Activation Mechanism of Meprins, Members of the Astacin Metalloendopeptidase Family*

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Meprins are mammalian zinc metalloendopeptidases with protease domains structurally related to astacin, the prototype of the “astacin family” of metalloproteases. Mature, active astacins are produced by proteolytic removal of an activation peptide to generate a new NH2-terminal residue. Structural studies indicate that the NH2-terminal ammonium group inserts into a water-filled cavity adjacent to the active site to form a salt bridge with a Glu residue that is conserved in all astacins. A similar interaction is known to play a crucial role in the activation of trypsin, resulting in the hypothesis that this salt bridge is required for the activation of astacin-like proteases. In this study, we have used the mouse meprin α subunit as a model to test this hypothesis of zymogen activation of the astacins. Mutants were generated to vary the NH2-terminal residue of the mature meprin α subunit (Asn78) and its putative salt bridge partner (Glu178). In addition, mutants creating NH2-terminal extensions and truncations were expressed in human embryonic kidney 293 cells. The recombinant proteins were activated by limited protease digestion and assayed for enzymatic activity and thermal stability. Point mutations of Asn78 resulted in enzymes with activity comparable to the wild-type enzyme, indicating that the structure of this side chain is not essential for activity. NH2-terminal extension mutants of meprin α retained partial activity, with greater decreases against peptide relative to protein substrates. A mutant with a deletion of Asn78 to disrupt salt bridge formation with Glu178 had full activity, indicating that the putative salt bridge with Glu178 is not essential for enzyme activity. However, all changes in meprin α subunit NH2-terminal structure were found to decrease the thermal stability of the enzyme. These observations and additional data indicate that the zymogen activation mechanism of meprin and other astacins differs from that of the trypsin family of enzymes, and has some features in common with matrixins. It is proposed that prosequence removal of astacins allows the formation of hydrogen bonds involving the two NH2-terminal residues that are critical for enzyme structure.

Meprins are oligomeric zinc metalloendopeptidases composed of α and/or β subunits that are evolutionarily related, but differ in function (1–3). Meprin β subunits are integral mem-

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brane proteins; α subunits are secreted from cells unless associated with β subunits (3, 4). Meprin A (EC 3.4.24.18) can exist as secreted α homooligomers (5) or as membrane-bound α/β heterooligomers (3, 4). Meprin B (EC 3.4.24.63) is an ectoenzyme composed exclusively of membrane-bound β subunits (1, 2). Meprins are highly expressed in the microvillar membranes of the mammalian kidney and intestine (1, 6, 7) and are especially abundant in rodent brush border membranes (8). Meprins A and B participate in the processing and degradation of peptides and proteins at the cell surface (1). Meprins may also be involved in the remodeling of the extracellular matrix in response to renal injury (9). It has been shown that meprin B has an activity identical to that of the kinase splitting membrane protease, an enzyme that inactivates protein kinase A by a cleavage in its catalytic domain (10).

The isolation and nucleotide sequence analysis of meprin α and β subunit cDNAs revealed that the two subunits are 42% identical in amino acid sequence and share a similar arrangement of structural domains, including a protease domain related to astacin, a crayfish digestive protease (1, 11, 12). The “astacin family” of metalloendopeptidases comprises a group of proteins involved in a wide range of biological functions in organisms as diverse as the sea urchin, Drosophila, and mammals (1). Examples include Tolloid, a protein involved in dorsal/ventral patterning in Drosophila (13), and bone morphogenetic protein-1 (procollagen C-proteinase), an enzyme required for the post-translational processing of procollagen types I–III (14). Other astacin-like enzymes (“astacins”) have been identified by the cloning of their cDNAs, but have yet to be characterized at the protein level (1).

