Structural Basis for Accelerated Cleavage of Bovine Pancreatic Trypsin Inhibitor (BPTI) by Human Mesotrypsin*

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Human mesotrypsin is an isoform of trypsin that displays unusual resistance to polypeptide trypsin inhibitors and has been observed to cleave several such inhibitors as substrates. Whereas substitution of arginine for the highly conserved glycine 193 in the trypsin active site has been implicated as a critical factor in the inhibitor resistance of mesotrypsin, how this substitution leads to accelerated inhibitor cleavage is not clear. Bovine pancreatic trypsin inhibitor (BPTI) forms an extremely stable and cleavage-resistant complex with trypsin, and thus provides a rigorous challenge of mesotrypsin catalytic activity toward polypeptide inhibitors. Here, we report kinetic constants for mesotrypsin and the highly homologous (but inhibitor sensitive) human cationic trypsin, describing inhibition by, and cleavage of, BPTI, as well as crystal structures of the mesotrypsin-BPTI and human cationic trypsin-BPTI complexes. We find that mesotrypsin cleaves BPTI with a rate constant accelerated 350-fold over that of human cationic trypsin and 150,000-fold over that of bovine trypsin. From the crystal structures, we see that small conformational adjustments limited to several side chains enable mesotrypsin-BPTI complex formation, surfacing the predicted steric clash introduced by Arg-193. Our results show that the mesotrypsin-BPTI interface favors catalysis through (a) electrostatic repulsion between the closely spaced mesotrypsin Arg-193 and BPTI Arg-17, and (b) elimination of two hydrogen bonds between the enzyme and the amine leaving group portion of BPTI. Our model predicts that these deleterious interactions accelerate leaving group dissociation and deacylation.

There are three human trypsins encoded by different genes; cationic trypsinogen (PRSS1) and anionic trypsinogen (PRSS2) are located at proximal loci on chromosome 7q35, while mesotrypsinogen (PRSS3) is found on chromosome 9p13 (1). All three isoforms are secreted as digestivezymogens in the pancreas and activated by enteropeptidase in the duodenum (2). A differentially spliced form of mesotrypsinogen termed trypsinogen 4, transcribed from an alternative promoter (3) and utilizing an unconventional CUG translation initiation codon (4), is highly expressed in brain tissue (5) and in some epithelial cell lines (6) and tumors (7). The two zymogen forms differ only at the N terminus, and processing of either form by removal of the prodomain results in active mesotrypsin of identical amino acid sequence (3). Trypsinogen 4 lacks a recognizable signal sequence and it is not known whether or how the enzyme might be secreted, though there is some evidence for processing of the prodomain and deposition of activated mesotrypsin in the extracellular neuronal matrix (8).

The most striking characteristic of mesotrypsin is its unique resistance to polypeptide trypsin inhibitors (9, 10). Canonical trypsin inhibitors feature characteristic binding loops that bind to the trypsin active site extremely tightly, mimicking a substrate, yet are cleaved extremely slowly (11–13). Mesotrypsin has reduced affinity to such inhibitors, yet surprisingly, has been shown to cleave several canonical inhibitors at an accelerated rate (14). The inhibitor resistance of this enzyme may be key to its biological roles. Although comprising only a minor component of pancreatic secretions, mesotrypsin may play a unique role in digestion through degradation of trypsin inhibitors in the diet (14), and premature activation of mesotrypsin may contribute to pancreatitis through degradation of the protective human pancreatic trypsin inhibitor SPINK1 (14). In epithelial and neural tissues, the inhibitor resistance of mesotrypsin may permit prolonged signaling activity through cleavage of protease-activated receptors (PARs) (6, 15, 16). Mesotrypsin may also contribute to neurodegeneration; studies demonstrating specific cleavage of myelin basic protein by mesotrypsin in vitro suggest a possible role in multiple sclerosis (17).

The reduced affinity of mesotrypsin for polypeptide trypsin inhibitors has been attributed to the presence of an arginine at active site residue 193, which in most trypsins and other serine proteases is a highly conserved glycine (9). Mutagenesis of mesotrypsin Arg-193 to Gly restored inhibitor sensitivity (14, 18). Furthermore, the crystal structure of mesotrypsin bound to the small molecule inhibitor benzamidine revealed that the presence of Arg-193 dramatically changes the electrostatic potential of the substrate binding cleft (18). A major unanswered question is how Arg-193 could favor productive binding and accelerated cleavage of canonical inhibitors.

