Multimeric Structure of the Secreted Meprin A Metalloproteinase and Characterization of the Functional Protomer*

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Meprins are zinc endopeptidases that are secreted from kidney and intestinal epithelial cells and capable of cleaving growth factors, extracellular matrix proteins, and biologically active peptides. The secreted form of meprin A is a homo-oligomer composed of α subunits, a multidomain protease of 582 amino acids coded for near the major histocompatibility complex of the mouse and human genome. Analyses of the recombinant homo-oligomeric form of mouse meprin A by gel filtration, non-denaturing gel electrophoresis, and cross-linking (with disuccinimidyl suberate or N-(4-azido-2,3,5,6-tetraflourobenzyl)-3-maleimidylpropionamide) indicate that the secreted enzyme forms high molecular weight multimers, with a predominance of decamers. The multimers are composed of disulfide-linked dimers attached noncovalently by interactions involving the meprin, A5 protein, receptor protein-tyrosine phosphatase μ (MAM) domain. The active protomer is the noncovalently linked dimer. Linkage of active protomers by disulfide-bonds results in an oligomer of ~900 kDa, which is unique among proteases and distinguishes meprin A as the largest known secreted protease. Electron microscopy revealed that the protein was present in two states, a crescent-shaped structure and a closed ring. It is concluded from this and other data that the covalent attachment of the protomers enables noncovalent associations of the native enzyme to form higher oligomers that are critical for hydrolysis of protein substrates.

Meprins are zinc endopeptidases that are secreted from or found in brush border membranes of kidney and intestinal epithelial cells of humans and rodents. They are members of the astacin family of metalloproteases and the metzincin superfamily (1, 2). The expression of meprins enables activation or degradation of bioactive peptides, growth factors, cytokines, hormones, and matrix proteins by limited proteolysis (3–5).

Meprins are multidomain, oligomeric glycoproteins with subunit masses of 85,000–110,000 Da (1). The subunits, α and β, may associate to form either homo- or hetero-oligomeric proteins (6). Multimers containing α subunits (both homo- and hetero-oligomers) are designated meprin A (EC 3.4.24.18), whereas homo-oligomers of β are designated meprin B (EC 3.4.24.63). The mouse α and β subunits are ~42% identical at the amino acid level, and their cDNA-deduced primary sequences predict a similar arrangement of functional domains (1, 5).

Meprin α cDNA (Structure I) encodes a 760-amino acid protein with an NH2-terminal signal sequence followed by a prosequence, the protease domain (containing the zinc active site), a MAM1 domain, a MATH (meprin and TRAF homology; TRAF, tumor necrosis factor receptor-associated factor) domain, followed by an AM (after MATH) domain, an EGF (epidermal growth factor)-like domain, a putative COOH-terminal transmembrane domain (TM), and a cytoplasmic (C) tail. The α subunit contains a 56-amino acid domain (I) inserted between the AM and EGF-like domains that is not present in the β subunit. The I domain has been shown to direct COOH-terminal proteolytic processing of the α subunit during biosynthesis (7). The mature α subunit lacks the domains COOH-terminal to the AM domain, including the transmembrane domain. As a result, meprin α homo-oligomers are secreted, and meprin α is only found membrane-bound via interactions with the β subunit (which does not undergo COOH-terminal proteolysis and is a type I integral membrane protein).

Studies of membrane-bound meprins indicated that the subunits are linked via disulfide bonds to form dimers (6–8). These dimers can further associate via noncovalent interactions to form tetramers (8). Meprins are unique among proteases in their oligomeric structure. Because hetero-oligomer formation requires interactions between the α subunits and membrane-bound β subunits, it was unclear whether the secreted homo-oligomer, which contains only α subunits, could form multimers of the same oligomeric state or even larger complexes.

Native gel electrophoresis of unpurified secreted meprin A followed by Western blot detection of the enzyme demonstrated that it migrated with a mobility larger than 700 kDa (9). Mutation of Cys-320, the cysteine residue in the MAM domain involved in an intersubunit disulfide bond, to an alanine (C320A) resulted in expression of a monomeric form of meprin.