Astacins are initially synthesized as inactive precursors with an NH2-terminal prosequence (1, 15). NH2-terminal sequence analyses of mature astacin, meprin A, and the procollagen C-proteinase indicate that the active forms of these enzymes have undergone a processing event that removes the prosequence (11, 14, 15). The NH2-terminal residue of mature astacins is a small, uncharged amino acid, usually Ala or Asn (1). The three-dimensional structure of crayfish astacin reveals that the NH2-terminal residue of the mature enzyme resides in a water-filled cavity adjacent to the zinc binding site, where its ammonium group forms a water-mediated salt bridge with a Glu side chain that is conserved in all astacins (15). A molecular modeling study predicted that this arrangement is also possible for the meprin α subunit catalytic domain (16), but the charged groups are bridged by two water molecules (Fig. 1). A similar interaction has been observed to be required for the activation of trypsin (17), leading to the prediction that formation of this salt bridge is essential for the activation of astacins (15, 16). To test this hypothesis, we have expressed and characterized a series of mutants of the mouse meprin α subunit. Point mutations have been introduced to change the side chain of Asn78, the NH2-terminal residue of mature meprin, and
Activation of Astacin Metalloendoproteinases

Aston

Meprin α

FIG. 1. Predicted water-mediated salt bridges in the internal water cavities of astacin and the meprin α subunit protease domain. Structural coordinates are from the Brookhaven Protein Data Bank files (1AST 5 (astacin) and 1IAF 8 (meprin α subunit protease domain molecular model (16)). Structures were imaged using the InsightII program on a Silicon Graphics workstation. Hydrogen bonds (dashed lines) were calculated and the distances (in Å) measured using the "H-bond" and distance measuring functions of InsightII. For astacin (1CP), the charged groups of Ala 1, Glu103, and Arg106 are able to participate in salt bridges mediated by a single structural water molecule (Wat505). For meprin α (right), Glu178 and Arg181 potentially form a salt bridge mediated by Wat550. Meprin’s Asn78 ammonium group can be linked to Glu178 indirectly through two water molecules (Wat289 and Wat256).

Glu178, the two residues implicated in salt bridge formation. Mutagenesis has also been used to extend and truncate the mature α subunit NH2 terminus to disrupt the interaction of the terminal ammonium group with the conserved Glu178. Recombinant proteins were expressed and tested for activity and thermal stability.

EXPERIMENTAL PROCEDURES

Cell Culture and Expression of Recombinant Proteins—Human embryonic kidney 293 cells (American Type Culture Collection CRL 1573) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, UT), 50 units/ml penicillin, and 50 μg/ml streptomycin (complete DMEM). Cells were incubated at 37 °C in an atmosphere containing 5% CO2. Unless otherwise noted, all media and cell culture reagents were purchased from Life Technologies, Inc. For the expression of meprin α subunits, wild-type and mutant cDNAs were cloned into the expression plasmid pCMV-1 generous gift of Dr. Colleen Brewer, University of Texas Southwestern Medical Center, Dallas, TX). pCB3 is a derivative of pCMV-1 (18), with transcription driven by the SV40 early promoter; pCB3 was obtained from Dr. William Brewer, University of Texas Southwestern Medical Center, Dallas, TX. The plasmids were transfected into 293 cells by a calcium phosphate precipitation procedure (19) and were cotransfected with the plasmid pVA1 (20) to enhance mRNA translation in the adenovirus-transformed 293 cells. After transfection, cells were grown for 24 h in complete DMEM, then switched to serum-free OptiMEM I (Life Technologies, Inc.) and cultured for an additional 36–48 h before the medium was collected for analysis of secreted meprin α subunits.

Analysis of Astacin and Meprin Three-dimensional Structures—The structural coordinates of astacin and a molecular model of the meprin α subunit protease domain were obtained from the Brookhaven Protein Data Bank. The files for astacin (1AST 5) and meprin (1IAF 8) were imaged and manipulated using InsightII™ software (Biosym/MSI, San Diego, CA) on a Silicon Graphics (Mountain View, CA) workstation. The files for astacin (1AST 5) and meprin (1IAF 8) were imaged and manipulated using InsightII™ software (Biosym/MSI, San Diego, CA) on a Silicon Graphics (Mountain View, CA) workstation.