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The atomic coordinates and structure factors (code 2R9P and 2RA3) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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‡ The abbreviations used are: PAR, protease-activated receptor; BPTI, bovine pancreatic trypsin inhibitor; Z-GPR-pNA, carboxybenzyl-Gly-Pro-Arg-p-nitroanilide; HPLC, high-pressure liquid chromatography; wt, wild type; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; r.m.s.d., root mean square deviation; PDB, Protein Data Bank.
Bovine pancreatic trypsin inhibitor (BPTI), an archetypal canonical trypsin inhibitor of the Kunitz family, forms a complex with bovine trypsin that is among the highest affinity protein-protein complexes characterized, with a 0.05 picomolar equilibrium dissociation constant (19, 20) and similarly impressive stability against proteolytic cleavage (see Table 1). Here, we employed BPTI to provide a rigorous challenge of the ability of mesotrypsin to catalyze hydrolysis of canonical inhibitors. We used human cationic trypsin as an ideal benchmark for comparison, since cationic trypsin is highly homologous to mesotrypsin, with 94% conserved and 87% identical residues, but possesses glycine at position 193 and is efficiently inhibited by polypeptide trypsin inhibitors. We describe kinetic studies measuring the ability of mesotrypsin and human cationic trypsin to cleave BPTI, as well as crystal structures of the mesotrypsin-BPTI and cationic trypsin-BPTI complexes.

**EXPERIMENTAL PROCEDURES**

*Expression, Purification, and Activation of Recombinant Human Trypsins—* Expression plasmids derived from pTrapT7, harboring genes for human cationic trypsinogen (*PRSS1*) and human mesotrypsinogen (*PRSS3*) (14, 21–23). Trypsinogens were activated by treatment with cationic trypsin and mesotrypsin were recombinantly expressed, refolded, and purified by electrophoretic chromatography essentially as described by Sahin-Töth and co-workers (14, 21–23). Trypsinogens were activated by treatment with bovine enteropeptidase (enterokinase, Roche Applied Sciences) in 0.1 M Tris-HCl and 1 mM CaCl₂ at 37 °C. For mesotrypsin activations featured 300:1 trypsinogen:enteropeptidase (w/w) and were typically completed in 1 h, as for cationic trypsinogen, activations featured 25,000:1 trypsinogen:enteropeptidase (w/w) and were typically completed in 1 h, as assessed by SDS-PAGE. Activated trypsins were purified on HiTrap Benzamidine FF affinity columns (GE Healthcare) by elution with a linear gradient from buffer A (50 mM Tris-pH 8.0, 0.5 M NaCl) to buffer B (25 mM HCl). Fractions containing trypsins were pooled and concentrated by ultrafiltration using Centricon-10 concentrators (Amicon). Typical yields were 10 mg of pure active trypsin per liter of bacterial culture. Enzyme concentrations were routinely measured by Nanodrop Spectrophotometer ND-1000 (NanoDrop Technologies USA) using calculated extinction coefficients at 280 nm (24) of 37,525 M⁻¹ cm⁻¹ for cationic trypsinogen or trypsin, and 41,535 M⁻¹ cm⁻¹ for mesotrypsinogen or mesotrypsin.

**Trypsinogen Mutants—** For crystallographic studies, the cationic trypsinogen and mesotrypsinogen expression constructs were mutated to produce catalytically inactive Ser-195 to Ala (S195A) mutants, using the PCR-based QuikChange method (Stratagene). For cationic trypsinogen, an Arg-117 to His (R117H) mutation was also introduced; this mutation removed a sensitive trypsinolytic site from a surface loop (14, 22), and the resulting double mutant trypsin gave improved stability against proteolytic cleavage (22), and the resulting double mutant trypsin gave improved stability against proteolytic cleavage (see Table 1). Here, we employed BPTI to provide a rigorous challenge of the ability of mesotrypsin to catalyze hydrolysis of canonical inhibitors. We used human cationic trypsin as an ideal benchmark for comparison, since cationic trypsin is highly homologous to mesotrypsin, with 94% conserved and 87% identical residues, but possesses glycine at position 193 and is efficiently inhibited by polypeptide trypsin inhibitors. We describe kinetic studies measuring the ability of mesotrypsin and human cationic trypsin to cleave BPTI, as well as crystal structures of the mesotrypsin-BPTI and cationic trypsin-BPTI complexes.

**Trypsin Inhibition Studies—** Active trypsin concentrations were quantified by active site titration using 4-nitrophenyl 4-guanidinobenzoate (Sigma) (25). BPTI (Aprotinin, Sigma) concentrations were determined by titration with bovine trypsin (Sigma), which had been previously titrated as described above. Bovine trypsin was mixed with a range of substoichiometric concentrations of BPTI and then assayed for residual activity with the substrate N₂-benzoyl-l-arginine 4-nitroanilide (Sigma); a plot of residual enzyme activity versus BPTI concentration allowed extrapolation to the stoichiometric equivalence point (26). Concentrations of the chromogenic substrate Z-GPR-pNA (Sigma) were determined by end point assay.

