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Characterization of meprin, a membrane-bound metalloendopeptidase from mouse kidney

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Meprin is an intrinsic protein of the brush border, a specialized plasma membrane, of the mouse kidney. It is a metalloendopeptidase that contains 1 mol of zinc and 3 mol of calcium per mol of the 85000-M_r subunit. The enzyme is isolated, and active, as a tetramer. The behaviour of the enzyme on SDS/polyacrylamide gels in the presence and absence of β-mercaptoethanol indicates that the subunits are of the same M_r (approx. 85000) and held together by intersubunit S—S bridges. Eight *S*-carboxymethyl-L-cysteine residues were detected after reduction of the enzyme with β-mercaptoethanol and carboxymethylation with iodoacetate. The enzyme is a glycoprotein and contains approx. 18% carbohydrate. Most of the carbohydrate is removed by endoglycosidase F, indicating that the sugar residues are *N*-linked. The isoelectric point of the enzyme is between pH 4 and 5, and the purified protein yields a pattern of evenly spaced bands in this range on isoelectric focusing. The peptide-bond specificity of the enzyme has been determined by using the oxidized B-chain of insulin as substrate. In all, 15 peptide degradation products were separated by h.p.l.c. and analysed for their amino acid content and *N*-terminal amino acid residue. The prevalent peptide-bond cleavages were between Gly^{20} and Glu^{21}, Phe^{24} and Phe^{25} and between Phe^{25} and Tyr^{26}. Other sites of cleavage were Leu^6–Cysteic acid^7, Ala^{14}–Leu^{15}, His^{10}–Leu^{11}, Leu^{17}–Val^{18}, Gly^8–Ser^9, Leu^{15}–Tyr^{16}, His^5–Leu^6. These results indicate that meprin has a preference for peptide bonds that are flanked by hydrophobic or neutral amino acid residues, but hydrolysis is not limited to these bonds. The ability of meprin to hydrolyse peptide bonds between small neutral and negatively charged amino acid residues distinguishes it from several other metalloendopeptidases.

INTRODUCTION

Proteinases as a group are considered to be among the best-characterized enzymes because of the extensive kinetic and structural studies of the small, monomeric, proteinases (e.g., trypsin, pepsin and thermolysin) [1–3]. The most thoroughly understood proteinases act extracellularly and are relatively stable enzymes. The intracellular lysosomal proteinases, too, are generally small (M_r 20000–40000) and monomeric [4]. However, recent studies of non-lysosomal cellular proteinases indicate that many of these are rather large (subunit M_r values in the range 50000–100000) and complex (oligomeric) enzymes, and comparatively little is known about their kinetic and structural properties [5,6]. Of the cell-associated proteinases, the plasma-membrane enzymes are relatively poorly characterized with regard to their structure and function, yet these proteinases have been postulated to function in many important physiological processes. Roles that have been implicated for these proteinases include the inactivation of biologically active peptides (e.g. neuropeptides and hormones), cell fusion, hormone action and neurotransmitter release [7–9]. Until recently, only one kidney plasma-membrane endopeptidase, neutral endopeptidase 24.11 (EC 3.4.24.11), had been purified and well-studied [10]. It is a metalloenzyme and is expressed in many cell types and species. Another distinct metalloendopeptidase, meprin, has now been purified from mouse kidney brush-border membranes (a specialized plasma membrane) [11]. This enzyme has several properties that distinguish it from endopeptidase 24.11 [12,13]. We report here on some of the physicochemical properties of meprin and on its peptide-bond specificity.

EXPERIMENTAL

Purification and assay of meprin

Meprin activity was determined by monitoring the digestion of azocasein. The reaction was initiated by addition of 5 μl (2.5 μg of a purified preparation) of meprin to 1.095 ml of solution containing 11 mg of azocasein and 0.15 M-NaCl in 20 mM-ethanolamine, pH 9.5, at 37 °C. At 30 min intervals, 250 μl samples were removed and placed immediately in 1 ml of 5% (w/v) trichloroacetic acid. The samples were centrifuged in a Beckman B Microfuge for 2 min. The absorbance of the supernatant fraction was determined at 340 nm and plotted as a function of time. By using linear regression, the slope of the line was determined; this slope was converted into units such that one unit was equivalent to an increase in absorbance of 0.001 at 340 nm in 1 min in the original reaction mixture. The enzyme was purified as previously described [11]. Briefly, the purification entails

Abbreviations: Endo F, endoglycosidase F; RNAase, ribonuclease A; -NA, 4-nitroanilide; -ONap, 2-naphthyl ester; -MCA, 4-methylcoumarinyl-7-amide; Bz-, benzoyl; Z-, carbobenzoxy; dansyl, 5-dimethylaminonaphthalene-1-sulphonyl; Suc, succinyl (3-carboxypropionyl).