Analysis of Recombinant Meprin α Subunits—For each recombinant protein preparation, serum-free conditioned medium from four 100-mm plates was concentrated 40-fold using Centricon 30 cartriges (Amicon, Inc., Beverly, MA); diluted 10-fold in 10 mM Tris-HCl, 0.15 mM NaCl, pH 8.0; and concentrated. Protein concentrations were measured using a bicinchoninic acid assay (Pierce) and found to be 0.8–1.0 mg/ml in the 40-fold concentrated media samples. Meprin α subunit zymogens were activated by a limited digestion with either 1-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (Sigma) or 1-nitro-5-fluoro-ketoproteinase Arg-C (Boehringer Mannheim). For trypsin activation, proteins were diluted to a concentration of 0.5 mg/ml in 10 mM Tris-HCl, 0.15 mM NaCl, pH 8.0; trypsin was added to 10 μg/ml, and the mixture was incubated at 24 °C for 30 min. Trypsin was then inhibited by the addition of soybean trypsin inhibitor (Sigma) to 40 μg/ml. Conditions for activation by endoproteinase Arg-C were as described previously (21), except that 0.1 mg/ml endoproteinase Arg-C was included in the reaction, and incubation was at 37 °C for 30 min. After the incubation, endoproteinase Arg-C was inhibited by the addition of 3,4-dichloroquinacrin (Sigma) to 200 μM.

Meprin protease activity was measured using the substrate azocasein (Sigma) as described previously (22). Peptidase activity was measured using the fluorogenic bradykinin analog BK+ (21). Assays were performed in the buffer 10 mM Tris-HCl, 0.15 mM NaCl, pH 8.0, at 24 °C in a total volume of 300 μl, using 10 μM BK+ as product. Fluorescence was measured with a Hitachi F2000 fluorescence spectrophotometer using an excitation wavelength of 320 nm and an emission wavelength of 417 nm. Arylamidase activity of meprin was determined using the substrate glutaryl-1-Ala-Phe-4-nitro-2-naphthylamide (gln-AAF-MNA, purchased from Sigma). Assays were as for BK+, except that the concentrations contained 50 μM glut-AAF-MNA, and the respective excitation and emission wavelengths were 340 nm and 425 nm. Estimates of Km were calculated using the equation: 

\[
K_m = \frac{V}{[S]} = \frac{V}{[E][S]}
\]

where Km is the initial rate observed under the assay conditions described above, in which [S] is the initial substrate concentration. [E] was calculated using a meprin α subunit molecular mass of 8.8 × 10^6. The specific activities reported are averages of triplicate assays of at least two independent protein preparations per recombinant protein.

Apparent Km values for the inhibition of meprin hydrolysis of BK+ by actinoin were determined as follows. The concentration of actinoin was varied from 0.05 μM to 0.5 μM in the presence of constant BK+ (10 μM) and constant amounts of meprin. Data were plotted as the inverse of initial rate versus actinoin concentration and fit to a straight line using a computer program. The x axis intercepts of the resulting lines were taken to be -Km.

Immunoblot Analysis of Recombinant Proteins—Samples of conditioned media from 293 cells were subjected to SDS-PAGE (24) using 7.5% polyacrylamide gels after reduction of samples with 2-mercaptoethanol. Proteins were then electrophoretically transferred to a polyvinylidene difluoride (PVDF) membranes (Fishier). PVDF membranes were successively incubated with an antibody raised against rat meprin A, horseradish peroxidase-conjugated donkey anti-rabbit IgG, and reagents for enhanced chemiluminescence (Pierce). The membranes were then exposed to x-ray film (NEN Life Science Products). Quantitation of recombinant meprin α subunits was aided by densitometry of films from immunoblots with known amounts of meprin A (purified from mouse kidney) electrophoresed alongside the recombinant samples. Gel scanning and densitometry software was from PDI, Inc. (Huntington Station, NY).

