Engineering Functional Antithrombin Exosites in α1-Proteinase Inhibitor That Specifically Promote the Inhibition of Factor Xa and Factor IXa

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We have previously shown that residues Tyr-253 and Glu-255 in the serpin antithrombin function as exosites to promote the inhibition of factor Xa and factor IXa when the serpin is conformationally activated by heparin. Here we show that functional exosites can be engineered at homologous positions in a P1 Arg variant of the serpin α1-proteinase inhibitor (α1PI) that does not require heparin for activation. The combined effect of the two exosites increased the association rate constant for the reactions of α1PI with factors Xa and IXa 11–14-fold, comparable with their rate-enhancing effects on the reactions of heparin-activated antithrombin with these proteases. The effects of the engineered exosites were specific, α1PI inhibitor reactions with trypsin and thrombin being unaffected. Mutation of Arg-150 in factor Xa, which interacts with the exosite residues in heparin-activated antithrombin, abrogated the ability of the engineered exosites in α1PI to promote factor Xa inhibition. Binding studies showed that the exosites enhance the Michaelis complex interaction of α1PI with S195A factor Xa as they do with the heparin-activated antithrombin interaction. Replacement of the P4-P2 AIP reactive loop residues in the α1PI exosite variant with a preferred IEG substrate sequence for factor Xa modestly enhanced the reactivity of the exosite mutant inhibitor with factor Xa by ~2-fold but greatly increased the selectivity of α1PI for inhibiting factor Xa over thrombin by ~1000-fold. Together, these results show that a specific and selective inhibitor of factor Xa can be engineered by incorporating factor Xa exosite and reactive site recognition determinants in a serpin.

The ubiquitous proteins of the serpin superfamily share a common structure and mostly function as inhibitors of intracellular and extracellular serine and cysteine-type proteases in a vast array of physiologic processes (1, 2). Serpins inhibit their target proteases by a suicide substrate inhibition mechanism in which an exposed reactive loop of the serpin is initially recognized as a substrate by the protease. Subsequent cleavage of the reactive loop by the protease up to the acyl-intermediate stage of proteolysis triggers a massive conformational change in the serpin that kinetically traps the acyl-intermediate (3, 4). Although it is well established that serpins recognize their cognate proteases through a specific reactive loop “bait” sequence, it has more recently become clear that serpin exosites outside the reactive loop provide crucial determinants of protease specificity (5–7). In the case of the blood clotting regulator antithrombin and its target proteases, physiological rates of protease inhibition are only possible with the aid of exosites generated upon activation of the serpin by heparin binding (5). Mutagenesis studies have shown that the antithrombin exosites responsible for promoting the interaction of heparin-activated antithrombin with factor Xa and factor IXa map to two key residues, Tyr-253 and Glu-255, in strand 3 of β-sheet C (8, 9). Parallel mutagenesis studies of factor Xa and factor IXa have shown that the protease residues that interact with the antithrombin exosites reside in the autolysis loop, arginine 150 in this loop being most important (10, 11). The crystal structures of the Michaelis complexes of heparin-activated antithrombin with catalytically inactive S195A variants of thrombin and factor Xa have confirmed that these complexes are stabilized by exosites in antithrombin and in heparin (12–14). In particular, the Michaelis complex with S195A factor Xa revealed that Tyr-253 of antithrombin and Arg-150 of factor Xa comprise a critical protein-protein interaction of the antithrombin exosite, in agreement with mutagenesis studies. Binding studies of antithrombin interactions with S195A proteases have shown that the exosites in heparin-activated antithrombin increase the binding affinity for proteases minimally by ~1000-fold in the Michaelis complex (15, 16).

In this study, we have grafted the two exosites in strand 3 of β-sheet C of antithrombin onto their homologous positions in a P1 Arg variant of α1-proteinase inhibitor (α1PI)2 and shown that the exosites are functional in promoting α1PI inhibition of factor Xa and factor IXa. The exosites specifically promote factor Xa and factor IXa inhibition and do not affect the inhibition of trypsin or thrombin. Moreover, mutation of the complementary exosite residue in factor Xa, Arg-150, largely abrogates the rate-enhancing effect of the engineered exosites in α1PI on factor Xa inhibition. Binding studies show that the exosites function by promoting the binding of α1PI and factor Xa in the Michaelis complex. Replacing the P4-P2 residues of the P1 Arg

2 The abbreviations used are: α, α1PI, α1-proteinase inhibitor; PEG, polyethylene glycol; NBD, 2-(acetyl)-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) ethylenediamine; SI, stoichiometry of inhibition.

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α₁PI with an IEG factor Xa recognition sequence modestly enhances the reactivity of the exosite mutant of α₁PI with factor Xa and greatly increases the selectivity of the mutant α₁PI for inhibiting factor Xa over thrombin. These findings demonstrate that a potent and selective inhibitor of factor Xa can be engineered by grafting exosite and reactive site determinants for the protease on a serpin scaffold.