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extraction of the enzyme from membranes of the kidney homogenates with toluene and trypsin, $(NH_4)_2SO_4$ fractionation, DEAE-cellulose chromatography and Sephadex G-200 gel filtration. The enzyme was purified 800–1000-fold to a specific activity of 10800 μg of azocasein hydrolysed/min per mg of protein.

Amino acid analysis

Purified meprin preparations (20 μg per analysis) were hydrolysed in 0.5 ml of constant-boiling HCl (Pierce, Rockford, IL, U.S.A.) containing 150 μmol of β-mercaptoethanol at 110 °C for 18 h in sealed, evacuated, tubes. Samples were then freeze-dried, dissolved in 0.2 M-sodium citrate, pH 2.2, and applied to a Durrum MBF amino acid analyser with an *o*-phthalaldehyde detection system.

Cysteine residues in meprin were measured (after reduction with β-mercaptoethanol) as carboxymethylcysteine after derivatization of the enzyme with iodoacetic acid. The purified enzyme (20 μg) was diluted to 200 μl with 50 mM-Tris/HCl, pH 8.5, and incubated at 37 °C for 5 h in the presence of 2 mM-β-mercaptoethanol and 6 M-guanidinium chloride. Iodoacetate was added to a final concentration of 5 mM and the reaction was allowed to proceed for 30 min at 37 °C. The sample was then dialysed exhaustively against water and acid-hydrolysed as described above.

Metal analyses

Purified preparations of meprin containing 0.29 μg of protein/ml were exhaustively dialysed against 1 mM-EDTA in 20 mM-ethanolamine/HCl, pH 9.0, and subsequently in the same buffer without EDTA. The water used for these dialyses was distilled, double-deionized and filtered through Chelex 100 (Bio-Rad, Richmond, CA, U.S.A.); its resistance was 18 MΩ. The Zn and Ca contents of samples were determined on a Perkin–Elmer atomic-absorption spectrophotometer (model 560) with an air/acetylene flame.

Endo F digestion

Samples of meprin (25 μg) for endo F treatment were denatured at 100 °C for 3 min and then incubated at 37 °C for 6 h with 5 units of endo F (New England Nuclear Corp., Boston, MA, U.S.A.). One unit of endo F will convert 1 nmol (14.8 μg) of RNAase B to RNAase A in 1 h at 37 °C.

SDS/polyacrylamide-gel electrophoresis

Enzyme preparations were boiled for 3 min in the presence of 1% (w/v) SDS and 1% (v/v) β-mercaptoethanol in 60 mM-Tris/HCl, pH 7.5, with 0.0013% Bromophenol Blue tracking dye. They were then subjected to SDS/polyacrylamide-gel electrophoresis in 10% (w/v) polyacrylamide slab gels (14 cm × 10 cm × 1.5 mm) by the method of Studier [14]. Electrophoresis was performed at a constant current at 25 mA per gel. Gels were stained with Coomassie Brilliant Blue R250.

Analytical isoelectric focusing

Isoelectric focusing was performed in tube gels according to a modification of the method of O'Farrell *et al.* [15]. Briefly, tube gels (70 mm × 1.5 mm) containing 7.5% (v/v) pH 3–10 Ampholines (Sigma) and 3.2% (v/v) pH 4–7 Ampholines were allowed to set overnight. They were then prefocused at 400 V for 30 min in a standard tube-gel-electrophoresis chamber (Hoefer Scientific Instruments, San Francisco, CA, U.S.A.) with the upper reservoir containing 0.02 M-NaOH and the lower chamber filled with 0.01 M-H_3PO_4. Samples of meprin (50 μg) were then applied to the gels and subjected to 250 V for 18 h under the same conditions described for the pre-focused gels. After electrophoresis, gels were fixed in 10% (w/v) trichloroacetic acid for 1 h and stained overnight with Coomassie Brilliant Blue R250.