Amino-terminal Sequence Analysis—Concentrated conditioned media samples containing the proteins N78del, N78,79del, N78NN, N78NN, or N78NAAN were activated by trypsin as described above. Each protein was then purified by chromatography on a MonoQ column (Pharmacia, Uppsala, Sweden) followed by preparative SDS-PAGE and transfer to PVDF membranes. The membranes were stained with Amido Black, and the meprin bands were excised and delivered to the Macromolecular Core Facility of this institution for six cycles of automated Edman degradation.

Mutagenesis—All mutagenesis of the mouse meprin α subunit cDNA (11) was done using polymerase chain reaction (PCR) with mutagenic oligonucleotide primers and Taq DNA polymerase (Fisher). Every PCR reaction made use of the antisense primer GGAGACTCTGGCGGTCAGAAG (complementary to residues 1905–1928 of the mouse meprin α cDNA) and the appropriate mutagenic primer. Before PCR products were subeloned into the meprin α subunit cDNA, they were screened for unintended errors and verified as correct by DNA sequence analysis using dye sequencing reactions performed under Amersham Life Science. DNA restriction and modification enzymes were purchased from Fisher. Prior to construction of mutations at Asn78, a mutation generating an AarII restriction site (GACGTC) upstream of this region was made to facilitate the cloning of other DNA fragments. This mutation converted Ile73 of the sequence to Val (ATC to GTC),

1 The abbreviations used are: DMEM, Dulbecco’s modified Eagle’s medium; BK+, bradykinin analog 2-aminoethyl-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Ph-Arg-Lys(Dnp)-Gly-OH, where Dnp is 2,4-dinitrophenyl; glut-AAF-MNA, glutaryl-1-Ala-Ala-Phe-4-methoxy-2-naphthylamide; MPP, matrix metalloproteinase; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PVDF, polyvinylidene difluoride.
and was found to have no effect on the expression level or specific activities of the meprin α subunit. The I71V mutant (having no changes in the mature form of the enzyme) is therefore considered to be equivalent to "wild-type" and was utilized for the cloning and expression of all Asn78 mutants.

The mutagenic primer "N78SN" (CCGGTCGACGTCCTCCTACCCAGCCACGGAGCCATGCGAGATCCCTCAAGC, where (X) represents codons inserted between Arg77, the trypsin cleavage point, and Asn78, the NH2 terminus of the wild-type meprin α subunits. For the primer "N78NN," (X) is AATGCC; for "N78NAAN," (X) is AACGCC. These primers generate NH2-terminal extensions of Asn, Asn-Ala, and Asn-Ala-Ala, respectively. All DNA fragments with mutations at codon Asn78 were subcloned into the meprin α cDNA (containing the I71V mutation) using AatII and SphI restriction sites.

Truncations of the NH2 terminus of the mature meprin α subunit were generated by mutagenic PCR primers deleting codons 78 ("N78del") and codons 78 + 79 ("N78,79del"). Primer N78del is: CGCGTCGACGTCCTCCTACCCAGCCACGGAGCCATGCGAGATCCCTCAAGC. Primer N78,79del is: CGCGTCGACGTCCTCCTACCCAGCCACGGAGCCATGCGAGATCCCTCAAGC. These primers generate NH2-terminal extensions of Asn, Asn-Ala, and Asn-Ala-Ala, respectively. All DNA fragments with mutations at codon Asn78 were subcloned into the pCBβ expression vector containing no cDNA insert.

The effects of the Asn78 point mutations were further examined by investigating the thermal stability of the mutants (Fig. 3). The activated wild-type and mutant proteins were incubated for varying periods of time at 53 °C, then assayed for activity using the BK+ substrate. It was observed that the wild-type enzyme is the most thermostable, retaining 84% of its initial activity after a 30-min incubation at 53 °C. The Asn78 mutants are not as stable at this temperature, and stability appears to decrease with increasing bulk of the mutant side chain. The values for inactivation calculated from the data are: 116 min for wild-type α, 54 min for the N78S mutant, 36 min for the N78T mutant, and 16 min for the N78I mutant. It is apparent that mutations at Asn78 having little effect on enzyme activity decrease the stability of the mature meprin α subunit.