For mesotrypsin inhibition studies, working stocks of enzyme, substrate, and BPTI were prepared at 40X the desired final concentrations. Stock solutions and dilutions of Z-GPR-pNA were prepared in 100% Me₂SO, BPTI dilutions were prepared in 0.01 M Tris·HCl, pH 8.0, and 0.1 mg/ml bovine serum albumin, and enzyme dilutions were prepared in 20 mM sodium acetate pH 4.5, 1 mg/ml bovine serum albumin, and 10 mM CaCl₂. Assays were conducted at 37 °C in a Varian Cary-100 spectrophotometer equipped with a multiscell transporter and a circulating water bath. Assay buffer (740 µl; 100 mM Tris-HCl pH 8.0, 1 mM CaCl₂), substrate (20 µl), and BPTI (20 µl) were mixed and equilibrated in cuvettes prior to reaction initiation by the addition of enzyme (20 µl). Reactions were followed spectrophotometrically for 3–5 min, and initial rates were determined from the absorbance increase caused by the release of p-nitroaniline (ε₄10 = 8480 M⁻¹ cm⁻¹ (27)). Data were globally fitted by multiple regression to Equation 1, the classic competitive inhibition equation, using Prism (GraphPad Software, San Diego CA). Reported inhibition constants are average values obtained from multiple independent experiments.

\[
\frac{v}{v_0} = \frac{k_{cat}[E][S]}{K_m(1 + [I]/K_I) + [S]} \tag{1}
\]

The cationic trypsin-BPTI inhibition studies were carried out in a similar manner, but the observation of slow, tight-binding behavior required an alternative kinetic treatment. Z-GPR-pNA concentration was fixed at 150 µM, BPTI concentrations ranged from 10–100 nM and enzyme concentration was 0.1 nM. Reactions were initiated by addition of substrate to quartz cuvettes containing trypsin and BPTI. Reactions were run at 25 °C, and were followed spectrophotometrically for 16 h so that reliable steady-state rates could be obtained. Conversion of substrate to product did not exceed 10% over the reaction time course. Data were analyzed as described previously for a similar case of slow, tight-binding inhibition (28). For such a case, when inhibitor is in large excess over enzyme (as here), the relationship described by equation 2 is valid (28), where \(v\) and \(v_0\) are the steady-state rates in the presence and absence of inhibitor, \(K_{m}\) is the Michaelis constant for trypsin cleavage of Z-GPR-pNA, and \([S_0]\) and \([I_0]\) are the initial concentrations of substrate and
inhibitor. Equation 2 predicts that a plot of \((v_o - v_i)/v_i\) versus \([I_o]\) will yield a straight line passing through the origin, with a slope of \(1/K_i(1 + [S_o]/K_m)\), allowing calculation of \(K_i\) (28, 29). For these calculations, we used a \(K_m\) value of 36.5 ± 0.7 \(\mu M\) determined from multiple independent Michaelis-Menten kinetic studies.

\[
(v_o - v_i)/v_i = [I_o]/K(1 + [S_o]/K_m)
\]  

**(Eq. 2)**

**BPTI Hydrolysis Studies**—The depletion of intact BPTI in time course incubations with active trypsins was monitored by HPLC and/or 16% SDS-Tricine polyacrylamide gels (30, 31). Trypsin and BPTI were incubated in 0.1 M Tris-HCl, pH 8.0 and 1 M \(\text{NaCl}\) at 37 °C. Aliquots for HPLC analysis were withdrawn at periodic intervals, adjusted to 6 M urea and 2 mM dithiothreitol, incubated for 10 min at 37 °C, quenched by acidification to pH 1 with HCl and then frozen at ~20 °C until analyzed. Enzyme, BPTI, and hydrolysis products were resolved on a 50 × 2.0 mm Jupiter 4 μm 90 Å C12 column (Phenomenex) with a gradient of 0 to 100% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 0.6 ml/min over 50 min. For each time point, HPLC injections were performed in triplicate. Intact BPTI was quantified by peak integration of absorbance traces monitored at 210 nm. Time point aliquots for gel analysis were quenched by acidification to pH 1 and stored at ~20 °C until analyzed. Thawed samples were mixed with an equal volume of 2× Tricine sample buffer containing 2% v/v 2-mercaptoethanol (Bio-Rad), heat-denatured at 95 °C for 5 min, and separated on 16% SDS-PAGE gels in Tris-Tricine buffer (30).

**Crystallization of Trypsin-BPTI Complexes**—Cationic trypsin and mesotrypsin complexes with BPTI were crystallized by vapor diffusion. Catalytically inactive mutant trypsins dissolved in 1 M HCl and BPTI dissolved in 10 M NaOAc pH 6.5 were mixed in a 1:1 stoichiometric molar ratio, to achieve a total protein concentration of 3–6 mg/ml. Crystals were grown at 22 °C in hanging drops, over a reservoir of 0.1 M Tris, pH 8.5 and 1.6 M (NH₄)₂SO₄ for cationic trypsin-BPTI complexes, and 1.6 M (NH₄)₂SO₄ for mesotrypsin-BPTI complexes. Drops (4 μl) were prepared by mixing equal volumes of protein and reservoir solutions. Crystals (0.4 × 0.2 × 0.2 mm) appeared within a day or two and grew over the course of several weeks. Crystals were harvested, soaked in a cryoprotectant solution (75 mM Tris, pH 8.5, 1.8 M (NH₄)₂SO₄ and 25% glycerol for cationic trypsin-BPTI complexes; 1.6 M (NH₄)₂SO₄ and 25% PEG400 for mesotrypsin-BPTI complexes) and flash-frozen in liquid N₂.