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The abbreviations used are: MAM, meprin, A-5 protein, receptor protein-tyrosine phosphatase μ; MALDI, matrix-assisted laser desorption/ionization; TCEP, Tris-(2-carboxyethyl)phosphine hydrochloride; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography; BK+, 2-aminobenzoyl-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-Lys(Dnp)-Gly-OH, where Dnp is dinitrophenyl.
The mutant had little or no ability to hydrolyze proteins, although it retained activity against peptide substrates and showed decreased thermostability and an increased susceptibility to tryptic degradation (9). This work indicated that the oligomeric state is critical for activity and stability of the enzyme but could not determine whether the properties exhibited by the monomeric mutant were due to lack of formation of the disulfide-linked dimer or the inability of the subunits to associate noncovalently. Moreover, the oligomeric state of meprin could not be accurately determined by native gel electrophoresis of the unpurified protein because the band observed could have been due to meprin subunits in association with other proteins. Thus, the oligomeric structure of the purified meprin protein was investigated, and the importance of this structure to function was examined.

EXPERIMENTAL PROCEDURES

Purification of the Meprin A Homo-oligomer—Recombinant mouse meprin A was purified from the media of human embryonic kidney 293 cells (American Type Culture Collection) stably transfected with full-length mouse meprin α cDNA (10). Briefly, the purification involved mono-Q and superose-12 fast protein liquid chromatography column chromatography; the protein was activated by trypsin after mono-Q chromatography (5, 10).

Photo-cross-linking of Meprin—An 8 μm meprin solution was labeled with 10 molar equivalents of N-(4-azido-2,3,5,6-tetrafluorobenzyl)-3-maleimidylpropionimid (Molecular Probes) for 12 h at 4 °C in the dark. Excess label was removed by passing the mixture through a Sephadex G-25 column. Cross-linking was initiated by subjecting the protein to ultraviolet light for 7 min, as described elsewhere (11), and quenched with 5 μl of gel loading buffer.

Mapping the Location of the Biotin-labeled Cysteine Residue—Fifty μl of an 800 μM N-biotinyl-N'-[6-maleimidohexanoyl]-hydrazide (biotin-maleimide) solution dissolved in dimethylformamide was added to 500 μl of an 8 μM meprin solution. The protein was incubated for 10 h at 4 °C, and excess label was removed with a Sephadex G-25 column. The labeled protein was subsequently deglycosylated with peptide-N-glycosidase F (PNGase F; New England BioLabs), and proteins were separated from carbohydrates by gel filtration. Deglycosylated, labeled protein was incubated with 50 μg of trypsin at pH 8.5, 37 °C for 14 h. The resulting peptides were separated by a reverse phase C-18 HPLC column (11), and fractions were analyzed by reflector mode MALDI mass spectrometry using an α-cyano-4-hydroxycinnamic acid matrix.

Electron Microscopy and Image Analysis—Samples from different preparations of meprin were diluted with deionized water to ∼25 μg/ml and negatively stained with 1% uranyl acetate using a double carbon method (12). Electron micrographs were obtained with a LEO 912AB microscope operated at 100 kV at an absolute magnification of 31,500. The spectrometer slit of the energy filter was used to remove inelastically scattered electrons. For preparation of figures, negatives were digitized at an optical resolution corresponding to 6.4 A/pixel on an Agfa Duscan flatbed scanner, and then composites were prepared using Adobe Photoshop. Statistics of particle measurements were compiled from enlarged prints on a digitizing tablet with SigmaScan (Jandel).

Tris-2-carboxyethylphosphine Hydrochloride (TCEP) Reduction and Analysis of Meprin—Eight μm meprin was reduced with 5 mM TCEP (Molecular Probes), pH 8.0, for 30 min at 4 °C. Excess reducing agent was removed by a Sephadex G-25 column, and the enzyme was subjected to native gel electrophoretic analysis to determine the dissociation of the oligomer and nonreducing SDS-PAGE to assess the amount of disulfide bond reformation. Proteolytic activity was determined with an internally quenched fluorescent bradykinin substrate analog, BK+ (2-aminobenzoyl-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-Lys(Dnp)-Gly-OH, where Dnp is dinitrophenyl), and the protein substrate azocasein as described previously (5).

