The Serpin MNEI Inhibits Elastase-like and Chymotrypsin-like Serine Proteases through Efficient Reactions at Two Active Sites†

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ABSTRACT: MNEI (monocyte/neutrophil elastase inhibitor) is a 42 kDa serpin superfamily protein characterized initially as a fast-acting inhibitor of neutrophil elastase. Here we show that MNEI has a broader specificity, efficiently inhibiting proteases with elastase- and chymotrypsin-like specificities. Reaction of MNEI with neutrophil proteinase-3, an elastase-like protease, and porcine pancreatic elastase demonstrated rapid inhibition rate constants $>10^7$ M^{-1} s^{-1}, similar to that observed for neutrophil elastase. Reactions of MNEI with chymotrypsin-like proteases were also rapid: cathepsin G from neutrophils ($>10^6$ M^{-1} s^{-1}), mast cell chymase ($>10^5$ M^{-1} s^{-1}), chymotrypsin ($>10^6$ M^{-1} s^{-1}), and prostate-specific antigen (PSA), which had the slowest rate constant at $\sim 10^4$ M^{-1} s^{-1}. Inhibition of trypsin-like (plasmin, granzyme A, and thrombin) and caspase-like (granzyme B) serine proteases was not observed or highly inefficient (trypsin), nor was inhibition of proteases from the cysteine (caspase-1 and caspase-3) and metalloprotease (macrophage elastase, MMP-12) families. The stoichiometry of inhibition for all inhibitory reactions was near 1, and inhibitory complexes were resistant to dissociation by SDS, further indicating the specificity of MNEI for elastase- and chymotrypsin-like proteases. Determination of the reactive site of MNEI by N-terminal sequencing and mass analysis of reaction products identified two reactive sites, each with a different specificity. Cys^{344}, which corresponds to Met^{358}, the P_1 site of α1-antitrypsin, was the inhibitory site for elastase-like proteases and PSA, while the preceding residue, Phe^{343}, was the inhibitory site for chymotrypsin-like proteases. This study demonstrates that MNEI has two functional reactive sites corresponding to the predicted P_1 and P_2 positions of the reactive center loop. The data suggest that MNEI plays a regulatory role at extravascular sites to limit inflammatory damage due to proteases of cellular origin.

MNEI (monocyte/neutrophil elastase inhibitor)[1] is a 42 kDa serpin protein of known sequence (*1*) identified initially as a fast-acting elastase inhibitor found at high levels in neutrophils and monocytes (*2*). MNEI reacts with elastase by the stoichiometric mechanism unique to serpin (SERine Protease INhibitor) superfamily molecules (*3, 4*). These are single-chain proteins and glycoproteins with a shared structure defined first for α1-AT (*5*), consisting of a stressed arrangement of nine alpha helices and three beta sheets with the reactive center in an exposed flexible loop on the surface of the molecule (*6*). Serpins regulate a wide range of physiological processes including blood coagulation, complement activation, inflammation, extracellular matrix remodeling, and tumor suppression (*7*). The reactive centers, in addition to exposed location, are highly divergent, and these cumulative characteristics contribute to the selectivity and high rates of interactions with cognate proteases. Particularly important are the P_1-P_1' residues[2] in the reactive center loop, with P_1 playing a dominant role in defining specificity (*9*). Whereas some serpins are specialized for single proteases, others inhibit more than one, at least in vitro, and in these cases, quantitative information about the rate and efficiency

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[1] Abbreviations: MNEI, monocyte/neutrophil elastase inhibitor; α1-AT, α1-antitrypsin, α1-proteinase inhibitor; α1-ACT, α1-antichymotrypsin; DFP, diisopropyl fluorophosphate; IL, interleukin; PAGE, polyacrylamide gel electrophoresis; PSA, prostate-specific antigen; r, recombinant; SCCA2, squamous cell carcinoma antigen-2; SDS, sodium dodecyl sulfate; SI, stoichiometry of inhibition; SLPI, secretory leukoprotease inhibitor, also called MPI, mucus proteinase inhibitor; pNA, *p*-nitroanilide; ONp, *p*-nitrophenyl ester; SBzl, thiobenzyl ester; Suc, succinyl; MeOSuc, methoxysuccinyl.

[2] Residues within the reactive site loop are numbered analogous to protease substrates as follows: $P_n \cdots P_3-P_2-P_1-P_1'-P_2'-P_3' \cdots P_n'$ (*8*) with reaction and cleavage occurring at the P_1-P_1' bond.

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of interaction can be useful in identifying physiologically relevant inhibitory reactions.

Previous studies suggested that MNEI functions as a physiological inhibitor of the serine proteases of neutrophil azurophil (primary) granules, enzymes that are important in antimicrobial defense (*10*) but, when present in excess, function as major agents of inflammation by destroying matrix proteins as well as immune defense molecules (*11*, *12*). Two of the enzymes, elastase and proteinase-3, have related specificities, preferentially cleaving target proteins on the C-terminal side of small aliphatic amino acids (*13*). It was thus not surprising that the first study of recombinant MNEI found that it inhibits proteinase-3 in addition to elastase (*14*). Cathepsin G, the third neutrophil azurophil granule protease, has a different specificity, preferentially cleaving after phenylalanine residues (chymotrypsin-like specificity) (*15*). Here we report that MNEI also efficiently inhibits cathepsin G[3] and other proteases with chymotrypsin-like specificity and that the specificity of MNEI for elastase- and chymotrypsin-like protease is mediated by two different reactive sites located at the predicted P_1 and P_2 positions of the reactive center loop.

EXPERIMENTAL PROCEDURES

MNEI. Recombinant human MNEI was produced in insect cells, purified by anion exchange and gel filtration chromatography, and stored at −80 °C in 20 mM Tris-HCl, pH 7.4, 500 mM NaCl, 2 mM mercaptoethanol, 1 mM EDTA (*17*). Preparations were dialyzed at 4 °C against phosphate-buffered saline, assayed (described below), and stored until use in aliquots at 1−3 mg/mL at −80 °C.