**Structure Determination of Trypsin-BPTI Complexes**—Synchrotron x-ray data were collected from crystals at 100 K using an ADSC Quantum-4 CCD detector at beam line X12-B, National Synchrotron Light Source, Brookhaven National Laboratory. For the cationic trypsin-BPTI complex, crystals belong to the space group C222, with unit cell parameters of \(a = 91.18\), \(b = 186.54\), \(c = 90.19\), and \(\alpha = \beta = \gamma = 90°\). Diffraction data were measured to 1.46-Å resolution, and the automation pack-

\[R = \sum_{i} |I_o - F_i|/\sum_{i} |I_o|\]

was calculated with data from 20° to 120° 2θ (1/2λ = 1.28 Å). This data was used for initial structure solution. Diffraction data were measured to 1.40 Å, and the automated software HKL-2000 (42) was used for data reduction. The structure was solved by molecular replacement using the program Phaser (35), using search models derived from previous structures of mesotrypsin (PDB ID 1H4W) (18) and BPTI (PDB ID 2PTC, chain I) (37). The successful solution contained 4 copies of each protein in the asymmetric unit, forming 2 canonical trypsin-BPTI complexes. Cycles of manual rebuilding in COOT (38) were alternated with automated refinement using the refinement module of the PHENIX software suite (39). A test set comprised of 10% of the total reflections was excluded from refinement to allow calculation of the free \(R\) factor. TLS refinement was employed, with each protein chain assigned to a separate TLS group. Waters, ions, and alternative conformational states of protein residues were added using COOT (38), and restraints on the geometry of the BPTI Lys-15–Ala-16 bond were omitted. For the mesotrypsin-BPTI complex, crystals belong to the space group P2₁, with unit cell parameters of \(a = 74.22\) Å, \(b = 109.72\) Å, \(c = 81.17\) Å, \(\alpha = \gamma = 90°\), and \(\beta = 117.15°\). Crystals of this form were pseudomorphically twinned, with twin fractions varying from 0.34 to 0.48, and approximated the higher symmetry of the C222 space group. Initial attempts at structure solution failed due to misindexing. Twinging was diagnosed and crystal twin fractions estimated using the program Xtriage (41), and a crystal with minimal twinning was selected for structure solution. Diffraction data were measured to 1.40 Å, and the automated software HKL-2000 (42) was used for data reduction. The structure was solved by molecular replacement using the program Phaser (35), using search models derived from previous structures of mesotrypsin (PDB ID 1TRN, chain A) (36) and BPTI (PDB ID 2PTC, chain I) (37). The successful solution contained 2 copies of each protein in the asymmetric unit, forming 2 canonical trypsin-BPTI complexes. Cycles of manual rebuilding in COOT (38) were alternated with automated refinement using the refinement module of the PHENIX software suite (39). A test set comprised of 10% of the total reflections was excluded from refinement to allow calculation of the free \(R\) factor. TLS refinement was employed, with each protein chain assigned to a separate TLS group. Waters, ions, and alternative conformational states of protein residues were added using COOT (38). In the later stages of refinement, hydrogen atoms were added in the riding positions using the program REFE (40), and restraints on the geometry of the BPTI Lys-15–Ala-16 bond were omitted.

**Superpositions and Figures**—All superpositions and structure figures were created using the graphics software Pymol (43).

**RESULTS**

**BPTI Is a Weak Competitive Inhibitor of Mesotrypsin but a Strong Inhibitor of Human Cationic Trypsin**—To assess the binding of BPTI to human cationic trypsin and mesotrypsin, we carried out enzyme inhibition experiments, monitoring cleavage of the chromogenic substrate Z-GPR-pNA by trypsin in the presence of varying BPTI concentrations. For mesotrypsin, anticipating that the interaction would be weak, we employed a classic competitive inhibition study to determine the inhibition constant of 14 ± 3 μM (Fig. 1A and Table 1). This value is much higher than that observed for most complexes of proteases with...
TABLE 1

| Kinetic constants describing interactions of trypsins with BPTI |
|--------------------------------------|------------------|------------------|
| Bovine trypsin                       | Human cationic trypsin | Human mesotrypsin |
| Kinetic parameters                   |                   |                   |
| $K_i^*$                              | $K_i^*$           | $K_i$             |
| $k_{cat}$                            | $k_{cat}$         | $k_{cat}$         |
| $5 \times 10^{-14}$ M                 | $2.0 \times 10^{-11}$ M | $1.4 \times 10^{-5}$ M |
| $8.7 \times 10^{-10}$ s$^{-1}$        | $3.7 \times 10^{-7}$ s$^{-1}$ | $1.3 \times 10^{-4}$ s$^{-1}$ |
| Turnover time                        |                   |                   |
| 36 years                             | 31 days           | 2.2 hours         |

*Experimental values determined at pH 8.2 (19) and pH 8.0 (20).
*Calculated value; pH 7.5 (46).

canonical inhibitors, which are typically in the nanomolar to picomolar range, but is qualitatively consistent with previous reports of mesotrypsin resistance to inhibition by canonical inhibitors (9, 10, 14, 18). We note, however, that our $K_i$ value is approximately an order of magnitude greater than that of a previous report (18). While we cannot fully explain the discrepancy, it may be related to the differing methods employed; the study of Katona et al. used progress curve analysis of BPTI-inhibited reactions performed at a single fixed substrate concentration.

