Structure of Homo- and Hetero-oligomeric Meprin Metalloproteases:

Dimers, Tetramers, and High Molecular Mass Multimers*

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Running Title: Oligomerization of Rat Meprins.
Meprin A and B, metalloproteases consisting of evolutionarily-related α and/or β subunits, are membrane-bound and secreted enzymes expressed by kidney and intestinal epithelial cells, leukocytes and cancer cells. Previous work established that the multidomain meprin subunits (each ~80 kDa) form homo and hetero disulfide-bridged dimers, and differ in substrate and peptide bond specificities. The work herein clearly demonstrates that meprin dimers differ markedly in their ability to oligomerize. Electrophoresis, light scattering, size exclusion chromatography and electron microscopy were used to characterize quaternary structures of recombinant rat meprins. Meprin B, consisting of meprin β subunits only, was dimeric under a wide range of conditions. By contrast meprin α homodimers formed heterogeneous multimers (ring, circle, spiral and tube-like structures) containing up to 100 subunits, with molecular masses at protein peaks ranging from approximately 1.0 to 6.0 MDa. The size of the meprin α homooligomers was dependent on protein concentration, ionic strength and activation state. Meprin αβ heterodimers tended to form tetramers but not higher oligomers. Thus, the presence of meprin β, which has a transmembrane domain in vivo, restricts the oligomerization potential of meprin molecules, and localizes meprins to the plasma membrane. By contrast, the propensity of secreted meprin α homodimers to self-associate concentrates proteolytic potential into high molecular weight multimers, and thus allows for autocompartmentalization. The work indicates that different mechanisms exist to localize and concentrate the proteolytic activity of membrane-bound and secreted...
meprin metalloproteinases.
INTRODUCTION

Proteolytic enzymes are essential components of many cellular and extracellular processes from maturation of proteins to cell death (1). Their activities and localities, however, must be highly regulated because of their destructive potential. Regulation of proteases is accomplished through several mechanisms including zymogen formation, inhibition, localization to specific compartments and transcriptional regulation. The structures of proteases themselves have revealed mechanisms to regulate proteolytic activity, and this has been amply demonstrated in the high molecular mass oligomeric structures of the proteasome, tripeptidyl peptidase II, and the tricorn protease (2-4). These multimeric serine and threonine proteolytic complexes are homo- or hetero-oligomeric, have molecular masses of 0.7 to 9 MDa, and are found intracellularly. The structures serve to restrict, localize and concentrate proteolytic activity. Proteases at the cell surface are known to form transient oligomeric complexes, such as those between MT1-MMP (membrane-type 1 matrix metalloproteinase), TIMP-2 (tissue inhibitor of metalloproteinase 2) and MMP-2 (matrix metalloproteinase 2) that lead to the activation of MMP-2 (5). However, stable secreted, multimeric proteolytic complexes were not described until recently, when homooligomers of meprin A were found to form multimers of approximately 0.9 MDa (6). These observations established meprin A as one of the largest known secreted proteases.

Meprins, zinc-dependent metalloendopeptidases of the ‘astacin family’ and ‘metzincin superfamily’, consist of multidomain, evolutionarily-related α and β subunits
The subunits are 42% identical at the amino acid level, highly glycosylated, and form disulfide-linked homo- or hetero-dimers (7, 9). Meprin A (EC 3.4.24.18) is a heterooligomer of $\alpha$ and $\beta$ subunits or a homooligomer of $\alpha$ subunits; meprin B (EC 3.4.24.63) is a homooligomer of $\beta$ subunits (10). The nascent subunits each have a signal peptide that directs the protein to the lumen of the endoplasmic reticulum during biosynthesis, and a propeptide that inhibits activity (11). Each subunit has an astacin-like catalytic domain, and several protein interaction domains including a MAM (meprin, A5 protein and protein-tyrosine phosphatase $\mu$), a MATH (meprin and tumor necrosis factor receptor-associated factor (TRAF) homology), and an AM (after MATH) domain (12). The MAM and MATH domains are found in cell-adhesion superfamily proteins and in adapter proteins in signal transduction, respectively, and permit homo- and hetero-philic selective associations with self and other proteins (13, 14). The MAM, MATH, and AM interaction domains are essential for the biosynthesis of active, stable meprins (12). The nascent meprin subunits are both synthesized with COOH-terminal EGF (epidermal growth factor)-like domains, putative transmembrane spanning domains and short cytoplasmic tails. However, the nascent meprin $\alpha$ subunit, but not the $\beta$ subunit, has a 56 amino acid inserted (I) domain between the AM and EGF-like domains, and the presence of this domain allows for a proteolytic event during maturation that liberates the meprin $\alpha$ subunit from the membrane (15). Because of this COOH-terminal processing, homooligomers of meprin A are secreted proteins, whereas heterooligomers of meprin A and homooligomers of meprin B are membrane-bound.

In mammals, meprins A and B are highly concentrated in kidney and intestinal...
brush border membranes; for example, these proteins are estimated to compose 5% of the mouse brush border membrane of proximal tubule juxtamedullary nephrons (16, 17). Further, membrane-bound and secreted meprins are expressed in leukocytes and cancer cells, implicating these enzymes in inflammation and tumor biology (17-19). The homooligomeric form of meprin A is found in rodent urine and the media of transfected cell lines and colon cancer cells (18, 20, 21). While meprin B and heterooligomeric meprin A are predominantly membrane-bound proteins \textit{in vivo}, there is some evidence that the membrane-bound form of human and rat meprin \(\beta\) can be shed from the cell surface (9, 22, 23).

Meprins can cleave diverse polypeptides including cytokines, basement membrane proteins, growth factors, protein kinases, gastrointestinal peptides and peptide hormones (10, 24-27). Recent studies demonstrated that the individual subunits have marked differences in their peptide bond and substrate specificities and conditions for optimal activity (26, 28). The meprin \(\beta\) subunit has an acidic pH optimum, prefers low ionic strength and has a distinct preference for acidic residues flanking the scissile bond in substrates. In contrast, the meprin \(\alpha\) subunit has a neutral to alkaline pH optimum, prefers small or hydrophobic residues flanking the scissile bond and has a stronger preference for proline residues proximal but not flanking the scissile bond. These substrate and activity differences imply different functions for the meprin isoforms.