Proteases. Human neutrophil elastase and porcine pancreatic elastase were from Elastin Products (Owensville, MO); proteinase-3 and cathepsin G from human neutrophils and chymotrypsin (human pancreas) from Athens Research and Technology (Athens, GA); and bovine chymotrypsin, trypsin, and thrombin from Sigma (St. Louis, MO). Human mast cell chymase in which a lysine replaced phenylalanine-127 to protect against autolytic cleavage (*18*) was expressed in insect cells with a vector-encoded pro-sequence, activated by enterokinase, and purified (*19*). Human seminal plasma-derived prostate-specific antigen (PSA) (>97% purity) was from Scripps Laboratories (San Diego, CA). Recombinant human PSA, expressed in *E. coli* as the cDNA-encoded proenzyme, was isolated, purified, and converted by trypsin to a 27 kDa active enzyme lacking the native *N*-glycan (*20*). Human plasmin was generated from plasminogen by treating with streptokinase immediately before use. Human granzyme A, provided by Drs. Paul Beresford and Judy Lieberman (Center for Blood Research), was expressed in *E. coli* with a vector-encoded pro-sequence and C-terminal His-tag, purified by nickel chromatography, and activated by enterokinase (*21*). Human granzyme B, provided by Dr. Christopher Froelich (Evanston Hospital and Northwestern University Medical School, Evanston, IL), was purified from the NK-like cell line YT (*22*). Human caspase 1 (interleukin-1β-converting enzyme) (*23*) expressed in *E. coli* was provided by Dr. Nancy Thornberry (Merck Research Laboratories, Rahway, NJ); human recombinant caspase 3 was from Upstate Biotechnology (Lake Placid, NY). Human macrophage elastase, also called MMP-12 (*24*), was generated in *E. coli* and purified as the 22 kDa processed enzyme (*25*).

Table 1: Proteases and Peptide Substrates[a]

protease (concentration)	substrate (concentration)
elastase, human neutrophil (1.9 μM)	MeO-Suc-AAPV-pNA (0.8 mM)
proteinase-3, human neutrophil (1.9 μM)	N-Boc-Ala-ONp (0.5 mM)
cathepsin G, human neutrophil (1.5 μM)	Suc-AAPF-pNA (1 mM)
chymase, r, human mast cell (0.9 μM)	Suc-AAPF-pNA (1 mM)
elastase, porcine pancreas (1.9 μM)	Suc-AAA-pNA (0.8 mM)
chymotrypsin, human pancreas (1.5 μM)	Suc-AAPF-pNA (1 mM)
chymotrypsin, bovine pancreas (1.0 μM)	Suc-AAPF-pNA (0.2 mM)
PSA, human (7.7 μM)	Suc-FLF-SBzl (0.24 mM)
trypsin, bovine pancreas (0.2 μM)	pyro-EGR-pNA (0.6 mM)
thrombin, bovine (2.5 μM)	benzoyl-FVR-pNA (0.2 mM)
plasmin, human (0.4 μM)	pyro-EFK-pNA (0.8 mM)
granzyme A, r, human (0.5 μM)	Boc-LT-SBzl (0.2 mM)
granzyme B, human (1.2 μM)	Boc-AAD-SBzl (0.2 mM)
caspase 1 r, human (1.3 μM)	Ac-YVAD-pNA (0.1 mM)
caspase 3 r, human (1.7 μM)	Ac-DEVD-pNA (0.1 mM)
MMP-12, r, human macrophage (2.3 μM)	Ac-PLG-S-LLG-OEt (0.1 mM)

[a] Protease and MNEI were incubated in 20 μL at 37 °C and then diluted to 1 mL with the indicated substrate. References are the following: proteinase-3 (*13*), chymase (*26*), trypsin (*27*), thrombin (*28*), plasmin (*29*), granzyme a (*30*, *31*), granzyme B (*32*, *33*), MMP-12 (*34*), and PSA (this study).

Substrates and Active Site Titrants. Methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide (MeO-Suc-AAPV-pNA), succinyl-Ala-Ala-Ala-pNA (Suc-AAA-pNA), Suc-Ala-Ala-Pro-Phe-pNA (Suc-AAPF-pNA), acetyl-Asp-Val-Glu-Asp-pNA (Ac-DEVD-pNA), *N*-butyloxycarbonyl-Ala-*p*-nitrophenyl ester (Boc-A-ONp), *N*-benzoyl-Phe-Val-Arg-pNA (benzoyl-FVR-pNA), and Boc-Leu-Tyr-thiobenzyl ester (Boc-LT-SBzl) were from Sigma; acetyl-Tyr-Val-Ala-Asp-pNA (Ac-YVAD-pNA), Suc-Phe-Leu-Phe-SBzl (Suc-FLF-SBzl), Suc-Ala-Ala-Pro-Phe-sBzl (AAPF-sBzl), Ac-Pro-Leu-Gly-S-Leu-Leu-OEt (Ac-PLG-S-LLG-OEt), and Boc-tyrosyl-*p*-nitrophenyl ester (Boc-Tyr-ONp) were from Bachem (King of Prussia, PA); Boc-Ala-Ala-Asp-SBzl (Boc-AAD-SBzl) and MeO-Suc-Ala-Ala-Pro-Val-SBzl (MeO-Suc-AAPV-SBzl) were from Enzyme Systems (Livermore, CA); and pyro-Glu-Gly-Arg-pNA (pyro-EGR-pNA) and pyro-Glu-Phe-Lys-pNA (pyro-EFK-pNA) were from Chromogenix (West Chester, OH). *N*-Acetyl-alanyl-alanyl-azaleucyl-*p*-nitrophenyl ester (Ac-Ala-Ala-AzaLeu-ONp) was provided by Dr. James Powers (Georgia Institute of Technology, Atlanta, GA).

Treatment of Protease with MNEI and Assays for Peptidase Activity. Proteases were incubated with MNEI in 20 μL of phosphate-buffered saline with 0.05% Tween-20 for 5−45 min at 37 °C. The reactions were diluted to 1 mL with specific assay buffers (defined below) and 10 μL of *p*-nitroanilide or *p*-nitrophenyl ester substrate (defined in Table 1). For thiobenzyl ester substrates, the protease−MNEI reaction was diluted to 1 mL with buffer, substrate, and Ellman's reagent [5,5′,-dithiobis(2-nitrobenzoic acid)] (Sigma)

[3] A preliminary report of a portion of these findings has appeared in abstract form (*16*).

at 500 mM, or 300 mM (granzyme B) or 1000 mM (MMP-12). Absorbance increases at 410 nm (*p*-nitroanilides), 412 nm (Ellman's reagent), or 347 nm (nitrophenyl ester) were monitored at 15 s intervals for 5 or 10 min (PSA) at room temperature. Concentrations of protease were in the linear activity range (data not shown), and thus the percent activity remaining after treatment was equal to the percent active enzyme. Linear regression analysis was used to determine the stoichiometry of inhibition.

