Partial Activation of Antithrombin without Heparin through Deletion of a Unique Sequence on the Reactive Site Loop of the Serpin

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Native antithrombin (AT) has an inactive reactive site loop conformation unless it is activated by a unique pentasaccharide fragment of heparin (H5). Structural data suggests that this may be due to preinsertion of two N-terminal residues of the reactive site loop of the serpin into the A-β-sheet of the molecule. Relative to α1-antitrypsin, the reactive site loop of AT has three additional residues, Arg399, Val400, and Thr401 at the C-terminal P' end of the loop. To determine whether a longer reactive site loop of AT is responsible for loop preinsertion in the native conformation, mutants of the serpin were expressed in which these residues were individually or in combination deleted. Kinetic analysis suggested that deletion of two residues, Val400 and Thr401, changed the solution equilibrium of the serpin in favor of the active conformation, thereby enhancing the inhibition of factor Xa by an order of magnitude independent of H5. Interestingly, the reactivity of this mutant with thrombin was impaired by the same order of magnitude in the absence, but not in the presence of H5. These results suggest that a longer reactive site loop in AT is responsible for its inactive native conformation toward factor Xa, while at same time AT requires this feature to regulate the activity of thrombin.

Antithrombin (AT) is the primary serpin inhibitor in plasma that regulates the activities of the serine proteinases of both the intrinsic and extrinsic pathways of the blood coagulation cascade (1–5). Similar to other inhibitory serpins, AT inhibits its target proteinases by binding to their active sites through an exposed reactive center loop and undergoing a conformational change which traps the enzymes in inactive, stable complexes (4, 5). Unlike most other inhibitory serpins, however, AT has a reactive site loop that has an inactive conformation (6–9).

A unique high affinity pentasaccharide (H5) fragment of heparin can bind and allosterically activate AT to promote its reactivity with factor Xa (fXa) by several hundred-fold (6, 10). Surprisingly, however, the allosteric activation of AT by H5 has no effect on the reactivity of the serpin with thrombin. In this case, longer chain heparins containing H5 plus at least 13 additional saccharides are required to efficiently accelerate the inhibition reaction by an alternative ternary complex bridging or template mechanism (11, 12). The molecular basis for differential reactivity of fXa and thrombin with the activated conformation of AT is not known.

Structural data suggest that the inactive native conformation of the reactive site loop of AT is caused by preinsertion of two N-terminal P14 and P15 (nomenclature of Schechter and Berger (13)) residues of the loop into the A-β-sheet of the serpin and that the binding of the cofactor to AT causes the expulsion of this inserted region and, thereby, activation of the serpin (6, 8, 14, 15). The structural feature(s) in the reactive site loop of the serpin that may be responsible for preinsertion of the two N-terminal residues into the A-β-sheet of the molecule has not been identified. Moreover, it is not known why activation of AT by H5 specifically promotes AT inhibition of fXa, but not that of thrombin.

To address these questions, the amino acid sequence of the reactive site loop of AT was compared with those of other inhibitory serpins to determine whether a unique feature exists in the reactive site loop of AT that may be responsible for its inactive native conformation. It was noted that relative to α1-antitrypsin, the archetypical inhibitor of the serpin superfamily, and other inhibitory serpins that have a basic heparin-binding D-helix (Fig. 1), the reactive site loop of AT has three insertion residues, Arg399, Val400, and Thr401, at the most C-terminal end of the loop between the P5 site and s1C-sheet (16). To determine the contribution of these residues to the inactive, partially buried reactive site loop conformation of AT, several mutants of the serpin were generated in which these residues were individually or in combination of two and three deleted from human AT cDNA and expressed in mammalian H293 cells. Following purification to homogeneity, the properties of these mutants were characterized with respect to their ability to bind heparin and react with fXa and thrombin in both the absence and presence of high affinity heparin and the H5 fragment of high affinity heparin. The results suggest that mutagenesis of all three residues leads to loss of affinity of the mutant for heparin, as well as its inhibitory property toward both fXa and thrombin. However, deletion of one or two residues shifts the conformational equilibrium of the AT mutants toward the activated state, thereby promoting the inhibition of fXa independent of H5. Interestingly, however, the abilities of mutants to inhibit thrombin are impaired in the absence, but...
not in the presence of $H_5$, suggesting that these residues in the native conformation of AT enable the serpin to interact with the catalytic pocket of thrombin. Consistent with the conformational equilibrium model for AT (6, 8, 17), improvement in the reactivity of a double mutant lacking Val$^{400}$ and Thr$^{401}$ of both fXa and thrombin. These results suggest that a longer reactive site loop of AT at the P$^1$ end of the loop is responsible for the partially buried loop of the serpin in the native conformation, which renders it inactive toward fXa. AT, however, requires this feature to regulate the activity of thrombin in the native conformation, which explains the differential reactivity of two proteinases with the native and H$_5$-activated conformers of the serpin. The significance of this unique structural feature with respect to the physiological function of AT is discussed.

