Characterization of Protein Z-Dependent Protease Inhibitor/ Antithrombin Chimeras Provides Insight into the Serpin Specificity of Coagulation Proteases

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ABSTRACT: Protein Z (PZ)-dependent protease inhibitor (ZPI) and antithrombin (AT) are two physiological serpin inhibitors involved in the regulation of proteolytic activities of the blood coagulation cascade. ZPI has restricted protease specificity capable of inhibiting factors Xa (FXa) and XIa (FXIa) but exhibiting no reactivity with other coagulation proteases. Unlike ZPI, AT is a general inhibitor of all coagulation proteases and the only physiological inhibitor of factor IXa (FIXa). To understand the molecular determinants of protease specificity of the two serpins, we engineered two ZPI mutants in which the P12-P3′ residues of the reactive center loop of ZPI were replaced with either P12-P3′ or P12-P7′ residues of AT (ZPI–ATP12-P3′ and ZPI–ATP12-P7′). The reactivity of chimeras with FXa was improved ~4–25-fold in the absence of PZ. Both chimeras inhibited FIXa with rate constants that were ~2 orders of magnitude higher than the rate of the AT inhibition of the protease. PZ improved the reactivity of chimeras with FIXa by another 2 orders of magnitude, rendering the chimeras potent inhibitors of FIXa so that the PZ-mediated inhibitory activity of the ZPI–AT chimeras toward FIXa was ~20-fold higher than that of the fondaparinux-catalyzed inhibition of FIXa by AT. Further studies revealed that the substitution of P1-Tyr of ZPI with an Arg is sufficient to convert the serpin to an effective inhibitor of FIXa. The potential therapeutic utility of the serpin chimeras as specific inhibitors of FIXa was diminished by findings that the chimeras function as effective substrates for other coagulation proteases.

INTRODUCTION

The two serpin inhibitors antithrombin (AT) and protein Z (PZ)-dependent protease inhibitor (ZPI) regulate the proteolytic activity of coagulation proteases of the blood clotting cascade.1–4 In contrast to AT which is a universal serpin inhibitor of all coagulation proteases of both intrinsic and extrinsic pathways,1,2 ZPI is a specific inhibitor of factors Xa (FXa) and XIa (FXIa) and exhibits no significant reactivity with other coagulation proteases.3,4 Both AT and ZPI require cofactors for their optimal inhibitory activity toward their specific target proteases. Whereas heparin functions as a cofactor to promote the inhibitory activity of both serpins,1,2 PZ functions as a cofactor to specifically enhance the reactivity of ZPI with FXa on negatively charged phospholipids in the presence of calcium.3,4 AT is the only physiological inhibitor of factor IXa (FIXa). The reactivity of FXIa with AT in the absence of heparin cofactors is low, exhibiting a second-order rate constant that is ~40-fold lower than that of the reactivity of the serpin with FXa.5 Nevertheless, the cofactor function of the therapeutic heparins improves the reactivity of FXIa with AT by several orders of magnitude by both conformational activation of the serpin and a template mechanism.6,7 It has been hypothesized that a small fraction of glycosaminoglycans lining the vasculature contains 3-O-sulfate containing heparin-like sequences that can also function as cofactors to activate the serpin, thereby improving the reactivity of AT with FIXa, FXa, and other coagulation proteases by similar mechanisms.8 In the case of ZPI, PZ functions as a vitamin K-dependent cofactor to promote the inhibitory activity of the serpin with FXa on negatively charged phospholipid vesicles and calcium.9 The complex formation of ZPI with PZ improves the reactivity of the serpin with FXa by at least 3 orders of magnitude under these conditions.10,11 In a study published several years ago, it was reported that ZPI can also inhibit FIXa, although subsequent studies did not confirm the initial findings.12 In a recent study, we investigated the molecular basis for the lack of reactivity of FIXa with ZPI and demonstrated that residues of the 39-loop (also referred to as 37-loop) restrict the ZPI specificity of FIXa.13 Thus, we discovered that a FIXa mutant, in which the residues of this loop were replaced with the corresponding residues of FXa, reacted with the ZPI–PZ complex with a similar second-order rate constant as did FXa.
Table 1. Second-Order Rate (k_{2(app)}) Constants for the Inhibition of FXa by ZPI Derivatives in the Absence and Presence of PZ, PC/PS, and Calciuma

