Disulfide engineering of human Kunitz-type serine protease inhibitors enhances proteolytic stability and target affinity toward mesotrypsin

Serine protease inhibitors of the Kunitz-bovine pancreatic trypsin inhibitor (BPTI) family are ubiquitous biological regulators of proteolysis. These small proteins are resistant to proteolysis, but can be slowly cleaved within the protease-binding loop by target proteases, thereby compromising their activity. For the human protease mesotrypsin, this cleavage is especially rapid. Here, we aimed to stabilize the Kunitz domain structure against proteolysis through disulfide engineering. Substitution within the Kunitz inhibitor domain of the amyloid precursor protein (APPi) that incorporated a new disulfide bond between residues 17 and 34 reduced proteolysis by mesotrypsin 74-fold. Similar disulfide engineering of tissue factor pathway inhibitor-1 Kunitz domain 1 (KDI1-TFFI) and bikunin Kunitz domain 2 (KDI2-bikunin) likewise stabilized these inhibitors against mesotrypsin proteolysis 17- and 6.6-fold, respectively. Crystal structures of disulfide-engineered APPi and KDI1-TFFI variants in a complex with mesotrypsin at 1.5 and 2.0 Å resolution, respectively, confirmed the formation of well-ordered disulfide bonds positioned to stabilize the binding loop. Long all-atom molecular dynamics simulations of disulfide-engineered Kunitz domains and their complexes with mesotrypsin revealed conformational stabilization of the primed side of the inhibitor-binding loop by the engineered disulfide, along with global suppression of conformational dynamics in the Kunitz domain. Our findings suggest that the Cys-17–Cys-34 disulfide slows proteolysis by dampening conformational fluctuations in the binding loop and minimizing motion at the enzyme–inhibitor interface. The generalizable approach developed here for the stabilization against proteolysis of Kunitz domains, which can serve as important scaffolds for therapeutics, may thus find applications in drug development.

Intramolecular disulfide bonds in proteins are covalent cross-links formed between two Cys residues within the macromolecule. The formation of such disulfide bonds, a common post-translational modification of secreted proteins, promotes thermal stability, correct folding, and biological activity (1–3). Disulfide bonds also contribute to maintaining protein integrity, because by stabilizing the protein structure and preventing unfolding, they also reduce the susceptibility of the protein to enzymatic degradation by proteases (4). Consequently, protein disulfide engineering, namely the introduction of non-native disulfide bonds to enhance protein function and robustness, has been explored as a means to improve the stability and half-life of protein therapeutics and to expand the operational range of enzymes for many industrial applications (5).

A functional class of proteins that are naturally highly enriched in disulfide bonds are the canonical inhibitors of serine proteases (6), which belong to a variety of convergently evolved protein families (6, 7). In these proteins, nature has taken full advantage of the stabilizing potential of disulfide linkages to produce molecules that are both highly stable to proteolysis and thermodynamically stable to unfolding processes. Common to these inhibitors is a protease-binding loop of highly characteristic “canonical” backbone conformation (8), which, in most cases, is encompassed within at least one disulfide-bridged loop or knot in the protein structure (9). They inhibit target proteases via the Laskowski mechanism (6, 9) by “poking” as ideal substrates, i.e. by binding extremely tightly with a specific reactive site peptide bond positioned for cleavage by the target enzyme and yet undergoing proteolysis many...
orders of magnitude more slowly than a typical substrate. An additional feature of the Laskowski mechanism is that cleavage of the reactive site bond is reversible; the cleaved inhibitor, held together by disulfide bonds and other intramolecular interactions, can rebind to the protease and undergo peptide bond resynthesis (6, 9). In vitro, the cleavage-resynthesis competition may eventually achieve thermodynamic equilibrium, and thus the interconvertible inhibitor forms may be considered equivalent thermodynamic inhibitors. However, such equilibration is often much slower than experimental or biological time scales, and the poorer inhibition kinetics of the cleaved inhibitor (9) can compromise its inhibitory activity for practical purposes. Furthermore, cleavage at the reactive site can render the inhibitor susceptible to inactivating cleavages at other sites by the same protease, and in biological settings it may lead to degradation by alternative proteases (10).

A well-studied family of such canonical inhibitors comprises the Kunitz-BPTI4 (MEROPS family I2 (11)) inhibitors, which are highly represented in humans and most other animal species. Kunitz-BPTI inhibitors are compact 58-residue globular protein domains that are highly stabilized by the presence of three native disulfide bonds. Despite the impressive resistance of Kunitz domains to proteolysis, they do undergo slow cleavage by target serine proteases at the reactive site bond, resulting in diminished inhibitory capability. For example, the Kunitz protease inhibitor domain of amyloid precursor protein (APPI) inhibits target proteases, such as trypsin and factor Xla, >100-fold more weakly after its cleavage at the reactive site bond (12). Furthermore, a subset of serine proteases, most notably among them human mesotrypsin, has evolved enhanced capability for proteolysis and inactivation of these inhibitors (12–17). In a previous study that identified structural and dynamic determinants of resistance versus susceptibility of different Kunitz domain inhibitors to mesotrypsin cleavage, we found that proteolysis rates correlate closely with the conformational dynamics of the inhibitors (18). We also found that a variant of APPI, evolved through directed evolution for improved affinity and resistance to mesotrypsin proteolysis, exhibited subtle backbone shifts, resulting in improved intramolecular and intermolecular stabilizing interactions (19), and hence reduced amplitude of conformational fluctuations, with consequent stabilization against proteolysis (18).

Our recent study thus illustrated the important role of substrate dynamics in proteolysis, revealing that even a tightly structured Kunitz domain with three disulfide bonds can undergo extensive acrobatic contortions, involving partial unfolding, as a potentially rate-limiting step in the process of proteolysis (18). On the basis of these observations, we hypothesized that it may be possible to further stabilize the Kunitz domain structure against proteolysis by the introduction of a fourth disulfide bond. In this study, we demonstrate that a novel engineered disulfide bond, placed so as to rigidify the canonical binding loop, can improve both affinity and proteolytic stability toward serine protease targets, such as mesotrypsin. Here, we incorporated an additional disulfide bond into the Kunitz inhibitors, APPI, tissue factor pathway inhibitor-1 Kunitz domain 1 (KD1TFPI1), and bikunin Kunitz domain 2 (KD2bikunin), and found that the mutant proteins exhibited reduced proteolysis by mesotrypsin. By solving the crystal structures of APPI and KD1TFPI1 variants in a complex with mesotrypsin, we demonstrated that the new engineered disulfide bonds are well-structured and positioned appropriately to stabilize the binding loop. In addition, molecular dynamics (MD) simulations based on our crystal structures provided support for a suppressive effect of the engineered disulfide bonds on the dynamics of the binding loops of APPI and KD1TFPI1 variants, thereby explaining their enhanced resistance to proteolysis. This type of disulfide engineering may be used to improve many Kunitz domain scaffolds that are currently used in approved and developmental protein therapeutics targeting serine proteases of pharmacological interest, thereby highlighting the potential value of this approach for biopharmaceutical applications.