EXPERIMENTAL PROCEDURES

Proteins—Recombinant α₁PI exosite mutants were produced from an α₁PI background containing two mutations, a P1 Met-358 change to Arg and a Cys-232 change to Ser, as in past studies (17). α₁PI variants were produced in Escherichia coli BL21 cells using a T7 expression system from Invitrogen and refolded from inclusion bodies as described (17, 18). After refolding, α₁PI was purified by ion exchange chromatography on DEAE-Sepharose at pH 6.5 and then on Monobeads-Q (GE Healthcare) at pH 7.0, with elution of the protein by a linear sodium chloride gradient, similar to past studies (17). Protein concentration was obtained from the 280 nm absorbance using an extinction coefficient of 27,000 M⁻¹ cm⁻¹ (19). All mutations of the α₁PI gene were done by PCR using specifically designed oligonucleotides from Sigma and Phusion DNA polymerase from Stratagene (La Jolla, CA). All mutations were confirmed by DNA sequencing. Coagulation factors IXa and Xa were purchased from Enzyme Research Laboratories (South Bend, IN), thrombin from U. S. Biochemical Corp., and trypsin from Sigma. The β-form of trypsin was purified from the commercial protein as described (20). Recombinant Gla domainless factor Xa zymogens for the mutants S195A and R150A were produced in embryonic human kidney cells, activated by proteolytic treatment with RVV snake venom activator, and purified by affinity chromatography using immobilized soybean trypsin inhibitor (10). Anhydrotrypsin was prepared by alkaline β-elimination of the phenylmethylsulfonyl fluoride adduct from trypsin, as described previously (21, 22). Concentrations of inactive proteases were determined from the 280 nm absorbance using extinction coefficients of 53,900 M⁻¹ cm⁻¹ for S195A factor Xa (10) and 36,800 M⁻¹ cm⁻¹ for anhydrotrypsin (22).

Protease Activity Assays—All inhibition and activity assays involving proteases were done in 0.15 M ionic strength, pH 7.4, buffer at 25 °C. Factor Xa and thrombin inhibition reactions were performed in 20 mM sodium phosphate, 100 mM NaCl, 0.1 mM EDTA, 0.1% PEG 8000; reactions with trypsin were done in 100 mM HEPES, 100 mM NaCl, 0.1% PEG 8000; and those with factor IXa were done in 100 mM HEPES, 93.5 mM NaCl, 5 mM CaCl₂, 0.1% PEG 8000. Assays of factor Xa reactions in the calcium-containing buffer used for factor IXa reactions gave results indistinguishable from those obtained in the phosphate buffer. Assays of residual protease activity were done in the same buffers as inhibition reactions except for factor IXa, in which case the buffer additionally contained 33% ethylene glycol to enhance factor IXa activity. The peptidyl-p-nitroanilide substrates Spectrozyme FXa from American Diagnostica (Stamford, CT) and S-2238 and S-2222 from Chromogenix (Milano, Italy) were used with factor Xa, thrombin, and trypsin, respectively. The peptidyl-7-amido-4-methylcoumarin substrate Pefafluor FIXa from Pentapharm (Norwalk, CT) was used with factor IXa. Active concentrations were obtained using specific activity values measured with active site-titrated proteases (23) or by titrations with antithrombin in the case of factor IXa (24). Protease amidolytic activity was assessed by measuring the initial linear rate of substrate hydrolysis monitored by changes of absorbance (405 nm) or fluorescence (λex = 380 and λem = 460).

Stoichiometry of α₁PI-Protease Reactions—The stoichiometry of inhibition for the reaction of proteases with α₁PI was determined from reactions containing a constant concentration of protease (100 nM) and increasing concentrations of the inhibitor (0–400 nM). After allowing enough time to reach at least 95% reaction completion, the remaining proteolytic activity was measured by diluting an aliquot of the reaction mixture into a solution of the appropriate substrate. The stoichiometry values were determined from the abscissa intercepts of linear regression fits of the decrease in protease activity as a function of the increase in molar ratio of inhibitor to protease (25).

SDS-PAGE—Protein purity and the ability of α₁PI variants to form SDS-stable covalent complexes with factor Xa were analyzed by SDS-PAGE under nonreducing conditions using the Laemmli discontinuous buffer system (26).

Association Rate Constants for α₁PI-Protease Reactions—The kinetics of the bimolecular association of α₁PI with proteases was studied by following the rates of protease inhibition under pseudo-first order conditions (i.e., with the concentration of the inhibitor at least 10 times larger than that of the protease) (25). Reactions of factor Xa and trypsin with wild-type and variant α₁PIs were monitored continuously in the presence of a reporter chromogenic substrate by following the time-dependent decrease in rate of substrate hydrolysis at 405 nm as in previous studies. The amplitudes of exponential progress curves for inhibition reactions were kept within a range over which the rate of substrate hydrolysis was linear in the absence of inhibitor to ensure that substrate depletion was minimal. Observed pseudo-first order rate constants (k_{obs}) were obtained by fitting the decrease in substrate hydrolysis rate by an exponential decay to a nonzero end point rate to account for minor amounts of degraded protease that were less susceptible to inhibition. In the case of reactions of thrombin and factor IXa with wild-type and variant α₁PIs, k_{obs} values were obtained from exponential fits of the decrease of active protease with time measured by discontinuous assays of residual protease activity. Second order association rate constants (kₐ) were obtained from slopes of linear regression fits of the dependence of k_{obs} on the inhibitor concentration. In the case of continuous assays, corrections for the competitive effect of substrate on k_{obs} were made by dividing the inhibitor concentration by the factor, 1 + [S]/K_m where [S] is the substrate concentration and K_m is the Michaelis constant for substrate hydrolysis by the protease in the absence of inhibitor. Measured K_m values of 33 μM for trypsin and 125 μM for wild-type and R150A factor Xa were used for these corrections.