Although it is known that dimerization of meprin A is important for stability and activity toward protein substrates, little is known about the propensities of meprin subunits to oligomerize (21). The recent finding that secreted mouse homooligomeric
meprin A was primarily decameric, contrasted with previous observations that indicated heterooligomeric meprin A isolated from mouse kidney was primarily tetrameric (6, 9). Little is known about the structure of meprin B, partially because only small amounts have been available. In addition, to date there has not been an accurate determination of the molecular masses of meprin subunits, or oligomeric forms. The work herein was conducted to determine definitively meprin subunit masses and to characterize the oligomeric forms of meprin A and B. For these studies, truncated and histagged recombinant rat meprin subunits were prepared, allowing for the secretion and subsequent large-scale production of meprins. The recombinant proteins were found to have similar properties to the native enzymes (28).
EXPERIMENTAL PROCEDURES

Expression and Purification of Meprins – Meprins were stably expressed in human embryonic kidney 293 cells by transfecting cells with pcDNA 3.1 (+) expression vectors containing meprin sequence using the calcium phosphate precipitation method. Recombinant rat meprin α was truncated at the putative mature carboxy terminus (R603 of the AM domain) and a histidine tag was added to the carboxy terminus. Meprin β was truncated at K648 which is at the EGF-transmembrane border and a GGGS linker and histidine tag were added to the carboxy terminus (28). The truncation of meprin proteins at R603 and K648 resulted in the secretion of the subunits into the media and the addition of histidine tags allowed for a facile purification scheme. Nickel nitrilotriacetic acid affinity chromatography was used to purify meprins. Homooligomeric meprin A and B were produced by transfecting cells with meprin α or β cDNA alone. A third cell line was transfected with both cDNAs to allow for the production of heterooligomeric meprin A. All proteins were secreted into the media as proenzymes. Active forms of meprins were produced by limited digestion of the purified latent meprin with trypsin as described (28). Meprin was treated with a 1:20 w/w ratio trypsin to meprin and meprin activity was monitored over time. Trypsin was inhibited with a 20-fold excess of soybean trypsin inhibitor when meprin activity no longer increased. Trypsin and soybean trypsin inhibitor were subsequently removed by size exclusion chromatography (SEC) using a Superose 6 column. Meprin protein was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and gels were stained with Coomassie blue to assess purity and
verify complete activation.

**PAGE and Immunoblotting** – Protein samples, boiled in sample buffer with SDS and 2-mercaptoethanol, were routinely subjected to electrophoresis on 7.5% Ready gels (Bio-Rad) unless indicated. For native PAGE, 3-8% NuPAGE Tris-acetate gels in the absence of SDS and reducing agent were used (Invitrogen). Individual meprin α and β subunits were detected using the subunit specific polyclonal antibodies, HMC52 and PSU56 respectively that were developed by our laboratory. HMC52 and PSU56 antibodies did not cross-react with the incorrect subunit with the amounts used in these studies.

**Collection of Rat Urine** Two female rats were placed in metabolic cages and urine was collected for 6 h. Urine was kept on ice. After collection, urine was filtered through a 0.2 μm cellulose acetate filter to remove particles. The filtered urine (6 ml) was buffer exchanged into 8 ml of 20 mM Tris-HCl, 150 mM NaCl, pH 7.5 using Econo-Pac 10 DG chromatography columns (Bio-Rad) and then concentrated to 600 μl using a Centriplus YM-50 concentrator (Millipore).

**Mass Spectroscopy Determination of the Monomeric and Dimeric Molecular Masses of Meprin A and B** – Purified recombinant rat latent and active meprins in the presence or absence of 10 mM dithiothreitol (DTT) were used for matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass spectroscopy studies. Molecular masses were determined using a Voyager-DE RP/Pro BioSpectrometry Workstation (PerSeptive Biosystems). The machine was set in the linear, delayed positive mode. The accelerating voltage was 25000 V, grid voltage 89%, guide wire
0.15% and extraction delay time 1200 nsec. At least 32 laser shots were taken per spectra and at least five spectrums were used to give the molecular mass. Proteins were diluted in a saturated matrix solution of sinapinic acid (Sigma) in 30% acetonitrile, 0.3% trifluoroacetic acid in water and then spotted on a stainless steel plate (PerSeptive Biosystems). Standards were the Sequazyme BSA (bovine serum albumin) and IgG1 mass standard kits (PerSeptive Biosystems). The low mass gate was set at 10 kDa. Data were analyzed using Data Explorer software Version 4.0.0.0 (Applied Biosystems).

Determination of the Molecular Masses of Meprin Proteins in Solution by Size Exclusion Chromatography Light Scattering – The molecular masses of native meprin proteins were determined using SEC light scattering (LS) in the HHMI Biopolymer Facility and W. M. Keck Foundation Biotechnology Resource Laboratory by Ewa Folta-Stogniew as described (29, 30). Briefly, a 100 µg sample of each form of meprin in a 500 µl volume was filtered through a 0.22 µm Durapore membrane (Millipore). The filtrate was applied to a Superose 6 HR 10/30 column upstream of Dawn DSP LS (Wyatt Technology), model 733 variable wavelength KRATOS UV (Applied Biosystems) and OPTILAB DSP refractive index (Wyatt Technology) detectors. The column was equilibrated in 20 mM HEPES, 150 mM NaCl, pH 7.5 at a flow rate of 0.3 ml/min. The weight average molecular masses ($M_W$) of rat meprins were calculated at peak maxima using three independent analyses, the two and three detector method and ASTRA analysis. A second order Berry fit was used for latent and active homooligomeric meprin A and a zero order Debye fit was used for the other meprins. The $M_W$ was estimated throughout the entire eluting peak at 5 µl intervals using ASTRA software.
Computations were performed as described (29, 30). For analysis of meprin molecular masses under different conditions a Superose 6 10/30 HR column was calibrated using the molecular mass data obtained from the SEC-LS analyses. The column was equilibrated in 20 mM Tris-HCl, 150 mM NaCl, pH 7.5 and the flow rate was 0.3 ml/min.