Thermolability and Proteolytic Susceptibility of Meprin—An 8 μm solution of native or reduced meprin was incubated at 47 °C for 5–55 min at 5-min intervals. The protein was cooled to 25 °C, and the activity toward BK+ was determined. Susceptibility to trypsin digestion was assessed by mixing 2 μl of an 800 μM solution of either the native protein or the reduced protein with trypsin at final concentrations of 20–100 ng/ml. After 15 min at 25 °C, the reaction was stopped by the addition of a 3-fold excess of soybean trypsin inhibitor, and the protein was subjected to SDS-PAGE followed by Western blotting to determine the extent of proteolysis.

RESULTS AND DISCUSSION

The Meprin A Homo-oligomer Is Capable of Forming High Molecular Weight Structures—When purified recombinant mouse meprin A homo-oligomer was subjected to nondenaturing, nonreducing gel electrophoresis, the native protein migrated as a species larger than the 669,000 molecular weight marker (Fig. 1A). The monomeric molecular weight of a meprin α subunit is ∼90,000, indicating that the multimeric protein is capable of forming at least octamers. Cross-linking the protein with disuccinimidyl followed by SDS-PAGE in the presence of 2-mercaptoethanol resulted in dimers and higher order oligomers (Fig. 1B). The largest cross-linked species observed migrated above the 669,000 molecular weight marker, confirming that meprin can form an octamer or even higher order complexes. When the protein was subjected to analytical size exclusion chromatography to obtain more precise molecular weight information, the elution volume of meprin corresponded to a molecular weight of 905,000, indicating that the protein is primarily decameric (Fig. 1C). The width of the meprin peak was subjected to SDS-PAGE followed by Western blotting to determine the extent of proteolysis.

FIG. 1. Determination of the molecular weight of meprin A. A, the purified meprin A homo-oligomer (14 μg) was subjected to native polyacrylamide gel electrophoresis in a 4%–20% polyacrylamide gradient gel at 200 V for 12 h in a buffer containing 25 mM Tris and 192 mM glycine, pH 8.8. The gel was stained with the Gel Code Blue stain to visualize protein bands. B, meprin (14 μg) in 20 mM HEPES, pH 8.0, and 150 mM NaCl was cross-linked with 20 molar equivalents of disuccinimidyl suberate added in 0.1 volume of dimethylformamide. The cross-linking reaction was allowed to proceed for 15 min and quenched with 2 μl of a 1 M glycine solution, pH 8.5, and the products were separated by SDS-PAGE in the presence of 2-mercaptoethanol on a 4%–20% gradient gel. C, the molecular weight of meprin was determined by using an analytical superose-6 gel filtration chromatography column calibrated with the following standards (indicated by arrows): 1, blue dextran, 2,000,000; 2, thyroglobulin, 669,000; 3, ferritin, 440,000; 4, catalase, 232,000; and 5, aldolase, 158,000. Each standard was injected onto the column separately at a flow rate of 0.5 ml/min and eluted in a buffer containing 20 mM Tris, pH 7.5, and 150 mM NaCl. The molecular weight of meprin (905,000) was calculated by fitting its elution volume to a standard curve generated by plotting the log molecular weight versus the ratio of the elution volume to the void volume of each standard.
Ethanol (5 mM) was used to reduce the meprin protein in the absence of 2-mercaptoethanol followed by SDS-PAGE. C, 2-mercaptoethanol (5 mM) was used to reduce the meprin protein (8 μM) in the absence of SDS, and the protein was subjected to native gel electrophoresis.

was comparable to that of thyroglobulin (669 kDa), the protein standard closest in mass. However, the meprin peak was more asymmetric than any of the monodisperse standard proteins. As shown in the elution profile (Fig. 1C), there appears to be some trailing on the right side of the peak, indicating the presence of smaller oligomeric forms.

The large size of the complex distinguishes the meprin A homolog as the largest known secreted protease. The ability of the secreted protein to form decameric complexes, compared with the tetrameric structure of hetero-oligomeric meprins (8), may result from the lack of the potential conformational barriers imposed by being membrane-bound.

