Purification and characterization of a metallo-endoproteinase from mouse kidney

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A metallo-endoproteinase was purified from mouse kidney. The enzyme was solubilized from the 100 000 g sediment of kidney homogenates with toluene and trypsin, and further purified by fractionation with $(\text{NH}_4)_2\text{SO}_4$, DEAE-cellulose chromatography and gel filtration. The molecular weight of the metalloproteinase was estimated by gel filtration on Sepharose 6B to be 270 000–320 000. On sodium dodecyl sulphate/polyacrylamide-gel electrophoresis in the presence of 2-mercaptoethanol, a single major protein with a mol.wt. of 81 000 was observed. Thus the active enzyme is an oligomer, probably a tetramer. It is a glycoprotein and has an apparent isoelectric point of 4.3. Kidney homogenates and purified preparations of the metalloproteinase degraded azocasein optimally at pH 9.5 and at I 0.15–0.2. The activity was not affected by inhibitors of serine proteinases (di-isopropyl phosphorofluoridate, phenylmethanesulphonyl fluoride), cysteine proteinases (4-hydroxymercuribenzoate, iodoacetate), aspartic proteinases (pepstatin) or several other proteinase inhibitors from actinomycetes (leupeptin, antipain and phosphoramidon). Inhibition of the enzyme was observed with metal chelators (EDTA, EGTA, 1,10-phenanthroline), and thiol compounds (cysteine, glutathione, dithioerythritol, 2-mercaptoethanol). The metalloproteinase degraded azocasein, azocoll, casein, haemoglobin and aldolase, but showed little or no activity against the synthetic substrates benzoylarginine 2-naphthylamide, benzoylglycylarginine, benzyloxycarbonylglutamyltyrosine or acetylphenylalanyl 2-naphthyl ester. This metalloproteinase from mouse kidney appears to be distinct from previously described kidney proteinases.

The considerable proteolytic capacity of extracts of mammalian kidney has been the subject of numerous investigations, and much information is available on the lysosomal cathepsins, renin and kallikrein in kidney (Barrett, 1977). Endopeptidases that have been isolated from kidney, other than the lysosomal cathepsins, generally display a high degree of substrate specificity. Accordingly, authors have suggested a distinct functional role for these kidney endopeptidases and have used, as substrates, polypeptides that are in keeping with their postulated role. Thus renin (EC 3.4.99.19) converts angiotensinogen to angiotensin and has little or no

general proteolytic activity (Hackenthal *et al.*, 1978); kallikrein (EC 3.4.21.8) liberates bradykinin and kallidin from precursors, but has little general proteolytic activity (Pisano, 1975); and a 'post-proline cleaving enzyme' has been shown to be a serine endopeptidase with limited activity towards large proteins (Koida & Walter, 1976). In addition, four metallo-endopeptidases from kidney have been described, all of which have limited or no activity towards large proteins such as haemoglobin, albumin or casein: (1) Kerr & Kenny (1974*a,b*) isolated a neutral metallo-endopeptidase (EC 3.4.24.11) from rabbit kidney brush border that hydrolyses insulin B chain and glucagon; (2) Varandani & Shroyer (1977) purified a membrane-bound metallo-endopeptidase from rat that hydrolyses insulin B chain; (3) Maruyama *et al.* (1970) described a rat microsomal metallo-endopeptidase that degrades parathyrin preferentially; and (4) Aswanikumar & Radhakrishnan (1975) purified P_z -peptidase, a

Abbreviations used: NNap, 2-naphthylamide; -ONap, 2-naphthyl ester; Pz-, 4-phenylazobenzoyloxycarbonyl; Z-, benzyloxycarbonyl; Bz, benzoyl; Ac-, acetyl; -NMec, 4-methyl-7-coumarylamide.

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monkey metalloproteinase that hydrolyses Pz-L-Pro-L-Leu-Gly-L-Pro-D-Arg, a synthetic substrate for bacterial collagenase.

During an investigation of the expression of proteolytic activity in diabetes in mouse tissues, we observed considerable proteolytic activity of kidney homogenates at alkaline pH values towards azocasein (Bond, 1980). The present paper reports the purification and characterization of one of the enzymes responsible for that activity in mouse kidney and describes important differences between this enzyme and other previously described kidney proteinases.