| ZPI derivatives | FXa | FXa + PZ and PC/PS |
|-----------------|-----|-------------------|
|                 | k_{2(app)} (×10^3 M^{-1} s^{-1}) | SI (mol l/mole) | k_{2(app)} × SI (×10^6 M^{-1} s^{-1}) | k_{2(app)} (×10^3 M^{-1} s^{-1}) | SI (mol l/mole) | k_{2(app)} × SI (×10^6 M^{-1} s^{-1}) |
| WT              | 3.3 ± 0.13 | 4.7 ± 0.14 | 1.6 ± 0.12 | 29.3 ± 2.2 | 2.8 ± 0.15 | 8.2 ± 0.18 |
| ATP\textsuperscript{P12-P7} | 21.1 ± 1.5 | 20.6 ± 1.1 | 43 ± 1.9 | 3.8 ± 0.41 | 31.2 ± 0.76 | 10.6 ± 0.26 |
| ATP\textsuperscript{P12-P7} | 2.6 ± 0.14 | 24.3 ± 1.5 | 63.6 ± 0.13 | 0.46 ± 0.03 | 36.1 ± 2.5 | 1.7 ± 0.42 |
| ATP\textsuperscript{P10} | 0.30 ± 0.08 | ND | ND | 0.38 ± 0.03 | 6.2 ± 0.64 | 0.24 ± 0.014 |

\(k_{2(app)}\) and SI values for the inhibition of FXa by ZPI derivatives in the absence and presence of PZ and PC/PS vesicles in TBS/Ca\textsuperscript{2+} were determined as described under Experimental Procedures. All values are the average of at least three measurements ± SD. ND, not determined.

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Expression and Characterization of ZPI–AT Chimeras.

