The Molecular Weight and Properties of a Neutral Metallo-Endopeptidase from Rabbit Kidney Brush Border

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1. Some properties of a brush-border neutral endopeptidase purified from rabbit kidney were investigated. The peptidase was assayed by its ability to hydrolyse [¹²⁵I]iodoinsulin B chain. 2. The enzyme was found to be homogeneous when studied in the analytical ultracentrifuge and stained as a single glycoprotein band after electrophoresis in polyacrylamide gels. 3. The molecular weight was estimated by gel filtration in columns of Sephadex G-200, by polyacrylamide-gel electrophoresis in the presence of 2-mercaptoethanol and sodium dodecyl sulphate and by sedimentation equilibrium in the ultracentrifuge. The estimates fell within the range 87000–96000. The mean from two sedimentation equilibrium experiments was 93000, though this estimate may be slightly inflated because of the carbohydrate component of the enzyme. No evidence of dissociation into smaller subunits was obtained in the presence of thiol, sodium dodecyl sulphate or guanidine hydrochloride. 4. The endopeptidase was maximally active at pH 6.0, although in phosphate buffer, which was strongly inhibitory, an optimum above pH 8 was observed. 5. The enzyme was not affected by di-isopropyl phosphofluoridate nor by several thiol reagents. It was, however, strongly inhibited by many thiols and by EDTA and other chelating agents. 6. Although activity of the EDTA-treated enzyme could be partially restored by various bivalent metal ions, the optimum concentration for its reactivation by Zn²⁺ was lower than that for other ions. This metal was detected in the enzyme preparation by atomic absorption spectrophotometry in an amount equivalent to approximately one atom/mol. 7. The enzyme is the only endopeptidase shown to be located in the kidney brush border and is the first mammalian example of a neutral Zn²⁺-activated endopeptidase to be characterized.

The preceding paper (Kerr & Kenny, 1974) described the purification of a neutral endopeptidase from rabbit kidney. The enzyme was shown to attack peptide bonds involving the amino function of hydrophobic amino acids, provided that the residue was not C-terminal. The enzyme therefore exhibited no dipeptidase activity but was capable of hydrolysing tripeptides, oligopeptides and polypeptides, including insulin B chain. The endopeptidase was assayed as a routine by its ability to release trichloroacetic acid-soluble radioactivity when incubated with [¹²⁵I]iodoinsulin B chain as substrate. This provides a convenient and reproducible assay even though the release of radioactivity from this substrate may not exactly parallel the hydrolysis of peptide bonds. In the present paper, we report on some of the properties of the purified enzyme. The effects of inhibitors and activators were generally similar to those observed with membrane-bound preparations of the peptidase that had been investigated in microsomal fractions (Wong-Leung & Kenny, 1968) and in purified preparations of kidney brush border (George & Kenny, 1973). This enzyme, which is the only endopeptidase so far identified in kidney brush border, appears

to belong to a class of neutral metal-chelator-sensitive peptidases comparable in specificity and metal ion-dependence with certain microbial proteases typified by thermolysin.

Methods

The neutral endopeptidase

The preparation of the purified enzyme and the assay with [¹²⁵I]iodoinsulin B chain as substrate have been described (Kerr & Kenny, 1974). The amount of enzyme was adjusted in each experiment so that the response fell on the linear part of the curve relating enzyme concentration to the percentage release of trichloroacetic acid-soluble radioactivity.

Effect of inhibitors and activators. Unless otherwise stated, all effectors were preincubated with the enzyme at 37°C for 30 min in 0.2 M-Tris-HCl, pH 7.0, in a volume of 0.4 ml. The substrate (0.1 ml) was then added and the assay mixture incubated for a further 15 min.

Assay of trypsin and papain. These enzymes were assayed fluorimetrically with benzoylarginine 2-naphthylamide as substrate. The incubation mixture

(3 ml) contained 0.2 mM substrate and 0.1 M-Tris-HCl, pH 8.0. The increase in fluorescence at 410 nm (excitation at 310 nm) was followed in an Aminco Bowman spectrophotofluorimeter model SP. 125 equipped with a thermoconstant cell compartment and chart recorder.

