forms were maintained in media containing 0.1% Triton X-100 and were of the same specific activity. The tt-form did not require the presence of detergent. It had a specific activity about 45% of that of the other forms and was only obtainable in very low yield (recovery, 0.2%).

Molecular properties of the three forms of the endopeptidase

Relative molecular mass. Gel filtration (two experiments on each form) on a calibrated column of Ultrogel AcA 22 revealed clear differences between the three forms (Fig. 3). The d-form eluted in two peaks, one of which contained a small and variable amount that appeared to be aggregated, of M_r approx. 800000. The main component appeared in a peak calculated to correspond to M_r 320000 and this peak eluted at the same volume as the sole component in the dt-form. In five experiments the dt-form never contained any of the higher aggregates. The tt-form differed from the detergent forms: it eluted as a molecule of M_r 216000. The d- and
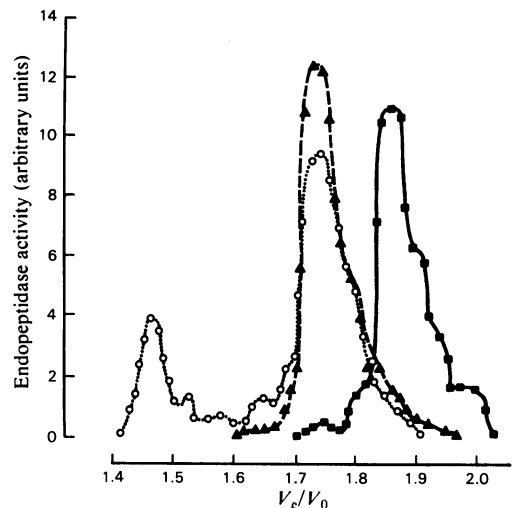
dt-forms were also chromatographed on Ultrogel AcA 34 (four experiments on each form) with similar results: 321000 ± 2000 (s.e.m.) and 318000 ± 10000 (s.e.m.) respectively.

SDS/polyacrylamide-gel electrophoresis. The apparent subunit M_r of each form was assessed as 89000. The d-form appeared to migrate slightly slower than the dt-form, but the difference was too small to quantify and the forms were not resolvable if run in the same track (Fig. 2). The tt-form was heterogeneous in this system. The 89000-M_r band accounted for 41% by a densitometric trace. The faster-migrating bands (mainly of M_r 48000, 28000 and 22000) were probably proteolytic fragments that retained antigenic determinants. Crossed immunoelectrophoresis into the specific antiserum revealed multiple arcs, only one of which was enzymically active and this migrated faster than the d- and dt-forms. Hence the 89000-M_r band is considered to represent the active tt-form.

Electrophoresis, under the same conditions, in a 7%-polyacrylamide gel, revealed the same pattern; the trypsin-treated forms migrated only slightly ahead of the d-form.

Amphipathic properties. Charge-shift crossed immunoelectrophoresis showed that only the d- and dt-forms were affected by substitution of ionic detergents for Triton X-100. In this experiment, a marker protein, transferrin, possessing only hydrophilic properties, was added to each form of the endopeptidase. Anti-transferrin was mixed with the antibody to the endopeptidase before running the second dimension. The marker therefore provided an internal standard to exclude effects attributable to variations in the electrophoretic conditions. The mobilities are expressed relative to this standard. The results (Table 2) show that the mobility of the tt-form was unaffected by cetyltrimethylammonium bromide or deoxycholate. However, the d- and dt-forms were markedly affected by the ionic detergent: deoxycholate enhanced the mobility; cetyltrimethylammonium bromide reversed the direction of movement towards the cathode.

A second criterion for hydrophobicity of the enzyme forms was the binding to phenyl- and octyl-Sepharose CL-4B. These results are shown in Table 3. The samples were applied in a buffer containing 10mM-sodium phosphate, 150mM-NaCl and 0.1% (w/v) Triton X-100, pH 7.4. After being washed in the same buffer without detergent, the columns were eluted by a medium containing 1mM-sodium phosphate and 2.5% (w/v) Triton X-100, pH 7.4. Both the d-and dt-forms showed substantial binding to both types of column matrix. The tt-form showed only 4–10% binding under these conditions. On both columns, the d-form showed slightly higher binding than the dt-form, but this may not be statistically significant.