The ZPI–AT chimeras were expressed in Escherichia coli using the small ubiquitin-related modifier (SUMO) fusion expression system and purified to homogeneity on a nickel column as described.\textsuperscript{15,16} The homogeneity of all expressed proteins was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (see below). The \(k_2\) values for the inhibition of FXa by the ZPI chimeras in both the absence and presence of PZ are presented in Table 1. In agreement with previously published results,\textsuperscript{16} wild-type ZPI (ZPI-WT) inhibited FXa with an apparent \(k_2\) (\(k_{2(app)}\)) of 3.3 \(×\) 10\(^{-3}\) M\(^{-1}\) s\(^{-1}\) and an inhibition stoichiometry (SI) value of 4.7, yielding an overall second-order rate constant (\(k_{2(app)}\) × SI) of 1.6 \(×\) 10\(^{-3}\) M\(^{-1}\) s\(^{-1}\). ZP promoted the ZPI inhibition of FXa (29.3 \(×\) 10\(^{-3}\) M\(^{-1}\) s\(^{-1}\)) by \(2\) orders of magnitude in the presence of PC/PS vesicles and decreased the cleavage rate of the serpin with FXa \(2\)-fold, yielding an overall second-order rate constant of 8.2 \(×\) 10\(^6\) M\(^{-1}\) s\(^{-1}\) (Table 1). ZPI–AT\textsuperscript{P12-P7} exhibited \(7\)–\(8\)-fold improved \(k_{2(app)}\) of inhibition and \(4\)-fold higher SI value; thus, the overall rate constant for the ZPI–AT\textsuperscript{P12-P7} inhibition of FXa (43 \(×\) 10\(^4\) M\(^{-1}\) s\(^{-1}\)) relative to ZPI-WT (and ZPI–AT\textsuperscript{P12-P7}) was enhanced \(19\)–\(20\)-fold. This enhancement in the reactivity of the chimeric serpin with FXa is primarily due to the presence of a P1-Arg on the RCL of chimera, rather than a P1-Tyr on the RCL of ZPI-WT. The substitution of P1-Tyr with an Arg (P1-Y/R) by itself has been shown to dramatically improve the reactivity of the mutant with FXa; however, the serpin mutant cannot inhibit FXa but functions as an effective substrate for the protease.\textsuperscript{17} The same results were observed in this study (data not shown). Thus, P1-Arg in the context of the AT RCL can endow inhibitory function to the chimeric serpin in the reaction with FXa, although also markedly increasing its reactivity in the substrate pathway. In contrast to the ZPI–AT\textsuperscript{P12-P7} chimera, the \(k_{2(app)}\) for the ZPI–AT\textsuperscript{P12-P7} inhibition of FXa was not improved but rather slightly decreased, suggesting that the three P5′-P7′ insertion residues of the longer RCL of AT impede its optimal interaction with FXa. This finding is consistent with the mutagenesis data showing that the deletion of these insertion residues from the AT RCL improves the reactivity of the AT mutant with FXa independent of pentasaccharide, suggesting that these insertion residues may be responsible for trapping the RCL of AT in a low inhibitory conformation. The native low inhibitory conformation of the AT RCL is thought to be due to the partial insertion of the hinge region of the loop (P14 and P15 residues) into \(\beta\)-sheet A of the serpin and that the cofactor function of heparin leads to expulsion of the loop and thus conformational activation of AT.\textsuperscript{18} It should be noted that a longer RCL is required for the serpin inhibition of thrombin because deletion of these residues impairs the reactivity of the AT mutant with thrombin.\textsuperscript{18} It appears that the three insertion residues of the AT RCL confer a canonical conformation for this loop to enable AT to interact with thrombin and that the low inhibitory activity of the longer RCL in AT in the reaction with FXa and FIXa is overcome by the cofactor function of heparin through conformational activation of AT, thereby allowing the serpin to make exosite-dependent interactions with both FXa and FIXa. Exosite interactions appear to be also important for the ZPI reaction with FXa and therefore modifying the RCL length in ZPI might be expected to affect such interactions and account for differences in the reactivity. In support of this hypothesis, grafting the three P5′-P7′ insertion residues of AT to the same site of ZPI (ZPI–AT\textsuperscript{P10}) dramatically reduced the reactivity of the serpin mutant with FXa without affecting the magnitude of
the cofactor function of PZ (Table 1). It is interesting to note that the overall rate constant for the ZPI−AT P12-F3+ reaction with FXa (43 × 10^4 M^-1 s^-1), including the increased reactivity in the substrate pathway) is similar to the reactivity of AT with FXa in the presence of pentasaccharide. Nevertheless, the improved reactivity of the ZPI−AT P12-F3+ chimera with FXa (relative to ZPI-WT) vanished in the presence of PZ, suggesting that the cofactor function of PZ may primarily be responsible for overcoming the nonoptimal binding of a P1-Tyr of ZPI to the primary specificity pocket of FXa.