### Table I

**Enzymatic activities of meprin α subunits with point mutations at the NH2-terminal residue**

Prior to assays, meprin zymogens were activated by limited trypsin digestion as described under "Experimental Procedures." One unit of azocasein activity is defined as the degradation of 1.1 μg of azocasein/min. BK+ and glut-AAF-MNA activities are expressed as both specific activity (μmol/min · mg) and as the rate constant (kcat/Km) (μ · s⁻¹). BK+ mutants contained 10 μM substrate; glut-AAF-MNA assays contained 50 μM substrate. The values are averages of triplicate assays obtained from at least two independent protein preparations. The amount of meprin included in assays was determined by densitometry as described under "Experimental Procedures." WT, wild-type.

|             | Azocasein activities | BK+ activities | glut-AAF-MNA activities |
|-------------|----------------------|----------------|-------------------------|
|             | units/mg             | μmol/min · mg | kcat/Km (μ · s⁻¹)       | μmol/min · mg | kcat/Km (μ · s⁻¹) |
| WT α        | 6.3 × 10³            | 3.2           | 4.8 × 10⁵                | 0.10         | 3.0 × 10³      |
| N78S        | 5.1 × 10³            | 2.5           | 3.7 × 10⁵                | 0.07         | 2.1 × 10³      |
| N78T        | 6.2 × 10³            | 2.1           | 3.1 × 10⁵                | 0.05         | 1.6 × 10³      |
| N78I        | 5.7 × 10³            | 3.6           | 5.4 × 10⁵                | 0.12         | 3.5 × 10³      |
NH₂ terminus after activation by limited trypsin digestion. It is expected that these mutants will change the position of the NH₂-terminal ammonium group relative to Glu₁⁷⁸ in the mature enzyme, thereby preventing formation of the salt bridge proposed to be required for zymogen activation. The NH₂ termini expected for the mature enzymes after trypsin activation are diagrammed in Fig. 5 (top). NH₂-terminal extensions of 1, 2, and 3 residues were generated by N78NN, N78NAN, and N78NAAN, respectively. All three mutations retain Asn as the NH₂-terminal residue, with Ala spacers making up the remainder of the extensions for N78NAAN and N78NAAN. The mutants N78del and N78,79del delete residue Asn⁷⁸ and the dipeptide Asn⁷⁸-Ala⁷⁹, respectively, generating NH₂ termini of Ala⁷⁹ and Met⁸⁰ in the mature enzymes after limited trypsin digestion. The NH₂-terminal structures of all five activated truncation and extension mutants were determined by NH₂-terminal sequence analysis of the purified recombinant enzymes (data not shown). The expected NH₂ termini were observed for all mutants, but species resulting from trypsin cleavage at Arg⁸¹ were detected for the three extension mutants, accounting for 10% (N78NN) to 25% (N78NAN and N78NAAN) of the activated proteins.

### Table II

| Azocasein | BK⁺ | Kₑᵦ/Kₘ |
|----------|-----|--------|
| WT α     | 7.8 x 10⁺³ | 3.4 | 5.2 x 10⁵ |
| E178Q    | 7.7 x 10⁺³ | 0.28 | 4.1 x 10⁴ |
| E178A    | ND  | ND   | ND   |
| E178D    | ND  | ND   | ND   |

The activated NH₂-terminal extension and truncation mutants were assayed for activity with the substrates azocasein, BK⁺, and glut-AAF-MNA (Table III). Of this group of mutants, none were found to be completely inactive, but only N78del had...
activity comparable to wild-type for all three substrates tested. N78,79del and all three extension mutants retained a substantial amount of azocasein degrading activity, with specific activities ranging from 36% to 46% of the wild-type value. This group of four mutants displayed an even greater decrease in activity against the peptide substrates, with specific activity values only 10–17% of wild-type, depending on the substrate and the mutant considered. The greater loss of activity against peptide compared with the protein substrate is interesting, indicating that the two activities are affected to different degrees by changes in enzyme structure.

