The Hydrolysis of Parathyroid Hormone by Papain-Solubilized Rat Meprin Differs from that of Detergent-Solubilized Meprin

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Introduction

Meprin (EC 3.4.24.18) is a membrane-bound metalloprotease that is abundant in the microvilli of mouse and rat kidneys and mouse, rat and human intestines. It has an oligomeric structure composed of α and/or β subunits (*Johnson* and *Hersh* 1994; *Kenny* and *Ingram* 1987; *Marchand, Tang* and *Bond* 1994). Meprin has been solubilized and purified from the microvillar membranes of rat kidney after treatment with either detergent or papain (*Kenny* and *Ingram* 1987; *Yamaguchi, Kido, Fukase, Fujita* and *Katunuma* 1991). Detergent-solubilized rat meprin has an ability to hydrolyze various hormones, such as parathyroid hormone (PTH), atrial natriuretic peptide, endothelin-1 and the insulin B-chain; the cleavage sites on these hormones were chiefly restricted to peptide bonds involving a hydrophilic amino acid (*Yamaguchi* et al. 1991; *Yamaguchi, Kido* and *Katunuma* 1992). Neuropeptide hydrolysis by meprin was also investigated using papain-solubilized preparations. *Stephenson* and *Kenny* (1988) reported that papain-solubilized rat meprin hydrolyzed luteinizing hormone-releasing hormone, substance P, bradykinin and angiotensins at bonds involving a hydrophobic amino acid. This hydrolytic property was different from that observed in our previous experiments, in which longer peptides and detergent-solubilized meprin were used.

Recently, it has been shown that papain-solubilized meprin consists of α β tetramers, whereas the detergent-solubilized enzyme consists of α_2 homodimers (*Marchand, Tang* and *Bond* 1994). In addition, the α subunit has been found to be more active than the β subunit against synthetic peptide substrates but less active toward azocasein (*Johnson* and *Hersh* 1994). Thus, the discrepancy in the substrate specificities of the two enzyme preparations may depend on their subunit composition, but not on the length of the peptide sequences used in the experiments. To clarify this, we investigated the hydrolysis of hPTH-(39-84) by papain-solubilized rat meprin in order to compare it with the previously described hydrolysis of the hormone by the detergent-solubilized enzyme.

Materials and Methods

Materials

Kidneys were obtained from male Wistar rats weighing 200–250 g. Papain and N-benzoyl-L-tyrosyl-*p*-aminobenzoic acid (Bz-Tyr-pAB) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Human (h) PTH-(39-84) was purchased from Peptide Institute (Osaka, Japan). All other chemicals were of the highest grade commercially available.

Enzyme assays

Papain-solubilized rat meprin was assayed with Bz-Tyr-pAB according to the method of *Kenny* and *Ingram* (1987). The incubation mixture (300 µl volume) contained, in addition to the enzyme sample, 3.3 mM Bz-Tyr-pAB and 0.1 M triethanolamine/HCl buffer, pH 7.1. After incubation at 37 °C for 1 h, the reaction was terminated by the addition of 2 ml dimethyl sulfoxide. Fluorescence was determined at 350 nm using 303 nm excitation. One unit of enzyme activity was defined as that catalyzing the degradation of 1 µmol synthetic substrate/min.

Enzyme purification

Meprin was purified from the papain-released fraction of rat kidney membranes as described by *Kenny* and *Ingram* (1987), except that chromatography was done with phenyl-Superose (Pharmacia LKB Biotechnology Inc.) rather than with hydroxyapatite chromatography, and the second ion-exchange chromatography was omitted (*Johnson* and *Hersh* 1994). Briefly, the kidney membrane fraction was digested with papain to release the enzyme. Subsequent purification steps included chromatography on columns of Mono Q, Superose 12 and phenyl-Superose (Pharmacia). Pooled Superose 12 fractons were adjusted to 1.8 M ammonium sulfate for loading onto phenyl-Superose. Proteins were then eluted with a linear gradient from 1.8 to 0 M ammonium sulfate. In each chromatographic procedure, meprin activities were assayed with Bz-Tyr-pAB as described above.

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Protein determination

Protein was measured with bovine serum albumin as a standard using a bicinchroninic acid (BCA) protein assay kit (Pierce, Rockford, IL) according to the manufacturer's instructions.