When either the d- or dt-forms were subjected to gel filtration on Ultrogel AcA 34 and in the absence of Triton X-100 all the activity appeared in the void volume. On Ultrogel AcA 22, both forms eluted in these conditions as aggregates of approx. M_r 800000. In contrast, the tt-form showed no tendency to aggregate in the absence of detergent and the elution volume of this form was also unaffected by the presence of detergent.

Determination of bound Triton X-100

The quantity of Triton X-100 bound to the d- and dt-forms was determined by exchange with [^{3}H]-Triton X-100. This was accomplished by rate-zonal centrifugation in a sucrose density gradient containing the labelled detergent. The dt-form bound Triton X-100 in a ratio (detergent/protein, w/w) of 0.49 ± 0.02 (s.e.m., three experiments). The value for the d-form was 0.48 ± 0.01 (two experiments). In molecular terms, these values correspond to 67 and 66 Triton X-100 monomers per subunit (M_r 89000) respectively and hence the detergent contributes 87000 Da to each dimeric molecule.

Ionic differences between the three forms

Each form was chromatographed on the same column of DEAE-cellulose in conditions that were, as far as possible, identical for each run. The elution

Table 3. *Hydrophobic chromatography of different forms of endopeptidase on columns of octyl- and phenyl-Sepharose CL 4B*

See the Experimental section for details. About 1.8 m-units of each form were applied to the columns. The results are expressed as the percentage of the applied activity that was unbound (i.e. recovered in the effluent during loading and washing) and that was eluted by 2.5% Triton X-100 in a buffer of low ionic strength.

| | | Endopeptidase activity | | |
| | Endo-peptidase form | Unbound (%) | Eluted (%) | Total recovered (%) |
Column				
Octyl-Sepharose	d	15.1	79.9	95.0
	dt	26.2	74.4	100.6
	tt	89.8	10.2	100.0
Phenyl-Sepharose	d	28.0	80.6	108.6
	dt	36.9	70.7	107.6
	tt	96.0	3.6	99.6

Table 2. *Crossed charge-shift immunoelectrophoresis of three forms of endopeptidase*

See the Experimental section for details. The mobilities in the first dimension are expressed relative to the mobility of a hydrophilic marker protein, human transferrin; the negative sign indicates migration towards the cathode. In all other cases, the endopeptidase migrated in the same direction as transferrin. Abbreviation used: CETAB, cetyltrimethylammonium bromide.

| | | Mobility relative to human transferrin | | |
Endopeptidase form	Detergent ...	Triton X-100	Triton X-100 + deoxycholate	Triton X-100 + CETAB
d		0.45	1.75	−0.18
dt		0.45	1.75	−0.18
tt		0.99	1.02	1.00

patterns are shown in Fig. 4. The d-form was eluted in a broader peak than the others. Activity appeared at a lower concentration of NaCl and a pronounced shoulder was observed, together with marked trailing. The dt-form was more sharply eluted, but a shoulder was also present. The tt-form eluted as a more symmetrical peak at the same concentration of NaCl.

The three forms migrated differently in the Ornstein–Davis gel system. R_F values (relative to Pyronine Y) were 0.16, 0.59, 0.70 for the d-, dt- and tt-forms respectively. The relative contribution of charge and molecular size to these differences is difficult to assess.