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant Proteins—Recombinant human antithrombin (rAT) and its deletion derivatives in which the three insertion residues, Arg$^{399}$, Val$^{400}$ and Thr$^{401}$, were individually (des-R, des-V, and des-T), or in combination of two (des-VT) or three (des-VT) deleted by standard PCR mutagenesis methods, expressed in H293 cells using pSV-P4 expression/purification vector system as described previously (18, 19). Accuracy of the mutations was confirmed by sequencing prior to expression. Wild type and mutant serpins were purified from cell culture supernatants by immunoaffinity chromatography using the HPC4 antibody linked to Affi-Gel 10 (Bio-Rad) followed by a HiTrap-Heparin (Amersham Biosciences, Inc.) ion exchange chromatography with a gradient elution from 0.1 to 2.0 M NaCl in 20 mM Tris-HCl, pH 7.4 as described previously (19). Concentrations of the AT derivatives were determined from the absorbance at 280 nm using a molar absorption coefficient of 37,700 $\text{mM}^{-1} \text{cm}^{-1}$ and by stoichiometric titration of the serpins with calibrated heparin as monitored from changes in intrinsic protein fluorescence (20).

Human plasma AT (pAT), the active AT-binding $H_5$ fragment of heparin and full-length high affinity heparin containing the $H_5$ with an average molecular mass of $\sim 21,000$ Da ($\sim 70$ saccharides, $H_5$) were generous gifts from Dr. Steven Olson (University of Illinois, Chicago). Concentrations of heparins were based on the AT binding sites and homogeneity and that all migrate with relative molecular masses identical to human pAT (Fig. 2).

FIG. 1. Alignment of the amino acid sequences of the heparin-binding serpins from the P1–P6 positions. $\alpha_1$-AT, $\alpha_1$-antitrypsin; PAI-1, plasminogen activator inhibitor-1; PN-1, protein nexin-1; HC-II, heparin cofactor-2.

RESULTS

Expression and Purification of the Antithrombin Derivatives—Wild type and mutant antithrombin derivatives were expressed in H293 cells and purified to homogeneity by a combination of HPC4 immunoaffinity and HiTrap-Heparin column chromatography as described previously (18, 19). For des-RVT, which eluted at $\sim 0.3$ M NaCl, recombinant wild type (rAT) and all other mutants of AT were eluted at $\sim 0.7$–$0.8$ M NaCl from the HiTrap-Heparin column (data not shown). Since des-RVT did not bind heparin with high affinity and exhibited insignificant inhibitory activity toward either fXa or thrombin in both the absence and presence of heparin, it was not included in the further studies described below. SDS-PAGE analysis of all other mutants under nonreducing conditions suggested that the AT derivatives have been purified to homogeneity and that all migrate with relative molecular masses identical to human pAT (Fig. 2).

Binding to Heparin—It is known that the binding of high affinity heparin to AT is associated with an $\sim 30$–$40$% intrinsic protein fluorescence enhancement (21). The dissociation constants ($K_D$) of the heparin binding to each mutant were determined by monitoring the fluorescence emission spectra as described above.
The Reactive Site Loop of Antithrombin

Fig. 3. Binding of heparin to plasma-derived wild type and recombinant AT derivatives. Heparin-induced spectral changes were monitored at room temperature by stepwise addition of 2–5 μl of a concentrated stock solution of heparin to 50 nM of each AT derivative in 20 mM Tris-HCl, 0.1 mM NaCl, and 0.1% PEG 8000, pH 7.4. The dissociation constant of heparin for each derivative was calculated from the changes of the intrinsic protein fluorescence by nonlinear regression analysis using the quadratic equation for the tight binding inhibitors as described under “Experimental Procedures.” The symbols are: ○, pAT; ●, rAT; □, des-R; ▼, des-V; Δ, des-T; and ▲, des-VT.