Experimental

Purification of the metalloproteinase

Male Balb/c Dub mice (21–26 g), obtained from Flow Research Animals, Dublin, VA, U.S.A., were given free access to water and food (Charles River Laboratory Chow; Charles River, Syracuse, N.Y., U.S.A.). Mice were killed by cervical dislocation and the kidneys were excised and dissected free of fat, adrenal glands and ureters before being weighed. Initial experiments showed that perfusion of the kidneys with 0.15 M-NaCl to remove blood had no effect on the proteolytic activity of kidney homogenates and thus perfusions were generally not performed. Kidneys were either stored frozen or homogenized immediately. Kidneys were diced and homogenized in 5 vol. of water (w/v) in a glass tissue grinder fitted with a Teflon pestle. Homogenates could be stored frozen at -20°C with no measurable loss of proteolytic activity.

Homogenates were centrifuged at 600 g for 10 min at 4°C to remove debris, and the supernatant fluid was decanted. The residue was homogenized and centrifuged as above with 2.5 vol. of water per g of original tissue and the two supernatant fractions were combined and centrifuged at 100 000 g (Beckman Ti 50.2 rotor) for 1 h at 4°C . The residue of the 100 000 g centrifugation was suspended in 5 vol. (per g of original tissue) of 20 mM-Tris/HCl, pH 7.5. Proteolytic enzymes were solubilized by the toluene/trypsin treatment described by Kerr & Kenny (1974a). $(\text{NH}_4)_2\text{SO}_4$ was then added to 20% saturation (4°C), the solution was stirred for 60 min and then centrifuged at 19 000 g for 20 min. The supernatant fluid was fractionated further to produce 20–40%-, 40–80%- and 80–90%-satd. $(\text{NH}_4)_2\text{SO}_4$ precipitates. Proteolytic activity was associated primarily with the 40–80%-satd. $(\text{NH}_4)_2\text{SO}_4$ precipitate. Samples were applied to a DEAE-cellulose (Whatman DE 52; Whatman, Clifton, NJ, U.S.A.) column (1.5 cm $\times$ 16 cm) equilibrated with 20 mM-Tris/HCl, pH 7.5, at 22°C . Eluted fractions containing proteolytic activity were pooled and concentrated with a Millipore Immers-

ible-CX ultrafilter (nominal molecular-weight exclusion limit 10 000; Millipore Corp., Bedford, MA, U.S.A.). Samples were then applied to a Sephadex G-200 (Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.) column (2.5 cm $\times$ 36.4 cm) equilibrated with 0.2 M-NaCl in 20 mM-Tris/HCl, pH 7.5, at 22°C .

Assay of proteolytic activity

Proteolytic activity was generally determined with azocasein as the substrate by monitoring at 340 nm the release of dye-containing peptides that were soluble in 4% (w/v) trichloroacetic acid (Beynon & Kay, 1978). A unit of proteolytic activity was defined as a rate of increase in A_{340} of $0.001 \cdot \text{min}^{-1}$ and was equivalent to the solubilization of $1.26 \mu\text{g}$ of azocasein $\cdot \text{min}^{-1}$.

Protein determinations

Protein content was generally determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard. For more purified preparations of the metalloproteinase, protein was determined by the method described by Kalb & Bernlohr (1977).

Molecular-weight and isoelectric-point determinations

The Sepharose 6B (Pharmacia) column (1.5 cm $\times$ 26 cm) was equilibrated with 0.2 M-NaCl in 20 mM-Tris/HCl buffer, pH 7.5, at 22°C . Flow rate of the column was $0.2 \text{ ml} \cdot \text{min}^{-1}$. The column was calibrated with rabbit muscle fructose biphosphate aldolase (mol.wt. 160 000), bovine liver catalase (mol.wt. 210 000), ferritin (mol.wt. 440 000) and thyroglobulin (mol.wt. 669 000).

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed as described by Laemmli & Favre (1973). After electrophoresis, gels were stained for carbohydrate with dansyl(5-dimethylaminonaphthalene-1-sulphonyl)hydrazine (Eckhardt *et al.*, 1976) and/or for protein with Coomassie Brilliant Blue R.

Preparative isoelectric focusing was performed in an LKB 8101 column, 120 ml volume, maintained at 4°C , with a 1% solution of LKB Ampholines, pH 3.5–10.