**Results**

*Introduction of an additional novel disulfide bond into the APPI Kunitz domain improves stability against proteolysis by mesotrypsin*

Based on our previous work in which we showed that the susceptibility to proteolysis of different Kunitz domains depends on the magnitude of their conformational fluctuations (18), we hypothesized here that proteolysis can be intentionally slowed by design, i.e. through the introduction of an additional disulfide bond to reduce conformational mobility. Examination of the structures of APPI and APPI(M17G/I18F/F34V), a triple variant previously optimized to enhance affinity and proteolytic stability toward mesotrypsin (19), suggested several possible residues for mutation. We focused initially on residue 34, an important residue within the inhibitor scaffold that lies below the binding loop (Fig. 1A). In the APPI(M17G/I18F/F34V) variant, the F34V mutation optimizes intramolecular packing, resulting in an H-bond between the Val-34 carbonyl and the Thr-11 side chain and a closer packing arrangement between this side chain and binding loop residue Gly-17 (Fig. 1A) (19). We reasoned that these contacts, which potentially contribute to the increased conformational stability and proteolytic resistance of APPI(M17G/I18F/F34V), could be further strengthened by engineering them to be covalent disulfide bonds (Fig. 1, B and C).

To test this hypothesis, we used site-directed mutagenesis to generate two new mutant constructs based on APPI(M17G/I18F/F34V) one incorporating a pair of Cys residues at positions 11 and 34 (APPI(T11C/M17G/I18F/F34C)), and the other incorporating a pair of Cys residues at positions 17 and 34 (APPI(M17C/I18F/F34C)). Notably, the Ca separations of the residue pairs targeted for mutagenesis (5.9 and 5.5 Å for residues 11–34 and 17–34, respectively) lie well within the range typical of natural disulfide

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4 The abbreviations used are: BPTI, bovine pancreatic trypsin inhibitor; APPI, amyloid precursor protein Kunitz protease inhibitor domain; KD1TFPI1, tissue factor pathway inhibitor-1 Kunitz domain 1; KD2bikunin, bikunin Kunitz domain 2; MD, molecular dynamics; RMSD, root-mean-square deviation; RMSF, residue root-mean-square fluctuation; BisTris, 2-(bis(2-hydroxyethyl) amino)-2-(hydroxymethyl)propane-1,3-diol; pNa, p-nitroanilinium; Z, benzylloxycarbonyl; BOC, t-butoxycarbonyl; AMC, aminomethylcoumarin; HAE, hereditary angioedema.
binding loop and stabilize it in a conformation with a less compatible fit to the enzyme. This explanation is also consistent with the much faster dissociation rate $k_{off}$ measured for APPIT11C/M17G/I18F/F34C relative to other APPI variants (Fig. 2C and Table 1).

In contrast, the APPIM17G/I18F/F34V variant retained the extremely potent inhibitory activity of the parental APPIM17G/I18F/F34V variant toward mesotrypsin and even showed modest improvement. Affinity of this variant toward mesotrypsin exceeded the practical limit of determination using the classical competitive inhibition treatment, and we therefore used an alternative kinetic treatment suitable for the quantification of slow, tight-binding behavior (Fig. 2, D–F). The $K_i$ value of 61 nM so obtained was ~1.5-fold stronger than that of the parental APPIM17G/I18F/F34V variant measured by the same procedure (Table 1). The improved inhibition relative to APPIM17G/I18F/F34V appears to be driven by a faster $k_{cat}$ (Table 1), suggesting that the presence of the Cys-17–Cys-34 disulfide bond may stabilize the binding loop in a conformation better pre-configured for association with the enzyme. Compared with WT APPI (APPIWT) or with APPIM18F, a single mutant that differs from APPIM17C/I18F/F34C only at the two introduced Cys positions, APPIM17C/I18F/F34C showed improved inhibition by >2000-fold or >50-fold, respectively (Table 1).

We then went on to determine whether incorporation of engineered disulfide bonds into the APPIT11C/M17G/I18F/F34C and APPIM17C/I18F/F34C variants had indeed succeeded in conferring improved resistance to proteolysis by mesotrypsin. To test the proteolytic stability of the inhibitors, we measured their rates of cleavage ($k_{cat}$) by mesotrypsin in an HPLC-based time-course hydrolysis assay (Fig. 3). APPIWT was previously shown to undergo cleavage by mesotrypsin specifically at the Arg-15–Ala-16 reactive site bond (12), and because the disulfide mutant inhibitors bind and inhibit mesotrypsin even more strongly than does APPIWT, the same bond is expected to be targeted for cleavage in the mutants. APPIT11C/M17G/I18F/F34C was ~12-fold more susceptible to mesotrypsin proteolysis than the parental APPIM17G/I18F/F34V variant, although still ~7-fold more resistant than APPIWT (Table 1). In contrast, the APPIM17C/I18F/F34C variant displayed a mesotrypsin cleavage rate that was 250-fold slower than that of APPIWT, 74-fold slower than that of APPI18F, and 3-fold slower than that of the parental APPIM17G/I18F/F34V variant, which had previously been optimized for resistance to proteolysis using a directed evolution strategy (Table 1) (19). Thus, introduction of a disulfide linkage between residues 17 and 34 of APPI appears to be an effective approach for stabilization against proteolysis by mesotrypsin, while also maintaining and enhancing binding affinity toward mesotrypsin.

**Disulfide bridging between residues 17 and 34 offers a general approach to stabilize Kunitz domain serine protease inhibitors against proteolysis**