_Electron Microscopy and Image Analyses_ – For electron microscopic analyses, samples of purified recombinant rat latent and active heterooligomeric meprin A and homooligomeric meprin A and B were diluted to 10 µg/ml with 20 mM Tris-HCl, pH 7.5 with or without 150 mM NaCl. Samples were negatively stained with 1% uranyl acetate and imaged at absolute magnifications of 50,000 or 63,000. For image analyses, micrographs were digitized on an Agfa Duoscan flatbed scanner at an optical resolution on the image scale corresponding to either 4.06 or 3.22 Å/pixel, respectively. Composites of typical fields and galleries of individual images were prepared using Adobe Photoshop. Statistics of individual particle measurements were compiled from enlarged prints on a digitizing tablet using SigmaScan (Jandel).

All image analysis was performed using the SPIDER/WEB software package (31). For two-dimensional averaging, each data set of untitled images was processed by reference-free alignment and hierarchical ascendant classification using principal component analysis. Three-dimensional volumes were calculated by iterative back projection. Those for activated meprin B and latent heterooligomeric A were constructed _de novo_ from tilt pairs of micrographs. These volumes were used as references for determining reconstruction angles by projection mapping for the volumes of latent
meprin B and activated heterooligomeric A, respectively. The numbers of images in each data set were: 7382 for latent meprin B, 5850 for active meprin B, 4366 for latent heterooligomeric meprin A, and 4470 for active heterooligomeric meprin A. In all instances, the number of images was limited so that angular coverage would be as even as possible. There were no significant areas of missing information for any of the volumes. Resolution limits were determined from the 50% cutoff of the Fourier shell coefficient between volumes of half data sets. Volume surfaces were created using IRIS Explorer (Numerical Algorithms Group). They are shown after filtering to their resolution limits and at the threshold for 100% mass as calculated using the molecular masses shown in Table I and a partial specific volume of 0.71 g/cm$^3$ (Dr Faoud Ishmael, personal communication).
RESULTS

Purification and Initial Characterization of Recombinant Histidine Tagged Rat Meprins – All forms of meprin proteins were purified to homogeneity (Fig. 1; left panel). The latent meprin β used in these studies is predicted to be larger than meprin α due to the presence of the EGF-like and a portion of the AM domain in meprin β and indeed it appeared to be larger by SDS-PAGE. Meprin bands were diffuse as previously published and as expected for highly glycosylated proteins (9). Rat meprin β has eight potential asparagine-linked glycosylation sites compared to six in the rat meprin α sequence; additional glycosylation in the meprin β subunit could contribute to the higher molecular mass compared to meprin α as well as the occurrence of the more diffuse band.

The band for the latent heterooligomeric meprin A migrated between that of homooligomeric meprin A and B. In some instances two bands were visible under reducing conditions corresponding to each subunit. However, the subunits in the heterooligomeric protein did not resolve well on the gels (Fig. 1; left panel). In order to obtain more accurate molecular masses, MALDI-TOF was employed (Table I). Using MALDI-TOF the molecular masses of latent meprin β and α subunits were found to be 85.5 and 77.7 kDa, respectively.

Quantitative Western analysis was employed to determine the ratio of the meprin α and β subunits in the purified rat heterooligomeric meprin A (21). Subunit specific antibodies were used to quantify the amount of each subunit present. Known amounts of purified homooligomers were used as standards, run on the same gels and calibration
curves were constructed. The amount of each standard and meprin subunit in each sample was determined by densitometry. The protein consisted of an approximately equal amount of subunits; a 1 : 1.2 ratio of the meprin β and α subunits was calculated.

The activation of meprin subunits by trypsin resulted in mobility shifts by SDS-PAGE (Fig. 1; left panel). The molecular mass losses were 3.4 and 9.4 kDa for the meprin α and β subunits, respectively, as determined by MALDI-TOF (Table I). Trypsin treatment removes the propeptide of both subunits (11, 22, 32). The greater loss of molecular mass in the meprin β subunit is probably due to the additional loss of amino acids within the EGF domain. Indeed, trypsin is used to remove meprin β subunits from brush border membranes (32).

All six forms of recombinant meprins were subjected to PAGE in the presence of SDS and absence of 2-mercaptoethanol to ensure that histidine-tagged proteins were also able to covalently dimerize in a similar manner to wild-type meprins (9, 33). All forms of meprins migrated in a manner consistent with the formation of disulfide-linked dimers (Fig. 1; right panel). The molecular masses of homooligomeric latent meprin A and B were 156 and 171 kDa respectively (Table I). The heterooligomeric form of latent meprin A had a molecular mass of 166 kDa. The molecular mass and densitometry data are consistent with a disulfide-linked heterodimer of meprin α and β subunits as expected (33). Molecular masses of 148, 154 and 152 kDa were determined for the active forms of homooligomeric meprin A and B and heterooligomeric meprin A respectively (Table I).

Native PAGE Demonstrates Evidence of Meprin Oligomers – Coomassie staining
of native PAGE gels indicated that the latent and active forms of homooligomeric meprin A had a molecular mass considerably greater than 669 kDa (Fig. 2). Therefore the activated homooligomer of recombinant rat meprin A forms high molecular mass complexes, analogous to the mouse homologue (6). The latent homooligomeric meprin A was not able to enter the gel indicating that it was larger than the active counterpart. PAGE in the presence of SDS and absence of 2-mercaptoethanol yielded dimers (Fig. 1; right panel). Therefore, the formation of the large complexes was dependent on noncovalent interactions. In contrast, the latent and activate forms of homooligomeric meprin B had electrophoretic mobilities that corresponded to molecular masses of approximately 200 kDa, consistent with the formation of dimers (Fig. 2). Analytical ultracentrifugation studies also demonstrate that rat meprin B exists as a dimer (Dr Faoud Ishmael, personal communication). Dimers were also formed under denaturing conditions (Fig. 1; right panel). Therefore, rat meprin β subunits do not interact noncovalently to form larger complexes under these conditions. The active form of meprin B was slightly smaller than the latent form as assessed by native PAGE as expected. For heterooligomeric meprin A two bands were visible for both the latent and active forms. The mobilities of the bands corresponded to molecular masses of approximately 200 and 400 kDa. Thus, latent and active heterooligomeric meprin A existed as a mixture of dimers and tetramers under these conditions (Fig. 2). Heterooligomeric meprin A existed as dimers in the presence of SDS and absence of 2-mercaptoethanol (Fig. 1; right panel). Thus noncovalent interactions were involved in the putative dimer to tetramer transition. Interestingly, the active tetramer had a larger
apparent molecular mass than the latent form by this technique.