Reaction of ZPI−AT Chimeras with FIXa. The examination of the reactivity of the ZPI−AT chimeras revealed that both chimeras are capable of inhibiting FIXa with a rate constant that is nearly 2 orders of magnitude higher than that of the AT inhibition (6 × 10^5 M^-1 s^-1) of the protease (Table 2). To further characterize the extent of the reactivity of FIXa with the chimeric serpins, first the SI values for the protease inhibition by ZPI chimeras were determined. The results presented in Figure 1 suggest that the two ZPI−AT P12-F3+ and ZPI−AT P11-F7 chimera inhibit FIXa with SI values of ∼4 and ∼6, respectively. Similar values have been observed for the ZPI-WT inhibition of FXa. Interestingly, the substitution of P1-Tyr of ZPI with an Arg (P1-Y/R) was sufficient to dramatically improve the reactivity of the ZPI mutant with FIXa (k(app) = 2.1 × 10^4 M^-1 s^-1), thus yielding an overall k(app) × SI of ∼1 × 10^6 M^-1 s^-1, a rate constant that nearly approaches the reactivity of FIXa with the pentasaccharide-activated conformation of AT. Furthermore, PZ accelerated the reactivity of the chimeric serpins with FIXa by another 2 orders of magnitude in the presence of PC/PS vesicles, thus the overall k(app) value approaching 10^6 M^-1 s^-1 (Table 2). These results clearly suggest that the P1-Tyr of ZPI is responsible for restricting the specificity of the serpin with FIXa. It is however surprising to note that PZ cannot overcome, at least partially, the restrictive function of P1-Tyr in the ZPI inhibition of FIXa. In a previous study, we showed that the substitution of the 39-loop of FIXa with the corresponding loop of FXa renders the FIIXa mutant susceptible to rapid inhibition by ZPI-WT with a rate constant similar to that observed for the ZPI inhibition of FIXa in both the absence and presence of PZ and negatively charged phospholipid vesicles. This observation suggests that the 39-loop of FIXa impedes the entrance of the bulky hydrophobic Tyr ring of ZPI-WT into the active-site pocket of the protease. The ZPI−AT NR chimera, similar to ZPI-WT, was not reactive with FIXa because it retained the P1-Tyr.

In light of the high inhibitory activities of the ZPI−AT chimeras toward FIXa, we postulated that the chimeric serpins might have therapeutic value in inhibiting FIXa in thrombosis patients. Nevertheless, before initiating translational research, we first assessed the protease inhibitory activity of one of the chimeras (ZPI−AT P11-F7) in the aPTT clotting assay. Surprisingly, no differences between the anticoagulant activities of ZPI-WT and ZPI−AT P11-F7 could be observed in normal plasma (data not shown). To make the aPTT assay more sensitive for monitoring FIXa inhibition, the anticoagulant activity of FIXa was calculated by an amidolytic assay described under Experimental Procedures. Results of the titration of FIXa (50 nM) with increasing concentrations of ZPI were carried out in the presence of PZ (2-fold in molar excess of ZPI) in TBS/Cal2+ containing PC/PS vesicles (50 μM). Symbols are as follows: ZPI−AT P12-F3+ (○); ZPI−AT P11-F7 (□); and ZPI-Y/R (□). The solid lines in both panels are linear regression fits of the inhibition data.

*Table 2. Second-Order Rate Constants (k_{app}) for the Inhibition of FIXa by ZPI Derivatives in the Absence and Presence of PZ, PC/PS, and Calcium*

| ZPI derivatives | FIXa | FIXa + PZ and PC/PS |
|-----------------|------|---------------------|
|                 | k_{app} (×10^5 M^-1 s^-1) | Sl (mol I/mol E) | k_{app} × Sl (×10^4 M^-1 s^-1) | k_{app} (×10^5 M^-1 s^-1) | Sl (mol I/mol E) | k_{app} × Sl (×10^4 M^-1 s^-1) |
| WT              | ND   | ND                  | ND                     | ND                       | ND                   | ND                     |
| ATP12-F3        | 2.5 ± 0.21 | 3.7 ± 0.15 | 0.93 ± 0.06 | 7.5 ± 0.65 | 2.2 ± 0.12 | 1.6 ± 0.05 |
| ATP11-F7        | 4.5 ± 0.46 | 6.0 ± 0.28 | 2.7 ± 0.11 | 1.4 ± 0.15 | 3.4 ± 0.21 | 0.48 ± 0.02 |
| P1-Y/R          | 2.1 ± 0.12 | 4.5 ± 0.27 | 0.95 ± 0.07 | 3.1 ± 0.4 | 3.5 ± 0.18 | 1.1 ± 0.04 |

Note: k_{app} and SI values for the inhibition of FIXa by ZPI derivatives was determined as described under Experimental Procedures. All values are the average of at least three measurements ± SD.
the ZPI−AT chimera may be cleaved by a protease upstream of FXa (most likely by FXIa) in the intrinsic pathway. To investigate this question further, the reactivity of the ZPI−AT chimeras with FXa was evaluated. Results suggested that the chimeras cannot inhibit FXIa and that no SI values for the inhibition of the protease by either one of the ZPI chimeras can be determined. Thus, the complex formation of chimeric serpins with FXIa and other coagulation proteases was assessed by SDS-PAGE.