Several human Kunitz protease inhibitor domains have been studied as promising scaffolds for drug development, yet stability against proteolysis in vivo remains a concern, as it does for therapeutic proteins in general. We hypothesized that incorporation of an engineered disulfide bond between residues 17 and...
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Figure 2. Disulfide bond engineering preserves the mesotrypsin inhibitory capability of APPI variants. Representative examples are shown for determination of kinetic constants for mesotrypsin inhibition by APPI disulfide variants. A and B, Kinetic profiles fitting the competitive model of inhibition. Concentrations of APPI variants are given at the top of each plot; concentration of mesotrypsin was 0.25 μM. A, data were fitted globally to the competitive inhibition equation to obtain the equilibrium constant $K_i$, $B$, Lineweaver-Burk transform of the data shown in A converges on the y axis, as is characteristic of the competitive model of inhibition. C, progress curve is shown for the recovery of mesotrypsin activity due to dissociation from APPIT11C/M17G/I18F/F34C upon 60-fold dilution into assay buffer containing 150 μM Z-GPR-pNA substrate. Experimental values are shown in black, and the fitted curve is shown as a dashed red line. Curve fitting was performed as described under "Experimental procedures" to determine first-order rate constant $k_{cat}$, which describes the transition from complete inhibition to a steady-state rate of partial inhibition. D–F, Slow, tight-binding inhibition of mesotrypsin by APPIT11C/M17G/I18F/F34C. Experimental values are shown in black and fitted curves, as dashed red lines, with APPIT11C/M17G/I18F/F34C concentrations indicated on the right. $V_0$ and $V_\infty$ are the steady-state rates in the presence and absence of inhibitor, and $K_{obs}$ is the observed first-order rate constant, which describes the transition from $V_0$ to $V_\infty$ from which the kinetic constants were calculated as described under "Experimental procedures." E, Values of the equilibrium inhibition constant $K_i$ were calculated from the steady-state portion of the progress curves from D as described under "Experimental procedures." F, Plot showing the linear dependence of $K_{obs}$ on APPIT11C/M17G/I18F/F34C concentration facilitated the calculation of $k_{on}$ and $k_{off}$ as described under "Experimental procedures."

### Table 1

Kinetic constants of the reactions between mesotrypsin and different Kunitz type inhibitors

| Inhibitor          | $K_i$ (fold decrease) | $k_{on}$ $s^{-1}$ | $k_{cat}$ $s^{-1}$ | $K_{cat}$ $\mu M$ |
|--------------------|-----------------------|------------------|------------------|-----------------|
| APPIT11C/M17G/I18F/F34C | (9.8 ± 0.1) $\times$ $10^{-11}$ | 34 $^b$ | (5.0 ± 0.1) $\times$ $10^{8}$ | (6.6 ± 0.1) $\times$ $10^{-3}$ |
| KD1TFPI1/K15R     | (6.1 ± 0.1) $\times$ $10^{-11}$ | 54 $^b$ | (6.0 ± 0.4) $\times$ $10^{4}$ | (5.2 ± 0.1) $\times$ $10^{-3}$ |
| KD2bikunin        | (4.5 ± 0.1) $\times$ $10^{-10}$ | 113 $^c$ | (5.1 ± 0.1) $\times$ $10^{-3}$ | (6.6 ± 0.4) $\times$ $10^{-3}$ |
| KD1TFPI1/K15R/I17C/I34C | (2.0 ± 0.1) $\times$ $10^{-7}$ | 215 $^c$ | (8.9 ± 0.1) $\times$ $10^{-1}$ | (6.6 ± 0.4) $\times$ $10^{-3}$ |
| KD2bikunin/F17C/P34C | (5.1 ± 0.1) $\times$ $10^{-10}$ | 17 $^c$ | (5.1 ± 0.1) $\times$ $10^{-3}$ | (6.6 ± 0.4) $\times$ $10^{-3}$ |

$^a$ $k_{cat}$ was determined by HPLC-based inhibitor cleavage time course.

$^b$ Data were determined using Equation 1 (see "Experimental procedures").

$^c$ Results were taken from Ref. 19.

$^d$ Data were determined using Equation 2 (see "Experimental procedures").

$^e$ Results were taken from Equation 3 (see "Experimental procedures").

$^f$ Data were determined using Equation 7 (see "Experimental procedures").

$^g$ Fold decrease relative to the parent protein scaffolds APPIT11C/KD1TFPI1/K15R, or KD2bikunin.

$^h$ Data were determined using Equation 8 (see "Experimental procedures").

34 into other Kunitz domain scaffolds of pharmaceutical interest might offer a general method for stabilization against proteolysis. To test this idea, we expressed and purified new disulfide-containing and WT proteins of two additional representative Kunitz domains, namely KD1TFPI1 and KD2bikunin. For KD1TFPI1, a particularly weak inhibitor of mesotrypsin (17), we also introduced a K15R mutation at the P1 primary specificity site in an attempt to strengthen its preexisting affinity for mesotrypsin, as the enzyme is known to prefer Arg over Lys at this position (21). We then evaluated the equilibrium inhibition constants ($K_i$) for mesotrypsin and rates of proteolysis ($k_{cat}$) by mesotrypsin, using approaches similar to those employed for the APPI variants above. Consistent with our hypothesis, both Kunitz domains were considerably stabilized against proteolysis by mesotrypsin upon incorporation of the X17C/X34C disulfide mutations (Table 1). Intriguingly, for the weak inhibitor KD1TFPI1 (K15R), the engineered disulfide also resulted in greatly enhanced $K_i$ of KD1TFPI1 (K15R) for mesotrypsin.
observed initial rate of hydrolysis averaged over at least three independent runs. APPIT\textsubscript{11C/M17G/I18F/F34C} was quantified by integration of HPLC peaks over the time course. Note the higher ratio of mesotrypsin to APPIM17C/I18F/F34C hydrolysis by mesotrypsin in a reaction containing 50 μM APPIT\textsubscript{11C/M17G/I18F/F34C} and 200 nM enzyme. Disappearance of intact APPIT\textsubscript{11C/M17G/I18F/F34C} was quantified by integration of HPLC peaks over the time course. B, time course showing APPIM17C/I18F/F34C hydrolysis by mesotrypsin in a reaction containing 50 μM APPIM17C/I18F/F34C and 7.14 μM enzyme. Disappearance of intact APPIM17C/I18F/F34C was quantified by integration of HPLC peaks over the time course. Note the higher ratio of mesotrypsin to APPIM17C/I18F/F34C required for cleavage of this more proteolytically-resistant variant on a similar time scale. For each variant, \( k_{cat} \) was calculated from the observed initial rate of hydrolysis averaged over at least three independent experiments.

whereas affinity of KD\textsubscript{2}bikunin for mesotrypsin was barely affected by the engineered disulfide (Table 1).