Electrofocusing of the three forms in an agarose gel containing Ampholines of range pH 4–6 revealed some charge heterogeneity. The pI values may be summarized as follows: the d-form major band 5.30,
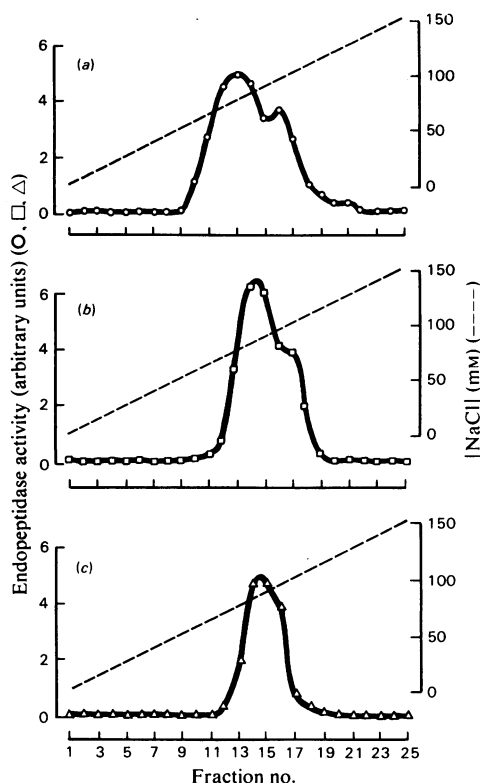
other bands 5.15, 5.45; dt-form major band 5.00, other bands 4.90, 5.10; tt-form major bands 5.10, 5.25, other bands 5.00, 5.13. However, the tt-form revealed more heterogeneity than either of the others.

Inhibition studies

The catalytic properties of the rabbit kidney endopeptidase have been studied in some detail (Kerr & Kenny, 1974b). Inhibition curves for thiorphan, phosphoramidon and puromycin for the pig kidney endopeptidase have been given (Fulcher et al., 1982). The results with inhibitors need only be summarized here. Table 4 gives the IC_{50} (concentration of inhibitor giving 50% inhibition) values for a variety of effective inhibitors. It also lists a number of other inhibitors, most exhibiting group specificity, that showed no inhibitory properties at the concentrations used.

Kinetic studies with glutaryl-Gly-Gly-Phe-2-NNap as substrate

Insulin B-chain, the usual substrate, was cleaved at ten sites by the purified rabbit endopeptidase (Kerr & Kenny, 1974a). The radioactive assay using the iodinated substrate is quick and quantitative provided not more than about 25% of the radioactivity is released (Kenny, 1977b), but is not ideal for kinetic experiments. Glutaryl-Gly-Gly-Phe-2-NNap has only one susceptible bond (Gly–Phe) and hence has a clear advantage in calculating kinetic constants. The assay requires a second enzymic step, accomplished by aminopeptidase N, to hydrolyse Phe-NNap to liberate 2-naphthylamine that can

Fig. 4. *DEAE-cellulose chromatography of three forms of endopeptidase*
The bed volume of the DEAE-cellulose column was 100 μl. Elution was by a gradient of 0–150 mM-NaCl in 5 mM-Tris/HCl (pH 7.4)/0.1% Triton X-100 (20 ml in total). In each experiment, 5 μg of endopeptidase was loaded: (a) d-form (O); (b) dt-form (□); (c) tt-form (△).

Table 4. *Inhibitor properties of endopeptidase*
Inhibition was determined in assays using 5.7 μM-^{125}I-insulin B-chain as substrate. Inhibitors were added to the incubation mixture in a range of concentrations: 0.1 μM–1 mM for EDTA, 1,10-phenanthroline and puromycin; 10 μM–5 mM for the thiols; 0.1 nM–100 μM for phosphoramidon and thiorphan. No inhibition was observed with di-isopropyl phosphorofluoridate, phenylmethanesulphonyl fluoride, 4-chloromercuribenzoate, N-ethylmaleimide and Captopril tested as concentrations of 1 μM–1 mM. Antipain, elastinal, chymostatin and pepstatin did not inhibit at 2.5 μg/ml.