For cationic trypsin, for which initial studies showed evidence of slow, tight-binding behavior, it was necessary to pursue an alternative kinetic treatment. A variety of approaches have been used to measure inhibition constants in similar cases of slow, tight binding between serine proteases and protein protease inhibitors; we opted to use a method which has previously proven both sensitive and robust in our hands (28, 44, 45). A series of parallel reactions at varying BPTI concentration were followed spectroscopically as the trypsin–BPTI binding equilibrium was achieved and steady state rates were acquired. Binding curves were analyzed as described under "Experimental Procedures," giving an inhibition constant of $20 \pm 1$ μM (Fig. 1, B and C and Table 1).

**Mesotrypsin Cleaves BPTI with a Substantially Accelerated Rate Constant**—Like other canonical protease inhibitors that follow the "standard mechanism" of Laskowski (12), the binding loop of BPTI features a peptide bond, Lys-15–Ala-16, that is targeted for slow proteolytic cleavage, leading to an equilibrium mixture of intact "virgin" inhibitor and cleaved "modified" inhibitor, comprised of two chains held together by three disulfide bonds (Fig. 2A). Cleavage of BPTI by bovine trypsin at neutral pH is extremely slow, with an estimated rate constant of $-9 \times 10^{-10}$ s$^{-1}$ (46, 47). We employed SDS-PAGE and HPLC methods to determine whether mesotrypsin catalyzes more rapid hydrolysis of this reactive site bond. In time course experiments, we detected cleavage within 2 h; the amount of intact BPTI diminished and cleavage product accumulated over time until a plateau was reached after several days of incubation (Fig. 2D). Following dithiothreitol-reduction of time point samples, HPLC peaks corresponding to mesotrypsin, intact BPTI, and two cleavage products were clearly resolved (Fig. 2C). Mass spectrometry identified the product peaks as fragments corresponding to BPTI residues 1–15 and 16–58, confirming that mesotrypsin cleaves BPTI selectively at the Lys-15–Ala-16 reactive site bond (data not shown).
Structures and Kinetics of Human Trypsin-BPTI Complexes

BPTI is a substrate for mesotrypsin, albeit one that is cleaved more slowly than a typical protease substrate. The competitive inhibition equation, employed above in determining $K_i$, also describes the enzymatic reaction of one substrate in the presence of an alternative, competing substrate, with the added condition that the observed $K_i$ for the alternative substrate must be equivalent to its $K_m$ (48). Thus, the $K_m$ for cleavage of BPTI by mesotrypsin is 14 $\mu$m. We designed a BPTI hydrolysis experiment at 70 $\mu$M BPTI ($5 \times K_m$) to approximate enzyme saturating conditions, and focused on the early stages of the reaction, where total BPTI hydrolysis did not exceed 10% conversion. Under these conditions, the linear initial rate of disappearance of intact BPTI, quantified by peak integration, gave a good estimation of the catalytic rate constant: $1.3 \times 10^{-4}$ s$^{-1}$ (Fig. 2E). Using similar methods over a much longer extended time course, we obtained an estimated catalytic rate constant for BPTI hydrolysis by human cationic trypsin of $3.7 \times 10^{-7}$ s$^{-1}$ (Fig. 2F). This places the catalytic rate for cleavage of BPTI by mesotrypsin at 350-fold that of human cationic trypsin, and 150,000-fold that of bovine trypsin (Table 1).

Structures of Mesotrypsin-BPTI and Human Cationic Trypsin-BPTI Complexes—To understand how mesotrypsin cleaves BPTI at an accelerated rate, we crystallized and solved structures for the mesotrypsin-BPTI and human cationic trypsin-BPTI complexes. We employed essentially isostructural but inactive mutants of mesotrypsin and human cationic trypsin featuring a Ser-195 to Ala mutation in the active site, to avoid heterogeneity associated with proteolysis of BPTI and/or trypsin autoproteolysis. Both structures were solved by molecular replacement and refined against data extending to 1.4–1.5 Å resolution; Table 2 summarizes the crystal, data collection, and refinement statistics. As discussed under “Experimental Procedures,” the mesotrypsin-BPTI structure was obtained from a pseudomerohedrally twinned crystal, requiring the use of twin-specific refinement procedures. The model contains four highly similar complexes in the asymmetric unit, each featuring a molecule of BPTI bound in the canonical fashion at the mesotrypsin active site. The only region in which the backbones of the multiple copies diverge substantially is the loop comprised of mesotrypsin residues 145–149, where two markedly different conformations are observed (not shown). All four of the complexes are well-defined and near identical throughout the region of the enzyme-inhibitor interface; in Figs. 3–5, only the representative complex of mesotrypsin chain “A” with BPTI chain “E” is shown. The structure for human cationic trypsin-BPTI contains two copies of the canonical enzyme-inhibitor complex in the asymmetric unit. While the trypsin molecules and the trypsin-interacting regions of BPTI are highly similar and well-ordered, one of the BPTI molecules (chain “C”) is partially disordered in the region distal to trypsin; the more highly ordered complex of trypsin chain “B” with BPTI chain “I” is used for the structural comparisons discussed below and displayed in Fig. 4.