Incubation of the oxidized insulin B-chain with meprin

Oxidized insulin B-chain (10 mg), purchased from Schwarz–Mann (Orangeburg, NY, U.S.A.), was dissolved in 0.5 ml of 10 mM-triethanolamine and the pH was adjusted to 7.5 with 1 M-acetic acid. NaCl was added to a final concentration of 150 mM. The reaction was started by the addition of 50 μl of meprin [2 mg/ml in 50 mM-Tris/HCl (pH 7.5)/150 mM-NaCl]. The mixture was incubated for 2 h at 37 °C and the reaction was stopped by the addition of an equal volume of 0.2% (w/v) trifluoroacetic acid.

Separation and analysis of the oxidized insulin B-chain degradation products

The reaction products were centrifuged in a Beckman Microfuge B for 10 min. Samples of the supernatant were subjected to h.p.l.c. on a Bio-Rad liquid chromatograph (Bio-Rad Laboratories, Richmond, CA, U.S.A.) employing a Waters μ-Bondapak C_{18} reverse-phase column (Waters Associates, Milford, MA, U.S.A.) that had been equilibrated with 0.1% (w/v) trifluoroacetic acid (Buffer A). A sample (100 μl) of the supernatant fluid was applied to the column, which was then washed with 5 ml of 0.1% (w/v) trifluoroacetic acid. Retained material was eluted from the column with a 90 min linear gradient [0–60% (v/v) acetonitrile (Buffer B)] at a flow rate of 1 ml/min. The eluate was monitored at 215 nm. Fractions (1 ml) of the eluate were collected in chromic-acid-washed tubes. The h.p.l.c. separation was repeated a number of times. Fractions of the column eluate representing single peaks of absorbance that eluted in the same region of the individual chromatographic separations were pooled and dried in a Savant Speed Vac concentrator. A portion of the material in each of these pooled samples was hydrolysed with 6 M-HCl in evacuated sealed tubes for 30 h at 110 °C and then subjected to amino acid analysis as described above. The same procedure was used for analysis of degradation products of angiotensin II and bradykinin.

The remaining material in each of the pooled samples was dansylated with dansyl chloride by the method of Gray [16]. The dansyl derivatives of the *N*-terminal amino acid residues were then identified by h.p.l.c. Briefly, samples of the derivatized amino acid residues were suspended in a minimal volume of 10% (v/v) acetonitrile in 30 mM-NaH_2PO_4 at pH 6.5 (Buffer A). These samples were then applied to a Bio-Sil ODS-5S column which had been flushed with Buffer A. The samples were then eluted from the column with a linear gradient (10–60%, v/v) of acetonitrile (Buffer B), at a flow rate of 1 ml/min. The eluate was monitored at 250 nm. The relative retention times of the unknown dansylated amino acid residues were compared with those of known dansylated amino acid residues that had previously been applied to the same column and eluted under the same conditions.

RESULTS AND DISCUSSION

Metal content of meprin

Atomic-absorption analyses of two different preparations of the purified enzyme, performed in triplicate, indicated the enzyme contained 0.96 and 0.80 μg of Zn per mg of protein. By assuming an M_r of 85000, this is equivalent to a mean of 1.1 mol of Zn per mol of the meprin subunit. Measurements of Ca by atomic absorption revealed 1.33 and 1.28 μg of Ca per mg of protein; this is equivalent to 2.75 mol of Ca per mol of the meprin subunit.

The metal ions in meprin are tightly associated with the enzyme; no metal ions need to be added to the enzyme during the course of purification or after prolonged dialysis against metal-free buffers. In addition, when the enzyme was incubated with EDTA or 1,10-phenanthroline at neutral or alkaline pH values so that it was 95% inhibited and the chelators were subsequently removed by exhaustive dialysis, the enzyme activity returned to 100% of its original value. These results indicated that the association constant between metal and enzyme, at neutral and alkaline pH, is greater than that between metal and chelating agent. Another example of such tight association between metal and enzyme is in RNA polymerase from *Bacillus subtilis*; in this instance, dialysis against 1 mM-EDTA for 7 days failed to remove the active-site zinc ion [17]. Also, in carbonic anhydrase the half-time for exchange between metal–enzyme and extraneous metal at neutral pH is 3 years [18]. It is probable that chelating agents would remove the zinc ion from meprin at acid pH values, as is the case with carbonic anhydrase [18]. However, meprin is rapidly and irreversibly denatured at acidic pH values, and thus it is not possible to perform the same chelation experiments below pH 7.