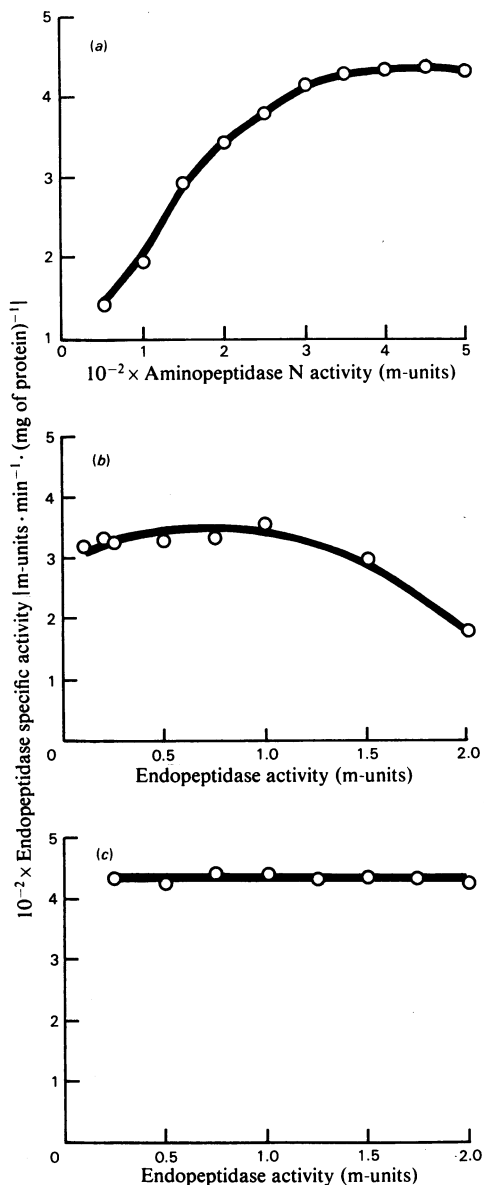| Inhibitor	IC_{50}
Phosphoramidon	13 nM
Thiorphan	13 nM
EDTA	71 μM
Puromycin	100 μM
1,10-Phenanthroline	126 μM
Dithiothreitol	0.89 mM
2,3-Dimercaptopropan-1-ol	1.26 mM

Fig. 5. *Fluorimetric assay of endopeptidase*
See the Experimental section for details. In each case the same sample of purified dt-form of endopeptidase was used and $200\,\mu$M-glutaryl-Gly-Gly-Phe-NNap. (*a*) Effect of increasing [aminopeptidase N] with constant endopeptidase ($210\,\mu$-units/tube); a continuous assay, rate determined when the increase in fluorescence had become linear (usually after 10 min). (*b*) Effect of increasing [endopeptidase] with constant aminopeptidase N (260 m-unit/tube); continuous assay, rate determined as in (*a*). (*c*) Two-step assay: first stage, 10 min incubation with endopeptidase; second stage contained $2.5\,\mu$M-phosphoramidon and 500 m-units of aminopeptidase N and was allowed to proceed to completion.

be determined fluorimetrically. However, such an assay can be performed in two ways: continuously, both enzymes present from the start, or as a two-step system, inhibiting the first before adding the second enzyme. The continuous assay seems temptingly simple, but is beset by a major weakness: the concentration of Phe-2-NNap produced by the endopeptidase is low in relation to the K_m of the aminopeptidase and, unless the latter is in great excess, the second step may be rate-limiting. This is illustrated in Fig. 5. The same sample of pure endopeptidase was assayed in a continuous system, with either increasing amounts of the aminopeptidase (Fig. 5*a*), or increasing endopeptidase (Fig. 5*b*), the other component being constant. In the first case, the true specific activity is attained only with a 1000-fold excess of aminopeptidase. In the second case, the true activity was never quite registered and the apparent activity fell away at higher concentrations of endopeptidase. The two-step assay (Fig. 5*c*) avoids these uncertainties, constant specific activity being observed over the whole range examined.

The two-step assay was used to determine the kinetic constants. These were determined by a double-reciprocal plot based on three experiments ($r^2 = 0.98$) and yielded the following values (means $\pm$ s.d.): K_m, $68 \pm 5\,\mu$M; $V_{max.}$, $484 \pm 7\,$nmol $\cdot$ min$^{-1}\cdot$ (mg of enzyme)$^{-1}$; $K_{cat.}$, $43.7\,$min^{-1}; $K_{cat.}/K_m$, $643\,$min$^{-1}\cdot$mM^{-1}.