Determination of cleavage products of hPTH-(39-84) liberated by papain-solubilized rat meprin

Ten micromolar hPTH-(39-84) was incubated with 2.17 nM papain-solubilized rat meprin purified as described above in 250 µl of 20 mM Tris/HCl pH 8.0 containing 100 µM amastatin, an inhibitor of aminopeptidases, for 16 h at 37 °C. The samples were then fractionated by HPLC on an ODS 120T reverse-phase column (C18; 4.6 × 250 mm; Toyo Soda, Tokyo, Japan) with a linear gradient of 9–26% acetonitrile in 0.1% trifluoroacetic acid for 36 min at a flow rate of 0.5 ml/min. Elution was monitored by the absorbance at 215 nm. Degradation products were collected in test tubes and freeze-dried. The NH_2-terminal amino acid sequences of these samples were analyzed by an automated gas-phase protein sequencer (Applied Biosystems model 470A gas-phase sequencer and model 120A HPLC analyzer system), and the cleavage sites were determined.

Results and Discussion

As shown in the HPLC profile in Fig. **1**, papain-solubilized rat meprin hydrolyzed hPTH-(39-84) into five major fragments.

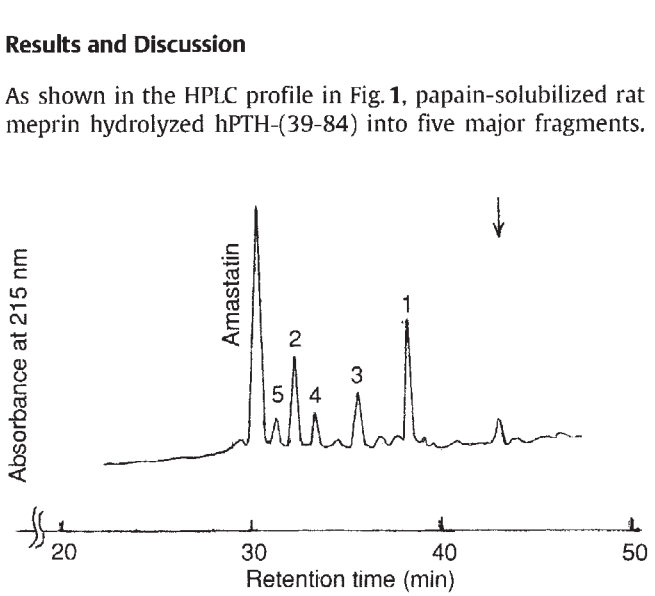

Fig. 1 HPLC analysis of peptide products formed by incubation of hPTH-(39-84) with papain-solubilized rat meprin. hPTH-(39-84) (10 µM) was incubated with 2.17 nM papain-solubilized meprin in 250 µl of 20 mM Tris/HCl, pH 8.0, for 16 h at 37 °C in the presence of 100 µM amastatin. Each sample was then fractionated by HPLC. The product peaks are numbered in order of yield, and numbers correspond to those in Table **1**. Arrow indicates the retention times of hPTH-(39-84).

The NH_2-terminal amino acid sequences of the cleavage peptides shown in Table **1** indicated that the major cleavage sites were Pro^{40}-Leu^{41}, Arg^{44}-Asp^{45}, Glu^{55}-Asp^{56}, Glu^{61}-Ser^{62} and His^{63}-Glu^{64} in order of yield of the products. The most prominent cleavage site, as shown by the NH_2-terminal sequences of three of the five major cleavage peptides (peaks 1, 3 and 4), was the hydrophobic Pro^{40}-Leu^{41} site. Other cleavage sites were at peptide bonds flanked by hydrophilic amino acid residues.

In a previous study (*Yamaguchi, Fukase, Kido, Sugimoto, Katunuma* and *Chihara* 1994), we showed that detergent-solubilized rat meprin hydrolyzed hPTH-(39-84) into five major fragments, with the Gly^{68}-Glu^{69}, Arg^{44}-Asp^{45}, Asp^{56}-Asn^{57} and Leu^{67}-Gly^{68} bonds being the most prominent cleavage sites, in that order. These cleavage sites were chiefly at peptide bonds involving a hydrophilic amino acid residue. When compared with the present study, the only cleavage site shared by the two enzyme preparations was the Arg^{44}-Asp^{45} bond. However, peak 2 in Fig. **1** consisted of 11 residues (Table **1**), whereas the peptides produced by detergent-solubilized meprin had more than 15 residues (*Yamaguchi* et al. 1994). Thus, none of the cleavage peptides of hPTH-(39-84) produced by papain-solubilized meprin corresponded to those produced by the detergent-solubilized enzyme, indicating that the mode of hydrolysis of hPTH-(39-84) by the two enzyme preparations was considerably different.