Gel filtration by Sephadex G-200

The molecular weight of the native enzyme was determined by gel filtration in columns (90 cm $\times$ 2.5 cm) of Sephadex G-200 (Andrews, 1965). The elution buffer contained 5 mM-Tris-HCl, pH 7.0. Fractions (10 ml) were collected. Blue Dextran 2000 was the marker for the void volume. Other protein markers, molecular weight shown in parentheses, were β -galactosidase from *Escherichia coli* (520 000), lactate dehydrogenase from rabbit muscle (135 000), aminopeptidase M from rabbit kidney (136 000; Kenny *et al.*, 1969; George & Kenny, 1973), bovine serum albumin (68 000), alkaline phosphatase from *E. coli* (78 000), chicken ovalbumin (45 000), horse heart cytochrome *c* (12 400) and insulin B chain (3500). Unless otherwise noted, the molecular-weight values and methods of detection of the marker are those of Andrews (1965).

Sodium dodecyl sulphate-polyacrylamide-gel electrophoresis

The endopeptidase was incubated at 0°C for 2 h with 1% (w/v) sodium dodecyl sulphate and 1% (w/v) 2-mercaptoethanol. The sample was then diluted 10-fold and applied to 10% polyacrylamide-0.15% methylenebisacrylamide-0.1% sodium dodecyl sulphate gels as described by Weber & Osborn (1969). Marker proteins (subunit molecular weights taken from Weber & Osborn, 1969) covered a range of 12 000-136 000.

Analytical ultracentrifugation

This was carried out in a Beckman Model E analytical ultracentrifuge equipped with rotor temperature indication and control facilities. Both schlieren and interference optics were available. For sedimentation-velocity studies the native enzyme was in a medium containing 5 mM-Tris-HCl buffer, pH 7.0, 0.15 M-KCl (η_{rel} 1.030 and ρ 1.010). Sedimentation coefficients were determined as described by Chervenka (1969). The Yphantis (1964) method for determination of the molecular weight by meniscus depletion sedimentation equilibrium was carried out as described by Chervenka (1969). Two samples of the purified endopeptidase were diluted to approximately 0.5 mg/ml in 5 mM-Tris-HCl buffer, pH 7.0, 0.15 M-KCl. Each was subjected to high-speed centrifugation in an AnD rotor fitted with a 12 mm double sector cell with interference window

holders, sapphire windows and aluminium-filled Epon double sector centrepiece. The first sample was run at 21 740 rev./min, the second at 23 150 rev./min. Sedimentation equilibrium was also carried out in guanidine hydrochloride. A sample of the purified enzyme was diluted in the same Tris-KCl buffer containing 6 M-guanidine hydrochloride and 0.1% (w/v) 2-mercaptoethanol. The solution was then dialysed exhaustively against the same buffer-guanidine-mercaptoethanol solution before centrifugation at a slightly higher speed (24 640 rev./min) to compensate for the greater viscosity of the medium.

Determination of carbohydrate

Gels were stained for glycoproteins by the method of Zacharius *et al.* (1969) with basic Fuchsin after periodate oxidation. Total hexose was determined on the purified enzyme by Dubois' phenol-H₂SO₄ method (Ashwell, 1966) with glucose as standard.

Determination of Zn, Ca, Co and Mn

These elements were determined by atomic absorption spectrophotometry by using either a Unicam SP. 90 Series 2, or a Perkin-Elmer 303 instrument.

Results

Molecular-weight determinations

Three distinct methods (involving gel filtration, gel electrophoresis in the presence of sodium dodecyl sulphate and the analytical ultracentrifuge) were used to estimate the molecular weight of the purified endopeptidase.

Two columns, prepared from the different batches of Sephadex G-200 and calibrated with various markers of known molecular weights were run. Although slight variations in elution volumes were found in the two experiments, the position of the neutral peptidase on the two curves corresponded to molecular weights of 87 000 and 95 500.

Electrophoresis in sodium dodecyl sulphate-polyacrylamide gels was performed in continuous buffer system. In each of the gels the endopeptidase migrated as a single protein band of low mobility in a position intermediate between bovine serum albumin (68 000) and β -galactosidase (130 000). From the curve relating mobility and subunit molecular weight of a number of marker proteins a value of 93 000 was obtained. This strongly suggests that the enzyme was not dissociated into subunits as a result of treatment with 2-mercaptoethanol and sodium dodecyl sulphate.