PSA Peptidase Assay. To develop a sensitive assay for PSA, Suc-Val-Pro-Phe-SBzl, Suc-Ala-Ala-Pro-Phe-SBzl, and Suc-Phe-Leu-Phe-SBzl (Suc-FLF-SBzl) (Bachem) in conjunction with Ellman's reagent were compared, and the latter was identified as the most sensitive. The V_{max} for hydrolysis of Suc-FLF−SBzl by PSA was 310 nmol min^{-1} mg^{-1} at ~22 °C, which compares favorably with a V_{max} of 50 nmol min^{-1} mg^{-1} at 37 °C for the best previously described substrate, MeO-Suc-Arg-Pro-Tyr-pNA (*35*). The relatively rapid rate of hydrolysis of Suc-FLF-SBzl by PSA and the higher molar extinction coefficient of Ellman's reagent increase the sensitivity of PSA detection by an approximate factor of 10.

Assay Buffers. Peptidase assay buffers are as follows: neutrophil elastase, pancreatic elastase, cathepsin-G, chymotrypsin, 20 mM Tris-HCl, pH 7.4, 500 mM NaCl, 0.1% poly(ethylene glycol); proteinase-3, 50 mM sodium phosphate, pH 7.5, 0.05% Triton X-100; chymase, 200 mM Tris-HCl, pH 8.0, 1 M NaCl; PSA, 100 mM Tris-HCl, pH 7.5, 500 mM NaCl; trypsin, 20 mM Tris-HCl, pH 7.8, 150 mM NaCl, 2 mM $CaCl_2$; thrombin and plasmin, 20 mM Tris-HCl, pH 7.8, 150 mM NaCl; granzyme A, 200 mM Tris-HCl, pH 8.0; granzyme B, 200 mM HEPES, pH 7.2, 0.5% Triton X-100, 300 mM NaCl, 1 mM EDTA; caspase 1 and caspase 3, 100 mM HEPES, pH 7.5, 20% glycerol, 0.5 mM EDTA, 0.1% bovine albumin, 5 mM dithiothreitol; macrophage metalloelastase, 50 mM HEPES, pH 6.8, 10 mM $CaCl_2$.

Complex Formation/Degradation of MNEI. MNEI was incubated with protease at 37 °C for 5 min (15 min for MMP-12 or 30 min for PSA), and the reaction was terminated by adding DFP (2 mM; Sigma) and incubating for 2 min at room temperature. An aliquot was solubilized for SDS−PAGE.

Identification of the P_1 Residue. An aliquot from the above complex formation reaction was stored at −80 °C until analyzed by automated Edman degradation at the Molecular Biology Core Facility of the Dana Farber Cancer Institute using an Applied Biosystems 477 pulsed liquid sequencer. Mass was determined for MNEI fragments by the MALDI-TOF method (matrix-assisted laser desorption ionization time of flight) using a Voyager DE-STR mass spectrometer (Perceptive Biosystems, Framingham, MA); the matrix used was α-cyano-4-hydroxycinnanamic acid.

SDS−PAGE. Samples were combined with an equal volume of 4% SDS, 120 mM Tris-HCl, pH 6.8, 20% glycerol, 4% mercaptoethanol, heated at 100 °C for 2 min, and fractionated by SDS electrophoresis (*36*) on 10% Tris−glycine−polyacrylamide gels (complex formation) or 10% NuPAGE Bis-Tris gels/MES buffer, pH 7.3 (MNEI degradation) (Novex, San Diego, CA). The gels were stained with Coomassie blue.

Quantitation of Active Enzyme. Active site concentration was determined for fresh stocks of bovine chymotrypsin by addition of 50 μg in 50 μL to 25 μM Boc-Tyr-ONp in 1 mL of 50 mM sodium citrate buffer, pH 3.2 (*13*, *37*), and measurement of $A_{345\ nm}$. The active chymotrypsin concentration calculated from the *p*-nitrophenol burst using an E_{345} at pH 3.2 of 6014 M^{-1} cm^{-1} was identical to the protein concentration based on an $A_{280}^{1\%}$ of 20.5 (*13*), indicating that the enzyme is 100% active. To determine the active concentration of neutrophil elastase, aliquots of 50 μg in 50 μL based on an $A_{280}^{1\%}$ of 9.85 (*38*) of frozen stock in phosphate-buffered saline were titrated using 25 μM Ac-Ala-Ala-azaLeu-ONp in 1 mL of 100 mM sodium phosphate buffer, pH 6.0, and an E_{345} of 6250 M^{-1} cm^{-1}. Neutrophil elastase was 93% active. MNEI activity was titrated against chymotrypsin and elastase and protein concentration calculated from amino acid content. MNEI was found to be 100% active. Other proteases were titrated against MNEI; protein concentrations were designated by the supplier or determined by optical density. Proteinase 3 was 100% active; cathepsin G, 100%; chymase, 98% and 93%; pancreatic elastase, 84%; human chymotrypsin, 80%; and PSA (seminal plasma-derived), 70% active. The titrated proteases were used the same day for determination of second-order rate constants.