**Crystal structures of mesotrypsin bound to APPI and TFPI disulfide variants reveal a well-ordered engineered disulfide bond positioned to stabilize the binding loop**

To gain insight into the impact of the engineered Cys-17–Cys-34 disulfide bond on the structure and function of the Kunitz domain, we co-crystallized both APPI\textsubscript{M17C/I18F/F34C} and KD\textsubscript{1}TFPI\textsubscript{K15R/I17C/I34C} with the catalytically inactive mesotrypsin-S195A mutant and solved the crystal structures of these complexes. X-ray data collection and refinement statistics are summarized in Table 2. The mesotrypsin/APPI\textsubscript{M17C/I18F/F34C} complex crystallized in space group P21 with one copy of the inhibited complex in the asymmetric unit. The structure was refined against data diffracting to 2.0 Å resolution. This structure represents the first reported structure for KD\textsubscript{1}TFPI\textsubscript{1} bound to a serine protease. As has been found for many protease complexes with other Kunitz inhibitor domains, the inhibitor-binding loop assumed a canonical conformation (6, 8), with residues Pro-13–Met-18 occupying the enzyme S3–S3′ subsites in a substrate-like manner (Fig. 4C). Similarly to the APPI\textsubscript{M17C/I18F/F34C} structure, the electron density for KD\textsubscript{1}TFPI\textsubscript{K15R/I17C/I34C} unambiguously confirmed the formation of a well-ordered engineered disulfide bond between residues Cys-17 and Cys-34 (Fig. 4C, inset). Superposition of the Kunitz domains from the mesotrypsin/APPI\textsubscript{M17C/I18F/F34C} and mesotrypsin/KD\textsubscript{1}TFPI\textsubscript{K15R/I17C/I34C} structures revealed that the engineered disulfide bonds had adopted very closely similar conformations in the two inhibitors (Fig. 4D). The highly similar structural impact of Cys-17–Cys-34 disulfide engineering on these two distantly related Kunitz domains (possessing only 36% sequence identity) is consistent with the idea that disulfide bridging between residues 17 and 34 may offer an engineering approach that is generally applicable to Kunitz domains.

**Molecular dynamics simulations reveal stabilization of the binding loop conformation by the engineered disulfide bond**

In earlier work, we showed that proteolysis rates of different Kunitz domains by mesotrypsin correlate closely with the global conformational dynamics of the inhibitors on the nanosecond–microsecond time scale (18). Based on these prior observations, we hypothesized here that the Cys-17–Cys-34 disulfide bond may stabilize Kunitz domains against proteolysis by reducing the flexibility and dampening the conformational fluctuations of the Kunitz domain fold. To explore this idea, we conducted a series of free all-atom MD simulations, based upon our crystal structures, in which we compared the conformational fluctuations undergone by APPI\textsubscript{M17C/I18F/F34C} and their complexes with mesotrypsin, versus APPI\textsubscript{M17C/I18F/F34C} (the parental Kunitz
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Table 2

X-ray data collection and refinement statistics

| Structure name | Mesotrypsin/APPIM17C/I18F/F34C | Mesotrypsin/KD1TFPI1K15R/I17C/I34C |
|---------------|--------------------------------|---------------------------------|
| PDB code      | 6HAR                          | 6BX8                           |
| Data collection |                               |                                |
| Resolution range (Å) | 46.47–1.50                | 60.06–1.98                      |
| Space group   | P21                           | P1                             |
| α, β, γ (°)   | 34.09, 82.78, 46.56           | 35.14, 87.20, 90.84            |
| Total/unique reflections | 78,755/40,015 | 116,262/70,450 |
| R-merge       | 0.048 (0.027)                 | 0.067 (0.249)                  |
| R-meas        | 0.068 (0.887)                 | 0.095 (0.352)                  |
| R-pim         | 0.057 (0.771)                 | 0.0674 (0.249)                 |
| CC1/2         | 0.997 (0.492)                 | 0.971 (0.887)                  |
| Multiplicity  | 4.3 (3.4)                     | 1.7 (1.7)                      |
| Completeness (%) | 96.8 (87.2)                  | 93.0 (92.2)                    |
| Mean I/σ(I)   | 7.3 (1.0)                     | 6.5 (2.5)                      |

Refinement

| Reflections used in refinement | 40,008 (3687) | 69,456 (6906) |
| Reflections used for R-free   | 2000 (172)    | 3375 (333)    |
| R-work/R-free                 | 0.169/0.193   | 0.192/0.221   |
| No. of non-hydrogen atoms     | 2437          | 9070           |
| Macromolecules                | 2182          | 8229           |
| Ligands                       | 5             | 20             |
| Solvent                       | 250           | 821            |
| Protein residues              | 278           | 1113           |
| RMSD bonds (Å)                | 0.005         | 0.019          |
| RMSD angles (°)               | 0.79          | 1.81           |
| Ramachandran favored (%)      | 98.54         | 97.72          |
| Ramachandran allowed (%)      | 1.46          | 2.28           |
| Ramachandran outliers (%)     | 0.00          | 0.00           |
| Average B-factor              | 19.93         | 30.91          |
| Macromolecules                | 19.10         | 29.84          |
| Ligands                       | 28.89         | 50.53          |
| Solvent                       | 27.03         | 41.1           |

* Numbers in parentheses indicate statistics for the highest resolution shell.

Domains lacking the engineered disulfide bond, and their complexes with mesotrypsin. Because we lack experimentally determined crystal structures for KD1TFPI1K15R and for its complex with mesotrypsin, the starting point for these simulations was based on a model derived from in silico back-mutation of the KD1TFPI1K15R/I17C/I34C structure, followed by energy minimization. After energy minimization and equilibration protocols, each Kunitz domain or complex simulation was run for >1000 ns, along with several shorter replicates to assess convergence. Subsequently, we evaluated differences in conformational dynamics between the Kunitz domains and their complexes by aligning MD simulation frames on backbone atoms and comparing root-mean-square deviation (RMSD) plots of backbone atom deviation from average positions for the Kunitz domain residues over the course of the simulations. We also examined per residue root-mean-square fluctuation (RMSF) plots that reveal which residues within the Kunitz domain are most impacted by the introduction of the engineered disulfide bond.

In simulations modeling the dynamics of free, unbound APPIM17C/I18F/F34C versus APPIM17G/I18F/F34V, RMSD plots did not reveal striking global differences in dynamics (Fig. 5A), possibly because the parental APPIM17G/I18F/F34V variant had already been engineered with mutations that broadly dampen global dynamics (18). However, RMSF comparisons did show a suppression of conformational dynamics in a few focused regions of APPIM17C/I18F/F34C relative to APPIM17G/I18F/F34V, most notably in residues 15–18 of the binding loop surrounding the G17C mutation (Fig. 5, B and C). Simulations of KD1TFPI1K15R/I17C/I34C versus KD1TFPI1K15R showed a modest suppressive effect of the engineered disulfide bond on the global dynamics, as illustrated by RMSD plots (Fig. 5D), and a more pronounced effect specifically dampening the motion of the apparent hotspot of dynamics surrounding Ile-17 of KD1TFPI1K15R (Fig. 5, E and F).