**TABLE 2**

Crystal statistics for trypsin-BPTI complexes

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<thead>
<tr>
<th>PDB ID</th>
<th>Mesotrypsin-BPTI</th>
<th>Cationic trypsin-BPTI</th>
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<tr>
<td>Complexes per ASU</td>
<td>2RP9</td>
<td>2RA3</td>
</tr>
<tr>
<td>Space group</td>
<td>P2, (pseudo-C222)</td>
<td>C222</td>
</tr>
<tr>
<td>Unit cell, Å</td>
<td>74.55, 109.72, 81.17</td>
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<td>Resolution range, Å</td>
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<td>26.11–1.46</td>
</tr>
<tr>
<td>Unique reflections</td>
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<td>115,118</td>
</tr>
<tr>
<td>Completeness, %</td>
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<tr>
<td>Multiplicity</td>
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<td>7.5 (2.3)</td>
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<tr>
<td>I/S.D</td>
<td>10.5 (3.2)</td>
<td>19.5 (1.9)</td>
</tr>
<tr>
<td>$R_{free}$</td>
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<td>0.057 (0.66)</td>
</tr>
<tr>
<td>$R_{merge}$</td>
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<td>15.66/17.83</td>
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<tr>
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<td>0.006</td>
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<tr>
<td>R.m.s.d. angles, °</td>
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<td>0.793</td>
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</table>

$^a$ Numbers in parentheses refer to the highest resolution bin.

**FIGURE 2.** Kinetics of BPTI hydrolysis by mesotrypsin and human cationic trypsin. A, BPTI sequence diagram. The Lys-15–Ala-16 reactive site bond, specifically targeted for cleavage by mesotrypsin and other proteases, is indicated by the red arrow; disulfide bonds are indicated by black brackets below the sequence. B, in a 24-h time course, cleavage of BPTI by mesotrypsin is contrasted with the stability of BPTI to digestion by bovine trypsin. Reactions contained 70 $\mu$M BPTI and 7 $\mu$M enzyme. Samples were resolved on a 16% SDS-Tricine polyacrylamide gel; arrows indicate the positions of trypsin (~24 kDa), BPTI (~6.5 kDa), and BPTI hydrolysis products. C, HPLC was used to quantify rates of BPTI hydrolysis. Representative traces from one time course are shown to illustrate the conversion of intact BPTI (peak shown in red) to two hydrolysis products; residues 1–15 (peak shown in green) and residues 16–58 (peak shown in cyan). The black peak to the right of BPTI represents mesotrypsin. Reactions contained 70 $\mu$M BPTI and 3.5 $\mu$M enzyme. Disappearance of intact BPTI from the time course illustrated in C was quantified by HPLC peak integration in D and E. D illustrates attainment of equilibrium between virgin and modified BPTI over the course of several days, while E shows the initial rate of hydrolysis, from which $k_{cat}$ is estimated (see text). F, similar HPLC methods were used to determine the initial rate of BPTI cleavage by human cationic trypsin (note the extended time frame for this time course). Reactions contained 70 $\mu$M BPTI and 7 $\mu$M enzyme.
Mesotrypsin Conformational Adjustments Enable Mesotrypsin-BPTI Complex Formation—Because structural modeling had revealed a steric clash between mesotrypsin Arg-193 and the P2/H11032 position of canonical trypsin inhibitors (18),4 we expected that conformational changes would be required for complex formation. Insight into these changes comes from superposing the mesotrypsin-BPTI structure onto the previously reported structure of mesotrypsin bound to benzamidine (1H4W, Ref. 18). Benzamidine is a small molecule that fills only the trypsin specificity pocket occupied by the P1 Arg or Lys side chain of a substrate or polypeptide inhibitor; the mesotrypsin-benzamidine complex is expected to closely approximate the free enzyme. The superposition reveals small displacements in multiple surface loops of mesotrypsin, but many of these alterations appear to result from differing crystal packing interactions rather than BPTI association. Structural changes involved in BPTI association are primarily confined to a few side chains, with more subtle adjustments in the backbone positions of the residues involved (Fig. 3A). Arg-193, displaced upwards by the Arg-17 P2/H11032 residue of BPTI, assumes a new conformation receding into the crevice between the two β-barrel domains. To accommodate this shift, His-40 swings outward to an alternative rotamer, and Asp-153 undergoes a more subtle side chain adjustment.