Association Rate Constants. For second-order rate reactions, equimolar protease and MNEI concentrations were incubated at room temperature, and aliquots were removed at varying times for addition of substrate. Remaining peptidase activity was determined as described above using the Table 1 substrates except for neutrophil elastase, which was assayed with MeO-Suc-AAPV-SBzl at 0.2 mM, cathepsin G with Suc-AAPF-SBzl at 0.6 mM, chymase with Suc-AAPF-pNA at 3 mM, and pancreatic elastase with Suc-AAA-pNA at 3.6 mM. Initial velocities were determined by measurement of product formation every 15 s over a short period of time. In some cases, aliquots were diluted 10-fold prior to addition of substrate. Inhibition reactions were sufficiently slow and/or substrate concentrations were sufficiently high [(2−13) × K_M] to effectively limit further inhibition during assays. The measurement of proteinase-3 with MNEI was the exception because substrate concentration was not high enough (0.5 × K_M) to reduce inactivation rates more than 2-fold. The rate constant for this reaction should be considered a maximal estimate. Second-order rate constants were calculated as described (*39*). The reciprocal of the activity (v) was plotted as a function of time. The plots were linear, and the slope of the line was obtained by linear least-squares regression analysis of the data. The slope (m) was used to calculate the time for half loss of enzyme activity according to eq 1, the relationship describing the loss of enzyme activity under second-order conditions (*39*), where v_0 is the activity of the enzyme without inhibitor. The second-order rate constant k_{assoc} (M^{-1} s^{-1}) was then calculated according to eq 2 where $[E]_0$ is the concentration of enzyme producing an activity of v_0 under experimental conditions of the measurement.

$$v^{-1} = {v_0}^{-1} + mt \quad (1)$$

$$k_{assoc} = (t_{1/2} \times [E]_0)^{-1} \quad (2)$$

Pseudo-first-order reaction of trypsin with MNEI was carried out by incubating trypsin with a 20-fold molar ratio of MNEI at room temperature. At various time points, the remaining peptidase activity was determined as in Table 1. A semi-

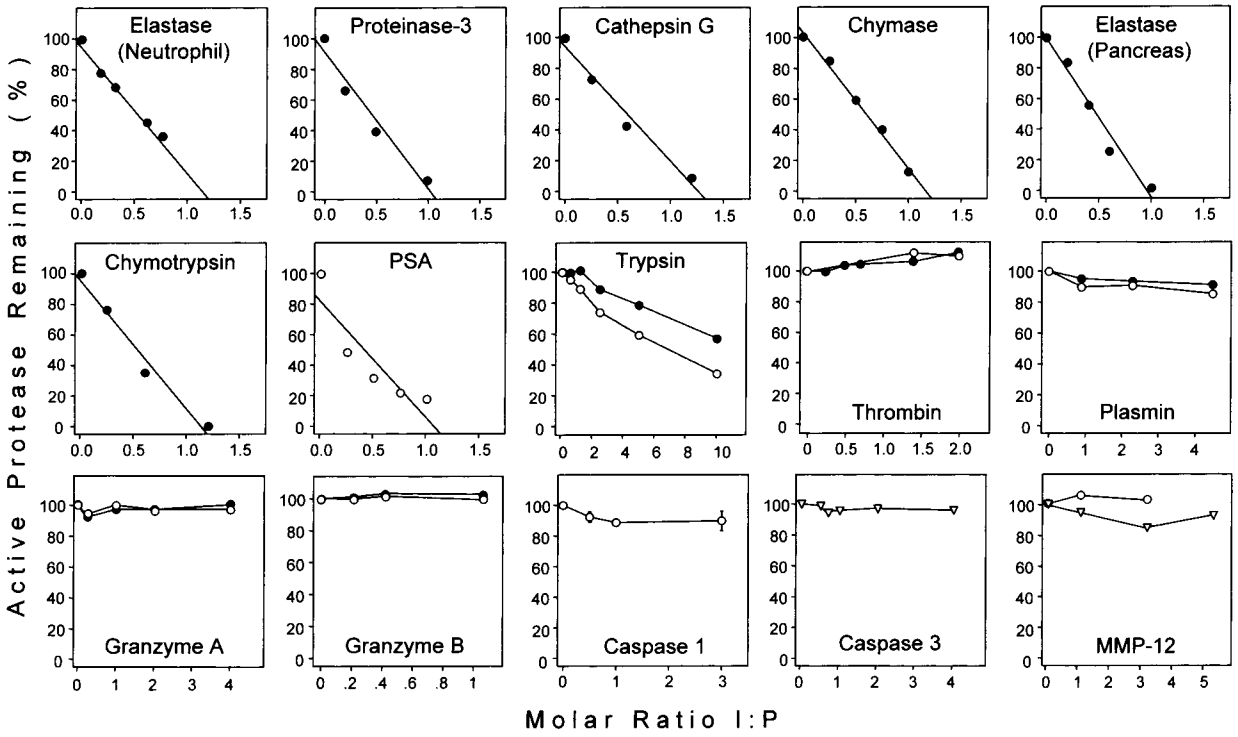


FIGURE 1: Inhibition of proteases by MNEI. Neutrophil elastase, proteinase-3, cathepsin G, chymase, pancreatic elastase, chymotrypsin, PSA, trypsin, thrombin, plasmin, granzyme A, granzyme B, caspase 1, caspase 3, and MMP-12 (macrophage elastase) were incubated at 37 °C with varying amounts of MNEI indicated by the inhibitor:protease (I:P) ratio for 5 (●), 15/20 (○), or 30 min (▽), and residual activity was assayed. Results are the average of duplicate experiments. Human chymotrypsin (shown) and bovine chymotrypsin (not shown) gave similar results, as did seminal plasma-derived PSA (shown) and rPSA (not shown). Note that MNEI efficiently inhibited neutrophil elastase, proteinase-3, cathepsin G, chymase, pancreatic elastase, chymotrypsin, and PSA, inefficiently inhibited trypsin, and did not inhibit thrombin, plasmin, granzyme A, granzyme B, caspase 1, caspase 3, or MMP-12.

logarithmic plot of activity (v) versus time was linear and used to calculate $t_{1/2}$; the association rate constant was then calculated as described (*39*) using eq 3 where $[I]_0$ is the concentration of inhibitor in the reaction.

$$k_{assoc} = 0.693 \times (t_{1/2} \times [I]_0)^{-1} \quad (3)$$

RESULTS

Spectrum of Inhibited Proteases. To define the inhibitory specificity of MNEI, we tested an enzyme panel that included 12 serine proteases, 2 cysteine proteases, and 1 metalloprotease. Each protease was titrated with MNEI to determine the completeness of inhibition and the stoichiometry of inhibition (SI). Analogous to the mechanism of suicide substrates, SI values may be greater than 1 in serpin−protease reactions if the trapping mechanism producing the inhibited complex is not efficient. MNEI was found to fully inhibit seven proteases including the three neutrophil azurophil granule proteases, neutrophil elastase and proteinase-3, as previously shown (*14*), and cathepsin G. MNEI also inhibited mast cell chymase, pancreatic elastase (*14*), chymotrypsin, and PSA (Figure 1). The SI values for all reactions were near 1, strongly suggesting that little or no MNEI enters the substrate pathway of these proteases. Each of these proteases was inhibited within 5 min, suggesting fast reactions, except for PSA, which was incubated for 15 min (Figure 1). Each inhibited protease formed a stable complex with MNEI with a molecular weight that approximated the combined molecular weight of the protease and inhibitor (shown below).