Most of the metalloproteinases (i.e. endopeptidases and exopeptidases that contain metals as an integral part of their structure) that have been analysed contain zinc as the essential metal cofactor. Among these are thermolysin, collagenases, endopeptidase 24.11, aminopeptidase N and peptidyl dipeptidase [19–22]. An exception to the rule is aminopeptidase A (EC 3.4.11.7), a kidney microvillar peptidase that contains Ca^{2+} at its active site. Metal-activated proteinases (those with a loose interaction between enzyme and metal), such as calpain, also appears to use Ca^{2+} as cofactor.

The occurrence of tightly bound zinc and calcium ions in meprin is similar to that in the metalloproteinase thermolysin. Thermolysin contains one zinc and four calcium ions per mol; the zinc is at the active site and plays an essential role in the catalytic activity, whereas the calcium ions play a structural role and are thought to contribute to the thermostability of the enzyme [23]. It seems reasonable to suggest similar roles for the cations in meprin. Interestingly, endopeptidase 24.11 isolated from pig kidney contains the one essential zinc ion per mol of enzyme but no calcium [19]. The occurrence of calcium in meprin and not in endopeptidase 24.11 may be one of the factors that accounts for the greater relative thermostability of meprin. In mouse strains where both meprin and endopeptidase 24.11 are present in the kidney brush border, meprin is clearly more stable; at 60 °C, pH 7.5, meprin activity decayed with a rate constant of 0.023 min^{-1} (half-life of 30 min), whereas endopeptidase 24.11 activity has a decay constant of 0.2 min^{-1} (half-life of 3.5 min) [12].

Physicochemical properties

Purified preparations of meprin have been estimated to have an M_r of 320000 according to gel-filtration behaviour [11]. Purified preparations yield a single Coomassie Blue-staining band after SDS/polyacrylamide-gel electrophoresis (Fig. 1). The M_r of the protein band was 85000 if β-mercaptoethanol was included in the SDS boiling mixture before application to the gel, whereas it was 320000 in the absence of β-mercaptoethanol. The enzyme, therefore, appears to be a tetramer and the subunits are linked by reducible covalent bonds, most likely disulphide bridges.

Intrasubunit S—S bonds are present in many of the monomeric extracellular proteinases (e.g. trypsin and chymotrypsin), the lysosomal cathepsins [24], and in human kidney endopeptide 24.11 [25]. By contrast, examples of proteinases containing disulphide bridges between subunits are rare. One possible example is a metalloendopeptidase present in human intestinal brush borders, *N*-benzoyl-L-tyrosyl-*p*-aminobenzoic acid ('PABA-peptide') hydrolase. The latter enzyme is active as a dimer (M_r 200000) and the subunits are linked by reducible bonds, presumably disulphide bridges (E. E. Sterchi, personal communication). Endopeptidase 24.11 has been purified and characterized from various species and tissues, but the occurrence of intersubunit S—S

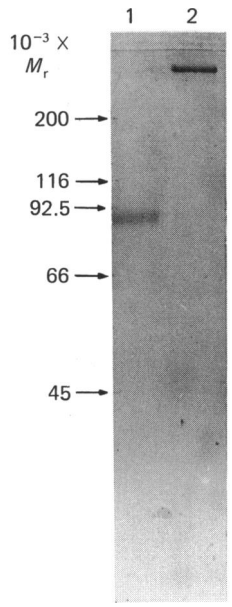

Fig. 1. SDS/polyacrylamide-gel electrophoresis of meprin prepared in the presence or absence of β-mercaptoethanol

Purified preparations of meprin (20 μg) were boiled for 3 min with 1% (w/v) SDS in the presence of 1% (v/v) β-mercaptoethanol (lane 1) or in the absence of this reducing agent (lane 2). Samples (10 μg) were then subjected to SDS/polyacrylamide-gel electrophoresis and stained for protein with Coomassie Brilliant Blue as described in the Experimental section. Gels were calibrated with myosin (M_r 200000), β-galactosidase (M_r 116000), phosphorylase *b* (M_r 92500), bovine serum albumin (M_r 66000), ovalbumin (M_r 45000) (all obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A.).

bridges has not been reported. Similarly, we have found no evidence of such covalent linkages in mouse kidney endopeptidase 24.11 [13]. There are, however, several examples of membrane receptors that display intersubunit disulphides. For example, follitropin receptors from ovarian cells contain intersubunit disulphides [26]. The occurrence of the disulphide bridges in the receptor is important to the interactions between receptor and the glycoprotein hormone follitropin. It is likewise possible that the S—S bridges have some significance in the interaction between protein substrates and meprin.