**Analysis of Stable Complex Formation.** SDS-PAGE analyses of complex formation of ZPI−AT chimeras with coagulation proteases are presented in Figures 3−6. As expected from the inhibition kinetic data, unlike ZPI-WT, both ZPI−AT chimeras formed stable complexes with FXa (Figure 3A,B under nonreducing and reducing conditions). In agreement with elevated SI values, some cleavage products were also observed with both chimeric serpins. In the case of FXa, stoichiometric incubation of FXa with the chimeras (3 μM each) did not yield any stable serpin−protease complex but the chimeras were cleaved by the protease. Thus, we titrated a fixed concentration of the chimeric serpins (3 μM) with substoichiometric concentrations of FXa (0−1000 nM). The results suggested that FXa effectively cleaves both serpin mutants (Figure 4). These results are consistent with markedly higher SI values obtained for FXa in the kinetic experiments (Table 1). Under similar conditions, no significant serpin cleavage products could be detected for the reactions of the serpin chimeras with FIXa (data not shown). Similar to FXa, thrombin effectively cleaved both chimeric serpins so that no significant stable protease−serpin complex could be detected if an equimolar concentration of thrombin and serpin (3 μM each) was analyzed on the SDS-PAGE (Figure 5A). Similarly, titrating a fixed concentration of the ZPI−AT chimeras (3 μM) with substoichiometric concentrations of thrombin (0−1000 nM) suggested that the fusion of the AT RCL to ZPI dramatically increases the reactivity of the chimeric serpin with thrombin in the substrate pathway of the reaction (Figure 5B,C). Interestingly, similar experiments with FXIa revealed that both chimeric serpins have become effective substrates for the protease such that a very low concentration of FXIa (1 nM) was sufficient to cleave the chimeric serpins (Figure 6), accounting for the inability to calculate the SI values for the inhibition of FXa by the chimeric serpins. The rapid cleavage of the serpin chimeras by FXIa also explains the basis for the inability of chimera to prolong the clotting time in the aPTT assay when compared to that of ZPI-WT (Figure 2). Thus, although the serpin chimeras exhibit high inhibitory properties toward FIXa, they, nevertheless, do not offer a therapeutic value in their current forms unless further mutagenesis strategies are developed to eliminate their high reactivity with FIXa in the substrate pathway of the reaction.