MNEI inefficiently inhibited trypsin. Inhibition required high inhibitor:protease ratios (10:1) and long time of incubation (45 min) (Figure 1). MNEI did not inhibit the serine proteases (thrombin, plasmin, granzyme A, granzyme B) or the cysteine proteases (caspase 1 and caspase 3), even at high inhibitor:protease ratios and/or long incubation times. MNEI also failed to inhibit MMP-12 (macrophage elastase), a metalloprotease that co-localizes with neutrophil elastase at inflammatory sites (*24*) (Figure 1, last panel). Included among the noninhibited serine proteases are those that preferentially cleave after basic amino acids (arginine/lysine), i.e., thrombin, plasmin, and granzyme A (Figure 1), and, as previously shown, urokinase-type plasminogen activator (u-PA) (*14*). MNEI also failed to inhibit granzyme B, a serine protease with specificity for cleavage after acidic residues (aspartic acid).

Effect of Noninhibited Proteases on MNEI. To determine whether the noninhibited proteases cleave MNEI, the inhibitor at 1.0 μM was incubated for 5 min at 37 °C with varying molar ratios (0.25−1.5) of each of the noninhibited proteases. The reactants were separated by SDS electrophoresis and detected by Coomassie blue staining. MNEI was unaffected by thrombin, plasmin, granzyme A, granzyme B, caspase 1, and caspase 3 (data not shown), but was cleaved by MMP-12. MMP-12 cleaved MNEI (42 kDa) to a 38 kDa species (Figure 2). Loss of approximately 4 kDa is consistent with cleavage of MNEI within the reactive center loop. Cleavage of MNEI by MMP-12 was dose- and time-dependent (Figure 2).

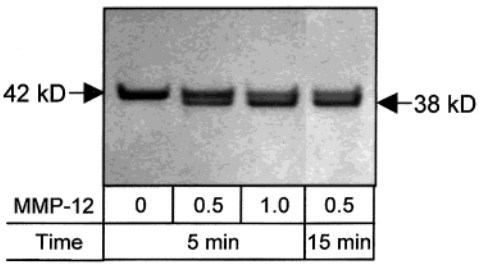


FIGURE 2: Cleavage of MNEI by MMP-12. MNEI (1.0 μM) was incubated at 37 °C with 0, 0.5, or 1.0 μM MMP-12 as indicated. Shown are Coomassie blue stained SDS gels. Note that MMP12 cleaves 42 kDa MNEI to a 38 kDa species.

On incubation of MNEI (1 μM) with trypsin (0.5−2 μM), trace levels were formed of an apparent covalent stoichiometric complex and trace levels of multiple MNEI fragments of 27−32 kDa (data not shown), suggesting slow and inefficient inhibition of trypsin by MNEI and slow nonspecific proteolysis of MNEI by trypsin.

Reactive Center P_1 Residue for the Interactions with Neutrophil Elastase, Proteinase-3, Cathepsin G, Chymase, Pancreatic Elastase, Chymotrypsin, and PSA. To determine how a single serpin inhibits seven proteases, we sought to identify the reactive center P_1 amino acid, which generally plays a dominant role in determining the specificity of inhibition. Inactivation of proteases by serpins initially involves a substrate-like cleavage of the reactive site (P_1−P_1' bond) to form the acyl enzyme. The acyl enzyme subsequently becomes trapped as the result of a conformational change that translocates the covalently bound protease to the opposite pole of the serpin (*40*−*42*). Thus, inhibition results in the production of a new N-terminus. Identification of the new N-terminus therefore marks the reactive site.

To generate the fragment of the inhibitory reaction, MNEI was incubated with neutrophil elastase, proteinase-3, or cathepsin G, and the formation of covalent higher molecular weight MNEI−protease complexes was verified by SDS/PAGE (Figure 3A). Aliquots of the reactions were subjected to automated Edman N-terminal sequencing. Two amino acids were found at each cycle, one from the N-terminus of the protease and one from the newly released MNEI fragment. Intact MNEI was not represented because its N-terminus is blocked (*2*). Thus, subtraction of the known protease sequence revealed the sequence of the released fragment beginning with P_1' (Figure 3C). These data identified P_1, the preceding residue of intact MNEI (Figure 3B), as Cys^{344}, as predicted by serpin alignment, for the reactions with neutrophil elastase and proteinase-3, but Phe^{343} for the reaction with cathepsin G (Figure 3C).

Similarly, the reactions of MNEI with chymase, pancreatic elastase, chymotrypsin, and PSA were analyzed by SDS/PAGE, which showed covalent complexes of MNEI formed with each protease (Figure 4A). For chymase, which has chymotrypsin-like cleavage specificity, and for chymotrypsin itself, the P_1 residue identified by N-terminal sequencing is Phe^{343} (Figure 4B). For pancreatic elastase, which has substrate specificity related to neutrophil elastase and proteinase-3, P_1 was found to be Cys^{344} (Figure 4B). For PSA, which is categorized as a chymotrypsin-like protease (*43*), P_1 was identified as Cys^{344}. Because the analysis of seminal plasma-derived PSA might have been adversely affected by