**The Meprin A Homo-oligomer Is Composed of Disulfide-linked Dimers that Associate Noncovalently**—The electrophoretic mobility of the protein under both reducing and denaturing conditions corresponds to the monomeric molecular weight (Fig. 2A). Under denaturing (in the presence of SDS) but nonreducing conditions (no 2-mercaptoethanol), the protein migrates as a dimer (Fig. 2B). When the enzyme was reduced with 2-mercaptoethanol and then subjected to native gel electrophoresis (no SDS), the protein migrated as a dimer of noncovalently linked subunits (Fig. 2C). These results indicate that the oligomer is composed of disulfide-linked dimers that associate noncovalently to form the native structure. The interaction between the covalently linked dimers does not appear to be strong because only dimers, not higher order oligomers, are observed after reduction of the S-S bond.

**The Functional Protomer of the Meprin A Homo-oligomer Is the Noncovalently Linked Dimer**—The secreted form of meprin A contains 11 cysteine residues per subunit (Structure II).

Previous work indicated that Cys-320 in the MAM domain is responsible for the covalent interaction of subunits (9). Mutation of Cys-320 to alanine (C320A) resulted in a meprin α monomer that exhibited altered proteolytic activity and stability (9). This mutant was incapable of forming not only disulfide-linked dimers but noncovalently linked oligomers as well.

To determine whether the changes seen in the mutant were due to the loss of the disulfide-linked dimer or instead to loss of interactions between the noncovalently linked subunits, we reduced wild type meprin with the phosphine TCEP and measured the activity and stability of the protein. TCEP was selected as a reducing agent because it does not contain a thiol group. Thiol-containing reagents such as 2-mercaptoethanol or dithiothreitol have been shown to inhibit meprin by either chelation of the active site zinc ion or reduction of conserved intrasubunit disulfide bonds in the protease, MAM, or AM domains (13). Reduction with TCEP resulted in dissociation of the oligomer into noncovalently linked dimers, as was the case with 2-mercaptoethanol (Fig. 2C); the disulfide bond did not reform when TCEP was removed, even after a period of days (data not shown). To determine the number of free thiols that are solvent-exposed, meprin protein (2.5 μM) was incubated with 2 mM 5,5′-dithio-bis(2-nitrobenzoic acid). 5,5′-Dithiobis(2-nitrobenzoic acid) reacts with free thiols, releasing thionitrobenzoic acid, which can be quantified (14). The 5,5′-dithiobis(2-nitrobenzoic acid) assay indicated that there was one free thiol per subunit before reduction and three additional thiols per subunit after reduction with TCEP; most likely one intrasubunit disulfide bond was reduced in addition to the intersubunit disulfide bridge.

When meprin was reduced with TCEP, subjected to Sephadex G-25 column chromatography to remove the reducing agent, and assayed for enzymatic activities, little or no activity loss was detected. The rate of hydrolysis of BK+ by the reduced enzyme was 95% that of the nonreduced form (5.95 × 10⁻⁹ versus 5.66 × 10⁻⁹ fluorescence units/s for the native and reduced form, respectively). This demonstrated that the reduction and formation of noncovalently linked dimers had not significantly altered the protease domain of the enzyme, which contains two intradomain disulfide bridges, and that the disulfide-linked dimer is not required for activity toward peptides. Similarly, the activity of the reduced enzyme toward the protein substrate azocasein was 90% that of the nonreduced protein, indicating that only the noncovalently associated dimer was necessary for hydrolysis of proteins.

The stability of the TCEP-reduced form of meprin was similar to that of the native enzyme, as assessed by heat inactivation and vulnerability to trypsin digestion. The thermostability was evaluated by incubating the proteins at 46 °C, removing samples at time intervals, and subsequently determining the ability of the protease to hydrolyze BK+. Unlike the monomeric C320A mutant (9), the noncovalent dimeric reduced form of meprin exhibited the same rate of heat inactivation as the native enzyme. Furthermore, both the wild type and TCEP-reduced forms of the enzyme were equally resistant to proteolytic digestion by trypsin. These results indicate that the changes exhibited by the C320A mutant were not due to the inability of the protein to form the disulfide-linked dimer but rather to an inability to form the noncovalent associations; thus, the noncovalently associated dimer is the functional protomer of the oligomer.