The Oligomeric Size of Meprins in Solution – SEC-LS was used to obtain additional oligomeric state information. SEC separates polydisperse mixtures of proteins before the determination of $M_W$. A single peak with a large tail was evident after SEC of the latent form of homooligomeric meprin A (Fig. 3A). The peak contained macromolecules with a $M_W$ range between 1.5 to 8.0 MDa (Table II). The signal saturated the LS detector at the peak maximum (7.9 ml). Nevertheless, it was clear from the $M_W$ distribution that homooligomeric meprin A formed a polydisperse pool of various molecular mass macromolecules. The large complexes were composed of up to more than 100 monomers based on a monomeric molecular mass of 77.7 kDa (Table I and II). The $M_W$ at 7.8 ml, near the peak maximum (7.9 ml) was 6.1 Mda (Table II). This value is consistent with this form of meprin existing as oligomers composed of approximately 39 dimers or 78 subunits (Table I and II). Using the two and three detector approach, a $M_W$ of 5.5 MDa was predicted for the peptide portion of the protein, indicating that on average 82 monomers are involved in the macromolecular structures. Berry analysis revealed that the macromolecules had root mean square (rms) radii ranging from 20 to 55 nm and an average of 26 nm (data not shown).

The SEC UV trace of the active form of homooligomeric meprin A indicated that this protein was less heterogeneous than the latent protein (Fig. 3B). The active protein peak contained macromolecules with $M_W$ values ranging from 1.0 to 1.7 MDa as compared to 1.5 to 8.0 MDa for the latent protein (Table II). The peak maximum was at
10.8 ml and had a MW of 1.5 MDa. It was clear that the peak contained a polydisperse mixture of macromolecules containing between 14 and 22 monomers with an average of 20 monomers (icosamers) based on a monomeric molecular mass of 74 kDa (Table I and II). The two and three detector approaches provided a MW of 1.3 MDa for the peptide portion of the protein. The molecular mass of the peptide portion is predicted to be 64.1 kDa per subunit indicating that this form of meprin exists as icosamers on average. The macromolecules had rms radii of gyrations ranging from 11 to 34 nm and an average of 17 nm (data not shown). The molecular mass value of active homooligomeric meprin A reported here of 1.5 MDa is higher than that reported for the mouse homologue which was 900 kDa (6). SEC-LS is a more accurate technique as compared to approaches used in previous studies for the measurement of molecular mass. However, there may be differences in multimerization of meprins from various species.

Single, well-resolved peaks were evident when the latent and active forms of homooligomeric meprin B were subjected to SEC. The peak maxima of latent and active meprin B were at 14.7 and 14.9 ml respectively indicating that meprin B was much smaller than homooligomeric meprin A (Fig. 3C and D). ASTRA computed Debye analysis revealed that the peaks contained macromolecules with MW values of 154 to 185 and 145 to 166 kDa demonstrating that both forms of meprin B existed in monodisperse dimeric states based on monomeric molecular masses of 85.5 and 76.1 kDa (Table I and II). The heterogeneity probably arises from differential glycosylation; the rat meprin β subunit has eight potential asparagine glycosylation sites, thus the dimer
would have sixteen potential sites. The $M_W$ values at the peak maxima for the latent and active proteins were 181 and 161 kDa respectively by Debye analysis; the two and three detector methods predicted $M_W$ values for the polypeptide portions of species to be 139 and 123 kDa. Based on the polypeptide portions having predicted monomeric molecular masses of 72 and 63 kDa, this is good evidence that the meprin B homooligomer existed as dimers; all three independent methods of light scattering analyses giving equivalent results.

After SEC of the latent form of heterooligomeric meprin A, two peaks were evident with maxima at 13.6 and 14.5 ml (Fig. 3E). The 13.6 ml peak contained macromolecules with $M_W$ in the range of 300 and 360 kDa. The maximum of the peak had a $M_W$ 333 kDa by Debye analysis. The two and three detector approaches predicted molecular masses of 268 and 269 kDa respectively. Based on the dimeric molecular mass of a heterodimer (166 kDa; Table I) and the polypeptide sequence-predicted molecular mass for the equimolar mixture of monomeric forms of the two subunits (140 kDa), this peak contained a tetramer, a noncovalent dimer of disulfide-linked heterodimers (Table II). Debye analysis indicated that the second peak, which eluted at 14.5 ml contained macromolecules with $M_W$ values between 181 and 240 kDa. The maximum of the peak had $M_W$ values of 228, 170 and 171 kDa by Debye, two and three detector approaches (Table II). The observed $M_W$ distribution by Debye calculations indicated that this peak contained a heterodimer of meprin $\alpha$ and $\beta$ subunits. The calculated values were probably higher than actual values due to poor resolution during
the SEC step between the dimer and tetramer. The rms radii of these molecules could not be determined by this method because the size fell below the accuracy limit of the machine.