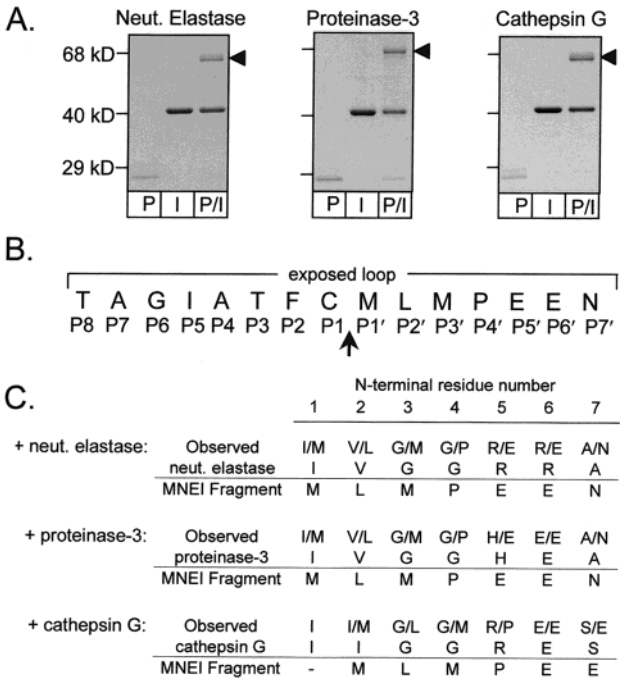


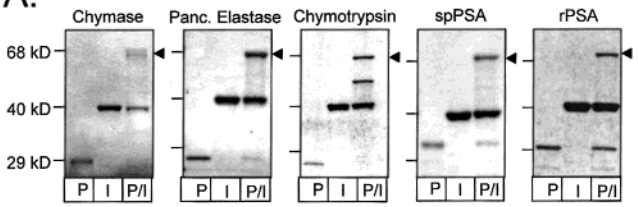
FIGURE 3: Analysis of MNEI in complex with neutrophil elastase, proteinase-3, and cathepsin G. MNEI (0.60 nmol) and protease (0.42 nmol) were incubated for 5 min at 37 °C. (A) Coomassie blue stained SDS gels show proteases and MNEI incubated separately (lanes P and I) and together (lanes P/I). Note that MNEI forms a complex (arrows) with neutrophil elastase (left panel), proteinase-3 (middle panel), and cathepsin G (right panel). (B) Sequence of the exposed reactive center loop region of intact MNEI. The putative numbering, P_8−P_7' (*8*), and the arrow indicating Cys^{344} as the P_1 residue are based on serpin superfamily alignment (*1*). (C) Sequencing results. Shown are 7 cycles of N-terminal sequence for each reaction, followed by the known protease sequence (MNEI has a blocked N-terminus), and the sequence of the newly generated MNEI fragment obtained by subtraction. The absence of an MNEI signal at the first position of the cathepsin G-generated fragment is understandable because cysteine residues are destroyed in the Edman reaction.

a contaminating protease, the sequencing experiments were repeated using recombinant PSA. P_1 was again identified as Cys^{344} (Figure 4B, last line).

To verify the P_1 residues, the released fragments of MNEI were analyzed by mass spectrometry. For neutrophil elastase, proteinase-3, pancreatic elastase, and PSA (seminal plasma-derived and recombinant), the measured mass values were close to the theoretical value of the Met^{345} to C-terminal MNEI fragment (4043 daltons) (Table 2), confirming that these proteases interact with Cys^{344} as P_1. For cathepsin G, chymotrypsin, and chymase, the measured mass values were close to the theoretical value of the Cys^{344} to C-terminal MNEI fragment (4147 daltons), confirming that these proteases interact with MNEI via Phe^{343} as P_1.

Kinetics of the MNEI Inhibitory Reactions. To quantify the rate of formation of the inhibitory complexes, inhibition rate constants were determined using second-order kinetics as described (*39*). These conditions could be used because MNEI inhibited all target proteases with a stoichiometry of inhibition near 1. Reactant concentrations were as low as possible in order to reduce inhibition rates to measurable levels. Substrate concentrations in assays used to measure residual activities of aliquots removed from reactions were as high as possible to adequately stop reactions at the

A.



B.

	MNEI Fragment N-terminal residue number 1	2	3	4	5	6	7
+ Chymase:	-	M	L	M	P	E	E
+ Pancreatic Elastase:	M	L	M	P	E	E	N
+ Chymotrypsin:	-	M	L	M	P	E	-
+ PSA (seminal plasma):	M	L	M	P	E	E	N
+ rPSA:	M	L	M	P	E	E	N

FIGURE 4: Analysis of MNEI in complex with chymase, pancreatic elastase, chymotrypsin, and PSA. MNEI (0.60 nmol) was incubated with 0.42 nmol of mast cell chymase, pancreatic elastase, chymotrypsin (human), and PSA (seminal plasma-derived and recombinant) for 5 min (30 min for PSA) at 37 °C and evaluated as in Figure 3. (A) Coomassie blue stained SDS gels show that MNEI forms complexes (arrows) with chymase, pancreatic elastase, chymotrypsin, and PSA. (B) Sequencing results. Shown are N-terminal sequences (7 cycles) for the MNEI fragment generated in each reaction obtained by subtracting the known protease sequences. For chymotrypsin, a two-chain enzyme, two sequences were subtracted.

Table 2: Mass Spectrometry of MNEI Fragments Generated by Neutrophil Elastase, Cathepsin G, Proteinase-3, Pancreatic Elastase, Chymotrypsin, Chymase, and PSA[a]

P_1 residue	fragment	calcd mass (Da)
Cys^{344}	Met^{345} to C-terminus	4043
Phe^{343}	Cys^{344} to C-terminus	4147

MNEI fragment generated by	obsd mass
neutrophil elastase ($n = 3$)	4057 ± 10
proteinase-3 ($n = 3$)	4076 ± 14
cathepsin G ($n = 3$)	4204 ± 28
chymase ($n = 3$)	4222 ± 17
pancreatic elastase ($n = 2$)	4064 (avg)
chymotrypsin ($n = 2$)	4216 (avg)
PSA (seminal plasma) ($n = 3$)	4022 ± 35
PSA (recombinant) ($n = 4$)	4061 ± 1

[a] The products of the MNEI−protease reactions (Figures 2 and 3) were examined by MALDI-TOF. Shown are theoretical and experimental mass values for MNEI fragments.