In further analyses of these simulations focusing specifically on alignments of the P3–P3’ residues 13–18, we found clear evidence of reduced dynamics throughout the binding loop of APPIM17C/I18F/F34C compared with APPIM17G/I18F/F34V (Fig. 6, A and B). In APPIM17G/I18F/F34V, the Gly-17 backbone fluctuates between different conformations, resulting in a considerable RMSF peak (Fig. 6, B and C). In contrast, this peak was completely eliminated in APPIM17C/I18F/F34C, as the protein backbone in this region assumed a single, more rigid conformation (Fig. 6, B and D). The suppressive effect of the engineered disulfide bond on the dynamics of the binding loop can also be seen in plots of the backbone φ and ψ angles sampled by binding loop residues: Ramachandran plots for residues 17 and 18, in particular, showed tighter clustering for APPIM17C/I18F/F34C compared with APPIM17G/I18F/F34V (Fig. 6, E and F). Similar observations were obtained for the KD1TFPI1 scaffold simulations: whereas the binding loop of KD1TFPI1K15R samples a variety of conformations, mobility throughout the binding loop was markedly suppressed in KD1TFPI1K15R/I17C/I34C (Fig. 7, A–D). Ramachandran plots revealed multiple populations of conformers at each binding loop residue of KD1TFPI1K15R, whereas throughout the simulations of KD1TFPI1K15R/I17C/I34C each residue remained better stabilized in a single major backbone conformation (Fig. 7, E and F). The stabilization of the engineered disulfide variants with a preconfigured binding loop...
conformation favorable for binding can help to explain the faster $k_{\text{on}}$ and improved $K_i$ values for these inhibitors toward protease targets.

All-atom MD simulations of Kunitz domain complexes with mesotrypsin likewise corroborate the role of the engineered disulfide bond in suppressing binding loop dynamics, although, as expected, the magnitude of the effect was somewhat reduced, because contact of the binding loop with mesotrypsin also restricted the conformations of these contact residues. When frames from MD simulations of mesotrypsin/APP1$\text{M}_{17/C_{118/F_{34}C}}$ complexes were aligned on the backbone atoms of the Kunitz domain alone, the RMSF peak for residues 16–19, evident in APP1$\text{M}_{17/C_{118/F_{34}C}}$ ($\text{G}17$/$\text{V}34$), was eliminated in APP1$\text{M}_{17/C_{118/F_{34}C}}$ ($\text{G}17$/$\text{V}34$) ($\text{Fig. S1}$). The region of suppressed dynamics revealed by parallel analyses of mesotrypsin/APP1$\text{K}_{15/R}$ complexes was more extensive, spanning binding loop residues 13–19 ($\text{Fig. S2}$). When frames from the simulations of mesotrypsin/Kunitz domain complexes were instead aligned on the backbone atoms of all protein residues, the enzyme chains aligned well, whereas the Kunitz domains displayed substantial displacements in the $\beta$-turn and $\alpha$-helical regions distal to the enzyme interface ($\text{Figs. S3 and S4}$). These displacements resulted from a rocking motion of the Kunitz domain relative to the enzyme, as described in our previous work, where we reported the amplitude of such intermolecular motions to correlate with proteolytic susceptibility (18). Here, we found that introduction of the engineered disulfide bond into APP1$\text{M}_{17/C_{118/F_{34}C}}$ subtly reduced the displacements of the $\alpha$-helix compared with those observed in the mesotrypsin/APP1$\text{M}_{17/C_{118/F_{34}C}}$ complex ($\text{Fig. S3}$); notably, APP1$\text{M}_{17/G}$ had already exhibited considerably suppressed global dynamics relative to APP1$\text{WT}$ due to stabilizing mutations (18). The suppressive effect of the engineered disulfide bond on Kunitz domain displacement was far more striking in MD simulations of mesotrypsin/APP1$\text{K}_{15/R}$ and mesotrypsin/APP1$\text{K}_{15/R}$ ($\text{Fig. S4}$). In summary, our MD simulations of both unbound Kunitz domains and complexes with mesotrypsin are consistent with the hypothesis that substantial conformational stabilization, conferred by the engineered Cys-17–Cys-34 disulfide bond, suppresses both local dynamics of the binding loop and global dynamics of the inhibitor within the complex, resulting in increased stabilization against proteolysis.

**Discussion**

By virtue of their three native disulfide bonds, the folds of Kunitz serine protease inhibitors are inherently highly stab-
lized against unfolding. Nonetheless, as a consequence of their mechanism of action for inhibition of serine proteases, these inhibitors remain susceptible to slow cleavage of a reactive site bond within the protease-binding loop, which diminishes their efficacy for inhibiting target enzymes (12, 15). Here, we showed that incorporation of an engineered disulfide bond between residues 17 and 34 in the inhibitor-binding loop represents a generalizable strategy for stabilization of Kunitz domain inhibitors against proteolysis by target serine proteases without impairing the inhibitors’ inhibitory potency or affinity for the proteases.

A rigorous validation of this idea may be drawn from the improved stability of the disulfide-engineered inhibitors against proteolysis by mesotrypsin, as this enzyme has a remarkable capacity for cleavage and inactivation of human Kunitz domain inhibitors. These properties of mesotrypsin are the result of multiple evolutionary mutations, the most critical being the substitution of Gly-193 by Arg, which clashes sterically with the inhibitors and weakens affinity of the complexes (14, 16). Additional mutations that contribute to mesotrypsin’s proteolytic capability are the substitution of Tyr-39 by Ser, Lys-97 by Asp, and Glu-74 by Lys, which alter interactions at the molecular interface within mesotrypsin/inhibitor complexes, eliminating hydrogen bonds and enhancing mobility (15, 16). These mutations are thus responsible for the unusually low affinity of mesotrypsin (relative to typical trypsins) for polypeptide trypsin inhibitors, and they also contribute to the unique ability of mesotrypsin to cleave several canonical trypsin inhibitors as substrates (13, 17, 21).

Our results show that introduction of a disulfide linkage between residues 17 and 34 of APPI, i.e., in the APPI_{M17C/I18F/F34C} variant, is an effective approach for stabilization against proteolysis by mesotrypsin. In particular, APPI_{M17C/I18F/F34C} displays a mesotrypsin cleavage rate that is 250-fold slower than that of APPI_{WT}, which makes it the most resistant APPI variant yet reported. We found similar disulfide bond placement to likewise stabilize the Kunitz domain inhibitors KD1TFPI and KD2bikunin against mesotrypsin proteolysis,

### Figure 5. Molecular dynamics simulations reveal suppressed dynamics in Kunitz domains with engineered disulfide bonds.