Two peaks were evident during the SEC of the active form of heterooligomeric meprin A with maxima at 13.8 and 14.8 ml (Fig. 3F). ASTRA computed Debye analysis indicated that the 13.8 ml peak contained macromolecules with $M_W$ values in the range of 250 to 331 kDa. The $M_W$ at the maximum of the peak was 304 kDa by Debye analysis; by the two and three detector approaches the $M_W$ were 245 and 246 kDa respectively (Table II). Based on the dimeric molecular mass of a heterodimer (152 kDa; Table I) and the polypeptide sequence-predicted $M_W$ for the equimolar mixture of monomeric forms of the two subunits (127 kDa), this peak contained a tetramer, presumably a noncovalent dimer of disulfide-linked heterodimers. The second peak eluted with a maximum at 14.8 ml and contained macromolecules with a range of $M_W$ values between 140 to 200 kDa (Table II). The observed $M_W$ distribution indicated that this peak contained a heterodimer of meprin $\alpha$ and $\beta$ subunits. The peak did not resolve well from the major peak that eluted at 13.8 ml. The maximum of the 14.8 ml peak had $M_W$ values of 182, 145 and 146 kDa by the Debye, two and three detector approaches (Table II). Although these values are higher than expected for dimers, the observed $M_W$ distribution indicates that this peak contained a heterodimer of meprin $\alpha$ and $\beta$ subunits. The estimated values are probably higher than the actual values due to poor resolution.
between the dimer and tetramer in the size exclusion chromatography step.

The Effect of Meprin Concentration and Ionic Conditions on the Formation of Higher Order Oligomers – SEC data indicated that the oligomeric states of homo- and hetero-oligomeric meprin A are dependent on the concentration of meprin (Summarized in Table III). The molecular masses and therefore oligomeric states of meprins were estimated using the LS-SEC data rather than traditional protein standards to avoid erroneous results due to shape effects, interaction with the resin and other problems associated with calibrations of this type. The multimeric state assigned to homooligomeric meprin A was based on the elution volume of the peak maximum. As the concentration of homooligomeric meprin A was increased, the elution volume at which the peak maxima appeared was lowered, therefore the apparent molecular mass of the protein complex increased. The peak maxima had apparent molecular masses that indicated on average, oligomers formed which had between 16 and 78 subunits in 150 mM NaCl. However it is clear that the samples had a broad range of molecular masses and oligomeric states. Thus, some oligomers exist that are composed of less than 16 monomers and some exist that have more than 78 monomers in the multimer. The range of elution volumes seen with latent homooligomeric meprin A was much larger than that for active protein (Table III). This implies that the propeptide is intimately involved in oligomerization. The average number of monomers involved in the oligomer at the peak maxima ranged from 16 to 78 for the latent and 16 to 20 for the active protein. A high NaCl concentration (1 M) was able to disrupt the noncovalent interactions in meprin A, resulting in smaller macromolecules. In the presence of 1 M NaCl, the latent form of
homooligomeric meprin A existed in tetrameric states on average, whereas the active protein existed in hexameric states on average.

The presence of homooligomeric meprin A has been reported in rodent urine (20). It was of interest to determine if urinary meprin A (α only) had the ability to form large oligomeric complexes. Therefore, rat urine was subjected to SEC on a Superose 6 column. The meprin in the injection sample was at a concentration of approximately 200 nM based on quantitative Western analysis. The elution volume was approximately 11.5 ml. Based on calibrations using the SEC-LS data this elution volume corresponds to a molecular mass at the peak maximum of approximately 1.6 MDa. Thus, native rat meprin A forms large oligomeric species in a similar manner to the recombinant protein.

The latent and active homooligomeric meprin B behaved in a completely different manner to homooligomeric meprin A by SEC. The elution volume was unaffected by meprin B concentration (Table III). In addition, the oligomeric state appeared to be independent of the concentration of NaCl from 150 mM to 1 M (data not shown). Therefore, dimeric species existed under all conditions studied.

It was evident that heterooligomeric meprin A formed two oligomeric species by SEC. These forms were dimers and tetramers based on the SEC-LS data (Fig. 3E and F; Table II). The dimer-tetramer was in a dynamic equilibrium. The oligomeric state was dependent on the concentration of meprin (Table III). The latent protein primarily formed tetramers under the conditions used except at low concentrations of meprin (20 nM) and with high amounts of NaCl (1 M), under these conditions the dimer was preferred. The active protein behaved in a similar manner although the dimer was only preferred in
the presence of 1 m NaCl. Therefore, heterooligomeric meprin A behaves in a similar manner to homooligomeric meprin A, however a tetramer was the highest oligomeric species observed under the conditions studied.

Ultrastructure of Meprin Oligomers – Transmission electron microscopy was used to visualize the structures of meprin oligomers. Both latent and active homooligomeric meprin A formed multiple types of structures (Fig. 4A and B). These included rings, crescents and spiral chains that are similar to those seen for the mouse homologue (6). However much longer chain lengths were observed for the latent form of the rat enzyme. In low salt conditions the latent form of homooligomeric meprin A existed as chains typically about 90–100 nm in length but they could extend up to 400 nm (Fig. 5A). In the presence of 150 mM NaCl, fewer of the extremely long polymers were seen and the majority measured 50–75 nm consistent with the SEC-LS data (Fig. 5B). As presented at higher magnification, the latent protein not only existed as circles and crescents but also as novel tube-like and long spiral-like structures (Fig. 6; rows 1-3). The tubes measured 30–40 nm in length and approximately 30 nm in width (Fig. 5C and D). The tube width corresponded to the diameter of the circular forms, and indicated that these are stacks of 3 to 6 rings or highly condensed spirals. The occurrence of tube-like structures was dependent on the presence of 150 mM NaCl.

The latent and active forms of meprin B were observed to be much smaller particles than homooligomeric meprin A (Fig. 4C and D). The predominant class of averaged images of the latent form of meprin B measured approximately 11 by 12 nm, while that of the active form was slightly smaller at 10 by 10 nm (Fig. 7; row A). Both
forms are characterized by discrete areas of high density surrounding a central cavity. In the particular orientation shown for the latent form, a surface opening is apparent. The latent and active forms of heterooligomeric meprin A have markedly different dimensions to homooligomeric meprin A and B (Fig. 4E and F). The most populated averaged images of these particles clearly show a distinctly barrel shape with a symmetric arrangement of two protomers connected by thin bridges (Fig. 7; rows B and C). The largest dimensions are approximately 18 by 11 nm for both the latent and active forms.