Serpins inhibit their target serine proteases by a branched pathway, suicide substrate inhibition mechanism in which an intermediate enzyme−serpin complex is stabilized in the form of an acylated covalent complex. Noting that the reaction mechanism of the serine proteases with their true substrates and serpins is nearly identical up to the acylation step of the reaction, the differences between the rates of RCL insertion into β-sheet A and deacylation determine the fate of the enzyme−serpin intermediate complex in the reaction. If the rate of RCL insertion is faster, the intermediate is trapped as a stable covalent complex, but if the deacylation rate is faster, the intermediate is cleaved as a substrate. Thus, SI values represent the relative rate of partitioning of the protease−serpin intermediate in the branched pathway to either a covalent stable complex or a cleaved serpin. The molecular basis for the conversion of ZPI−AT chimeras to effective substrates for FIXa and thrombin and their high reactivity with FXa in the substrate pathway is not known. The reactivity of wild-type AT with either FXIa (k(app) = 3 × 106 M−1 s−1) or FIXa (6 × 101 M−1 s−1) is rather low in the absence of heparin cofactors. Assuming that the primary determinants of the specificity of the protease recognition by AT is mediated through the RCL and the fact that the entire RCL of AT (with the exception of the proximal hinge region residues) is fused to ZPI (Figure 7), one would have expected that FIXa and FXIa would react with the ZPI−AT chimeras with similar low k(app) values as they do with AT.
However, the results are not in agreement with this expectation and they may suggest that interactions other than the RCL residues in ZPI also contribute to the specificity of the reaction with these proteases. ZPI has a highly acidic N-terminal tail (52 residues) on the A-helix that is not conserved in other serpins (4). The possibility that this acidic tail contributes to interaction of ZPI chimeras with these proteases is not supported by our previous results, showing that the deletion of the acidic tail of ZPI does not affect the reactivity of the serpin with its target proteases in either the inhibitory or substrate pathway.16 The fact that the SI values for the ZPI–AT chimeras with all coagulation proteases are markedly elevated clearly suggests that the rate of loop insertion in the chimeric serpins is slower than the rate of deacylation. In light of the importance of the residues of the hinge region and differences in the structure of these residues between the two serpins, it is possible that the hinge region residues of ZPI are not compatible with comparable rapid loop insertion in the chimeric serpins (Figure 7). Of particular note is the presence of P16-Glu in AT (25), which is an Arg in the corresponding site of ZPI.4 The role of this residue in the mechanics of loop insertion in the context of the AT RCL in ZPI needs to be investigated to determine whether it contributes to the slower rate of loop insertion in the chimeric serpins. Noting that the SI values for the ZPI–WT chimeras with all coagulation proteases are markedly elevated clearly suggests that the rate of loop insertion in the chimeric serpins is slower than the rate of deacylation. In light of the importance of the residues of the hinge region and differences in the structure of these residues between the two serpins, it is possible that the hinge region residues of ZPI are not compatible with comparable rapid loop insertion in the chimeric serpins (Figure 7). Of particular note is the presence of P16-Glu in AT (25), which is an Arg in the corresponding site of ZPI.4 The role of this residue in the mechanics of loop insertion in the context of the AT RCL in ZPI needs to be investigated to determine whether it contributes to the slower rate of loop insertion in the chimeric serpins. Noting that the SI values for the ZPI–WT chimeras with all coagulation proteases are also high (~5 and 10 for FXa and FXIa, respectively), one might postulate that, relative to AT and other serpins, ZPI has an intrinsically slower loop insertion rate during interaction with its target coagulation proteases. It is also worth noting that the conformation of the AT RCL is allosterically linked to the hinge region, the shutter region, and the heparin-binding D-helix of the serpin.17,26 These structural features in AT are known to control the flexibility of the RCL and its inhibitory mechanism. These structural features are not expected to be transferred to ZPI when the AT RCL is grafted on the chimeric serpins, possibly accounting for the inflexibility of the grafted RCL as well as its noninhibitory and substrate properties in the reaction with coagulation proteases. However, it was interesting to note that the serpin chimeras could effectively inhibit FXa in the presence of PZ, possibly suggesting that the deacylation step of the intermediate in the FIXa reaction with the chimeric serpins is slower than that of other coagulation proteases, thus compensating for the slower rate of loop insertion in the chimeric serpins.

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant Proteins. The expression, purification, and characterization of ZPI-WT, prepared in E. coli using the SUMO fusion expression system, have been described previously.15,16 The RCL chimeric mutant of ZPI in which the P12-P3′ residues of ZPI (Ala-Val-Ala-Gly-Ile-Leu-Ser-Glu-Ile-Thr-Ala-Tyr-Met-Pro) were replaced with the corresponding residues of AT (Ala-Ala-Ala-Ser-Thr-Ala-Val-Val-Ile-Ala-Gly-Arg-Ser-Leu-Asn-Pro-Asn-Arg-Val) (ZPI–ATP12-P3′) was constructed by standard PCR mutagenesis methods and expressed using the same vector system as described.16 The same vector system was used to express another ZPI chimera in which the P12-P3′ residues of the RCL were replaced with the P12-P7′ residues of AT (Ala-Ala-Ala-Ser-Thr-Ala-Val-Val-Ile-Ala-Gly-Arg-Ser-Leu-Asn-Pro-Asn-Arg-Val) (ZPI–ATP12-P7′).
Two other ZPI mutants were prepared, in one of which the P1-Tyr of the serpin was replaced with an Arg (P1-Y/R) and in the other the AT RCL residues from P5′-P7′ (Asn-Arg-Val) were inserted after the native P4′ (Pro) residue of ZPI (ZPI−ATNRV).18,25 The concentrations of ZPI derivatives were calculated from their absorbance at 280 nm using a molar absorption coefficient of 31 525 M−1 cm−1 as described.27 The expression, purification, and characterization of PZ in HEK-293 cells have been described.10 The homogeneity of all recombinant proteins was verified by SDS-PAGE.