In the BPTI-bound conformation, Arg-193 is more buried, but the hydrogen bonding potential of the guanidino moiety appears to be well satisfied, with direct coordination to the backbone carbonyl oxygens of Trp-141 and Pro-152, and two waters, the positions of which are conserved across the four copies of the mesotrypsin-BPTI complex (Fig. 3B). One water forms a salt bridge to the Asp-153 side chain, which has moved 1 Å nearer (relative to the benzamidine-bound structure) to participate in this stabilizing interaction, while the other water bridges an interaction with the Arg-17 side chain of BPTI. The two Arg residues pack side-by-side, with distances of 3.7 Å between their respective NH1 atoms. This concentration of positive charge does not appear to be well balanced by ion pairing interactions with any acidic residue other than Asp-153; sulfate ions in partial occupancy appear to contact BPTI Arg-17, but the density is weak and positions not conserved among the multiple copies of the protein complex, suggesting that such interactions would not persist in solution.

BPTI-bound Mesotrypsin and Human Cationic Trypsin Display Significantly Different Interactions at the Prime-side Binding Interface—We examined the mesotrypsin-BPTI and human cationic trypsin-BPTI interfaces for differences that might
account for the large differences in binding affinities and catalytic rates. Superposing the two structures, we found no significant differences in contacts involving the non-prime-side of the canonical binding loop (BPTI residues 13–15) or the secondary contact loop (BPTI residues 36–39). However, the region around the prime-side of the canonical binding loop revealed three hydrogen bonds present in the cationic trypsin complex that are absent in the mesotrypsin complex (Fig. 4): trypsin His-40 N\_e to the trypsin Gly-193 carbonyl, BPTI Arg-17 N\_e\_i\_i to the trypsin His-40 carbonyl, and trypsin Tyr-39 OH to the BPTI Ile-19 nitrogen. Two of these H-bonds represent direct stabilizing interactions between cationic trypsin and BPTI. In the mesotrypsin-BPTI structure, the presence of Arg-193 requires BPTI Arg-17 to occupy an alternative conformation too distant from His-40 to form a hydrogen bond; in this conformation, Arg-17 can only form the water-bridged interaction with like-charged Arg-193 described above and shown in Fig. 3B. Mesotrypsin possesses Ser rather than Tyr at residue 39, and is thus unable to form this second hydrogen bond bridging the interface, nor do we observe any compensating water-bridged interactions involving the Ser-39 O\_i. Human cationic and bovine trypsins also differ substantially in BPTI cleavage (Table 1). However, comparison of our cationic trypsin complex with the previously solved bovine trypsin-BPTI structure (49) showed no obvious differences at the inhibitor interface.

**DISCUSSION**

**Structural Insights into Mesotrypsin-BPTI Binding**—Inhibition studies reveal that mesotrypsin-BPTI association is 6 orders of magnitude weaker than that of human cationic trypsin-BPTI, and 9 orders of magnitude weaker than that of bovine trypsin-BPTI (Table 1). While mesotrypsin Arg-193 has been implicated previously in the reduction in affinity (14, 18), the structures reported here help to explain the role of this critical residue in binding. For binding to occur, Arg-193 must pack tightly into a crevice on the enzyme surface, burying the charged side chain. Furthermore, B-factor analysis suggests that this residue becomes more highly ordered upon BPTI complex formation: the atoms of the guanidino moiety have B-factors in the range of 14–19 (compared with a mean B-factor of 22.8) in the mesotrypsin-BPTI structure, while the same atoms show B-factors ranging from 25–28 (compared with a mean B-factor of 20.0) for the mesotrypsin-benzamidine structure. These observations suggest an entropic cost associated with complex formation, which, in aggregate with unfavorable steric and electrostatic interactions between Arg-193 and the P_2 Arg-17 residue of BPTI, results in the observed poor binding of the mesotrypsin-BPTI complex.

**Structural Insights into BPTI Cleavage by Mesotrypsin**—The mesotrypsin-BPTI structure offers insights into how mesotrypsin may accelerate cleavage of BPTI over that of bovine and human cationic trypsins (Table 1). We have demonstrated previously that canonical inhibitor complexes represent near-ideal Michaelis complexes, at least in some cases capable of undergoing acylation on the millisecond timescale (13, 44). The roadblock to reaction occurs at the deacylation step, when the leaving group amine, rather than dissociating from the enzyme, is tenaciously retained in the active site through interactions with the enzyme and the inhibitor scaffold, favoring religation of the scissile peptide bond. This mode of action, dubbed the clogged gutter mechanism, leads to an equilibrium in which the noncovalent Michaelis complex predominates over the acyl-enzyme, and typically only the former is observed crystallographically. While attempts to detect rapid acylation in the trypsin-BPTI complex have thus far proven inconclusive (49), the alignment of the nucleophilic Ser-195 relative to the scissile bond in trypsin-BPTI structures is characteristic of the productive geometry seen in other highly reactive complexes (13, 50), suggesting a conserved mechanism of inhibition.