Surface representations of the three-dimensional volumes provide additional support for the dimeric and tetrameric composition of meprin B and heterooligomeric meprin A, respectively (Fig. 7; rows D and E). The volumes of latent and active meprin B measure approximately 10 x 11 x 11 nm and have two distinct domains likely corresponding to the individual subunits (Fig. 7; row D). When comparing latent and active meprin B, the mass bridging the two halves of the latent form is no longer visible in the active form. The three-dimensional volumes of latent and active heterooligomeric meprin A (Fig. 7; row E) measure approximately 9 x 11 x 18 nm. The mass distributions are consistent with the four-ringed barrels seen in the two-dimensional projection (Fig. 7; rows B and C) and indicate a symmetric assembly of two protomers. Again, in the active form, less bridging mass is seen and the central opening is larger.

A model for the oligomerization of rat meprins is proposed in Fig. 8. The simplest form is meprin B, a disulfide-linked dimer of β subunits. Thus meprin β subunits are limited to covalent interactions and lack the ability to associate with other meprin α and β subunits by noncovalent interactions. A disulfide-linked homodimer of
α subunits is the building block of homooligomeric meprin A complexes. These dimers are able to associate further to produce the high molecular weight structures seen by electron microscopy. Oligomerization of homooligomeric meprin A is in a dynamic equilibrium whereby dimers continuously associate and disassociate with others dimers via noncovalent interactions. Finally heterooligomeric meprin A predominantly consists of a disulfide-linked heterodimer as the building block. The dimers are able to associate with other dimers, via noncovalent interactions of α subunits to form tetramers. This is the largest oligomer possible because the meprin β subunits reside on the outer surface of the oligomer.
DISCUSSION

The multiple technologies used in these studies establish definitively that the meprin \(\alpha\) and \(\beta\) subunits have vastly different propensities to form homophilic associations. It is clear that meprin B exists as a disulfide-linked dimer under a wide range of concentrations and has no tendency to form higher molecular mass complexes. This information about meprin \(\beta\) provides a rationale for the observation that the heterooligomeric meprin \(\alpha\beta\) dimer only forms tetramers in spite of the fact that the meprin \(\alpha\) subunits tend to self-associate into larger multimers. Thus, the solubilized recombinant form of heterooligomeric meprin A studied herein, as the meprin A isolated from mouse and rat kidney brush border membranes, is limited in the ability to multimerize because of the presence of meprin \(\beta\) \(9, 34\). The recombinant rat meprin \(\alpha\) homodimers associate into a polydisperse mixture of high molecular mass complexes (millions of Da), as homooligomeric meprin A in rat urine and recombinant mouse homooligomeric meprin A \(6\). The previous work demonstrating that mouse homooligomeric meprin A formed multimers was conducted with activated protein. The work with the rat enzymes herein establishes that latent homooligomers of meprin A form even larger complexes than activated forms, and the degree of multimerization is dependent on protein and salt concentration. We propose that the function of these very large latent proteolytic complexes is to enable the movement of these metalloproteinases through extracellular spaces in a nondestructive, concentrated ‘particle’ to areas where they are activated in order, for example, to destroy foreign material or undesirable protein
complexes. The self-association of the latent proteases would allow the delivery of a high concentration of proteolytic activity only at specific sites containing activating proteases. Sites of inflammation and metastasizing cancer cells, for example, are known to be associated with the presence of many types of trypsin-like proteases that are capable of activating meprins (35-37).

The kidney and intestine have the highest levels of expression of meprins in vivo, and under normal circumstances meprin B and heterooligomeric meprin A are membrane-bound while homooligomeric meprin A is secreted into the lumen of the proximal tubule and intestinal lumen. The concentration of secreted meprin A in these tissues has not been determined, however, our studies indicate that in rat urine the concentration is approximately 20 nM, where it exists as a multimer of approximately 16 subunits. Both protein and salt concentrations markedly change from the kidney proximal tubule to the urine as water and solutes are reabsorbed. Osmolarity, for example, varies from isotonic conditions in the proximal tubule fluid (300 mOsm/l; equivalent to 150 mM NaCl) to hyperosmotic in the descending loop of Henle and urine (1400 mOsm/l) and hypoosmotic in the ascending loop of Henle (80 mOsm/l; 38). Thus the multimeric structure of homooligomeric meprin A will vary markedly throughout the nephron. The activities of meprins are also salt dependent (28). The affects of salt concentration on homooligomeric meprin A activity are minor in the range between 150 and 1000 mM (k_{cat}/K_{M} specificity constants 77 and 86 x 10^{4} M^{-1} s^{-1} respectively) whereas the differences in oligomerization are marked (oligomer contains 16-20 subunits at 150 mM and six at 1000 mM). Thus, higher order oligomerization does not correlate
with altered specific activity.

The membrane-bound forms of kidney meprin are concentrated in the juxtamedullary region of the cortex (16). Based on the purification schemes of meprin (i.e. the total amount purified and the yield) the concentration of meprin at the kidney brush border membrane \textit{in vivo} is estimated to be in the high micromolar range (39). There is evidence that membrane-bound meprins associate with other proteins at the membrane, including other proteases (e.g., angiotensin converting enzyme and leucine aminopeptidase) and amino acid transporters (24, 32, 39). The data thus far indicate that meprin $\beta$ is the subunit involved in heterophilic interactions, in contrast to meprin $\alpha$ that is clearly involved with homophilic interactions. Heterophilic proteolytic complexes at the plasma membrane could coordinately degrade proteins to produce small peptides, dipeptides and amino acids that can then directly feed to the associated amino acid transporters. This would be an efficient link for proteolysis and recycling of protein building blocks.