P7-NBD Labeling of α₁PI—α₁PI with the reactive loop P7 Phe mutated to Cys was labeled with iodoacetamido-NBD fluorophore as in past studies (15). P7-Cys-α₁PI was concentrated (10 μM), buffer-exchanged by ultrafiltration into 20 mM HEPES.
buffer, pH 8.2, 100 mM NaCl, 25 µM dithiothreitol, and then incubated with iodoacetamido-NBD (400 µM) for 16 h at 4 °C. The buffer was then exchanged to 100 mM Hepes, pH 7.4, by repeated ultrafiltration, and the labeled protein was purified by ion exchange chromatography with Monobeads-Q as in the purification of the expressed/refolded protein. The yield of label incorporation was determined from the absorbance at 280 nm and an extinction coefficient of 25,000 M\(^{-1}\) cm\(^{-1}\), to the protein concentration determined as described above but correcting for the contribution of NBD to the 280 nm absorbance. Yields of about 90% incorporation were routinely obtained.

**Binding Studies**—The binding of inactive S195A and anhydrosproteases to \(\alpha\)-PI labeled with the fluorescent reporter NBD was quantified by titrating the proteases into 100 nm solutions of the labeled \(\alpha\)-PI in 100 mM Hepes buffer, pH 7.4, 5 mM CaCl\(_2\), 93.5 mM NaCl, and 0.1% PEG 8000 at 25 °C. Relative NBD fluorescence changes \((\Delta F_{\text{obs}}/F_o)\) at 540 nm, upon excitation at 480 nm, were fit to the quadratic equilibrium binding equation with the binding stoichiometry, relative maximal change in fluorescence, and equilibrium dissociation constant \((K_D)\) the fitted parameters. Binding of S195A factor Xa to wild-type and exosite mutant \(\alpha\)-PIs was also determined from the competitive effect of S195A factor Xa on the inhibition of factor Xa by \(\alpha\)-PI under pseudo-first order conditions. Increasing amounts of S195A factor Xa were added to inhibition reactions of active factor Xa (8 nm) with \(\alpha\)-PI (100 nm). After incubating for 1 min (\(\alpha\)-PI double exosite mutant) or for 10 min (\(\alpha\)-PI wild type), a time yielding 90% factor Xa inhibition in the absence of S195A protease, the remaining proteolytic activity was determined by adding factor Xa substrate solution and measuring the initial rate of substrate hydrolysis. Changes in \(k_{\text{obs}}\) as a function of S195A factor Xa concentration were calculated from the equation, 

\[
\Delta k_{\text{obs}} = \left(\ln\left(\frac{F_{\text{obs}}}{F_o}\right)\right)/t,
\]

where \(F_{\text{obs}}\) and \(F_o\) represent the uninhibited control factor Xa activity and the inhibited activity at time \(t\) respectively. Dissociation constants \((K_D)\) for S195A factor Xa-\(\alpha\)-PI interactions were determined from fits of the decrease in \(k_{\text{obs}}\) with increasing S195A factor Xa concentration by the hyperbolic equation for competitive inhibition (27),

\[
k_{\text{obs}} = k_{\text{obs,0}} \times \left(K_D/(K_D + [S195A FXa])\right) \quad \text{(Eq. 1)}
\]

where \(k_{\text{obs,0}}\) and \(k_{\text{obs}}\) represent \(k_{\text{obs}}\) values measured in the absence and presence of S195A factor Xa, respectively, and FXa represents factor Xa.

**RESULTS**

Engineering Antithrombin Exosites in \(\alpha\)-PI—Two residues in antithrombin, Tyr-253 and Glu-255 in strand 3 of \(\beta\)-sheet C, are principal determinants of an exosite that promotes the binding of the target proteases, factor Xa and factor IXa, when antithrombin is conformationally activated by heparin (9). To determine whether these exosite residues could function in another serpin that does not require heparin to activate its inhibitory function, we mutated the homologous strand 3C residues, Lys-222 and Leu-224, in the serpin \(\alpha\)-PI (Fig. 1A). Single and double mutations of these residues were produced on the background of a natural \(\alpha\)-PI variant, the “Pittsburgh” variant, in which the P1 Met is mutated to Arg. This substitution converts the serpin into an efficient inhibitor of trypsin-like blood clotting proteases (28, 29). Cys-232 was also mutated to Ser in the \(\alpha\)-PI-Pittsburgh background as in prior studies to avoid intermolecular disulfide bonding (17). The P1 Arg/Ser-232 \(\alpha\)-PI base molecule, referred to as wild-type \(\alpha\)-PI, and variants in which one or both antithrombin exosite residues were grafted on this background were expressed in E. coli, refolded, and purified as in past studies (17, 18). Wild-type and exosite variant \(\alpha\)-PIs yielded single bands on SDS-PAGE and were all capable of forming SDS-stable complexes with factor Xa (Fig. 2).

**Exosite Effects on \(\alpha\)-PI Reactivity with Factor Xa**—The rates of factor Xa inhibition by \(\alpha\)-PI wild type and exosite variants were measured continuously under pseudo-first order conditions in the presence of a reporter chromogenic substrate. Observed inhibition rate constants \((k_{\text{obs}})\) showed a linear dependence on inhibitor concentration for wild type and the two single exosite mutants, whereas the double exosite mutant

![Image](https://via.placeholder.com/150)