A, global dynamics of unbound APPI_{M17G/I18F/F34V} and APPI_{M17C/I18F/F34C}, compared according to backbone atom (NCuC) average RMSD from MD simulations, show only modest differences in amplitude. B, magnitudes of positional fluctuations per residue of APPI variants over the length of the MD simulation are plotted as CO average RMSF. C, positional RMSF amplitudes from B are heat-mapped onto the protein structures in the blue-white-red spectrum (scale bar shown). Note the peak of elevated mobility spanning binding loop residues 15–18 of APPI_{M17G/I18F/F34V} that is absent in APPI_{M17C/I18F/F34C}. D, global dynamics of unbound KD1TFPI_{K15R} versus KD1TFPI_{K15R/I17C/I34C}, compared according to backbone atom (NCuC) average RMSD from MD simulations, show lower amplitudes of backbone atom deviation from average positions in the engineered disulfide variant. E, magnitudes of positional fluctuations per residue of KD1TFPI variants over the length of the MD simulation are plotted as CO average RMSF. F, positional RMSF amplitudes from E are heat-mapped onto the protein structures in the blue-white-red spectrum (scale bar shown). Note that several peaks of elevated mobility in KD1TFPI_{K15R}, including binding loop residues 16–19, are reduced in KD1TFPI_{K15R/I17C/I34C}.
demonstrating applicability of the strategy across the Kunitz inhibitor family.

Mesotrypsin is a pancreatic enzyme transcribed from the PRSS3 gene, and several alternative splice isoforms of PRSS3 are transcribed by tumors and other tissues (22). Although active mesotrypsin has not been directly detected in tumors, indirect evidence suggests that PRSS3-encoded trypsinogen 4 may become activated to produce mesotrypsin and contribute to malignancy, driving cellular invasion and migration. The pro-metastatic potential of mesotrypsin has been suggested, for example, in cell culture and xenograft models of pancreatic (23) and prostate cancers (24), where silencing of the PRSS3 gene by RNA interference (RNAi) markedly reduced the invasiveness and metastasis of these cancers both in vitro and in vivo. The enhancement of tumor cell migration and invasion has been linked to the specific proteolytic activity of mesotrypsin,

because invasive behavior of prostate cancer cells could be stimulated by exogenously added mesotrypsin (but not a catalytically inactive mutant) and could be blocked by either PRSS3 knockdown or treatment with an inhibitor of mesotrypsin activity (19, 24). These studies suggest that mesotrypsin could be a potential target for therapeutic intervention in cancer, and thus, stabilization of the Kunitz domain against proteolysis by mesotrypsin may be regarded as a step toward the development of potent inhibitors to inhibit the progression and metastasis of various cancers.

A number of previous studies have reported attempts to stabilize proteins for both medical and industrial applications by applying structure-based design for engineering disulfide bonds into the molecules (5, 25). For example, in their recent work, Schultz and co-workers (26) genetically encoded noncanonical amino acids that are capable of forming disulfide bonds.
that bridge longer distances than the native disulfide bonds. These longer and more flexible linkages made it much easier to crosslink sites within the molecule to produce disulfide-engineered proteins with improved thermal stability vis-à-vis their WT counterparts (26). Indeed, disulfide engineering has achieved considerable success in a wide range of applications, yet contrary to expectations, many designed disulfide bonds have resulted in decreased stability of the modified protein, probably in cases where the introduction of cysteines disrupts or removes intramolecular interactions that stabilize the native structure (5, 27). These previous studies have most often focused on protein thermodynamic stability and rates of folding and unfolding (as fold stability is an important property for therapeutic proteins and for enzymes engineered for various industrial applications). In contrast, in this study, we focused on the impact of engineered disulfides on a different property that impacts protein activity and longevity in biological settings, namely resistance to proteolytic inactivation and degradation.

Our crystal structures and molecular dynamics simulations support the interpretation that engineered disulfides can slow proteolysis by suppressing conformational dynamics. Most studies of protein dynamics in enzyme catalysis have focused on conformational changes within the enzyme and their impact on substrate/inhibitor binding, progression along the catalytic reaction coordinate, and product release (28–33). However, for the many enzymes that catalyze reactions of protein substrates, we (18) and others (34) have shown that it is necessary to look beyond the enzyme to the protein substrate as well (in this case Kunitz inhibitor domain) if we are to fully appreciate the impact of protein dynamics on enzyme-catalyzed reactions. In our previous work, we found that rates of proteolysis of different Kunitz domains by mesotrypsin correlated closely with the global and local conformational dynamics of the Kunitz domain substrates in the nanosecond to microsecond time regime. Our current MD simulations of APP1M17C/I18F/F34C and KD1TFPIK15R/I17C/I34C Kunitz domains, both unbound and...
when complexed with mesotrypsin, confirm and extend these findings and are consistent with the premise that the well-ordered engineered disulfide bond between residues Cys-17 and Cys-34 suppresses both the local dynamics of the binding loop and the global dynamics of the inhibitor within the complex, resulting in increased stabilization against proteolysis.

This novel general method for extending the biological stability and potency of Kunitz domain scaffolds may be of particular importance for the development of new and second-generation pharmaceuticals. An illustrative example is the Food and Drug Administration-approved orphan drug ecballantide (Kalbitor or DX-88); this drug was engineered on the scaffold of KD1TFPI1 to target plasma kallikrein for the treatment of hereditary angioedema (HAE) (35). Treatment with ecballantide compensates for the insufficient quantity of the natural kallikrein inhibitor C1 in HAE patients and is expected to replace conventional therapy with C1 that is isolated from human plasma (36). Another drug that is based on the KD1TFPI1 scaffold is the plasmin inhibitor DX-1000, which is in preclinical development for blocking tumor growth and metastasis in breast cancer (37). Yet another example is a drug based on the KD2bikunin scaffold that targets neutrophil elastase. Known as depelestat (DX-890), this protein is currently in clinical trials for acute respiratory distress syndrome (38) and cystic fibrosis (39).

Although the application of our approach may be successful for biopharmaceuticals and other commercial proteins, we need to bear in mind several limitations. The utility of our approach may be limited to reducing target protease cleavage at the inhibitor reactive site and may not offer protection against other types of proteolytic attack. A second limitation relates to the potential susceptibility of cysteine disulfides to intra- or intermolecular disulfide bond exchange or to several pathways of chemical degradation, thereby rendering some disulfide-containing proteins susceptible to degradation, aggregation, and precipitation (2, 40, 41). In the future, further advantage might be gained by engineering redox-stable disulfide analogs, such as has been explored in the case of sunflower trypsin inhibitor (SI2) (42), and it is anticipated that such disulfide-engineered proteins with enhanced stability could lead to the development of a new generation of biopharmaceuticals.

**Experimental procedures**

**Protein expression and purification**

Kunitz domain inhibitors (including APP1, KD1TFPI1, and KD2bikunin variants) were cloned into a pPIC9K vector, transformed, expressed in *P. pastoris* (GS115 strain), and purified by nickel-affinity chromatography, followed by size-exclusion chromatography, as described for APPI in our previous work (19). The concentrations of the Kunitz inhibitors were determined by titration with pre-titrated bovine trypsin (Sigma, Poole, UK) and the substrate benzoyl-l-arginine-p-nitroanilide (Sigma), as described previously (14). Mesotrypsin and the catalytically inactive mutant mesotrypsin-S195A were expressed recombinantly aszymogen forms in *Escherichia coli*, isolated from inclusion bodies, refolded, purified, and proteolytically activated, essentially as described previously (14, 16). Active mesotrypsin was quantified by titration with 4-nitrophenyl 4-guanidinobenzoate (Sigma) (14), and mesotrypsin-S195A was quantified by UV-visible absorbance readings at 280 nm using a calculated extinction coefficient of 41,535 M⁻¹ cm⁻¹.