Reducing agents, such as cysteine, glutathione and dithiothreitol, completely inhibit meprin at concentrations between 5 and 10 mM [11]. The reducing agents may inhibit by chelating metal ions or by disrupting S—S bridges. Preliminary studies of brush-border preparations indicate that the meprin subunits are cross-linked by S—S bridges in microvilli also and that the covalent links are not artefacts of the purification procedure. There is some indication, however, that meprin is active as a dimer as well as a tetramer. During purification of the enzyme, some activity is observed at a mobility corresponding to M_r 180000, half that of purified meprin [11]. The proteolytic activity of M_r 180000 has not been purified to homogeneity; however, initial studies using several substrates and inhibitors indicate it is very similar catalytically to tetrameric meprin.

When the enzyme is treated with endo F, the M_r of the protein subunit is decreased to approx. 70000 (Fig. 2). Endo F removes *N*-linked sugar residues from both high-mannose and complex carbohydrate chains. These results indicated that meprin contained approx. 18% carbohydrate. Further analyses of the sugar residues indicated that a quarter of the carbohydrate was glucose residues; preliminary results (not shown) indicate that galactose and mannose were also present. The amount (18%) and types of carbohydrate associated with meprin are not unusual for proteins of eukaryotic plasma membranes [27].

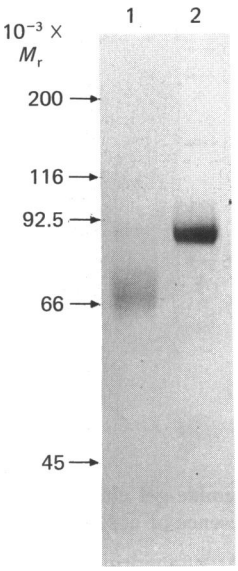

Fig. 2. SDS/polyacrylamide gel electrophoresis of meprin before and after treatment of the enzyme with endo F

Samples of meprin were incubated with endo F, then boiled in SDS containing β-mercaptoethanol and subjected to electrophoresis as described in the Experimental section. Lane 1 contained a preparation of meprin after endo F treatment; lane 2 contained the same meprin preparation before endo F treatment. Proteins were stained with Coomassie Brilliant Blue and gels calibrated as in Fig. 1.

Table 1. Amino acid composition of meprin

The values shown are the averages of three analyses. Tryptophan and proline were not determined.

Amino acid	Composition (mol/100 mol)	Composition (mol/mol of 85kDa subunit)
Asp/Asn	14.7	128
Glu/Gln	9.2	80
His	3.2	28
Lys	5.9	52
Arg	6.1	53
Thr	8.0	70
Ser	7.8	68
Gly	9.2	80
Ala	5.1	45
Val	5.5	48
Met	2.0	18
Ile	7.6	66
Leu	4.0	35
Tyr	4.1	36
Phe	6.1	53
½-CyS*	0.9	8

* The half-cystine residues were determined after first reducing the enzyme with dithiothreitol and then allowing the enzyme to react with iodoacetate; *S*-carboxymethyl-L-cysteine residues were determined on the amino acid analyser. No cysteine residues were detected if the enzyme was incubated with iodoacetate before reduction with dithiothreitol.

The amino acid composition of the enzyme is shown in Table 1. In general, the composition is similar to that of endopeptidase 24.11 from pig kidney and intestine [28]. No methionine or cysteine residues were reported for the pig metalloendopeptidases. For meprin, no methionine was detected unless β-mercaptoethanol was included in the acid hydrolysate; no cysteine residues were detected unless the enzyme was first reduced with β-mercaptoethanol and then carboxymethylated with iodoacetate. With the latter treatment, eight *S*-carboxymethylcysteine residues were detected per subunit. These results, coupled with the finding that no *S*-carboxymethylcysteine residues were detected if the enzyme was treated with iodoacetate as described above (without first reducing with β-mercaptoethanol), indicate that all the cysteine residues in meprin are involved in covalent, reducible, bonds. At least some of these bonds are intersubunit disulphide bonds (Fig. 1).