The sedimentation coefficient (*s*) was determined at four different enzyme concentrations. In each run the protein sedimented as a single symmetrical peak over the whole length of the cell. Photographs

Fig. 1. Ultracentrifuge pattern of the neutral endopeptidase in sedimentation velocity experiments

See the Methods section for details. The photographs were taken at phase plate settings of 60° and above. Sedimentation is from left to right.

Fig. 2. Concentration-dependent sedimentation velocity of the neutral endopeptidase

See the Methods section for details.

from one run are shown in Fig. 1. s_{20} values were obtained from these four runs. When corrected for density and relative viscosity the $s_{20,w}$ values, so obtained, were plotted as shown in Fig. 2 to give an $s_{20,w}^0$ value of 5.77S.

Two samples of the endopeptidase were subjected to sedimentation equilibrium. In each case a depletion of the meniscus was observed and plots of logarithm (fringe-displacement) against (radial distance)² were found to be linear (Fig. 3). From these data, and by using a partial specific volume ($\bar{v}$ value) of 0.725 (Bowen, 1970), the molecular weight was estimated to be 90100 and 95600 (mean 93000). Another sample of the endopeptidase was treated with 6M-guanidine hydrochloride and 0.1% 2-mercaptoethanol and the sedimentation-equilibrium run repeated. By assuming no change in the value of $\bar{v}$ and by calculating the density of the medium to be 1.15 (Kawahara & Tanford, 1966), a value of 96300 was obtained for the molecular weight in this system

Fig. 3. Sedimentation equilibrium of the neutral endopeptidase

Three different samples of the purified neutral endopeptidase were subjected to sedimentation equilibrium as described in the Methods section. Curves (I) and (II) (●) were obtained at rotor speeds of 21740 rev./min and 23150 rev./min respectively. Curve (III) (○) was from a sedimentation-equilibrium experiment in the presence of guanidine hydrochloride and 2-mercaptoethanol at a rotor speed of 24640 rev./min.

(curve III in Fig. 3). If one assumes that the partial specific volume, $\bar{v}$, was lowered by 0.015 in the presence of 6M-guanidine hydrochloride (Hade & Tanford, 1967) the molecular weight is 87200. Both estimates indicate a protein comprising a single polypeptide chain that was not further dissociated by guanidine hydrochloride and 2-mercaptoethanol.

Carbohydrate content of the endopeptidase

The purified endopeptidase migrated as a single protein band on disc electrophoresis in polyacrylamide gel (Kerr & Kenny, 1974). When similarly run gels were stained with basic Fuchsin after periodate oxidation the endopeptidase gave a strongly positive reaction for carbohydrate. Three preparations of the purified enzyme were also analysed for hexose by the phenol- H_2SO_4 method. These determinations gave values of 66, 190 and 240 (mean 165) $\mu\text{g}/\text{mg}$ of protein.

pH-activity curves and general ionic effects

The activity of the endopeptidase was found to vary in different buffer mixtures. The responses were generally similar to those observed with a crude kidney microsomal fraction (Wong-Leung & Kenny, 1968). Because of these buffer ion effects, the most reliable

Fig. 4. pH-activity curves of the neutral endopeptidase

One curve (●) was obtained in a pH-stat (Radiometer Autotitrator, 72, Emdrupvej, Copenhagen, Denmark). The incubation mixture (1.0ml) contained 25 μ g of [125 I]iodoinsulin B chain and 0.25M-NaCl. The initial pH was adjusted with dilute NaOH or HCl before addition of the enzyme (0.25ml) and the pH maintained by the autotitrator with 10mm-NaOH. After 15min of incubation at 37°C, samples (0.5ml) were removed and treated as in the standard assay. In the other curves the standard assay was followed except for a change in buffer (the concentrations noted were those in the incubation mixture): ○, 0.12M-Tris-HCl; ■, Pipes-NaOH buffer; △, McIlvaine's citric acid- Na_2HPO_4 buffer (Dawson *et al.*, 1969); ▲, 0.1M- Na_2HPO_4 - NaH_2PO_4 buffer (Dawson *et al.*, 1969). The peptidase activities are shown as a percentage relative to that obtained under the usual assay conditions, 0.15M-Tris-HCl, pH 7.0.