Discussion

Catalytic properties of the pig kidney endopeptidase

In comparing the effects of inhibitors on this preparation from pig with our results on the rabbit enzyme (Kerr & Kenny, 1974*b*; Kenny, 1977*a,b*) or with the partially purified pig intestinal endopeptidase (Danielsen *et al.*, 1980*a*), no important differences have emerged. Like the rabbit endopeptidase, the pig enzyme contains 1 zinc atom/subunit. Apart from the group of effective inhibitors that bind this metal ion, no other group-specific reagents inhibited the endopeptidase. The enzyme is therefore a zinc metalloendopeptidase correctly assigned as EC 3.4.24.11.

The specific activity using the radioactivity assay of the purified d-form was 177 m-units $\cdot$ (mg of protein)$^{-1}$. This is somewhat lower than that reported for the rabbit enzyme (301 m-units $\cdot$ mg^{-1}) (Kerr & Kenny, 1974*a*), but the two enrichment values are similar, both about 250-fold. This value is consistent with the view that the endopeptidase is a major protein in the microvillar membrane. From the specific activities we calculated that the endopeptidase was 3.7% of the proteins of the pig microvillus and 9% of the Triton X-100-extracted proteins. The conclusion conflicts with the results of

Mumford *et al.* (1981), who have reported a 3900-fold purification with a 748% yield of a membrane-bound metalloendopeptidase from pig kidneys. The properties of this enzyme have both similarities to and differences from the endo-peptidases that we have studied from rabbit and pig kidneys. Although the best substrates were cleaved at -Ala–Phe- and -Gly–Met- bonds, substantial activity was reported for -Ala–Ala- and -Phe–Ala- bonds, a specificity not observed in our study of the rabbit enzyme. Their enzyme was sensitive to phosphoramidon (though with a higher IC_{50} value, 94nM) as well as to chelators and to some hydroxamate compounds that are effective inhibitors of thermolysin (Nishino & Powers, 1979). One estimate of M_r was obtained from high-performance gel filtration, 88000, consistent with a monomeric form. We have observed activity only in forms that are either dimeric or in larger aggregates. But these conflicting findings are small compared with the large difference in specific activities reported by these authors. The microsomal fraction had an activity of $104 nmol \cdot min^{-1} \cdot mg^{-1}$ and the pure form $437 \mu mol \cdot min^{-1} \cdot mg^{-1}$ of protein, in continuous assays, at 25°C, with $200 \mu M$-succinoyl-Ala-Ala-Phe-NMec as substrate. This compound is analogous to our fluorogenic substrate, glutaryl-Gly-Gly-Phe-2-NNap, for which we obtained a $V_{max.}$ value, at 37°C, of $484 nmol \cdot min^{-1} \cdot mg^{-1}$, three orders of magnitude less. We have pointed out the problems that may arise from the continuous assay system and have argued the case for a two-step assay. It is possible that errors in the assay might produce the curiously high recovery value. Indeed it seems a more possible explanation than the presence of endogenous inhibitors in the starting material, a phenomenon that we have never encountered with this enzyme. But these considerations cannot explain the very high activity of their preparation. This point deserves further study to see whether it results from the presence of alanine residues in the substrate at positions P_2 and P_3.

Orlowski & Wilk (1981) have purified, though not to homogeneity, an apparently similar endo-peptidase from bovine pituitaries. Their fluorescence assay used benzoyl-Gly-Arg-Arg-Leu-NNap as sub-strate and they also emphasize the need for a large excess of aminopeptidase in the continuous assay if the endopeptidase is to be rate-limiting. They also studied 14 different substrates of this class and reported some surprising effects attributed to diffe-rent amino acid residues in positions P_2, P_3 and even P_4.