For the generation of the progress curves described below, the concentration of the chromogenic substrate Z-GPR-pNA (Sigma) was determined in an end-point assay from the change in absorbance caused by the release of p-nitroaniline (ε₄₅₀ = 8480 M⁻¹ cm⁻¹). All reactions were monitored with a Synergy2 microplate spectrophotometer (BioTek).

**Progress curves for human mesotrypsin with APPI-M17C/I18F/F34C were generated as we have previously described for mesotrypsin with APPI-M17C/I18F/F34V (43). Briefly, stock solutions of enzyme, substrate, and APPI proteins were prepared at 40× the desired final concentrations. Assays were performed in 96-well microplates at 37 °C as follows: trypsin buffer (296 μM Tris-HCl, pH 8.0, 1 mM CaCl₂), Z-GPR-pNA substrate (8 μM; 6 mM concentration), and APPI (8 μM; concentrations ranging from 0 to 40 nM) were mixed and incubated at 37 °C for 10 min. Reactions were then initiated by dilution of the enzyme (8 μM; 1 nM concentration) into the pre-equilibrated mixture and were followed spectroscopically (from the increase in absorbance at 410 nm) for 1 h.

**Progress curves for mesotrypsin with APPI-WT, APPI-I18F, and APPI-T11C/M17C/I18F/F34C for independent determination of kₐff were obtained in 96-well microplates at 37 °C as follows. APPI (10 μM) and mesotrypsin (60 nM) were mixed in a 1:1 ratio and incubated at 37 °C for 1 h. The substrate (Z-GPR-pNA) was prepared in trypsin buffer at a final concentration of 150 μM and incubated at 37 °C for 10 min. Reactions were then initiated by dilution of the enzyme/inhibitor mixture (5 μL) by 60× into the pre-equilibrated substrate (295 μL) and followed spectroscopically.

**Progress curves for mesotrypsin with APPI-T11C/M17C/I18F/F34C, KD1TFPI1-K15R, KD1TFPI1-K15R/I17C/I34C, KD2bikunin, and KD2bikunin-F17C/F34C for determination of the dissociation constant Kᵢ were generated as we have previously described (19). Briefly, stock solutions of enzyme, substrate, and inhibitors were prepared at 40× the desired final concentrations. Assays were performed in 96-well microplates at 37 °C in the presence of different concentrations of Z-GPR-pNA substrate (50, 100, 150, 200, and 250 μM). The final concentrations of the inhibitors were 0, 50, 100, 200, and 400 nM for APPI-T11C/M17C/I18F/F34C (0, 11, 22, 45, and 85 μM for KD1TFPI1-K15R, 0, 0.125, 0.25, 0.5, and 1 μM for KD1TFPI1-K15R/I17C/I34C), and 0, 30, 60, 120, and 250 nM for KD2bikunin and KD2bikunin-F17C/F34C. Trypsin buffer (296 μL), substrate (8 μL), and APPI (8 μL) were equilibrated together in a 96-well microplate prior to the addition of enzyme (8 μL of 10 nM mesotrypsin). Reactions were followed spectroscopically for 5 min, and initial rates were determined from the increase in absorbance due to the release of p-nitroanilide (ε₄₅₀ = 8480 M⁻¹ cm⁻¹).
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Progress curve analysis

The dissociation constants $K_i$ for APPIT11C/M17G/I18F/F34C, KD1TFPI1K15R, KD1TFPI1K15R/I17C/I34C, and KD2bikunin in complex with mesotrypsin were determined by fitting their progress curves by multiple regression to Equation 1, the classic competitive inhibition equation (Fig. 2, A and B),

$$V = \frac{k_{cat}[E]_0[S]_0}{K_m(1 + [I]/K_i) + [S]_0} \quad \text{(Eq. 1)}$$

where $V$ is the velocity of product formation at the start of the reaction; $K_m$ (Michaelis constant) and $k_{cat}$ are the kinetic parameters for substrate hydrolysis; $[E]_0$ is the total concentration of enzyme, and $[S]_0$ and $[I]_0$ are the initial concentrations of substrate and inhibitor, respectively.

Values of the equilibrium inhibition constant ($K_i$) of mesotrypsin with APPIM17C/I18F/F34C were calculated by using Equation 2 from the steady-state portions of the progress curves (Fig. 2, D and E), as described previously (45). Equation 2 describes an equilibrium state of reversible competitive inhibition with slow, tight-binding behavior,

$$\frac{(V_0 - V_i)}{V_s} = \frac{[I]_0}{K_i(1 + [S]_0/K_m)} \quad \text{(Eq. 2)}$$

where $V_i$ and $V_0$ are the steady-state rates in the presence and absence of inhibitor (Fig. 2, D and E).

Association ($k_{on}$) and dissociation ($k_{off}$) constants for slow inhibition of mesotrypsin by APPIM17C/I18F/F34C were obtained using Equations 3–6 (46). Data from the generated curves were first globally fitted by multiple regression to Equation 3, with the integrated rate equation describing slow binding inhibition,

$$[P] = \left(\frac{V_0 - V_i}{K_{obs}}\right)(1 - e^{-k_{obs}t}) + V_s \cdot t \quad \text{(Eq. 3)}$$

where $K_{obs}$ is the observed first-order rate constant that describes the transition from $V_0$ to $V_i$ (Fig. 2D), and $[P]$ is the concentration of product formed at any time, $t$.