**FIGURE 1. Location of antithrombin exosite residues and their homologues in \(\alpha\)-PI and other human serpins.** A, backbone representation of antithrombin (from the S195A factor Xa-antithrombin-heparin structure, Protein Data Bank code 2GD4) and \(\alpha\)-PI (from the S195A trypsin-\(\alpha\)-PI Pittsburgh structure, Protein Data Bank code 1OPH) showing the two exosite residues, Tyr-253 (red) and Glu-255 (yellow), in strand 3 of \(\beta\)-sheet C (cyan) in antithrombin and the homologous residues in \(\alpha\)-PI in stick representation with identical colors. The exposed reactive center loops are colored green, with the P1 Arg residues shown in stick representation (blue). B, amino acid sequences for strand 3C of all 34 human serpins were obtained from NCBI GenBank™ and aligned on the basis of sequence homology using the program GeneDoc. Light gray to black shading indicates increasing levels of sequence homology among the selected group of serpins. Full names of abbreviated serpins are as follows. PI-6, 8, and 9, proteinase inhibitors 6, 8, and 9; PAI-2, plasminogen activator inhibitor 2; MNEI, monocytic elastase inhibitor; ZPI, protein Z-dependent proteinase inhibitor.
showed evidence of a saturable dependence of $k_{o bs}$ on inhibitor concentration (Fig. 3A). Second order association rate constants ($k_a$) were determined from the slopes of the linear kinetic plots or from the initial linear dependence of a fit of the data by a rectangular hyperbolic function (Table 1). Introduction of the two exosite residues, individually or combined, into the wild-type $\alpha_1$PI-Pittsburgh resulted in enhanced rates of inhibition of factor Xa relative to the wild-type serpin reaction. Wild-type $\alpha_1$PI inhibited factor Xa with a $k_a$ of $2.4 \times 10^4$ M$^{-1}$ s$^{-1}$. The K222Y mutation alone increased $k_a$ 2.3-fold, and the L224E mutation alone increased $k_a$ 3.7-fold. Combining these mutations resulted in a greater than additive 14-fold increase in $k_a$ to $3.3 \times 10^5$ M$^{-1}$ s$^{-1}$. Evaluation of the stoichiometries of inhibition (SIs) of factor Xa by wild-type and mutant $\alpha_1$PIs yielded values of $\sim$1 mol of $\alpha_1$PI/mol of factor Xa in all cases (Table 1), indicating that the exosite mutations had no effect on the efficiency of trapping the covalent acyl-intermediate complex. This result was consistent with the dominant formation of an SDS-stable serpin-protease complex and appearance of minimal cleaved $\alpha_1$PI in the reactions of wild-type and mutant $\alpha_1$PIs with factor Xa by SDS-PAGE analysis (Fig. 2).

**Engineering Factor Xa-specific Exosites in a Serpin**

**Factor Xa Residues Mediating the $\alpha_1$PI Exosite Interaction**—To determine whether the reaction of the $\alpha_1$PI double exosite mutant with factor Xa was promoted by exosite-exosite interactions similar to those in the reaction of heparin-activated antithrombin with factor Xa, we mutated the complementary exosite residue in factor Xa, Arg-150, that interacts with the antithrombin exosite (10). The rates of inhibition of R150A factor Xa by wild-type and mutant $\alpha_1$PIs were measured in continuous assays under pseudo-first order conditions as with wild-type factor Xa. Plots of $k_{o bs}$ versus inhibitor concentration yielded linear dependences for all four serpin-protease reactions (Fig. 3B), from which second order association rate constants were obtained from the slopes (Table 1). $k_a$ for the reaction of wild-type $\alpha_1$PI with R150A factor Xa was slightly faster (1.8-fold) than $k_a$ for the wild-type $\alpha_1$PI reaction with normal factor Xa. Notably, the K222Y and L224E single mutant $\alpha_1$PIs showed much smaller enhancements in $k_a$ over wild type of 1.6- and 2.0-fold, respectively, for the reactions with R150A factor Xa than for the corresponding reactions with wild-type factor Xa. Moreover, combining the two exosite mutations in $\alpha_1$PI resulted in an approximately additive enhancement in $k_a$ of 3.1-fold over wild type for the reactions with R150A factor Xa. These alterations in $k_a$ were not due to increases in a substrate mode of reaction, since $k_2$ values for all reactions were unaffected by the mutation in factor Xa and had values of $\sim$1. These results indicate a marked attenuation of the exosite-dependent enhancement of the $\alpha_1$PI reaction with factor Xa when Arg-150 of the protease is mutated and are consistent with Arg-150 interacting with the exosite residues in the double mutant $\alpha_1$PI in a manner similar to its interaction with these exosite residues in heparin-activated antithrombin.

**Protease Specificity of the $\alpha_1$PI Exosite Interaction**—The antithrombin exosite residues specifically promote the inhibition of factor Xa and factor IXa but not other proteases. To determine whether the transfer of the exosite to $\alpha_1$PI retains this protease specificity, association rate constants were measured for the reactions of wild-type and exosite mutant $\alpha_1$PIs with factor IXa, thrombin, and trypsin (Table 1). The inhibition rates were determined under pseudo-first order conditions using either continuous or discontinuous assays of the time-dependent loss in enzyme activity. The inhibition of factor IXa, a protease with an autolytic loop similar to that of factor Xa, including Arg at position 150, was promoted by the exosites on $\alpha_1$PI in a manner similar to that observed in the reaction with factor Xa. $k_a$ was thus increased 2.2- and 4.0-fold for the K222Y and L224E single mutant
Engineering Factor Xa-specific Exosites in a Serpin

TABLE 1
Association rate constants and SI values for reactions of α1PI wild type and exosite mutants with proteases at $I = 0.15$, pH 7.4, 25 °C