estimate of the pH optimum was that obtained in a series of incubations in which the pH was controlled by a pH-stat. Tris-HCl buffer was present in the stock enzyme solution, but the concentration did not exceed 1mM in the incubation mixture; 0.2M-NaCl provided the main ionic component. The pH-activity curve showed a maximum at pH 6.0 (Fig. 4). Other curves obtained over limited pH ranges with Tris-HCl buffer and Pipes buffer [piperazine-*NN'*-bis-(2-ethanesulphonic acid)] indicated maxima that were not above this value. A curve obtained with sodium citrate-phosphate mixtures showed an optimum at or above pH 8.0. The phosphate-citrate buffer was strongly inhibitory, the maximum activity being less than 25% of that found in optimum conditions with Tris-HCl buffer.

The activity was influenced by the ionic strength of the assay medium. In Tris-HCl buffer, pH 7.0, the activity was increased by about 50% over the range 1–120mM. In the presence of 1mM-Tris-HCl, the addition of NaCl over the range 0–120mM increased the activity by about 35%. Some activation by 100mM-Tris-HCl was observed even in the presence of 200mM-NaCl.

Stability

The neutral peptidase is stable for long periods in both crude and purified states. When stored at -5°C in 50mM-Tris-HCl buffer, pH 7.0, it lost little activity over a period of several months. The enzyme was, however, heat-labile. Half of the activity was lost in 15min at 50°C and after 1min at 60°C . The presence of Ca^{2+} at 1 or 10mM concentration did not increase the heat stability of the enzyme.

Di-isopropyl phosphofluoridate

Incubation with di-isopropyl phosphofluoridate at either 100 or 10 μM for periods up to 3h had no effect on the neutral endopeptidase. Trypsin under the same conditions was completely inhibited. This excludes the kidney endopeptidase from the serine group of proteases (Hartley, 1960).

Thiol reagents

The neutral peptidase was insensitive to several inhibitors of thiol enzymes. For example, iodoacetate (1–10mM), *p*-hydroxymercuribenzoate (0.1–4mM) and *N*-ethylmaleimide (0.1–1mM) had no effect. Papain, a typical thiol peptidase, incubated under the same conditions was strongly inhibited by these reagents.

Thiol compounds

Several thiols were strongly inhibitory to the endopeptidase (Fig. 5). These, in decreasing order of effectiveness, were dithiothreitol, cysteine, 2,3-dimercaptopropanol (British anti-Lewisite), GSH, 2-mercaptoethanol and thioglycollate. GSSG was not inhibitory. It seems probable that the effect depended on the chelating properties of this group of reagents rather than on their ability to reduce protein disulphide bonds.

Inhibition by chelating agents and reactivation by metal ions

The crucial importance of a divalent metal ion for the activity of the enzyme was demonstrated by the inhibitory effects of many chelating agents. The inhibition curves are shown in Fig. 6. EDTA, 1,10-phenanthroline and dithizone gave similar pK_i values (near to 4) although the slopes of the three curves differed. $\alpha\alpha$ -Bipyridyl, KCN, imidazole and P_i gave pK_i values of 3, 2.1, 0.9 and 0.6 respectively (pK_i is used here in an operational sense and is the negative logarithm of the inhibitor concentration causing 50% inhibition).

The EDTA-inhibited enzyme did not regain activity when the EDTA was removed by dialysis. However, activity was partially restored by preincubation

Fig. 5. Inhibition of the neutral endopeptidase by thiols

See the Methods section for details. The concentrations shown are those in the preincubation mixture. Each point is the mean of two experiments. ●, Dithiothreitol; ○, cysteine; ▲, 2,3-dimercaptopropanol; △, 2-mercaptoethanol; ■, GSH; □, thioglycollate; ▼, GSSG.