The MAM Domain Is Involved in the Noncovalent Association Between the Disulfide-linked Dimer—Only 1 of the 11 cysteine residues present in the secreted form of the meprin subunit is a free thiol that is exposed to solvent as determined by 5,5′-dithio-bis(2-nitrobenzoic acid) (data not shown). This cysteine residue was specifically labeled with the thiol-reactive photocross-linker N′(4-azido-2,3,5,6-tetrafluorobenzyl)-3-maleimidylpropionamide, and the protein was subjected to ultraviolet light to initiate cross-linking (Fig. 3A). In the absence of cross-linker and 2-mercaptoethanol, the protein migrated as a single band corresponding to dimer (Fig. 3A, lane 1). In the absence of cross-linker but in the presence of reducing agent, the mobility of the protein was consistent with monomer only, indicating that the intersubunit disulfide bond was completely reduced (Fig. 3A, lane 2). In the presence of cross-linker and the absence of the reducing agent, a band corresponding to a cross-linked

**Structure II. Secreted meprin α subunit; lines above the domains indicate cysteine residues.**

![Oligomerization of Meprin A](http://www.jbc.org/)

**Fig. 2. Subunit organization of meprin. A, meprin was reduced with 2-mercaptoethanol (5 mM), denatured with 1% SDS at 25 °C, and subjected to SDS-PAGE. B, meprin was denatured with 1% SDS (in the absence of 2-mercaptoethanol) followed by SDS-PAGE. C, 2-mercaptoethanol (5 mM) was used to reduce the meprin protein (8 μM) in the absence of SDS, and the protein was subjected to native gel electrophoresis.**
tetramer was observed (Fig. 3A, lane 3). When both cross-linker and reducing agent were present, a cross-linked protein corresponding to the meprin dimer was observed (Fig. 3A, lane 4). This indicates that the cross-link must have occurred between the noncovalently linked subunits because cross-linking between the disulfide-linked dimers would result in only dimers after SDS-PAGE, regardless of whether reducing agent was present or not.

To determine the location of the cysteine residue that was labeled with N-(4-azido-2,3,5,6-tetrafluorobenzyl)-3-maleimidylpropionamide and thus one of the sites of interaction between the noncovalently linked subunits, meprin was labeled with the thiol-reactive probe biotin-maleimide. The site of the modified cysteine residue was determined by subjecting the biotin-labeled protein to tryptic digestion followed by MALDI mass spectrometry to identify the labeled peptide as described under “Experimental Procedures.” A monoisotopic mass of 1655.826 Da ([M+H]+) was observed by reflector mode MALDI mass spectrometry, which is consistent with the monoisotopic mass of the protonated fragment containing amino acids 353–361 modified with the biotin probe (expected mass of 1655.870 Da) (Fig. 3B). The labeled cysteine residue, Cys-355, resides in the MAM domain, which has been described as an adhesion domain that mediates protein-protein interaction. For instance, the MAM domains of receptor protein-tyrosine phosphatase μ and κ are required for specific homophilic cell-cell interactions in transfected nonadherent insect cells (15). The studies herein demonstrate that the MAM domain plays an important role in maintaining both covalent and noncovalent interactions in the meprin multimer. Removal of the MAM domain by truncation or deletion mutagenesis results in misfolding of the protein and subsequent degradation by the proteasome, demonstrating the importance of this domain in folding and assembly of the native protein (16).

**Fig. 3.** Cross-linking of meprin subunits and determination of the site of interaction. A, the photoactivatable cross-linker N-(4-azido-2,3,5,6-tetrafluorobenzyl)-3-maleimidylpropionamide was used to specifically label meprin and cross-link the subunits as described under “Experimental Procedures.” For lanes 1–4, the samples that were separated by SDS-PAGE contain the following: lane 1, meprin unexposed to UV light; lane 2, meprin unexposed to UV light but exposed to 2-mercaptoethanol; lane 3, meprin exposed to UV light; and lane 4, meprin exposed to UV light and 2-mercaptoethanol. The cross-link bands are identified with arrows. B, the site of the label was mapped by tryptic digestion of meprin labeled specifically with biotin maleimide, followed by HPLC purification of the peptide fragments and MALDI mass spectrometry to identify the labeled peptide.