Values of $k_0$ were determined from the slopes of linear plots or initial slopes of hyperbolic plots of $k_{\text{obs}}$ versus inhibitor concentration. Values of $k_{\text{obs}}$ were measured from continuous or discontinuous assays of protease inhibition by wild-type and mutant serpins under pseudo-first order conditions. Inhibitor concentrations were corrected for substrate competition in continuous inhibition assays as in Fig. 3. SI values were measured from kinetic end point titrations of inhibitor-protease reactions. Further details are given under "Experimental Procedures." Average and S.D. values from at least three independent determinations are reported. ND, not determined.

| Factor Xa | Wild type | K222Y + L224E | K222Y | L224E | Wild type | L224E | Wild type | L224E | Wild type | L224E |
|-----------|-----------|---------------|-------|-------|-----------|-------|-----------|-------|-----------|-------|
| $k_0$     | $2.4 \pm 0.1 \times 10^3$ | $3.3 \pm 0.2 \times 10^3$ | $5.5 \pm 0.1 \times 10^3$ | $8.9 \pm 0.3 \times 10^3$ | $4.9 \pm 0.2 \times 10^4$ | $4.9 \pm 0.1 \times 10^5$ |
| SI        | $1.0 \pm 0.0$ | $1.0 \pm 0.0$ | $1.1 \pm 0.1$ | $1.1 \pm 0.0$ | $0.96 \pm 0.01$ | $1.2 \pm 0.0$ |
| Factor IXa| $1.4 \pm 0.1 \times 10^3$ | $1.5 \pm 0.2 \times 10^3$ | $3.1 \pm 0.1 \times 10^3$ | $5.6 \pm 0.3 \times 10^3$ | $4.3 \pm 0.3 \times 10^4$ | $3.6 \pm 0.8 \times 10^4$ |
| $k_0$     | $0.90 \pm 0.04$ | $0.95 \pm 0.05$ | $0.95 \pm 0.05$ | $0.31 \pm 0.04$ | $26 \pm 8$ |
| SI        | $0.91 \pm 0.01$ | $1.0 \pm 0.0$ | $1.0 \pm 0.0$ | $1.3 \pm 0.0$ | ND |
| Thrombin  | $1.2 \pm 0.1 \times 10^3$ | $2.1 \pm 0.1 \times 10^3$ | $2.1 \pm 0.1 \times 10^3$ | $6.2 \pm 0.4 \times 10^3$ | $5.4 \pm 0.4 \times 10^3$ | $3.0 \pm 0.4 \times 10^3$ |
| $k_0$     | $1.0 \pm 0.0$ | $0.27 \pm 0.05$ | $0.48 \pm 0.07$ | $0.14 \pm 0.02$ | $1.2 \pm 0.2 \times 10^{-3}$ | $6.8 \pm 1.5 \times 10^{-4}$ |
| SI        | $0.91 \pm 0.01$ | $1.0 \pm 0.0$ | $1.0 \pm 0.0$ | $1.3 \pm 0.0$ | ND |
| Trypsin   | $3.4 \pm 0.1 \times 10^3$ | $2.5 \pm 0.1 \times 10^3$ | $3.3 \pm 0.3 \times 10^3$ | $3.1 \pm 0.4 \times 10^3$ | $3.1 \pm 0.3 \times 10^4$ | $1.7 \pm 0.2 \times 10^4$ |
| $k_0$     | $1.0 \pm 0.0$ | $0.74 \pm 0.05$ | $0.97 \pm 0.12$ | $0.91 \pm 0.14$ | $0.91 \pm 0.11$ | $0.50 \pm 0.07$ |
| SI        | $0.91 \pm 0.01$ | $1.2 \pm 0.1$ | $1.1 \pm 0.1$ | $1.2 \pm 0.0$ | $0.91 \pm 0.03$ | $1.1 \pm 0.1$ |
| R150A Factor Xa | $4.2 \pm 0.1 \times 10^3$ | $1.3 \pm 0.0 \times 10^3$ | $6.9 \pm 0.1 \times 10^4$ | $8.6 \pm 0.1 \times 10^4$ |
| $k_0$     | $1.0 \pm 0.0$ | $3.1 \pm 0.1$ | $1.6 \pm 0.1$ | $2.0 \pm 0.1$ |
| SI        | $0.93 \pm 0.08$ | $0.94 \pm 0.05$ | $0.95 \pm 0.03$ | $0.98 \pm 0.03$ |

α1PI reactions, respectively, and 11-fold for the double mutant α1PI reaction relative to the wild-type inhibitor reaction. In contrast, the inhibition of thrombin and trypsin, proteases with autolysis loops different from that of factor Xa, was not promoted by the exosites on α1PI. The exosites in fact had a detrimental effect on the inhibition of thrombin, causing $k_0$ to decrease by 2–7-fold. The SIs for these reactions were all ~1, indicating efficient inhibition with no significant substrate reaction (Table 1).