Studies on the specificity of the proteinase

Azocoll degradation was monitored by measuring the solubilization of material that absorbs at 580 nm; by using this procedure the specific activity of *Clostridium histolyticum* collagenase (Sigma type IV) was $17.6 A_{580} \cdot \text{h}^{-1} (\text{units}) \cdot \text{mg}^{-1}$ at pH 7 and 6.2 units/mg at pH 9. Casein or haemoglobin hydrolysis was determined by measuring the increase in trichloroacetic acid-soluble Folin-positive material (Lowry *et al.*, 1951). Fructose-bisphosphate aldolase (EC 4.1.2.13) was incubated with the metallo-

proteinase at pH 7.0 or 8.0 in 0.1 M-Tris/HCl buffer, and samples were removed at various intervals for assays of aldolase activity (Bond & Barrett, 1980). The first-order rate constant for aldolase inactivation was calculated from plots of $\log(\text{aldolase activity})$ against time. Bz-DL-Arg-Nap and Leu-Nap hydrolyses were measured as previously described (Barrett, 1977; Kirschke *et al.*, 1977), except that no cysteine or EDTA were present, the pH values were 7.0 or 9.0 rather than 6.0 and the stopping solution was prepared by using 2% (v/v) Brij 35 in 0.5 M-sodium acetate, pH 4.0, and 65 mM-HCl. The specific activities of trypsin (bovine pancreas, Sigma Type III) with Bz-Arg-Nap as substrate were 81 and $42 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ at pH 7.0 and 9.0 respectively; the limit for detection of hydrolysis of this substrate was $1 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$. Z-Glu-Tyr degradation was measured as described by Maruyama *et al.* (1970), except that 50 mM-sodium phosphate, pH 7.0, or 50 mM-sodium borate, pH 9.0, were used as buffers. Ninhydrin-positive material was assayed by the method of Rosen (1957). The limit for detection of hydrolysis of Z-Glu-Tyr was $25 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$. Bz-Gly-Arg hydrolysis was assayed as described by Folk (1970); the limit for detection of hydrolysis was $35 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$. Ac-Phe-ONap hydrolysis was assayed as described by Pacaud & Uriel (1971); the limit for detection with this substrate was $250 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$. Hydrolysis of Z-Phe-Arg-NMec (Peninsula Laboratories Inc., San Carlos, CA, U.S.A.) was assayed at pH 7.0 and 9.0 as described by Barrett (1980), except that cysteine was omitted.

Materials

Chymostatin, leupeptin and antipain were obtained from the Peptide Research Institute, Osaka, Japan. Pepstatin and phosphoramidon were generously given by Dr. A. J. Barrett (Strangeways

Laboratory, Cambridge, U.K.). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) except where noted.

Results

Purification of the metalloproteinase

The results of a typical purification procedure for mouse kidney endoproteolytic activity are summarized in Table 1. Most (at least 85%) of the proteinase activity in kidney homogenates was associated with the 100 000 g sediment prepared as described in the Experimental section. Preliminary studies indicated that the proteolytic activity could not be solubilized from the 100 000 g sediment by incubation for up to 17 h (4°C , 4 mg of protein $\cdot \text{ml}^{-1}$) in solutions of low NaCl concentration (0.005 M), high NaCl concentration (1.0 M), Triton X-100 (0.1%) or Brij-35 (0.1%) and thus harsher treatments were used. Approx. 80% of the activity was solubilized by the toluene/trypsin treatment and precipitated in the 40–80%-satd. $(\text{NH}_4)_2\text{SO}_4$ fraction (Table 1). During DEAE-cellulose chromatography, the major peak of proteolytic activity eluted between 0.05 and 0.075 M-NaCl (Fig. 1). The fractions that eluted from the DEAE-cellulose column with a proteolytic activity of more than 300 units $\cdot \text{ml}^{-1}$ were pooled and concentrated. On subsequent gel filtration on Sephadex G-200, two peaks of proteolytic activity were eluted (Fig. 2). The first, and major peak of activity was further characterized.