Molecular structure of the purified endopeptidase

The apparent subunit M_r estimated from SDS/polyacrylamide-gel electrophoresis was about 89000 for all forms of the endopeptidase. This value agrees

with the weight calculated to contain 1 g-atom of zinc and with the values previously obtained for the tt-form of the endopeptidase purified from rabbit kidneys (Kerr & Kenny, 1974*b*). However, estimates of the M_r under non-denaturing conditions gave values from 216000 to 320000. The former was that of the hydrophilic tt-form and is consistent with a dimeric structure. The higher value was obtained with the amphipathic d- and dt-forms. Amphipathic proteins bind detergent, in this case Triton X-100. Triton X-100 has an aggregation number of 140 and a micellar weight of 90000 Da (Helenius & Simons, 1975). Our estimate of the amount of Triton X-100 bound to the d- and dt-forms revealed 67 monomers/subunit, a number that is half that expected to form a micelle. A dimer therefore appears to bind one Triton X-100 micelle. Electron microscopy of the enzyme after reconstitution of the d-form [the following paper (Kenny *et al.*, 1983)] also revealed dimers when viewed *en face* on the surface of liposomes. If we can assume that the two subunits are identical, each possessing an anchor, it follows that a single detergent micelle can accom-modate both hydrophobic anchors.

The d-form often contained a small proportion of a larger aggregate of M_r 800000, and aggregates of about the same size were observed when the d- and dt-forms were subjected to gel filtration in the absence of detergent. These may be 'protein micelles' comprising about four dimers, similar to those described by Helenius *et al.* (1981). The tt-form never revealed any tendency to aggregate in this way.

Although the subunit sizes of the tt-forms of the rabbit and pig endopeptidases were similar, the rabbit endopeptidase differed in that no dimers were observed under non-denaturing conditions. In this respect the rabbit endopeptidase is like some other microvillar hydrolases that are monomeric in this species but dimeric when purified from pig micro-villi (e.g. see Kenny & Maroux, 1982).

Effect of trypsin on the endopeptidase

The tt-form was the only one wholly lacking in amphipathic properties. The action of toluene on the membrane, such that it permitted trypsin to re-lease a hydrophilic form of the enzyme, remains unclear. However, it also permitted some degradation of the enzyme by trypsin, or possibly other protein-ases in the kidney fraction. This was evident in the multiple bonds visible in Fig. 2. We attribute the 89000-M_r band to the tt-form because: (*a*) its abun-dance corresponds to the specific activity; (*b*) the apparent M_r correlates reasonably well with that assessed for the dimeric form; and (*c*) the value agrees with that of the rabbit tt-form. The next most abundant polypeptides (M_r 48000 and 28000) pose difficulties in these respects. The tt-form may be

regarded as a hydrophilic globular protein, lacking hydrophobic region(s) by which it was anchored to the membrane. This component cannot be large, indeed no bigger than that observable by the resolution of the SDS/polyacrylamide-gel system, namely less than 3000 Da.

It is unclear what structural changes were associated with the conversion of the d- into the dt-form. Both were amphipathic, hence the hydrophobic anchor domain was present in each. It seems likely that trypsin removed a small positively charged peptide. This would explain the lower pI values for the components of the dt-form and, also, the slightly different elution pattern observed by chromatography on DEAE-cellulose. The dt-form required a slightly higher concentration of NaCl for elution.

Topology of the endopeptidase in the kidney microvillar membrane

Although the endopeptidase cannot be released from the membrane by treatment with proteinases, one should not assume that its topology is radically different from other more typical microvillar enzymes (see, e.g., Kenny & Maroux, 1982). Our results are consistent with the endopeptidase being a 'stalked' dimeric protein. In such cases, most of the mass is a hydrophilic globular protein exposed at the luminal surface of the membrane and anchored by a hydrophobic region that constitutes only a very small proportion of the total mass and which is located near one end of the polypeptide chain. In other examples this has been shown to be adjacent to the N-terminus, but this has yet to be established for the endopeptidase. The globular model has, however, been confirmed by reconstitution in liposomes [see the following paper (Kenny et al., 1983)].

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