Fig. 6. Inhibition of the neutral endopeptidase by chelating agents

See the Methods section for details. The concentrations shown are those in the preincubation mixture. Each point is the mean of at least two experiments; those for EDTA, 1,10-phenanthroline and dithizone are the means of four experiments. ●, EDTA; ○, 1,10-phenanthroline; ▲, dithizone; △, $\alpha\alpha$ -bipyridyl; ■, KCN; □, imidazole; ▼, P_1 .

of the inhibited, dialysed enzyme with various metal ions (Fig. 7). The concentration-activity curves exhibited a variety of forms. Four metal ions were capable of causing substantial reactivation. One of these, Zn^{2+} , showed a sharp maximum at $10\mu M$, two others, Mn^{2+} and Co^{2+} plateaued at $0.1mM$, whereas Cu^{2+} showed no maximum up to $1mM$

Fig. 7. Reactivation of the EDTA-treated enzyme by metal ions

The endopeptidase (10 units) was preincubated (1.1 ml) at $37^\circ C$ for 30 min in 5 mM-Tris-HCl buffer, pH 7.0, and 10 mM-EDTA. EDTA was then removed by dialysis against the buffer for 5 h, with a change of buffer at each hour (final vol. 2 ml). The treated enzyme was then diluted 10-fold and samples (0.1 ml) were incubated for 30 min at $37^\circ C$ with 0.3 ml of 250 mM-Tris-HCl buffer, pH 7.0, containing metal ions at the appropriate concentrations. After this preincubation 0.1 ml of [^{125}I]iodoinsulin B chain was added and the peptidase activity determined. ●, $ZnSO_4$ (4); ○, $MnSO_4$ (4); ▲, $CoSO_4$ (4); △, $NiSO_4$ (4); ■, $CaCl_2$ (2); □, $MgCl_2$ (2); ▼, $CuSO_4$ (2); ▽, $HgCl_2$ (2). The number of experiments are shown in parentheses. The results are expressed as the activity (%) relative to that of the enzyme before EDTA treatment. The activity after EDTA and dialysis was in the range 5–10% in different experiments.

concentration. Although Zn^{2+} only partially reactivated the enzyme (55%) it was unique in that the optimum concentration was close to $10\mu M$. No other metal ion was as effective at this low concentration, and, unlike the other reactivating metal ions, Zn^{2+} was strongly inhibitory at concentrations above $30\mu M$.

Effect of metal ions on the native enzyme

Although several metal ions were capable of partially restoring activity to the EDTA-inhibited enzyme, none was found to activate the untreated peptidase when assayed in the usual Tris-HCl buffer. Several bivalent metal ions were strongly inhibitory (Fig. 8); these included Zn^{2+} , Ni^{2+} , Hg^{2+} , Cu^{2+} and Ca^{2+} , but Mn^{2+} and Co^{2+} had little or no effect on activity.

Fig. 8. Effect of metal ions on the untreated neutral endopeptidase

See the Methods section for details. ●, ZnSO_4 ; ○, MnSO_4 ; ▲, CoSO_4 ; △, NiSO_4 ; ■, CaCl_2 ; □, MgCl_2 ; ▼, CuSO_4 ; ▽, HgCl_2 .

Metal content of the purified endopeptidase

The sensitivity of the enzyme to chelating agents and the ability of four bivalent metals (Zn^{2+} , Ca^{2+} , Co^{2+} and Mn^{2+}) to reactivate the inhibited enzyme made it desirable to analyse the native enzyme for these metals. Since the purified enzyme withstood prolonged dialysis without loss of activity, it was clear that any such metal ion was firmly bound to the protein. Analyses were performed by atomic absorption spectrophotometry. No detectable amounts of Ca, Co or Mn were present. On the other hand, Zn was present in significant amounts. Two different preparations of the purified enzyme, analysed in triplicate, gave values of 0.75 and 0.80 μg of Zn/mg of protein respectively. By assuming a molecular weight of 93000, this is equivalent to a mean of 1.1 g-atom of Zn/mol.