When meprin was subjected to chromatofocusing, the enzyme activity was eluted in a symmetrical peak between pH 4 and 5 (Fig. 3). When the purified protein was subjected to isoelectric focusing on an analytical gel, the enzyme separated into multiple bands in the range of pH 4.1–5.0 (Fig. 4). There were five major protein bands fairly evenly spaced between pH 4.1 and 4.5 and another five bands between pH 4.6 and 5.0. Thus the 85000-M_r subunit is composed of proteins of different charges. This type of protein pattern on isoelectric focusing is similar

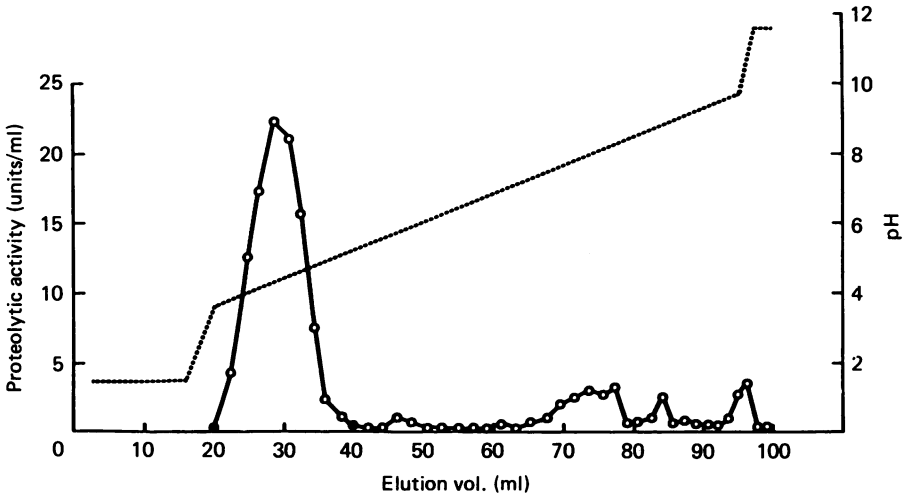

Fig. 3. Chromatofocusing of meprin

Preparative isoelectric focusing was performed at 4 °C by using an LKB 8101 vertical isoelectric-focusing column with a 0–50% (w/v) sucrose gradient containing 1% Ampholines of pH 3.5–10 (LKB). The sample (2 ml, 2170 units of proteolytic activity) was applied in the middle of the gradient. The column was focused at 200 V for 5 h, followed by 250 V for 10.5 h and finally 300 V for 3 h. Fractions were then collected from the bottom of the column; proteolytic activity against azocaein (○) and pH values (----) of the fractions were determined.

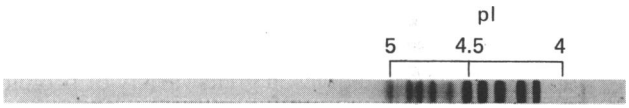

Fig. 4. Analytical isoelectric focusing of meprin

A purified preparation of meprin (50 μg) was applied to a cylindrical gel, which was subjected to 250 V for 18 h with 0.02 M-NaOH in the upper reservoir and 0.01 M-H_3PO_4 in the lower reservoir. The gel was then treated with 10% (w/v) trichloroacetic acid and stained with Coomassie Brilliant Blue R-250.

to that shown by other purified glycoproteins; the charge differences of the bands are likely due to differences in the number of sialic acid residues, although there are several other post-translational modifications that could account for the charge differences.

Peptide-bond specificity of meprin

Incubation of meprin with oxidized insulin B-chain (enzyme/substrate ratio 1:100, w/w) resulted in the appearance of at least 15 peptides as detected by A_{215} after separation by h.p.l.c. (Fig. 5). A series of experiments was conducted in which the enzyme was incubated with insulin B-chain for various periods of time to detect initial cleavages and to determine whether there were obvious preferred cleavages. However, the h.p.l.c. profiles of peptide products were very similar at all time points. Therefore the peptide products of a relatively long incubation (2 h), when 20% of the insulin B-chain had been converted into trifluoroacetic acid-soluble products, were analysed for amino acid content. Analysis of the total amino acid content and *N*-terminal amino acid of the peptides led to the results presented in Fig. 6. The most abundant peptide products were peptides I, II and VI as determined by the areas under the peaks and considering the number of peptide bonds in the peptide. These results indicated that there were major sites of cleavage at Phe^{24}–Phe^{25}, Phe^{25}–Tyr^{26} and Gly^{20}–Glu^{21}. Other peptide bonds cleaved, in order of the decreasing amounts of peptide product identified,