Physico-chemical properties

Molecular-weight determinations of the enzyme by using Sepharose 6B gel filtration yielded estimates of 270 000–320 000. The activity eluted as a single symmetrical peak from the Sepharose 6B column. In addition the enzyme contained one

Table 1. Purification of the metalloproteinase from mouse kidney

Mouse kidneys (20 g) were homogenized and fractionated as described in the Experimental section. Proteinase activity is expressed in units of azocasein hydrolysis at pH 9.5. Protein was measured by the method of Lowry *et al.* (1951) up to and including the $(\text{NH}_4)_2\text{SO}_4$ precipitation step and by the method of Kalb & Bernlohr (1977) after that step.

	Protein (mg)	Proteinase activity (units)	Specific activity (units/mg of protein)	Purification (fold)	Yield (%)
Homogenate	2936	27 028	9.2	1	100
100 000 g sediment	1041	22 846	21.9	2.4	85
Toluene/trypsin treatment and subsequent ammonium sulphate precipitation with 40–80%-satd. $(\text{NH}_4)_2\text{SO}_4$	32	18 314	572	62	68
DEAE-cellulose	4.3	10 118	2353	256	37
Sephadex G-200					
Peak I	1.3	4185	3219	350	15
Peak II	0.96	2137	2226	242	8

Fig. 1. DEAE-cellulose chromatography of toluene/trypsin-solubilized proteins from mouse kidney

A portion (14 ml) of the 40–80%-satd.-(NH₄)₂SO₄-precipitated protein (32 mg of protein) prepared after toluene/trypsin treatment as described in the Experimental section, was dialysed against 20 mM-Tris/HCl buffer, pH 7.5, and applied to a DEAE-cellulose column equilibrated with the same buffer. The column was then washed with 50 ml of equilibrating buffer and 320 ml of a linear gradient of 0–0.2 M-NaCl in equilibrating buffer. The flow rate was 27 ml·h⁻¹; 3.5 ml fractions were collected. Proteolytic activity (O) against azocasein was measured as described in the Experimental section; protein (●) was measured by the Kalb & Bernlohr (1977) method. The conductivity (....) is expressed in terms of concentrations of standard NaCl solutions. — indicates the fractions that were pooled for further purification.

Fig. 2. Gel filtration of pooled fractions eluted from the DEAE-cellulose column

Fractions from the DEAE-cellulose column that were pooled (indicated on Fig. 1) were concentrated to 4.3 ml and applied to a Sephadex G-200 column equilibrated with 20 mM-Tris/HCl buffer, pH 7.5, containing 0.2 M-NaCl. The column was eluted at the rate of 10 ml·h⁻¹; eluted fractions contained 2 ml. ●, Protein; O, proteolytic activity. — indicates fractions pooled for peaks I and II.

3); the molecular weight of that band was calculated to be 81000. A minor protein band, with mol.wt. 45000, and traces of two other bands, with mol.wts. between 45000 and 81000, were also observed. From the intensities of Coomassie Blue-stained bands at different concentrations of protein added to gels, we have estimated that the minor and trace bands together contained not more than 10% of the total protein. The major protein band stained positively for carbohydrate with dansylhydrazine (Fig. 3). Although the minor band does not show a reaction with dansylhydrazine in Fig. 3, in other gels both major and minor bands stained positively for carbohydrate. Thus the enzyme is a glycoprotein that exists as an oligomer in its active form, probably a tetramer containing identical subunits.

Isoelectric focusing of kidney proteins that had been solubilized with toluene/trypsin revealed that the metallo-endoproteinases had apparent isoelectric points of 4.3 (results not shown).

Enzymic properties

pH-dependence of activity. The enzyme degrades azocasein optimally between pH 9 and 10 (Fig. 4). The proteolytic activity of kidney homogenates was also optimal between pH 9 and 10 (results not shown).

Effects of ionic strength on metalloproteinase activity. The NaCl concentration in the reaction mixture markedly affected the proteinase activity in

major Coomassie Blue-staining band after sodium dodecyl sulphate/polyacrylamide-gel electrophoresis in the presence of 2-mercaptoethanol (Fig.