Discussion

The ultracentrifuge data confirm the high degree of purity of the enzyme. In sedimentation velocity runs, it sedimented as a single symmetrical peak and showed only a very slow rate of diffusion. In sedimentation equilibrium studies, the linearity of the plot of the logarithm (fringe displacement) against (radial distance)² also serves to support this conclusion (Bowen, 1970). The estimates of the molecular weight of the native enzyme in the ultracentrifuge and by gel filtration were in the range 87000–95600. No evidence of dissociation into

subunits was obtained by gel electrophoresis after treatment with 2-mercaptoethanol and sodium dodecyl sulphate and sedimentation equilibrium in a medium containing a thiol and 6M-guanidine hydrochloride. The enzyme is therefore a single polypeptide chain of about 93000 molecular weight.

The presence of carbohydrate in the purified enzyme was demonstrated by the staining reaction of the protein band on polyacrylamide gels and shown to be about 15% by analysis of three samples of the enzyme. Glycoproteins, because of their asymmetric or expanded structures, compared with globular proteins, may give anomalous estimates of molecular weight. In particular, the sedimentation characteristics of proteins in the ultracentrifuge are altered because the partial specific volume ($\bar{v}$) is decreased by the carbohydrate component. A value, typical of a globular protein, of 0.725 ml/g was assumed in our calculation. In a few instances, $\bar{v}$ has been determined for known glycoproteins. Thus caeruloplasmin (7% carbohydrate) and enterokinase (37% carbohydrate) have $\bar{v}$ values of 0.714 and 0.705 respectively (Morell *et al.*, 1969; Baratti *et al.*, 1973). If an interpolated value of 0.711 is assumed for the endopeptidase a molecular weight of 88000 rather than 93000 is obtained.

The finding that the endopeptidase is a glycoprotein should occasion no surprise, having regard to its origin in the microvillus membrane. Several other brush-border enzymes are known to be glycoproteins. γ -Glutamyl transpeptidase and aminopeptidase M each contain about 20% carbohydrate (Szewczuk & Baranowski, 1963; Orłowski & Meister, 1965; Wacker *et al.*, 1971). There is evidence too, that human kidney alkaline phosphatase contains sialic acid (Butterworth, 1966). No doubt there are others, since Glossmann & Neville (1971) could resolve 11 glycoprotein bands from rat kidney brush border by electrophoresis in sodium dodecyl sulphate–polyacrylamide gels. The carbohydrate component of the glycoproteins of membranes is orientated towards the outer surface (for review see Parsons & Subjeck, 1972). A glycocalyx, analogous to that on the surface of intestinal microvilli (Ito, 1965), can be demonstrated with carbohydrate-staining techniques on the surface of microvilli from the kidney proximal tubule (Groniowski *et al.*, 1969). From this it may be inferred that the endopeptidase is orientated in the microvillus membrane such that the glycopeptide region is exposed at the surface. Other regions of the molecule may penetrate into the hydrophobic region of membrane, perhaps more deeply than an enzyme such as aminopeptidase M which is very readily released from the membrane by papain (George & Kenny, 1973).

If the presence of carbohydrate is one feature common to several brush-border enzymes, the presence of Zn may well be another. The neutral

peptidase is clearly a metallo-enzyme from its inhibition by a variety of metal chelating agents. The special role of Zn was established by the partial reactivation of the inhibited enzyme at relatively low concentrations of Zn^{2+} and by the demonstration of about one atom of Zn/mol of enzyme. Aminopeptidase M is also a Zn metallo-enzyme. Wacker *et al.* (1971) found two atoms of Zn/mol of pig kidney aminopeptidase M (mol.wt. 280000). There is some evidence, too, that kidney alkaline phosphatase contains Zn (Mathies, 1958), but there seems to be little information about the role of Zn in other brush-border enzymes.

The kidney endopeptidase resembles a group of microbial proteases referred to as the metal chelator-sensitive neutral proteases (EC 3.4.24.4) (Matsubara & Feder, 1971). The resemblance in the specificity of the bond cleavage of insulin B chain has been pointed out (Kerr & Kenny, 1974). It is now apparent that the similarity extends to the presence of Zn at the catalytic site, which is also a common feature of the group. Thermolysin, for example, contains one atom of Zn/mol, which is essential for activity, together with four atoms of Ca, necessary for thermostability (Matthews *et al.*, 1972). Although the brush-border endopeptidase contains no Ca, it is closely analogous to this group in other respects and is the first mammalian example of a Zn-activated endopeptidase to be characterized.

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