Exosites Enhance Binding of α1PI to Inactive Factor Xa—To determine whether the accelerated inhibition of factor Xa by the α1PI exosite mutants relative to wild-type α1PI resulted from an enhanced Michaelis complex interaction of factor Xa with the mutant α1PIs, we engineered a Phe to Cys mutation at the P7 position of the reactive loop of wild-type and double exosite mutant α1PIs and stoichiometrically labeled the Cys with an NBD reporter fluorophore. The association rate constants and stoichiometries of inhibition for reactions of the NBD-labeled α1PIs with factor Xa were modestly affected by the labeling of the proteins. Values for $k_0$ and SIs of $1.5 \pm 0.2 \times 10^4$ M$^{-1}$ s$^{-1}$ and $1.8 \pm 0.3$ for the wild-type α1PI reaction and $1.5 \pm 0.2 \times 10^3$ M$^{-1}$ s$^{-1}$ and $1.7 \pm 0.1$ for the double exosite mutant α1PI reaction were obtained (compare with values in Table 1 for the unlabeled proteins). Titrations of both labeled α1PIs with increasing concentrations of catalytically inactive S195A factor Xa or anhydrotrypsin caused progressive enhancements of the NBD fluorescence, as observed previously in titrations of P7-NBD antithrombin-heparin complex with S195A factor Xa (15). Titrations with anhydrotrypsin resulted in saturable increases in fluorescence over the range of protease concentrations examined (Fig. 4A). From the fitting of the two binding curves by the equilibrium binding equation, dissociation constant values and stoichiometries of $3.1 \pm 0.4$ nM and $0.9 \pm 0.1$ for the wild-type and $30 \pm 2$ nM and $0.8 \pm 0.1$ for the double exosite mutant interactions with anhydrotrypsin were obtained. The former value is comparable with the $K_m$ of 5 nM reported previously for the unlabeled wild-type α1PI interaction with anhydrotrypsin (30), indicating that the label did not perturb the Michaelis complex interaction. The titration of the α1PI double exosite mutant with S195A factor Xa resulted in saturable increases in NBD fluorescence (Fig. 4B). From the fitting of the binding curve by the equilibrium binding equation and allowing a fixed value of 1 for the stoichiometry, a $K_D$ of $8.0 \pm 0.2 \mu M$ was obtained for the double exosite mutant α1PI-S195A factor Xa interaction. The titration of the wild-type protein produced linear increases in fluorescence over the same range of S195A factor Xa concentrations. Assuming a stoichiometry of 1 and a maximum fluorescence change equal to that obtained with the titration with the double mutant, a $K_D$ of ~70 $\mu M$ was estimated for the interaction of S195A factor Xa with the wild-type α1PI.

To confirm these differences in binding affinity, we examined the ability of S195A factor Xa to competitively inhibit the reaction of active factor Xa with wild-type and double exosite mutant α1PIs. S195A factor Xa produced concentration-dependent decreases in $k_{\text{obs}}$ for the reaction of factor Xa with wild-type and exosite mutant α1PIs in accordance with its action as a competitive inhibitor. S195A factor Xa was a more potent inhibitor of the exosite mutant reaction than of the wild-type reaction. $K_I$ values of 7.2 ± 0.7 and $=80 \mu M$ for exosite mutant and wild-type α1PI interactions, respectively, were determined from fits of the data by the hyperbolic equation for competitive inhibition (Fig. 4C). These results indicate a minimally 10-fold greater Michaelis complex affinity of factor Xa for the double exosite mutant than for the wild-type α1PI, in good agreement with the 14-fold greater $k_0$ for the mutant than for the wild-type α1PI reactions with factor Xa and consistent with $K_m$ values much greater than 1 $\mu M$ for these reactions (Fig. 3A).

Engineering a Factor Xa Substrate Recognition Sequence in the Reactive Loop of α1PI—To determine whether the double exosite mutant of α1PI could be further improved as a factor Xa inhibitor, we changed the substrate recognition sequence in the
α;PI reactive loop to enhance factor Xa recognition. The P4–P2 reactive loop residues of the wild-type and double exosite mutant α;PIs were changed to yield the amino acid sequence, IEGR, corresponding to the P4–P1 substrate recognition sequence of factor Xa in one of the activation cleavage sites of prothrombin. This change in reactive loop sequence increased about 2-fold the $k_a$ for the reactions of either wild-type or double exosite mutant α;PIs with factor Xa and had no significant effect on the stoichiometry of inhibition (Fig. 3A and Table 1). The measured $k_a$ of $4.9 \pm 0.1 \times 10^5 \text{M}^{-1} \text{s}^{-1}$ for the variant α;PI with both exosite and reactive loop changes represented a ~20-fold rate enhancement over the wild-type α;PI reaction lacking these changes. The reactivity of factor IXa was also surprisingly enhanced 2.4-fold by the IEGR reactive loop sequence change in the presence of the exosite residues, whereas reactivity was decreased ~3-fold in the absence of the exosites. However, the enhanced reactivity also elevated the inhibition stoichiometry by 10-fold (Table 1). The IEGR sequence was slightly detrimental for α;PI inhibition of trypsin when it was substituted in wild-type and exosite mutant α;PIs (Table 1). Notably, $k_a$ for α;PI inhibition of thrombin was severely depressed several hundred-fold by substituting the IEGR sequence in both the exosite mutant and wild-type α;PIs (Table 1). Although SIs of ~1 were retained for trypsin inhibition by the IEGR α;PI variants, SIs for the factor IXa reaction without exosites and for thrombin reactions with or without exosites were not determinable because of slow reaction rates.