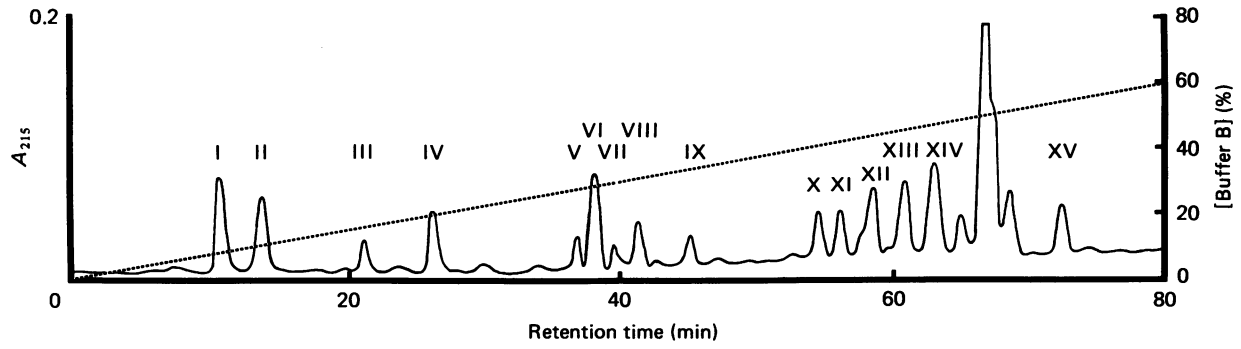

Fig. 5. H.p.l.c. of the soluble peptide fragments of the oxidized insulin B-chain produced by meprin

Samples (100 μl each) of the trifluoroacetic acid-soluble products of the oxidized insulin B-chain were subjected to h.p.l.c. as described in the Experimental section. A typical separation is shown. Buffer B was 75% (v/v) acetonitrile in 1% (w/v) trifluoroacetic acid. Corresponding absorbance peaks from several h.p.l.c. separations were pooled for peptide analyses.

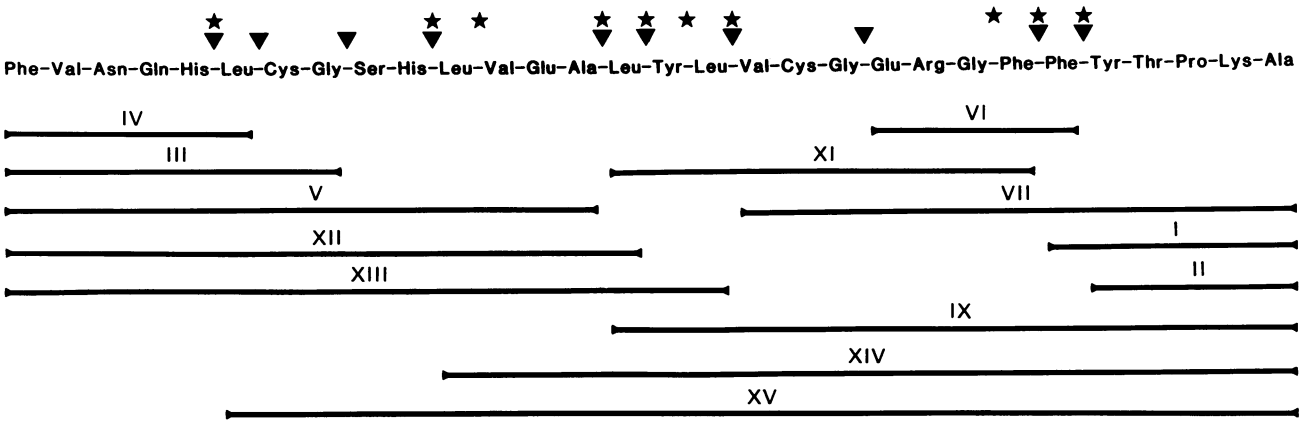

Fig. 6. Peptides produced by the action of meprin on the oxidized insulin B-chain