Fig. 3. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

Fractions containing peak I from the Sephadex G-200 column (Fig. 2) were pooled, dialysed against 5 mM-phosphate buffer, pH 7.0, and freeze-dried. Samples were solubilized and boiled in sodium dodecyl sulphate containing mercaptoethanol as described in the Experimental section. Track 1 contained 1.5 μ g of protein; tracks 2 and 3, 10 μ g of protein; track 4, 20 μ g of protein. Tracks 1 and 2 were stained with Coomassie Brilliant Blue R; tracks 3 and 4 were stained with dansylhydrazine. Gels were calibrated with myosin (mol.wt. 200 000), β -galactosidase (mol.wt. 130 000), phosphorylase *b* (mol.wt. 94 000), bovine serum albumin (mol.wt. 68 000), ovalbumin (mol.wt. 43 000), carbonic anhydrase (mol.wt. 30 000), soya-bean trypsin inhibitor (mol.wt. 21 000) and lysozyme (mol.wt. 14 300) (Bio-Rad Laboratories, Richmond, CA, U.S.A.).

kidney homogenates and in purified preparations (Fig. 5). Considerable stimulation of the digestion of azocasein was observed up to I 0.15–0.2. To determine whether sodium, chloride or other univalent ions specifically stimulated the proteolytic activity, reactions were performed at I 0.15 with a variety of sodium and chloride salts. No significant differences in activity were seen between the fluoride, chloride, bromide, or acetate salts of sodium. Similarly, there were no significant differences in proteolytic activity in the presence of lithium, sodium or potassium chlorides. Thus there were no specific univalent-anion or -cation effects and the stimulation of activity by NaCl appears to be due to

Fig. 4. Effect of pH on activity

Proteolytic activity was measured by using 1.5 μ g of the enzyme and 11 mg of azocasein in a final volume of 1.1 ml, at 37°C. The ionic strength of the assay mixture was maintained with NaCl at 0.15. The buffers used were: 20 mM-maleic acid/NaOH, (○); 20 mM-Tris/HCl (●); 20 mM-ethanolamine/HCl (Δ). Portions of the incubation mixture were removed at 0, 0.5, 1.0 and 1.5 h and proteolytic activity was measured as described in the Experimental section.

the ionic strength of the reaction mixture. CaCl_2 and MgCl_2 at I 0.14 stimulated activity similarly to the univalent salts. Enzyme activity was routinely assayed at a final ionic strength of 0.15 [20 mM-ethanolamine/HCl (pH 9.5)/0.14 M-NaCl] with 10 mg of azocasein $\cdot \text{ml}^{-1}$. The apparent K_m of the enzyme for azocasein under these conditions was 1.5 mg $\cdot \text{ml}^{-1}$.

Effects of potential inhibitors on activity. Several inhibitors were tested for their ability to diminish the alkaline-proteinase activity of homogenates and of purified preparations (Table 2). The proteolytic activity in kidney homogenates showed the same sensitivity to inhibitors as did purified preparations. These results indicated that the enzyme was a metalloproteinase.

Thiols, such as 2-mercaptoethanol, cysteine, dithioerythritol and reduced glutathione, were effective inhibitors of the kidney proteinase (Table 2 and Fig. 6). The thiol group was essential for the inhibitory effect, since cystine and oxidized glutathione were without effect.

Substrate specificity. The metalloproteinase was

Fig. 5. Effect of NaCl concentration on proteolytic activity

Proteolytic activity was measured as described in the Experimental section, except that the NaCl concentration in the incubation mixture was varied as indicated. Kidney homogenates were used as the source of proteolytic activity. Proteolytic activities of homogenates and the purified preparation had similar responses to NaCl concentration.

Fig. 6. Effect of thiol compounds on metalloproteinase activity

Kidney homogenates were assayed for proteolytic activity against azocasein, at pH 9.5, in the presence of indicated concentrations of 2-mercaptoethanol (○), dithioerythritol (□), cysteine (●), reduced glutathione (△), cysteine (■), and oxidized glutathione (▲). Results of a typical experiment are shown and expressed as a percentage of the activity measured in the absence of thiols.

active against several protein substrates in addition to azocasein, including azocoll, casein, haemoglobin and aldolase (Table 3). In general, it was more active at pH 9 than 7; however, the ratios of the activities at the two pH values varied with the substrate. No activity was observed against Bz-Arg-NNap, a trypsin substrate, or Ac-Phe-ONap, a chymotrypsin substrate. It also failed to hydrolyse the carboxypeptidase substrates Z-Glu-Tyr and Bz-Gly-Arg. Activity against the aminopeptidase substrate Leu-Nap and the cathepsin B substrate Z-Phe-Arg-NMec was observed; however, it is possible these activities are due to contaminating proteins in the preparation.