**DISCUSSION**

Our previous work demonstrated the critical role of antithrombin residues Tyr-253 and Glu-255 as exosites that enhance the reactivity of heparin-activated antithrombin with factors Xa and IXa by promoting the initial Michaelis complex encounter between the activated serpin and the proteases (8, 9, 15, 16). Heparin conformationally activates antithrombin by disrupting a reactive loop hinge-sheet A interaction that enables the loop to adopt the canonical conformational form of other serpins and allows factor Xa and factor IXa bound to the loop to productively engage the exosites on strand 3 of β-sheet C (12, 14, 31–34). Based on these findings, we hypothesized that the antithrombin exosite residues might similarly function to enhance factor Xa and factor IXa reactivity in another serpin whose reactive loop already exists in the canonical conformation. To test this idea, the residues homologous to Tyr-253 and Glu-255 in antithrombin, namely Lys-222 and Leu-224, were mutated

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**FIGURE 4. Binding of S195A factor Xa to α;PI.** Titrations of 100 nM P7-NBD α;PI wild type (●) or K222Y/L224E double mutant (▲) with anhydrotrypsin (A) or with S195A factor Xa (B). Titrations were monitored from increases in NBD fluorescence and plotted as observed fluorescence changes normalized to the fitted maximal fluorescence change, $\Delta F_{\text{obs}}/\Delta F_{\text{max}}$, as a function of the protease concentration. The fitted relative maximal fluorescence changes ($\Delta F_{\text{max}}/F_0$) were 0.14 and 0.27 for titrations of labeled wild-type and exosite mutant α;PIs with anhydrotrypsin and 0.65 for titrations of labeled exosite mutant α;PI with S195A factor Xa. Each data point represents the average from two independent determinations. The **solid lines** are fits of the data by the quadratic equilibrium binding equation. C, hyperbolic decrease produced by increasing S195A factor Xa concentrations on $k_{\text{obs}}$ for the reactions of 8 nM active factor Xa with 100 nM wild-type (●) and double exosite mutant (▲) α;PI. The **solid lines** are fits of the data by the equation for competitive inhibition from which $K_i$ values for the α;PI-S195A factor Xa interaction were determined.
to their antithrombin counterparts in the context of a天然 P1 Arg variant of α1PI that was an established inhibitor of thrombin and factor Xa (29).

The two exosite residues, individually and in combination, accelerated the inhibition of both factor Xa and factor IXa by α1PI, with combined effects of 11–14-fold that were greater than additive and thereby suggestive of cooperative actions of the two residues. These rate enhancements are remarkably close to the 11–12-fold factor Xa and factor IXa inhibition rate enhancements produced by the exosite residues in heparin-activated antithrombin when these residues are replaced with the corresponding residues in α1PI (Table 2) (9). That the tyrosine and glutamate residues function as exosites in α1PI, similar to the way they act in heparin-activated antithrombin, is strongly suggested by the observation that the rate enhancements produced by the exosite residues are specific for factor Xa and factor IXa reactions and are not observed for thrombin and trypsin reactions (Table 1). Moreover, the rate enhancement for the factor Xa reaction is significantly ablated by mutation of Arg-150 in the autolysis loop of factor Xa (Table 1), a residue shown to be principally responsible for the specific exosite interaction of heparin-activated antithrombin with factors Xa and IXa (10–12). This residue is present in the autolysis loop of factor IXa but absent in the loops of thrombin and trypsin (35).

Interestingly, of the two exosite residues, the glutamate residue produced the largest increase in the rate of either factor Xa or factor IXa inhibition by α1PI, whereas the tyrosine residue was of overriding importance in enhancing the rate of inhibition of both proteases by heparin-activated antithrombin. Moreover, the tyrosine and glutamate residues in antithrombin act cooperatively in that the glutamate residue is effective in promoting factor Xa inhibition only when paired with the tyrosine (Table 2) (9, 15). It would thus appear that the individual and combined effects of the serpin exosite residues on kₐ for reactions with factor Xa and factor IXa is context-dependent and influenced by surrounding residues in the contact interfaces between serpin and protease. This would be in keeping with proposed repulsive interactions of the exosite region in native antithrombin with factor Xa that are relieved upon heparin activation to favor the serpin-protease exosite interaction.³

Replacing both antithrombin exosite residues with Ala instead of the Lys and Leu residues in α1PI suggests that Tyr-253 and Glu-255 are responsible for even greater ~50 and ~260-fold enhancements in antithrombin reactivity with factor Xa and factor IXa, respectively, than those observed by replacing these residues with α1PI residues (Table 2) (9). Even the Lys and Leu residues of α1PI can thus promote to some extent the interactions with factor Xa and factor IXa, implying a certain plasticity of the exosite-exosite contact region and possible importance of hydrophobic interactions.

Our studies not only have demonstrated that the antithrombin exosite can function in α1PI; they also have shown that the efficiency and selectivity of the exosite mutant α1PI can be further improved by making the reactive loop recognition sequence better match the preference of factor Xa. Substituting the P4–P2 residues of the α1PI reactive loop to provide an IEGR factor Xa recognition sequence modestly improved α1PI reactivity with factor Xa ~2-fold but greatly depressed the reactivity of α1PI with thrombin either in the absence or presence of the exosite residues. This resulted in a marked improvement in the selectivity of the exosite mutant pro tease inhibitor for inhibiting factor Xa over thrombin, the selectivity ratio increasing from a ~3-fold to ~1600-fold faster kₐ for the factor Xa reaction relative to the thrombin reaction when the reactive loop P4–P1 sequence was changed from AIPR to IEGR (Table 1). The decrease in thrombin reactivity probably reflects the changes of the P2 Pro, a preferred residue for thrombin, to Gly and the P3 Ile to the unfavorable Glu (36, 37).

³ P. G. W. Gettins and S. T. Olson, manuscript in preparation.