While the mesotrypsin-BPTI structure was obtained using a mutant enzyme with Ala substituted for Ser-195, precluding direct examination of the nucleophilic attack trajectory in this complex, superposition of mesotrypsin-BPTI with bovine trypsin-BPTI structure 2FTL (49) indicated that atomic positions for the Ser/Ala-195 C alpha and C beta superposed within 0.05 Å, allowing us to model the expected orientation of Ser-195 in the mesotrypsin-BPTI complex (Fig. 5). Based on our model, the nucleophilic attack trajectory in mesotrypsin-BPTI is similar to that in bovine trypsin-BPTI and other canonical inhibitor complexes, and we detect no evidence for unique structural features likely to affect the acylation reaction. We do, however, expect the uniquely unfavorable interactions between mesotrypsin and the prime-side BPTI residues 16–19 to affect the deacylation reaction. We hypothesize that, upon acyl-enzyme formation, these prime-side residues of BPTI (the “leaving group” in the acylation reaction) will dissociate from the enzyme active site much more rapidly than in human or bovine cationic trypsin-BPTI complexes, as a result of (a) steric and electrostatic repulsion between Arg-193 and BPTI Arg-17, and (b) fewer stabilizing H-bonds at the prime-side interface. Expulsion of...
the leaving group will prevent inhibitor religation and allow access to water, accelerating deacylation.

**Insights into Mesotrypsin Interaction with Other Macromolecular Inhibitors and Substrates**—Here we have presented the first structure of mesotrypsin in complex with a polypeptide inhibitor, revealing that complex formation requires the Arg-193 residue to become more highly ordered in a new, buried position. This requirement may be common to mesotrypsin interaction with other polypeptide inhibitors. In the mesotrypsin-BPTI structure, electrostatic repulsion between Arg-193 and the $P_2'$ Arg-17 residue of BPTI appears to play a key role in reducing affinity and accelerating catalysis; however, mesotrypsin resistance to inhibition has also been observed with inhibitors featuring uncharged residues at the $P_2'$ position, such as Alzheimer precursor protein inhibitor APPi (with $P_2'$ Met) (18) and the human pancreatic trypsin inhibitor SPINK1 (with $P_2'$ Tyr) (14); SPINK1 is also cleaved by mesotrypsin at an accelerated rate (14). This suggests that bulky, uncharged $P_2'$ residues can also have a deleterious effect on binding and a rate-promoting effect on catalysis, perhaps through a similar mechanism to that described here, but deriving primarily from steric interactions. It also appears that there may be substantial variation in rates of catalytic cleavage of different canonical inhibitors by mesotrypsin, resulting from factors other than the chemical nature of the $P_2'$ residue. Comparing our results with BPTI to those of Szmola et al. (14) with the soybean trypsin inhibitor SBTI (with $P_2'$ Arg), cleavage of BPTI appears to proceed substantially more slowly. Kinetic differences may arise from differences between the inhibitor scaffolds, or from sequence differences at other binding loop positions and resulting differences in the structures or dynamics of the enzyme-inhibitor interfaces. The relative contributions of steric and electrostatic factors and the impact of inhibitor scaffold and reactive site primary sequence in cleavage of inhibitors by mesotrypsin remain to be addressed in future structure-function studies.

Because BPTI mimics a substrate, our findings also provide insight into mesotrypsin interaction with macromolecular substrates. The activity of mesotrypsin on short synthetic substrates lacking prime-side interactions is similar to that of other trypsins (14, 18), although subtle thermodynamic differences have been observed with the substrate analogue 4-methylumbelliferyl 4-guanidinobenzoate (51). However, cleavage of classically physiological trypsin substrates, such as pancreaticzymogens, is compromised or abrogated (14), indicating that mesotrypsin possesses enhanced substrate specificity. Mesotrypsin has been shown to display a restricted specificity for the substrate $P_1$ position, preferring small polar side chains (52). Trypsin-sensitive sites in proteins tend to be relatively flexible loops, and proteolysis requires that these sites undergo conformational reorganization to resemble the “ideal” trypsin-binding conformation of the canonical inhibitor loop (53). However, there is substantial entropic cost associated with this reorganization, resulting in substrate $K_m$ values in the micromolar range, as contrasted with canonical inhibitor dissociation constants in the low-nanomolar to sub-picomolar range. For mesotrypsin, with conformational change also required of the enzyme, we speculate that the additive energetic costs associated with binding an unstructured substrate may be too great, and the resultant affinity too low, for efficient catalysis; a more rigid, preconfigured substrate may be required to induce the necessary conformational changes in the residues surrounding Arg-193. This would be consistent with the proposal that mesotrypsin specifically targets canonical inhibitors (such as SPINK1) as substrates in vivo (21). Alternatively, a flexible substrate of ideal prime-side primary sequence may be able to adopt a conformation that is both complementary to the mesotrypsin active site and catalytically productive, alleviating the need for a conformational change in the enzyme. Further structural and kinetic studies, exploring a range of physiological inhibitors and substrates, are needed to clarify these possibilities.

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Structures and Kinetics of Human Trypsin-BPTI Complexes


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