It has become increasingly apparent that several key proteolytic processes depend on the formation of very large multiprotein complexes. For example, in apoptosis a central scaffold protein oligomerizes to form the apoptosome (700 to 1,400 kDa) and recruits and activates caspases (40). Also the tricorn protease, an icosahedral capsid of 5 to 9 Mda is proposed to interact with other proteases such as prolyl iminopeptidase and the 20S proteasome (700 kDa) with its 4 stacked heptamer rings to form giant proteolytic machines (4). Large macromolecular enzymatic assemblies have probably evolved as a means of autocompartmentalizing a reaction or series of reactions. The close proximity
of many active sites may act by engulfing large protein substrates and degrading them in a concerted fashion. Homo- and hetero-oligomeric meprin structures may represent novel molecular scaffolds that prevent inadvertent proteolysis by confining proteolytic active sites to localized regions.

The structure of meprin B and the heterooligomeric form of meprin A were elucidated for the first time in the work herein. It is clear that the structures of the covalently-linked dimers and tetrameric meprins have distinct overall shapes and subunit arrangements. Both latent and active dimeric meprin B are approximately square and each contains one large central cavity. In contrast, heterooligomeric meprin A is rectangular and somewhat resembles the barrel shape of the proteasome. Again, both latent and active forms are characterized by extremely large continuous central cavities. Activation of meprin B and heterooligomeric meprin A results in an expansion of the overall structures as indicated by decreases in central densities observed in the two- and three-dimensional images. The volume dimensions of dimeric meprin B were approximately 10 x 11 x 11 nm and tetrameric heterooligomeric meprin A were approximately 9 x 11 x 18 nm. Thus dimeric and tetrameric meprins are similar in size to the 20S component of the proteasome, a 700 kDa complex with dimensions of approximately 11 x 11 x 15 nm (2). The locations and structural contributions of the multiple glycans within the meprin proteases are as yet unknown and they may contribute to the large volume, saccharides having greater hydrodynamic volumes compared to polypeptides. Further, meprins appear to have a more open structure than the proteasome. The central cavity of the proteasome is narrow and represents less than 10%
of the entire volume. In contrast the cavities in meprins represent approximately 30% of the total volume.

It is likely that the oligomerization propensity of meprins lies in the noncatalytic domains (e.g., MAM, MATH and AM). None of the other astacin family members contain these interaction domains, and none of them are known to form dimers or multimers. Both MAM and MATH domains are implicated in homo- and hetero-phlic protein-protein interactions (6, 13, 14, 41). For example, the MATH domain in TRAF2 is involved in trimerization events (42). TRAF domains have been identified in a diverse set of proteins throughout eukaryotes (43). These domains permit homo- and hetero-phlic interactions with other proteins carrying TRAF domains or act as a scaffold on which other types of protein recognition elements are displayed. TRAF domains have been identified in a large number of intracellular proteins that are associated with signal transduction. Meprins are the only extracellular proteins identified thus far to contain TRAF domains, and therefore, broaden the potential role of these domains in cell physiology.

The meprins have emerged as unique proteinases with features/domains common to many other proteins. The differences between the evolutionarily-related meprin α and β subunits will provide insights into elements or motifs that drive oligomerization and this will no doubt have relevance to many protein-protein interactions and physiologic and pathological processes.
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FOOTNOTES

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1The abbreviations used are: MAM, meprin, A5 protein and protein-tyrosine phosphatase µ; MATH, meprin and tumor necrosis factor receptor-associated factor (TRAF) homology; AM, after MATH; EGF, epidermal growth factor; SEC, size exclusion chromatography; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; MALDI-TOF, matrix assisted laser desorption ionization time of flight; LS, light scattering; M_W, weight average molecular mass.
FIGURE LEGENDS

Fig. 1. Characterization of Purified Recombinant Rat Meprins by SDS-PAGE. Left panel, latent (-) and active (+) meprins were subjected to electrophoresis under reducing conditions (presence of 2-mercaptoethanol) on 7.5% SDS-PAGE gels. Active meprins were produced by limited trypsin hydrolysis as described (28). Right panel, latent and active meprins were subjected to electrophoresis under nonreducing conditions on 7.5% SDS-PAGE. Protein was stained by Coomassie Brilliant Blue R-250. Prestained standards were α2-macroglobulin (190 kDa), β-galactosidase (115 kDa), fructose-6-phosphate kinase (85 kDa), pyruvate kinase (66 kDa) and fumarase (59 kDa). Homo A, homooligomeric meprin A; Homo B, homooligomeric meprin B; Hetero A, heterooligomeric meprin A.

Fig. 2. Native PAGE of Recombinant Rat Meprins. The latent (-) and active (+) forms of recombinant homooligomeric meprin A (Homo A) and B (Homo B) and heterooligomeric meprin A (Hetero A) were subjected to electrophoresis on nonreducing and nondenaturing gels (native PAGE). NuPAGE 3-8% Tris-acetate gels were used in the absence of SDS and reducing agent. Meprins were stained with Coomassie blue. Thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa) and lactate dehydrogenase (140 kDa) were used as standards.

Fig. 3. Native Molecular Masses of Recombinant Rat Meprins by Size Exclusion
Chromatography Light Scattering (SEC-LS). Approximately 100 µg of each meprin was subjected to SEC on a Superose 6 column. The downstream refractive index, UV (Abs 280 nm, solid line) and light scattering detectors allowed for the determination of the weight average molecular mass (MW, dotted line) by ASTRA software using Debye (latent and active homooligomeric meprin A) or Berry analysis (all other forms of meprin) at 5 µl intervals. Every tenth datum point is shown on the MW line for clarity. Panel A and B, latent and active homooligomeric meprin A; Panel C and D, latent and active homooligomeric meprin B; Panel E and F, latent and active heterooligomeric meprin A.

Fig. 4. Electron Micrographs of Rat Meprins. Representative fields of negatively stained samples of each meprin isoform are shown. A and B, latent and active homooligomeric meprin A; respectively; C and D, latent and active homooligomeric meprin B, respectively; E and F, latent and active heterooligomeric meprin A, respectively.

Fig. 5. Histograms of Size Measurements of Latent Homooligomeric Meprin A Structures. The dimensions of selected latent homooligomeric meprin A structures are shown. A, chain lengths in absence of salt; B, chain lengths in presence of 150 mM NaCl; C, lengths of tubes; D, widths of tubes.