### Table 2

Comparison of the effects of exosite residue changes in α1PI with those in antithrombin on kₐ for reactions of the serpins with factors Xa and IXa

| Exosite | Factor Xa | α1-PI | Factor IXa | α1-PI |
|---------|-----------|-------|------------|-------|
| KL      | 4.2 × 10⁴ (1-fold) | 2.4 × 10⁴ (1-fold) | 1.8 × 10⁴ (1-fold) | 1.4 × 10³ (1-fold) |
| YL      | 2.0 × 10⁵ (4.8-fold) | 5.5 × 10⁴ (2.3-fold) | 1.1 × 10⁵ (6.1-fold) | 3.1 × 10³ (2.2-fold) |
| KE      | 2.3 × 10⁴ (0.55-fold) | 8.9 × 10⁴ (3.7-fold) | 1.9 × 10⁴ (0.11-fold) | 5.6 × 10⁴ (4.0-fold) |
| YE      | 4.6 × 10⁴ (11-fold) | 3.3 × 10⁴ (14-fold) | 2.1 × 10⁴ (12-fold) | 1.5 × 10⁴ (11-fold) |

Values of kₐ for the reactions of wild-type and exosite mutant α1PIs with factor Xa and factor IXa from Table 1 are compared with kₐ values for the corresponding reactions of heparin pentasaccharide-activated wild-type and exosite mutant antithrombins with these proteases as determined in previous studies (9). The -fold changes in kₐ due to replacing α1PI residues, lysine and leucine, with antithrombin exosite residues, tyrosine and glutamate, in the two serpins are shown in the top of the table. For antithrombin, the variant with α1PI residues contains an additional K257M mutation, which, when mutated alone, has no effect on antithrombin reactivity with factors Xa and IXa. The bottom of the table shows the -fold changes in kₐ when alanine substitutions of the two exosite residues in antithrombin are changed back to wild-type residues, as determined in previous studies (9).
It is interesting to note that $k_a$ values for the reactions of factor Xa with P1 Arg $\alpha_1$PI in the absence or presence of the exosites are close to the values observed for the corresponding reactions of constitutionally activated antithrombin with factor Xa (Table 2). This similarity extends to the $k_a$ values for $\alpha_1$PI and activated antithrombin reactions with factor IXa in the absence and presence of exosites (Table 2).

Such results confirm previous findings that $k_a$ values for $\alpha_1$PI-Pittsburgh reactions with the proteases, trypsin and thrombin, are similar to those for the reactions of constitutionally activated antithrombin with the same proteases, although only when P2 residues are matched in the case of the thrombin reactions (33). Indeed, the $k_a$ values for reactions of IEGR reactive loop $\alpha_1$PI mutants with factor Xa in which the P2 residues are matched become indistinguishable from corresponding heparin-activated antithrombin reactions (Tables 1 and 2).

Although the similar $k_a$ values of $\alpha_1$PI-Pittsburgh and heparin-activated antithrombin reactions with proteases were previously suggested to reflect similar canonical interactions of the proteases with the serpin reactive loop in the Michaelis complex, our findings do not support this proposal. Thus, the engineered exosites in $\alpha_1$PI enhance the Michaelis complex affinity with S195A factor Xa 10-fold to yield a $K_D$ of $\sim 7 \mu M$, whereas the exosites in heparin-activated antithrombin increase the Michaelis complex affinity with S195A factor Xa >30-fold to give a much stronger $K_D$ of $\sim 0.1 \mu M$ (15). The converse is true for $\alpha_1$PI-Pittsburgh and heparin-activated antithrombin interactions with trypsin. $\alpha_1$PI-Pittsburgh thus forms a high affinity Michaelis complex interaction with anhydrotrypsin with a $K_D$ of 3–5 nM, whereas heparin-activated antithrombin binds anhydrotrypsin $\sim 1000$-fold more weakly (22, 30).

How the exosites produce similar effects on $k_a$ but different stabilizing effects on the Michaelis complex can be accounted for by the multistep inhibitory mechanism of serpins. Exosite interactions may thus promote Michaelis complex formation as well as facilitate acylation of this complex, but these effects may be offset by the need to disrupt the exosite interactions to allow the conformational trapping of the acyl-intermediate (15, 27). The net effect of exosites on $k_a$ may therefore not be readily predicted from their effects on the Michaelis complex alone. The different contributions of the exosites in $\alpha_1$PI and heparin-activated antithrombin to stabilizing the Michaelis complex interaction may reflect the different contexts of these exosites as noted above, but an additional significant factor could relate to the different flexibilities of the reactive loops of the two serpins. $\alpha_1$PI has a shorter more rigid reactive loop than antithrombin (Fig. 1A). Although the rigid canonical loop favors a high affinity interaction with the trypsin active site, it could hinder the ability of bound factor Xa to engage both the loop and exosite optimally in the Michaelis complex and account for the weaker affinity. Conversely, the more flexible loop of antithrombin could disfavor the canonical loop interaction with trypsin but favor the joint engagement of the loop and the exosite with factor Xa.

It is interesting to note that of the 34 serpin-coding genes in humans (1), five of these contain tyrosine aligning with Tyr-253 of antithrombin, and two have phenylalanine at the same position (Fig. 1B). We have previously shown that antithrombin with a phenylalanine substitution at this position maintains a normal exosite function (9, 15). The intracellular serpins protease inhibitor-8 and -9 contain both Tyr/Phe and Glu at the homologous exosite positions of antithrombin. Interestingly, granzyme B, which is a natural target for protease inhibitor-9 (38), has a lysine in its autolysis loop at the homologous position to Arg-150 in factor Xa. Whether these residues function as exosites is an intriguing possibility that requires further investigation. More importantly, our findings have shown that reactive site and exosite motifs can be engineered in a serpin to create the specific recognition of a nontarget protease. Such findings reinforce the view that serpins can be tailor-made to specifically inhibit particular protease targets for various therapeutic applications.

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