Discussion

The proteolytic activity of mouse kidney homogenates at pH 9.5 can be largely attributed to membrane-bound metalloproteinases. This conclusion was reached on the basis of the observations that (a) most of the proteolytic activity was associated with particulate fractions from which it could be solubilized by toluene/trypsin treatment but not readily by detergents or by hypo- or hyper-

osmotic salt solutions, and (b) the activity was inhibited by compounds that typically inhibit metalloenzymes (EDTA, EGTA, 1,10-phenanthroline, thiols) but not by inhibitors of serine, cysteine and aspartic proteinases. There are at least two alkaline proteinases in kidney homogenates that can be separated by gel filtration on a Sephadex G-200 column. Comparisons of the properties of the activity investigated and the proteolytic activity associated with peak II of the Sephadex column indicate that the two alkaline proteinases in kidney are very similar with respect to pH optima, response to ionic strength and inhibitors, and behaviour on DEAE-cellulose, isoelectric focusing and hydroxyapatite columns.

The response of the enzyme to potential inhibitors of proteinases was generally typical of metalloproteinases (Barrett, 1977). It is noteworthy that phosphoramidon did not inhibit this enzyme because it is a very powerful inhibitor of several metalloproteinases such as thermolysin and the well-characterized metallo-endopeptidase from rabbit kidney (Kenny, 1977). The sample of phosphoramidon used in the present study was judged to be authentic in

Table 2. *Effect of potential inhibitors on proteolytic activity in homogenates and on purified preparations*

Kidney homogenates or purified preparations of the metallo-endoproteinase were preincubated with the potential inhibitor at the concentrations shown. For inhibitors of serine and cysteine proteinases, preincubations were for 3 h at 37°C, and 0.2 ml portions were added to azocasein incubation mixtures; for all other inhibitors, preincubation was for 9 min and 0.6 ml portions were added to azocasein incubation mixtures. Proteinase incubation mixtures had a total volume of 1.1 ml in ethanolamine/HCl buffer, pH 9.5, at a final ionic strength of 0.15. Abbreviation used: n.d., not determined.

Inhibitor	Concn. (mm)	Inhibition (%)	
		Homogenate	Purified enzyme
None	—	0	0
Inhibitors of serine endopeptidases			
Di-isopropyl phosphorofluoridate	2.0	0	n.d.
Phenylmethanesulphonyl fluoride	2.0	0	0
Inhibitors of cysteine endopeptidases			
Iodoacetate	1.0	0	0
4-Hydroxymercuribenzoate	1.0	0	0
Phenylmercurisulphonate	1.0	0	n.d.
Inhibitors of trypsin-like enzymes			
Soya-bean trypsin inhibitor	0.008	0	0
Benzamidine	1.0	0	0
Inhibitors of metalloproteinases			
EDTA	0.1	0	10
	1.0	25	90
	10.0	88	100
1,10-Phenanthroline	0.1	0	5
	1.0	65	72
	10.0	96	100
EGTA	1.0	n.d.	85
2-Mercaptoethanol	1.0	38	95
L-Cysteine	1.0	44	60
Other proteinase inhibitors	($\mu\text{g/ml}$)		
Pepstatin	27	0	0
Leupeptin	26	0	0
Antipain	42	0	0
Chymostatin	28	29	20
Phosphoramidon	0.00054–540	0	0

that, at a concentration of $10\mu\text{M}$, phosphoramidon totally inhibited thermolysin digestion of azocasein at pH 7.0 or 9.5. The inhibition of the enzyme by chymostatin is also notable in that this compound generally inhibits chymotrypsin-like proteinases. Chymostatin is actually a mixture of compounds that are not presently available at very high purity. Thus the significance of the partial inhibition observed with relatively high concentrations of chymostatin is not clear at present.