Fig. 6. Multiple Structures of Latent Homooligomeric Rat Meprin A Observed in
**Negatively Stained Electron Micrographs.** Row 1, closed rings and crescents; row 2, tubes; row 3, spirals.

**Fig. 7.** Images from Negatively Stained Electron Micrographs Illustrate Dimeric and Tetrameric Ultrastructure of Latent and Active Homooligomeric Meprin B and Heterooligomeric Meprin A. The left column contains images of the latent forms of the enzymes, while the right column depicts activated enzymes. Row A, the first and third panels are the most populated image averages of meprin B. The number of images in each average is in the lower right of the panels; the second and fourth panels depict corresponding images with density contour overlays where whiter areas show highest densities. Row B, image averages of heterooligomeric meprin A. Row C, corresponding images with density contour overlays. Row D, three-dimensional reconstructions of meprin B. Row E, three-dimensional reconstructions of heterooligomeric A. All three-dimensional volumes have been filtered to their resolution limits of 22–25 Å and are shown at a threshold calculated to correspond to 100% of the protein mass.

**Fig. 8.** Diagrammatic Representation of Rat Meprin Oligomerization. Meprin B exists as a disulfide-linked dimer. Homooligomeric meprin A exists in many forms including long chains, spirals, rings and tubes. The large complexes form via noncovalent interactions between disulfide-linked dimers. Heterooligomeric meprin A exists in a dimer-tetramer equilibrium. It is postulated that the noncovalent interface of the tetramer is from the meprin α subunit due to the inability of meprin β subunits to
interact noncovalently.
Table I: Monomeric and Dimeric Molecular Masses of Meprin A and B.

| Meprin Isoform          | Monomer       | Dimer       |
|-------------------------|---------------|-------------|
| Homooligomeric Meprin A | Latent        | 77.7 ± 0.4  | 156 ± 0.3   |
|                         | Active        | 74.3 ± 0.3  | 148 ± 0.2   |
| Homooligomeric Meprin B | Latent        | 85.5 ± 0.3  | 171 ± 0.4   |
|                         | Active        | 76.1 ± 0.4  | 154 ± 0.2   |
| Heterooligomeric Meprin A | Latent    | ND          | 166 ± 0.6   |
|                         | Active        | ND          | 152 ± 0.8   |

The monomeric and dimeric molecular masses of meprin subunits were determined by MALDI-TOF mass spectroscopy in the presence or absence of reducing agent, dithiothreitol, respectively. Noncovalent interactions between subunits were disrupted due to the presence of acetonitrile and trifluoroacetic acid. The mean of at least five spectra and the standard deviations are reported for each protein. ND, not determined.
Table II: Native Molecular Masses of Meprins.

| Molecular Mass (kDa) | Peak Maximum | Range               |
|----------------------|--------------|---------------------|
|                      | Two Detector | Three Detector | ASTRA | ASTRA            |
| **Homo A**           |              |                    |       |                  |
| Latent               | 5,500*       | 5,500* ± 500       | 6,100* | 1,500 – 8,000*1  |
| Active               | 1,300        | 1,300 ± 200        | 1,500  | 1,000 – 1,700    |
| **Homo B**           |              |                    |       |                  |
| Latent               | 139          | 139 ± 4            | 181    | 154 – 185        |
| Active               | 122          | 123 ± 4            | 161    | 145 – 166        |
| **Hetero A**         |              |                    |       |                  |
| Latent (Peak 1)      | 268          | 269 ± 11           | 333    | 300 – 360        |
| Latent (Peak 2)*2    | 170          | 171 ± 16           | 228    | 181 – 240        |
| Active (Peak 1)      | 245          | 246 ± 16           | 304    | 250 – 331        |
| Active (Peak 2)*2    | 145          | 146 ± 21           | 182    | 140 – 200        |

Size exclusion chromatography-light scattering (SEC-LS) was employed to determine the weight average molecular mass ($M_W$) of meprins. Three independent analyses, ASTRA software (Berry and Debye calculations) and the two and three detector methods were used to determine the $M_W$ at the peak of each meprin. ASTRA was used to determine the $M_W$ throughout the peak to give the entire $M_W$ range under the conditions
used. *Determined at 7.8 ml rather than the peak maximum (7.9 ml), due to saturation of LS signal at the peak; *1Selected slices calculated due to saturation of the LS detector; *2Peak 2 is poorly resolved from the major peak, therefore MW values shown appear to be higher.
### Table III: Oligomeric States of Meprin A and B: Effects of Meprin Concentration.

| Meprin Concentration (nM) | Homooligomeric meprin A* | Homooligomeric meprin B | Heterooligomeric meprin A*1 |
|---------------------------|--------------------------|--------------------------|-----------------------------|
|                           | Latent | Active | Latent | Active | Latent | Active |
| 20                        | 16     | 16     | 2      | 2      | 2 > 4  | 4 > 2  |
| 100                       | 22     | 18     | 2      | 2      | 4 > 2  | 4 > 2  |
| 500                       | 38     | 20     | 2      | 2      | 4 > 2  | 4 > 2  |
| 1000                      | 48     | 20     | 2      | 2      | 4 > 2  | 4 > 2  |
| 2000                      | 78     | 20     | 2      | 2      | 4 > 2  | 4 > 2  |

The relationship between meprin concentration and molecular mass was evaluated by subjecting various concentrations of meprins to SEC and analyzing the shifts in elution volumes for the peak maxima. The column was equilibrated in 20 mM Tris-HCl, 150 mM NaCl, pH 7.5. Data reported are based on the elution volume of the peak maxima. The oligomeric states were estimated using the SEC-LS M<sub>W</sub> data. *The oligomeric state reported is an estimated average for the peak maximum and not an absolute number.

*1 From chromatograms it is evident that two forms arise, a dimer and tetramer.
Structure of homo- and hetero-oligomeric meprin metalloproteases: Dimers, tetramers, and high molecular mass multimers
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