**Fig. 4.** Electron microscopy reveals flexible chain and ring forms of meprin oligomers. A, typical electron micrograph of negatively stained meprin. B, enlarged images that emphasize characteristic views. Row 1, orientation of chains with smooth outer faces and notched inner faces; row 2, orientation of chains with regularly notched sides and midline indentations; row 3, ~80-nm intrachain circles; row 4, large interchain circles. C, histogram of length measurements of meprin oligomer chains. D, histogram of circumference measurements of meprin oligomer circles.

to bend into a variety of crescent shapes and closed circular particles.

The open chains, which comprise the majority of multimers, consistently measure 8–10 nm in width. The length of the chains varies considerably. Whereas most measure between 30
and 60 nm, some are as small as 20 nm or as large as 90 nm. Two distinct views of the chains are visible. One is most common in crescent shapes and is characterized by a smooth outer face and a notched or toothed inner face (Fig. 4B, row 1). The second orientation of the chains is typical of the straighter and shorter oligomers (Fig. 4B, row 2). In this view, both sides have regularly spaced notches, and there are also distinct stain-filled holes along the midline.

The second population of meprin multimer structures is composed of rings. The majority of these structures measure ~80 nm in circumference (Fig. 4D; Fig. 4B, row 3). This indicates that they are formed from the longer chains, that is, when sufficient length is reached to allow interaction of the ends and closure of the crescents. The average measurement of the inner diameter of these rings is 13 nm. Occasionally, much larger circles are seen (Fig. 4B, row 4). These measure 100–120 nm in circumference and appear to be formed by end-to-end interactions of two chains.

Assembly of the complex may involve attaching subunits together to form a rigid structure, such that upon the addition of subunits, the curvature of the oligomer increases until enough subunits are added to close the ring. Formation of a ring from a chain containing lower numbers of subunits may be thermodynamically unfavorable due to the rigidity of the structure. Although ring closure would provide more protein-protein contact, the strain on the structure would make it less stable than the open form, requiring the association of a critical number of subunits for ring formation. Chains containing fewer subunits than this number would be unlikely to form a ring and would remain open, whereas those containing more subunits would favor ring formation.

A model of the multimer is proposed in which the protein is built from disulfide-linked dimers associated noncovalently to form either crescent-shaped structures or closed rings (Fig. 5). The subunit interactions are mediated by the MAM domain, which is involved in both the covalent linkage and noncovalent linkage of the subunits. Reduction of the rings with TCEP results in dissociation into the active protomer, the noncovalently linked dimer. Similarly, reduction of the open, crescent-shaped multimer yields only noncovalently linked dimers. As shown schematically in Fig. 5, reduction would yield monomers at the ends of the chain, which, in the absence of the restriction imposed by the quaternary structure, are free to associate noncovalently.

Meprin may function to degrade large complexes of proteins in vivo, which could require multiple copies of the active protomer in close proximity. The presence of multiple subunits may serve to increase the efficiency of proteolysis. Linkage of these units together in a specified geometry could serve to effectively digest proteins, in a manner reminiscent of the proteasome (18). Meprin also shares similarities to other proteases such as tripeptidyl peptidase II and aminopeptidase I. These enzymes are large multimeric complexes composed of homo-oligomers that function to digest polypeptides and play roles in protein turnover within the cell (19–21). Yeast aminopeptidase I is a dodecamer with a molecular mass of ~640 kDa (20). Tripeptidyl peptidase II forms even larger complexes, between 5000 and 9000 kDa (21). It is composed of subunits of 138 kDa that associate to form a rod-shaped structure about 50 nm in length and 17 nm in diameter (21). However, unlike these proteases, the meprin A homo-oligomer is secreted from the cell and degrades extracellular proteins and peptides.

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