The enzyme differs from previously described kidney metalloproteinases with regard to several parameters including substrate specificity, pH optimum and specific inhibitors. For instance, (1) the metallo-endopeptidase from rabbit kidney isolated by Kerr & Kenny (1974a) does not attack proteins (insulin, albumin, casein) and it has optimal activity, with insulin B-chain as substrate, at pH 6. Also, as mentioned previously, the rabbit metallo-endopeptidase is inhibited by phosphoramidon and the present enzyme is not. (2) The metallo-endopep-

tidase from rat kidney that degrades the insulin B-chain, purified by Varandani & Shroyer (1977) has weak or no activity towards proteins (denatured haemoglobin, albumin, insulin, ribonuclease) and a pH optimum of 6.5. This enzyme is reportedly inhibited by phosphate (Phelps *et al.*, 1979). The inhibition by phosphate may, however, be a consequence of high ionic strength, as it can be calculated that the inclusion of 0.5 M-potassium phosphate in the assay was sufficient to raise the ionic strength from approx. 0.08 to 1.1. A specific phosphate effect may therefore be unlikely in view of the results described here. Another difference between the rat and mouse metalloproteinase is that the rat kidney activity decreases in response to diabetes (Phelps *et al.*, 1979), whereas the mouse kidney activity is not affected by the disease (Bond, 1980). (3) The metallo-endopeptidase from rat kidney that preferentially hydrolyses parathyrin, described by Maruyama *et al.* (1970), has some activity towards protein substrates but, in contrast with the

Table 3. *Substrate specificity*

Substrates were incubated at 37°C with the enzyme ($10\mu\text{g}\cdot\text{ml}^{-1}$) in 0.1M-sodium phosphate buffer, pH 7.0, 0.1M-ethanolamine/HCl, pH 9.0, or 0.1M-sodium borate buffer, pH 9.0, except where noted in the Experimental section. The specific activities of the enzymes are expressed in the following units: for azocasein, μg hydrolysed $\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$; for azocoll, $\Delta A_{280}\cdot\text{h}^{-1}\cdot\text{mg}^{-1}$; for casein and haemoglobin, μg of tyrosine equivalents $\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$; for aldolase, first-order rate constant for inactivation/amount of proteinase ($\text{min}^{-1}\cdot\text{mg}^{-1}$); for the synthetic substrates, nmol hydrolysed $\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$. Abbreviation used: n.d., not determined.

Substrate (concn.)	Rate of hydrolysis of substrate	
	pH 7	pH 9
Azocasein (11 mg/ml)	394	820
Azocoll (5 mg/ml)	6.3	8.1
Casein (8 mg/ml)	20.4	30.5
Haemoglobin (8 mg/ml)	7.4	9.3
Aldolase (0.89 mg/ml)	0.18	0.19*
Bz-Arg-Nap (5 mM)	0	0
Ac-Phe-ONap (3 mM)	0	n.d.
Z-Glu-Tyr (1 mM)	0	0
Bz-Gly-Arg (1 mM)	n.d.	0*
Leu-NNap (5 mM)	22	12.1
Z-Phe-Arg-NMec (5 μM)	15.9	56.8

* Determined at pH 8.0

mouse enzyme, also hydrolyses Z-Glu-Tyr and Bz-Arg-NNap. In addition, the parathyrin hydrolysing enzyme has a broad pH optimum, pH 7–10, is partially inhibited by 4-hydroxymercuribenzoate and iodoacetate and is activated by Ca^{2+} . (4) The metallopeptidase from monkey kidney (P_z -peptidase), described by Aswanikumar & Radhakrishnan (1975), hydrolyses a synthetic substrate for bacterial collagenase, but has no activity against azocasein, clearly distinguishing it from the present enzyme.

The molecular properties of the enzyme also seem to differ from previously described kidney metallo-endopeptidases. The rabbit kidney endopeptidase is a monomer with a mol.wt. of 93 000 (Kerr & Kenny, 1974b), the rat kidney endopeptidase has a mol.wt. of 213 000 (Varandani & Shroyer, 1977), the rat parathyrin-hydrolysing enzyme has a mol.wt. of 800 000 (Maruyama *et al.*, 1970), the monkey peptidase a mol.wt. of 56 000 (Aswanikumar & Radhakrishnan, 1975), whereas the mouse enzyme has a mol.wt. of 270 000 to 320 000 and a subunit of 81 000. All the kidney metallo-endopeptidases, on the other hand, required harsh treatments to solubilize the enzyme from membranes and thus they all seem to be integral parts of membranes. The mouse enzyme and the rabbit kidney endopeptidase described by Kerr & Kenny (1974b) are glycoproteins. Whereas the previously described

kidney metallo-endopeptidases seem to be uniquely suited to hydrolyse specific compounds, especially peptide hormones, the present enzyme seems to be capable of degrading many proteins and may, therefore, play a role in protein degradation.

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