Peptides recovered from the h.p.l.c. column (Fig. 5) were analysed for amino acid content and the *N*-terminal amino acid residue. The amino acid sequences were deduced from the known structure of the insulin B-chain. ▼, Bonds cleaved by meprin; ★, bonds cleaved by pig kidney endopeptidase 24.11 reported by Kenny [27].

were Leu^{6}–Cysteic acid^{7}, Ala^{14}–Leu^{15}, His^{10}–Leu^{11}, Leu^{17}–Val^{18}, Gly^{8}–Ser^{9}, Leu^{15}–Tyr^{16}, His^{5}–Leu^{6}. These results indicated that meprin has a preference for peptide bonds flanked by neutral or hydrophobic amino acids. The preference for cleavage at hydrophobic amino acids is shared by thermolysin and pig endopeptidase 24.11 (see the stars on Fig. 6). The preference for hydrophobic bonds is not absolute for meprin, however, in that there was one major site of cleavage (Gly^{20}–Glu^{21}) and one other site (Leu^{6}–Cysteic acid^{7}) in which negatively charged residues occupied the S_1' enzyme subsite [29]. Thus meprin can accommodate negatively charged residues at the leaving-group site (S_1'), a property not common to other metalloendopeptidases. There were two instances where histidine residues occupy the P_1 site of the peptide. However, at pH 7.5, the pH of incubation of meprin with insulin B-chain, the histidine residues were probably unprotonated and therefore uncharged. The ability of meprin to cleave peptide bonds flanked by neutral amino acids and bonds where the amino group is contributed by a negatively charged amino acid would enable the enzyme to hydrolyse bonds on the surface of proteins and therefore to digest a great variety of protein substrates. Cleavage at an acidic amino acid residue by a minor enzyme contaminant in the meprin preparation cannot be discounted. However, preincubation of the enzyme with either bestatin (an aminopeptidase inhibitor) or phosphoramidon (an inhibitor of some metalloendopeptidases) had no effect on the elution profile of peptide fragments from the h.p.l.c. column.

Table 2. Digestion of synthetic peptide substrates by meprin

All incubations contained meprin at a concentration of 10 μg/ml. Key to letters in column 3: a, spectrometric assay of NA or ONap group; b, fluorescence assay of MCA; c, h.p.l.c. assay for detection of peptide products. The analyses of degradation products of angiotensin II and bradykinin were performed as described in the Experimental section.

Substrate	[Substrate] (mM)	Group measured	Hydrolysis (nmol/min per mg of enzyme)
Leu-NA	0.8	a	0
Bz-Arg-NA	0.8	a	0
Bz-Tyr-NA	0.8	a	0
Ac-Phe-ONap	0.8	a	0
Suc-Ala-NA	0.8	a	0
Arg-MCA	0.005	b	0
Z-Phe-Arg-MCA	0.005	b	0
Bz-Phe-Val-Arg-MCA	0.005	b	0
Bz-Gly-Arg	1.0	c	0
Z-Glu-Tyr	1.0	c	0
Gly-Gly-Phe-Leu	0.64	c	0
Tyr-Gly-Gly-Phe-Leu	0.45	c	0
Tyr-Gly-Gly-Phe-Met	0.44	c	0
Leu-Arg-Arg-Ala-Ser-Leu-Gly	0.32	c	0
Ala-Phe-Pro-Leu-Glu-Phe	0.35	c	0
Bz-Phe-Val-Arg	0.37	c	0
Angiotensin II ↓ Asp-Arg-Val-Tyr-Ile-His-Pro-Phe	0.24	c	398
Bradykinin ↓ Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	0.24	c	393

To establish further the substrate specificity of meprin, the enzyme was incubated with several peptide substrates (Table 2). No hydrolysis of small substrates (ranging in size from two to seven amino acids) was detected by using a number of spectrophotometric or fluorimetric methods. Furthermore, hydrolysis of these substrates was not observed with increased enzyme concentrations and/or incubation times. Cleavage of some of the peptides (e.g. the enkephalins) by meprin would be predicted from the peptide-bond specificity observed with the oxidized B-chain of insulin. The absence of such hydrolysis indicated that the active site of meprin requires an extended polypeptide chain for binding and hydrolysis of substrates.

The bioactive peptides angiotensin II and bradykinin are cleaved rapidly by meprin at the positions shown (Table 2). Hence, proteins and peptides eight amino acids in length or longer are substrates for meprin. The cleavage of such substrates may be an important inactivation/reabsorption function of meprin *in vivo*.

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