The NH₂-terminal truncation and extension mutants were also tested for their thermal stability. Initial experiments showed that these mutants are highly unstable at 53 °C, so the experiments were conducted at 49 °C to obtain inactivation rates. After incubation at 49 °C for various times, the samples were assayed using BK⁺ as the substrate. The wild-type enzyme is extremely stable at 49 °C, having a calculated t₁/₂ for inactivation of 1030 min. As shown in Fig. 6, the N78del mutant is considerably less stable, with a t₁/₂ of 15 min. The other mutants exhibit even lower stability at 49 °C, with t₁/₂ values of approximately 3 min. These values are: 3.5 min for N78NN, 2.6 min for N78NA, 4.3 min for N78NAAN, and 3.0 min for N78,79del. Therefore, this group of four mutants was found to have nearly identical properties when both catalytic activity and thermal stability are considered. Comparison of the mutant proteins before and after heat treatment by immunoblot analysis confirmed that the observed losses of activity were due to denaturation, not to autolysis or degradation by other proteases present in the samples.

The above deletion and truncation mutants were also compared for their sensitivity to inhibition of BK⁺ hydrolysis by actininon, a peptide hydroxamate. Apparent Kᵢ values for actininon of 0.10 μM and 0.13 μM were obtained for wild-type α and the N78del mutant, respectively; higher Kᵢ values of 0.29, 0.33, 0.25, and 0.27 μM were obtained for N78,79del, N78NN, N78NAAN, and N78NAAN. The similarities in enzymatic activity, thermal inactivation rates, and inhibition constants for the latter group of four mutants is an indication that they have all undergone a similar change in structure, even though they possess different mutations.

**DISCUSSION**

The results herein for meprin are inconsistent with a trypsin-like mechanism of zymogen activation, because neither the structure of the NH₂-terminal side chain nor conservation of a salt bridge involving this residue were found to be essential for enzymatic activity. The activation of trypsinogen is well characterized by both structural and mutagenesis studies. A region termed the “activation domain,” including the S1 binding site and the oxyanion hole, is disordered in trypsinogen, but completely formed in mature, active trypsin (17, 25). The crucial conformational change is triggered by cleavage of the zymogen activation peptide, creating a new NH₂-terminal residue (Ile16, chymotrypsin numbering system). The new terminal ammonium group forms a salt bridge with Asp94, stabilizing the protein by 3 kcal/mol (17, 26). A recent study has shown that the contribution of the Ile side chain to stability is critical, accounting for 5 kcal/mol of stabilization energy to the activation domain; trypsin mutants I16A and I16G have greatly decreased rates of acylation in amide bond hydrolysis (26). This contrasts with the full activity seen for the meprin α Asn⁷⁸ point mutants. Thus, although there are several structural features of mature astacins that resemble the NH₂-terminal structure of trypsin, the crucial features for activation differ.

Point mutations of meprin Glu¹⁷⁸ were found to have marked effects on enzyme structure. The mutants E178A, E178D, and E178Q were all substantially degraded by the limited trypsin digestion generally used to activate meprin zymogens. Of the mutants activated by digestion with endoproteinase Arg-C, only E178Q had detectable activity, 10% that of the wild-type mutant, E178Q were all substantially degraded by the limited trypsin digestion generally used to activate meprin zymogens. Of the mutants activated by digestion with endoproteinase Arg-C, only E178Q had detectable activity, 10% that of the wild-type mutant.