Recent studies with mice and rats revealed that papain-solubilized meprin mainly consisted of α β tetramers, whereas detergent-solubilized meprin consisted of α_2 homodimers (*Marchand, Tang* and *Bond* 1994). We also have shown that detergent-solubilized meprin mainly composed of α subunits by the analysis of its N-terminal amino acid sequence (*Yamaguchi* et al. 1994). Thus, the difference in the substrate specificities of the two enzyme preparations presented above might be because the meprin α subunit has a preference for a hydrophilic amino acid residue in peptide hydrolysis, whereas the β subunit has a preference for a hydrophobic residue. Since our previous studies showed that a microvillar membrane preparation of rat kidney hydrolyzes PTH-(1-84), PTH-(39-84) and PTH-related peptide (PTHrP)-(109-141) and that the hydrolytic activities were quite similar to those of detergent-solubilized rat meprin (*Yamaguchi* et al. 1994; *Yamaguchi, Fukase, Sugimoto* and *Chihara* 1995), it is likely that, *in vivo*, the microvillar membrane-bound meprin metaboilzes the hormones chiefly through its α subunits. Indeed, it has been proposed that the α subunit is a totally extracellular component that is attached to the β subunit, and that the β subunit is an intrinsic microvillar membrane protein that could act as an anchor for the α subunit (*Johnson* and *Hersh* 1994; *Marchand, Tang* and *Bond* 1994). Hence, *in vivo*, the α subunits appear to be exposed to

Peak	Amino acid sequence	Cleavage site
1	Leu-Ala-Pro-Arg-Asp-Ala-Gly-Ser-Gln-Arg-Pro-Arg-Lys-Lys-	Pro^{40}-Leu^{41}
2	Asp-Ala-Gly-Ser-Gln-Arg-Pro-Arg-Lys-Lys-Glu	Arg^{44}-Asp^{45} Glu^{55}-Asp^{56}
3	Leu-Ala-Pro-Arg-Asp-Ala-Gly-	Pro^{40}-Leu^{41}
4	Leu-Ala-Pro-Arg- Ser-His-Glu-Lys-Ser-Leu-Gly-	Pro^{40}-Leu^{41} Glu^{61}-Ser^{62}
5	Glu-Lys-Ser-Leu-	His^{63}-Glu^{64}

Table 1 Amino acid sequences of degradation products of hPTH-(39-84) liberated by papain-solubilized rat meprin. The individual peak fractions, designated in Fig. **1**, were collected and their amino acid sequences were determined in an automated gas-phase protein sequencer.

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the tubular lumen and thus can more readily interact with and hydrolyze the hormones in the urinary flow than can the β subunits.

Meprin is one of the most abundant endopeptidases in the renal microvillar membranes (*Kenny* and *Ingram* 1987). Another such endopeptidase is neprilysin (EC 3.4.24.11), which plays a major role in the metabolism of atrial natriuretic peptide and endothelin-1 in the kidney and in the metabolism of neuropeptides in the brain (reviewed in *Erdos* and *Skidgel* 1989). Although the role of neprilysin is well known, the physiological role of meprin has long been unclear. Papain-solubilized rat meprin has been reported to hydrolyze neuropeptides, such as bradykinin and substance P, but less efficiently than neprilysin does (*Stephenson* and *Kenny* 1988). Our recent studies using detergent-solubilized meprin, however, showed that meprin, rather than neprilysin, was involved in the metabolism of PTH and the C-terminal peptide of PTHrP in the microvillar membranes of the kidney (*Yamaguchi* et al. 1994; *Yamaguchi* et al. 1995), suggesting the physiological importance of meprin in the degradation of these calcitropic hormones *in vivo*. The present study has shown that the substrate specificity of meprin could be affected by the solubilization procedure, and together with our previous results, suggests that the detergent-solubilized enzyme is more suitable than papain-solubilized enzyme for investigating hormone metabolism.

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