Human plasma protein factors IXa (FIXa), Xa (FXa), XIa (FXIa), AT, and thrombin were purchased from Haematologic Technologies Inc. (Essex Junction, VT). Phospholipid vesicles containing 80% phosphatidylcholine and 20% phosphatidylserine (PC/PS) were prepared as described.28 Normal pooled human plasma and FX-deficient plasma were purchased from George King Bio-Medical, Inc. (Overland Park, KS). Human AT-deficient plasma was purchased from Affinity Biological Inc. (Ontario, Canada). The activated partial thromboplastin time (aPTT) reagent (Alexin) was purchased from Sigma (St. Louis, MO). Chromogenic substrates S2276, S2238, and S2366 were purchased from Diapharma (West Chester, OH), and CBS 31.39 was purchased from Midwest Bio-Tech. Inc. (Fishers, IN).

**Inhibition Assays.** A discontinuous assay method was used to measure the second-order associate rate constants (k2) for the ZPI inhibition of all coagulation proteases under pseudofirst-order conditions in both the absence and presence of PZ as described.13,16 Briefly, each protease (1 nM FXa, 10 nM FIXa, 2 nM thrombin, and 1 nM FXIa) was incubated with ZPI (500–

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**Figure 5.** SDS-PAGE analysis of the reaction of thrombin with ZPI derivatives. (A) Thrombin (3 μM) was incubated with an equimolar concentration (3 μM) of each ZPI derivative in TBS/Ca2+ at room temperature for 5 min. Five microliters of a 5× nonreducing loading buffer was added to each reaction, and following boiling for 5 min, the reaction mixtures were loaded on a 10% polyacrylamide gel. Lane 1, thrombin; lane 2, ZPI-WT; lane 3, thrombin + ZPI-WT; lane 4, ZPI−ATP12-P3′; lane 5, thrombin + ZPI−ATP12-P3′; lane 6, ZPI−ATP12-P7′; lane 7, thrombin + ZPI−ATP12-P7′, and lane 8, molecular mass standards in kDa. (B) ZPI−ATP12-P3′ (3 μM) was incubated with increasing concentrations of thrombin (0–1000 nM, lanes 2–9) in TBS/Ca2+ at room temperature for 5 min. Five microliters of a 5× nonreducing loading buffer was added to each reaction, and following boiling for 5 min, the reaction mixtures were loaded on a 10% polyacrylamide gel. Lane 1, thrombin (1000 nM) alone; lane 2, ZPI−ATP12-P3′ (3 μM) alone; lanes 3–9, ZPI−ATP12-P3′ plus 16, 31, 62.5, 125, 250, 500, and 1000 nM thrombin, respectively; and lane 10, molecular mass standards in kDa. (C) Same as (B) except that ZPI−ATP12-P7′ was used in the reactions.

**Figure 6.** SDS-PAGE analysis of the reaction of FXIa with ZPI derivatives. (A) Time course of the reaction of FXIa (1 nM) with ZPI-WT or ZPI−ATP12-P7′, (3 μM) was monitored in TBS/Ca2+ at room temperature. Five microliters of a 5× nonreducing loading buffer was added to each reaction, and following boiling for 5 min, the reaction mixtures were loaded on a 10% polyacrylamide gel. (B) Same as (A) except that the time course of the reaction of FXIa (1 nM) with ZPI−ATP12-P3′ (3 μM) was monitored.