### Table III

| Enzymatic activities of meprin α subunits with NH₂-terminal truncations and extensions |
|-------------|-----------|---------------|---------------|
|             | Azocasein | BK⁺ | glut-AAF-MNA |
|             | units/mg  | μmol/min ∙ mg | 1/2 | μmol/min ∙ MNA | 1/2 |
| WT α        | 6.3 × 10³ | 3.2 | 4.8 × 10⁻² | 0.10 | 3.0 × 10⁻³ |
| N78del      | 6.1 × 10² | 2.4 | 3.7 × 10⁻² | 0.11 | 3.3 × 10⁻³ |
| N78,79del   | 2.3 × 10² | 0.33 | 5.2 × 10⁻⁴ | 0.010 | 3.1 × 10⁻⁴ |
| N78NN       | 2.5 × 10² | 0.51 | 7.7 × 10⁻⁴ | 0.015 | 3.9 × 10⁻³ |
| N78NAAN     | 2.9 × 10² | 0.44 | 6.6 × 10⁻⁴ | 0.014 | 4.0 × 10⁻³ |
| N78NAAN     | 2.6 × 10³ | 0.54 | 8.1 × 10⁻⁴ | 0.015 | 4.5 × 10⁻³ |

**Fig. 5.** Design and expression of meprin α subunit NH₂-terminal truncation and extension mutants. Top, NH₂-terminal amino acid sequences of the wild-type meprin α subunit and mutants after activation by limited trypsin digestion. Bottom, concentrated conditioned media samples (2.0 μg of protein) from 293 cells transfected with plasmids encoding wild-type meprin α (WT) or mutant meprin α subunits were activated by limited trypsin digestion prior to SDS-PAGE and immunoblot analysis, as described under “Experimental Procedures.” The migration of protein molecular mass standards (in kDa) is shown at the left.
Asn78 located 4.88 Å and 5.32 Å from the oxygen atoms of the cavity. This cavity is of comparable size, with the ammonium group of the NH₂-terminal extension and truncation mutants were designed to determine from these experiments exactly which of these interactions are disrupted by the Glu178 mutations. The NH₂-terminal extension and truncation mutants were designed to more directly assess the importance of the NH₂-terminal salt bridge forzymogen activation.

The astacin NH₂ terminus is inserted into an internal cavity containing eight ordered water molecules. The charged groups of astacin residues Glu103 and Arg106 participate in salt bridges mediated by some of the ordered water molecules (15, 16). The residues Ala¹ and Ala² are also completely inserted into this cavity. In the meprin α model described by Stöcker et al. (16), this cavity is of comparable size, with the ammonium group of Asn78 located 4.88 Å and 5.32 Å from the oxygen atoms of the Glu178 side chain. The mutant N78NN would extend the meprin α NH₂ terminus by about 3.5 Å. It is possible that the water cavity can accommodate the extra residue and allow a conformation that conserves the salt bridge, but this seems unlikely for the extensions N78NAN and N78NAAN, which would extend the NH₂ terminus by some 7–10 Å. Similar mutations of the plasmin (trypsin-like) protease domain apparently disrupted the crucial salt bridge, decreasing activity about 10⁴-fold (27). The relatively minor decreases in activity observed for these meprin mutants indicate that the proposed salt bridge is not critical for active site structure and activity. The results also demonstrate that processing of the enzyme’s NH₂ terminus to a precise residue is not absolutely required for catalytic activity, because the mutant proteins retain significant activity, particularly against the substrate azocasein. The NH₂-terminal truncation mutant N78del was found to have catalytic activity virtually identical to the wild-type enzyme. This mutation is expected to position the NH₂ terminus an additional 3.6 Å away from Glu178, making the formation of a salt bridge virtually impossible without gross rearrangements in enzyme structure. The analogous mutation of trypsin (deletion of Ile¹⁶) decreased activity against amide substrates 10⁴-fold, and similar truncations of the plasmin protease domain lowered activity to undetectable levels (27). Therefore, we conclude that the formation of a salt bridge between the NH₂ terminus and Glu178 is not essential for the activation of meprin, and such an interaction is probably not required for the activation of other astacins. Despite activity comparable to wild-type meprin α, evidence for a structural change in the N78del mutant lies in its decreased thermal stability. The decreased stability of this protein could be explained by the loss of one or more of several interactions that could be contributed by the wild-type NH₂-terminal residue. Besides the potential salt bridge with Glu178, the molecular model of meprin α (16) shows that a hydrogen bond forms between the backbone carbonyl oxygen of the terminal residue (Asn78) and the backbone amide group of residue Phe176 (Fig. 7). This hydrogen bond is also present in the astacin structure (between Ala¹ and Tyr101), possibly stabilizing the mature forms of both enzymes.