indicated times. Plots of 1/residual activity versus time were linear in all determinations. Data extrapolated to zero-time predicted control activities (1/activity without inhibitor) reasonably well. Values established from duplicate measurements were in good agreement. We estimate that the reported values are accurate to within 2−4-fold. The k_{assoc} values for the interactions of MNEI with the neutrophil granule proteases are very high: for MNEI−neutrophil elastase, 3.4×10^7 M^{-1} s^{-1}; for MNEI−proteinase-3, 1.7×10^7 M^{-1} s^{-1}; and for MNEI−cathepsin G, 2.3×10^6 M^{-1} s^{-1}. The k_{assoc} for the interaction MNEI−pancreatic elastase is also high, 1.9×10^7 M^{-1} s^{-1}, as is the k_{assoc} for the interaction MNEI−chymotrypsin, 2.8×10^6 M^{-1} s^{-1}. Even the lowest k_{assoc} values determined, 2.2×10^5 M^{-1} s^{-1} for the interaction MNEI−chymase and 8.2×10^3 M^{-1} s^{-1} for the interaction MNEI−PSA, are the highest reported rate constants for inhibition of these proteases (summarized in Figure 5, left column) (also see Discussion). Finally, an approximate rate constant for the low level inhibition of trypsin by MNEI was determined by the use of pseudo-first-order kinetics. The rate constant measured for this reaction is approximately 3.6×10^2 M^{-1} s^{-1}.

DISCUSSION

This study defines the specificity of MNEI, a serpin protein found at high amounts in neutrophils and monocytes. MNEI was found to be a highly efficient inhibitor of proteases with elastase-like specificity. Three elastase-like proteases, neutrophil elastase, proteinase-3, and pancreatic elastase, were inhibited with k_{assoc} values greater than 10^7 and with an SI near 1. This makes MNEI comparable to α1-AT in terms of ability to inhibit neutrophil elastase (Figure 5). MNEI also exhibited a secondary specificity for proteases with chymotrypsin-like specificity. Demonstrated was the inhibition of cathepsin G, mast cell chymase, bovine chymotrypsin, and PSA. K_{assoc} values for these proteases ranged from 10^4 to 10^6, and SI values in all cases were near 1. Uninhibited or inefficiently inhibited by MNEI were proteases with trypsin-like specificity including plasmin, granzyme A, thrombin, and trypsin; proteases with Asp-ase-like activity were represented by granzyme B and cellular caspases (caspase-1 and caspase-3). MNEI also did not show any inhibitory activity toward a metalloprotease (MMP-12) with elastase-like activity from macrophages. Among the noninhibited proteases, only MMP-12 cleaved MNEI.

The ability to inhibit elastase-like and chymotrypsin-like proteases makes MNEI effective against the three major serine proteases of neutrophils: elastase, proteinase-3, and cathepsin G. These proteases are stored in high quantity within the azurophil (primary) granules of the neutrophil. They are released via vesicle fusion into phagosomes to help digest engulfed microbes and into the extracellular environment by secretion and cell death (*12, 56*). Neutrophil proteases, especially elastase, have been implicated in several destructive tissue diseases such as cystic fibrosis, emphysema, and chronic obstructive pulmonary disease (COPD) (*11, 57*). MNEI may help to counter the destructive effect of these proteases. Finding MNEI in monocytes, which also express neutrophil elastase and cathepsin G, supports the protective role of this inhibitor.

The ability of MNEI to inhibit human mast cell chymase and PSA and the pancreatic enzymes elastase and chymotrypsin suggests that this inhibitor may be expressed in other cells as well. Human chymase is not very effectively inhibited by serpins found in plasma, α1-AT and α1-ACT, since the rate constants are low, ranging from 2000 to 20 000 M^{-1} s^{-1} (Figure 5), and SI values for each reaction are unusually high, greater than 4. SCCA-2, a cellular serpin, was shown to inhibit chymase (*46*). The rate constant for this reaction is 30 000 M^{-1} s^{-1} (Figure 5). Thus, MNEI is the most effective serpin inhibitor toward chymase so far identified. Like chymase, PSA is also not rapidly inhibited by α1-ACT (*58*). Furthermore, complex formation between PSA and other inhibitors, protein C inhibitor (*59*) or α2-macroglobulin (*60*), is also not rapid and has not been well characterized. In the current study, MNEI was shown to be

Protease	k_{assoc} (Molar^{-1} x sec^{-1}) for the reaction with:					
	MNEI	α1-AT	SLPI/MPI	α1-ACT	SCCA2	PI-6
Neutrophil Elastase (human)	3.4×10^7	$6.5 \times 10^{7,a}$ $1.9 \times 10^{7,b}$	$6.4 \times 10^{6,c}$	no inhibition[b]	no inhibition[d]	no inhibition[e]
Proteinase 3 (human)	1.7×10^7	$8.1 \times 10^{6,f}$	no inhibition[g]	no inhibition[f]		
Cathepsin G (human)	2.3×10^6	$4.1 \times 10^{5,a}$	$1.5 \times 10^{6,h}$	$5.1 \times 10^{7,a}$ $8.1 \times 10^{5,b}$ $7.0 \times 10^{5,i}$	$1.0 \times 10^{5,d}$	$6.8 \times 10^{6,j}$
Mast Cell Chymase (human)	2.2×10^5	$7.5 \times 10^{3,k}$	$2.0 \times 10^{4,l}$	$2.1 \times 10^{4,k}$	$3.0 \times 10^{4,d}$	
Pancreatic Elastase (porcine)	1.9×10^7	$1.0 \times 10^{5,a}$				
Chymotrypsin (human)	2.8×10^6	$5.4 \times 10^{6,a}$	$1.4 \times 10^{6,m}$	$1.0 \times 10^{4,a}$	no inhibition[d]	
(bovine)	1.2×10^6	$5.9 \times 10^{6,a}$	$1.6 \times 10^{6,i}$	$6.0 \times 10^{4,a}$ $5.5 \times 10^{5,b}$		$6.0 \times 10^{6,n}$
PSA (human)	8.2×10^3		no inhibition[o]	slow inhibition[p]	no inhibition[d]	