Slow, tight-binding inhibition can be described by two alternative general mechanisms (46). In brief, one mechanism is a two-step process involving the formation/accumulation of an initial inhibitor–enzyme complex, followed by slow kinetics to form a tighter complex. In this mechanism, $k_{on}$ and $k_{off}$ are both first-order kinetic constants that are characterized by a nonlinear relationship between $K_{obs}$ and the inhibitor concentration. In contrast, the second mechanism is a direct, single-step process in which the final complex is formed slowly. In this mechanism, $k_{on}$ and $k_{off}$ are second- and first-order kinetic constants, respectively, that are characterized by a linear dependence between $K_{obs}$ and the inhibitor concentration $[I]$, as shown in Equation 4. For the inhibition of mesotrypsin by APPIM17C/I18F/F34C, a plot of $K_{obs}$ versus inhibitor concentration displayed a linear dependence, consistent with the single-step mechanism (Fig. 2F).

$$K_{obs} = k_{off} + \frac{k_{cat}[I]}{1 + [S]_0/K_m} \quad \text{(Eq. 4)}$$

$k_{on}$ and $k_{off}$ were calculated from the linear curve generated by Equation 4 and by using the following relationships shown in Equations 5 and 6.

$$k_{on} = \text{slope} \cdot (1 + [S]_0/K_m) \quad \text{(Eq. 5)}$$

$$k_{off} = K_{obs} \cdot [I] = 0 \quad \text{(Eq. 6)}$$

Because the inhibition kinetics of mesotrypsin with APPIT11C/M17G/I18F/F34C and APPIM17C/I18F/F34C were relatively fast, estimation of $k_{off}$ from Equation 4 would be inaccurate. Therefore, $k_{off}$ was calculated independently of the inhibitor concentration by using a multiple regression curve fit to Equation 7 (Fig. 2C (46)).

$$[P] = V_s \cdot t - \frac{V_s}{k_{off}}(1 - e^{-k_{off}t}) \quad \text{(Eq. 7)}$$

The calculated association rate constant was then obtained from their measured $K_i$ by using Equation 8.

$$k_{on} = k_{off}/K_i \quad \text{(Eq. 8)}$$

Calculations were performed using $K_m$ values of 24.7 ± 1.3 $\mu M$ for mesotrypsin as determined from at least three Michaelis-Menten kinetic experiments that were previously performed in our laboratory (19). All calculations were performed using Prism (GraphPad Software, San Diego). Results (means ± S.D.) were obtained from at least three independent experiments.

The cleavage of intact inhibitors (including APP1, KD1TFPI1, and KD2bikunin variants) in time course incubations with catalytically active mesotrypsin was monitored by HPLC. Mesotrypsin was incubated at 37 °C with the inhibitors in trypsin buffer (100 mM Tris-HCl, pH 8.0, 1 mM CaCl2) for APPIT11C/M17G/I18F/F34C and APPIM17C/I18F/F34C. The reaction was performed for 6 h in the presence of 50 $\mu M$ inhibitor using 0.2 and 7.14 $\mu M$ enzyme, respectively; for KD1TFPI1K15R/I17C/I34C, KD2bikunin, and KD2bikunin(17C/F34C), the reaction was performed for 4 h in the presence of 50 $\mu M$ inhibitor using 0.033, 0.25, and 1 $\mu M$ enzyme, respectively; for KD1TFPI1K15R the reaction was performed for 1 h in the presence of 300 $\mu M$ inhibitor using 0.05 $\mu M$ enzyme; inhibitor and enzyme concentrations are given in Table S1. For HPLC analysis, aliquots of 30 $\mu l$ were withdrawn at periodic intervals (Table S1), added to a solution containing 80 $\mu l$ of 6 $M$ urea and 2 $\mu M$ DTT, incubated for 10 min at 37 °C, quenched by acidification to pH 1, and then frozen at −20 °C until analyzed. Samples were resolved on a 50 × 2.0-mm Jupiter 4 µ 90-Å C12 column (Phenomenex) with a gradient of 0–100% acetonitrile in 0.1% trifluoroacetic acid (TFA) at a flow rate of 0.6 ml/min over 50 min. Intact inhibitors were quantified by peak integration of absorbance traces monitored at 210 nm. Initial rates were obtained by linear regression using a minimum of seven data points within the initial linear phase of the reaction. Hydrolysis rates reported for each inhibitor represent the average of three independent experiments.
Disulfide engineering of Kunitz protease inhibitors

Initial structural models for MD of mesotrypsin/Kunitz domain complexes were generated from the following crystallographic structures: (a) mesotrypsin/APPI_{M17G/I18F/F34V} (PDB code 5C67 (19)); (b) mesotrypsin/APPI_{M17G/I18F/F34C} (PDB ID 6HAR; present study); and (c) mesotrypsin-S195A/KD1TFPI{K15R/I17C/I34C} (PDB code 6BX8; present study). Additionally, a model for the mesotrypsin/KD1TFPI{K15R} complex was generated via in silico back-mutation of the mesotrypsin/KD1TFPI{K15R/I17C/I34C} structure, followed by energy minimization. Initial structural models for MD of the same four Kunitz inhibitor domains in the unbound state were generated by extracting the coordinates only for the inhibitor chains from the models of mesotrypsin complexes described above. All models were further energy-minimized, validated, and prepared for MD, as described previously (18). Well-known MD methodologies and capabilities were used (18, 62–65). Free (unbiased) all atom MD simulations were run in explicit solvents using methods that we have described in detail previously (18, 66–68). Models were solvated with TIP3P water molecules, salt ions (Na\(^{+}\), Cl\(^{-}\)), and counter-ions (neutralizing the enzyme) resembling a biological environment to a depth of 15–18 Å from the edge of the molecule forming a simulation box, which was subjected to a particle-mesh Ewald algorithm and periodic boundary conditions for complete electrostatic treatment (69). Simulations were run using NAnoscale Molecular Dynamics 2 engine (NAMD2) and AMBER force-field parameters, which were exported to Yasara for very long runs on a Linux Beowulf cluster. We compared NAMD2 pre-equilibration using CHARMM 27 with CMAP correction force field parameters to check for any inconsistencies in the structure prior to production run simulations. Simulations were carried out using the particle mesh Ewald technique with repeating boundary conditions with a 9 Å nonbonded cutoff, using SHAKE with a 2-fs time step. A constant temperature of 310 K was maintained using the Berendsen weak-coupling algorithm with a time constant of 1.0 ps. Following equilibration cycles of minimization and heating under restrained and unrestrained MD, equilibration was determined from a flattening of RMSD over time. Production runs were subsequently carried out with constant pressure boundary conditions (relaxation time of 1.0 ps) for >1000 ns. Our protocols for equilibration and production runs are described in greater detail elsewhere (18, 66, 67). Following simulations, individual frames were superposed back to the origin to remove rotation and translation effects.
Disulfide engineering of Kunitz protease inhibitors

Author contributions—I. C., M. C., A. S., B. S., and S. L. data curation; I. C., M. C., A. S., B. S., T. R. C., E. S. R., and N. P. formal analysis; I. C., M. C., A. S., T. R. C., E. S. R., and N. P. validation; I. C., M. C., B. S., A. H., T. R. C., E. S. R., and N. P. investigation; I. C., M. C., B. S., T. R. C., and N. P. methodology; I. C., E. S. R., and N. P. writing—original draft; I. C., M. C., A. S., S. L., T. R. C., E. S. R., and N. P. writing—review and editing; E. S. R. and N. P. conceptualization; B. S., A. H., T. R. C., and E. S. R. resources; E. S. R. and N. P. supervision; E. S. R. and N. P. funding acquisition; E. S. R. and N. P. visualization.

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