Astacins are part of a superfamily of structurally-related metalloproteinases known as the “metzincins” (28). Matrixins (matrix metalloproteinases or MMPs), reprolysins (e.g. snake venom metalloproteinases and tumor necrosis factor-α-converting enzyme), and serralysins (bacterial enzymes) are also metzincins. The latency of proforms of matrixins and reprolysins is maintained by a “cysteine switch” mechanism (29–31), in which a cysteine residue of the prosequence coordinates the active site zinc ion to prevent catalysis. Astacins do not possess a recognizable cysteine switch peptide; the meprin α subunit prosequence contains no cysteine residues at all. Therefore, the
latency of astacins must be maintained by a different mecha-
nism. Several activated forms of both MMP-1 (interstitial col-
lagenase) and MMP-8 (neutrophil collagenase) have been ob-
served, corresponding to NH2 termini beginning with residues 79, 80, and 81 (32–35). All of these enzyme forms are active, but enzymes beginning with residue 79 have specific activities 3–12-fold higher than the proteins having shortened NH2 ter-
mini (34, 35). Partial activity (15–20%) of collagenases and stromelysin retaining up to 13 residues of the prosequence has been observed (34, 36). It is evident that both astacins and matrixins can exist as partially active NH2-terminally ex-
tended and shortened forms, and are fully active only when the
NH2 terminus is precisely processed. The structural basis of
the above observations has been addressed by determination of
the three-dimensional structures of neutrophil collagenase pro-
tease domains having NH2 termini beginning with Phe79 and
Met80 (37, 38). No obvious differences were noted in the active
sites of the two proteins, however it was noted that the NH2-
terminal seven residues of the less active Met80 form were
ordered, whereas they were ordered in the Phe79 protein
(37). A similar loss of NH2-terminal structure could explain the
decreased activity of the extended and truncated meprin mu-
tants observed in this study.

It was observed that the mature N78,79del, which lacks the
first two residues of the mature α subunit, displays phenotypes
indistinguishable from the three NH2-terminal extension mu-
tants. Nearly identical values were obtained for activity
against protein, peptide and arylamide substrates, thermal
stability, and sensitivity to inhibition by actinonin, raising the
possibility that all four mutants have undergone the same
structural change. Examination of the astacin three-dimen-
sional structure and the meprin α catalytic domain structural
model reveals that for the second residue of the mature en-
zymes (Ala2 of astacin, Ala79 of meprin α) the backbone
carbonyl oxygen and amide groups make two hydrogen bonds with
the backbone atoms of Arg135 (astacin) and Thr210 (meprin α, Fig. 7). Thus, the two terminal residues of both enzymes con-
tribute three hydrogen bonds linking the NH2 terminus to
distant regions of the polypeptide chain (residues 101 and 135
in the astacin numbering system). Because both terminal res-
ides are deleted in the N78,79del mutant, these three hydro-
gen bonds cannot form. It is possible that localized structural
perturbations in the NH2-terminal extension mutants elimi-
nate these hydrogen bonds in the proteins, resulting in struc-
tures and phenotypes for all three extension mutants that are
similar to the N78,79del mutant. If so, this observation could
explain why astacins are processed to a precise residue in their
mature forms, a processing event producing an enzyme with
maximal activity and stability.

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