**Figure 7.** X-ray crystal structures of ZPI and AT. The P12-P3′ residues of the RCL in ZPI are colored in purple. The P12-P7′ residues of the RCL in AT including the three insertion residues (N-R-V) are shown in purple. The P1 residue and the hinge region for both serpins are marked. The coordinates (Protein Data Bank accession code 2BEH for AT and 3H5C for ZPI) were used to prepare the figure.
2000 nM) in 0.1 M NaCl, 0.02 M Tris–HCl, pH 7.5, and 5 mM Ca²⁺ containing 0.1 mg/mL BSA and 0.1% poly(ethylene glycol) (PEG) 8000 (TBS/Ca²⁺) at room temperature. All reactions were carried out in 50 μL volumes in 96-well plates and at different time points (15–120 min depending on the rate of the reactions) and microliters of the chromogenic substrate specific for each protease (S2276 for FXa, CBS 31.39 for FXIa, S2366 for FXIa, and S2238 for thrombin) in TBS was added to each well; the remaining activities of enzymes were measured by a Vmax Kinetics Microplate Reader (Molecular Devices, Menlo Park, CA). The rate constants (kₚ) were determined from the values of observed pseudo-first-order rate constants (kobs) divided by the concentration of serpins as described.13,16 Reactions in the presence of PZ were the same except that the proteases were incubated with ZPI (100–200 nM) in complex with PZ (2.5–5.0 nM) on PC/PS vesicles (50 μM) in the same TBS buffer. The inactivation reactions were stopped by addition of 50 μL of the chromogenic substrate in TBS containing 50 mM EDTA, and kₚ values were measured from the remaining enzyme activity as described.13 All values are presented as the average of at least three independent measurements ± SD.

**Determination of SI.** SI values for the inhibition of coagulation proteases by the ZPI derivatives were determined by titration of 10–250 nM of active-site-titrated protease with increasing concentrations of the serpin–cofactor complex corresponding to serpin/protease molar ratios of 0–20 and a PZ concentration equal to or 2-fold in molar excess of ZPI. The reactions were carried out in TBS/Ca²⁺ containing 50 μM PC/PS and the residual amidolytic activity of proteases was measured using the specific chromogenic substrates as described above. After completion of the inhibition reactions, the serpin/protease ratios were plotted versus the residual activity of the protease and the SI values were determined from the x-intercept of the linear regression fit of the inhibition data as described.13

**Analysis of the Stable Serpin–Protease Complex Formation.** Complex formation of coagulation proteases with the serpins was monitored by SDS-PAGE as described.13 The reaction was carried out in 20 μL volume using 3 μM ZPI and equimolar concentration of the protease in TBS/Ca²⁺. Following incubation at different times at room temperature, 5 μL of a 5X reducing or nonreducing loading buffer was added and the samples were loaded on a 10% SDS-PAGE and stained with Coomassie Blue R-250 as described.13 Under the conditions where the SI values were determined to be very high, the cleavage of the serpin chimeras was monitored at lower concentrations of coagulation proteases (1 nM to 1 μM).

**Plasma Clotting Assay.** The anticoagulant activity of the serpin chimeras in plasma was evaluated in an aPTT assay using normal plasma, FX-deficient plasma, and AT-deficient plasma and a STart 4 fibrinogen (Diagnostica/Stago, Asnieres, France). Briefly, 0.050 mL of TBS containing 2.5 μM final concentrations of ZPI-WT or the chimera was incubated with a mixture of 0.05 mL normal plasma, AT-deficient plasma, or FX-deficient plasma, supplemented with an autolysis loop mutant of FX, which when activated exhibits very poor reactivity with ZPI,12 plus 0.05 mL of the aPTT reagent (Alexin) for 5 min before the initiation of clotting by the addition of 0.05 mL of 35 mM CaCl₂ at 37 °C. The FXa autolysis loop mutant has a normal prothrombinase activity.20

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