FIGURE 5: Apparent second-order rate constants for the reactions of human MNEI, α1-AT, SLPI/MPI, α1-AT, SCCA2, PI-6, and PI-9 with various proteases. The rate constants are taken from the following: *a*, Beatty et al. (*39*); *b*, Lomas et al. (*44*); *c*, Boudier and Bieth (*45*); *d*, Schick et al. (*46*); *e*, Sun et al. (*47*); *f*, Rao et al. (*13*); *g*, Rao et al. (*48*); *h*, Fath et al. (*49*); *i*, Duranton et al. (*50*); *j*, Scott et al. (*51*); *k*, Schechter et al. (*26*); *l*, Walter et al. (*52*); *m*, Belorgey et al. (*53*); *n*, Riewald and Schleef (*54*); *o*, Ohlsson et al. (*55*); and *p*, Christensson et al. (*35*). Measurements were made at 20−25 °C except for the PI-6−cathepsin G reaction, which was measured at 17 °C.

the best inhibitor thus far of PSA. Although both chymase and PSA are well-accepted chymotrypsin-like proteases, it is intriguing that their respective reactive center P1 residue for MNEI interaction differs, Phe[343] for chymase and Cys[344] for PSA.

Dual Reactive Sites. Whereas Cys[344], the residue predicted by serpin alignment, was identified as P_1 for inhibition of neutrophil elastase, proteinase-3 and pancreatic elastase (elastase-like enzymes), and PSA, the preceding residue, Phe[343], was identified as P_1 for the reactions with chymotrypsin, cathepsin-G, and chymase (chymotrypsin-like enzymes). The demonstration of separate reactive sites provides a molecular explanation for the ability of MNEI to function as a dual specificity inhibitor highly reactive with each group of proteases.

Dual reactive sites, although infrequent in the overall serpin superfamily (*61*), are not uncommon among ov-serpins (ovalbumin-related serpins) (*62, 63*). In addition to MNEI, ov-serpins with dual reactive sites include PI-6, which has sites for thrombin/trypsin and chymotrypsin (*54*), PI-8, which has sites for thrombin and chymotrypsin (*64*), and PI-9, which has sites for granzyme B (*65, 66*) and neutrophil elastase (*67*). Although dual sites per se are not unique, the highly reactive nature of the two MNEI sites is noteworthy because second sites often have low rate constants of inhibition and/or high SI values, indicating preference for the substrate pathway. In contrast, for MNEI and also for PI-6 (*54*), each reactive site supports rapid inhibition of a group of proteases with related specificity, and all reactions are highly efficient, having SI values close to 1.

MNEI should be compared also with leukocyte elastase inhibitor (LEI), an ov-serpin from horse leukocytes (*68*). These might be orthologues since their sequences are 82% identical and horse LEI also inhibits elastase ($k_{assoc} = 4.4 \times 10^7$ M^{-1} s^{-1}) (*68*) and chymotrypsin-like enzymes (k_{assoc} for cathepsin G inhibition, 2.0×10^6 M^{-1} s^{-1}) (*69*). However, for putative orthologues, MNEI and horse LEI showed a puzzling difference in their reactive center loop (RCL) sequences in that the P_5 to P_1 region of MNEI is Ile-Ala-Thr-Phe-Cys and that of horse LEI is Thr-Ile-Met-Leu-Ala. In light of the present findings, it is of note that the predicted P_1 of horse LEI is Ala[344] (*70*), which although different from Cys[344] of MNEI, is not surprising for an elastase inhibitor, and the preceding amino acid, Leu[343] (*70*), might serve as P_1 for inhibition of chymotrypsin-like enzymes. Thus, horse LEI may also use adjacent reactive sites.

A different perspective is provided by comparing MNEI and α1-AT, which have largely shared inhibitory function (Figure 5). α1-AT, unlike MNEI, has only one active site. The single P_1 residue of α1-AT, Met[358], functions in the inhibition of both elastase- and chymotrypsin-like enzymes (chymotrypsin and cathepsin G) (and also trypsin) (*71, 72*). Thus, the two serpins, α1-AT and MNEI, have evolved substantially shared biochemical activity through different structures.

Reactive Center Loop Length. In a recent study, the first crystallographic structure was defined for a serpin−protease complex (α1-AT-Pittsburgh complexed to trypsin) (*41*). As predicted by earlier findings (*42, 73, 74*), the entire serpin reactive center loop N-terminal to the reactive site, $P_{15}-P_1$, was found inserted as an additional strand in the A β-sheet, and the protease was displaced to the opposite pole of the serpin. The structure of the complex showed the displaced protease tightly packed against the serpin with the catalytic site profoundly distorted (*41*), providing an explanation for the irreversibility of serpin inhibition and suggesting that the fixed length of the N-terminal portion of the RCL is a tightly regulated critical component of the mechanism.

In particular, reactive loops longer than the 17 residues in α1-AT, measured from the hinge glutamic acid residue [Glu-342 in standard numbering (*6*)] to the P_1 residue, are not found in physiological inhibitors, and 16-residue reactive loop lengths are infrequent (*75*). Mutations in α1-AT-Pittsburgh that lengthened the loop were found to destabilize

the complex, and mutations that shortened the loop enhanced the stability of the complex, but lowered the rate constant for complex formation, providing a default advantage to the substrate pathway. However, the quantitative effects of lengthening and shortening the RCL varied greatly depending on the protease being inhibited, an effect thought to be caused by steric features of the particular protease (*75*).

Within this framework, we can conclude that MNEI inhibits neutrophil elastase, proteinase-3, and pancreatic elastase by forming a complex via Cys^{344} that has a canonical 17-residue-inserted RCL, and MNEI inhibits cathepsin-G, chymase, and chymotrypsin by forming a complex via Phe^{343} with a generally less favorable 16-residue-inserted RCL. For the PSA−MNEI reaction, we speculate that steric features of PSA prevent formation of a 16-residue complex, and the reaction defaults to the biochemically surprising but sterically permissible reaction via Cys^{344} to form an inhibitory complex with a 17-residue-inserted RCL. These data suggest that crystallographic structure analysis of MNEI complexes would reveal additional features of the serpin mechanism of inhibition.

Conclusion. Overall, the findings characterize MNEI as a dual specificity inhibitor with two adjacent reactive sites that support rapid efficient inhibitory reactions with cellular proteases including the three neutrophil granule proteases.

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