Inactivation of FXa and Thrombin—$k_2$ values for the association of the wild type and mutant serpins with FXa and thrombin in both the absence and presence of H5 or H70 are presented in Tables I and II. As expected from the purity on SDS-PAGE, rAT and pAT exhibited identical reactivities with both proteinases in either the absence or presence of cofactors. Except for des-R, which exhibited a wild type reactivity with FXa, all other mutants inhibited FXa better than the wild type serpin in the absence of the cofactors (Table I). The improvement in the reactivities of the mutants with FXa ranged from ~2–3-fold for des-V and des-T to ~12-fold for the double mutant des-VT. Interestingly, the 200–300-fold rate-accelerating effect of H5 in AT inhibition of FXa was reduced by a similar extent in inhibition by the mutant serpins. Thus, in contrast to ~300-fold catalytic effect of H5 in rAT inhibition of FXa (Table I), this value was reduced to only 35-fold in inhibition by the des-VT mutant. Similarly, the extent of the cofactor effect of the full-length heparin, H70, in inhibition of FXa by the mutant serpins was decreased (Table I). We previously demonstrated that the cofactor effect of H70 in AT inhibition of FXa is mediated by ~300-fold enhancement through activation and ~200–300-fold enhancement through template mechanism in the presence of a physiological concentration of Ca$^{2+}$ (22, 23). Thus, analysis of data in Table I suggests that the observed decrease in the rate-accelerating effect of H70 in inhibition of FXa by the mutant serpins is not due to a decrease in the extent of the template effect of heparin, but rather due to mutants being in constitutively activated conformations. This is derived from the observation that the ratio of the cofactor effect of H70 to that of H5 is not significantly affected with the mutant serpins (Table I).

Unlike the reactions with FXa, the ability of mutant serpins to inhibit thrombin was impaired 4–15-fold (Table II). However, the cofactor function of H5 and H70 partially or completely restored the impairment in the reactivities of the mutants with thrombin. The extent of impairment in the reactivity of des-VT with thrombin was nearly identical to the extent of improvement in the reactivity of this mutant with FXa. Such a property resulted in the interesting observation that the rate accelerating effect of H5 on FXa (35-fold) and thrombin (44-fold) inhibition by this mutant was essentially similar (Tables I and II). Consistent with the results observed for FXa, the extent of the template effect of H70 (ratio of H70 to H5) in thrombin inhibition by the AT derivatives was not significantly altered. Taken together, these results suggested that reducing the length of the reactive site loop of AT by one and two residues has a reciprocal effect on the ability of the serpin to inhibit the two proteinases in the absence of cofactors, thus improving the inhibition rates with FXa and impairing them with thrombin.

Unlike all other mutants, the reactivity of des-R with FXa was not improved and was impaired most with thrombin. Based on previous results of our own (24) as well as those of others (25), it is believed that Arg$^{299}$ interacts productively with the acidic Glu$^{39}$ (chymotrypsin numbering) of both FXa and thrombin, thus its deletion impairs the rate of reaction with both proteinases. In support of this, the reactivity of wild type AT with the Glu$^{299}$ → Lys mutant of thrombin (24) was impaired ~5–10-fold independent of H5 (data not shown). Thus, the lack of improvement in the reactivity of this mutant with FXa is irrelevant for the conformational state of the reactive site loop of the mutant serpin.

Consistent with previous results (10, 22), a bell-shaped dependence on the H5 concentrations (optimal 20–200 nM) was observed for all AT derivatives in reaction with both FXa and thrombin (data not shown). Similar to previous results with the wild type AT (10), inhibition stoichiometries of ~1 in the absence of heparin, and ~1–1.5 in the presence of heparin, were observed for all mutant serpins in reaction with both FXa and thrombin. This was consistent with the observation that both proteinases formed SDS-stable complexes with all AT derivatives to a similar extent (data not shown), suggesting that the mutagenesis has not affected the substrate pathway of the reactions.

DISCUSSION

It has been hypothesized that AT exists in equilibrium between two conformationally active and inactive states (6, 8, 17). In the inactive state, which predominates the solution equilibrium of the serpin, the two N-terminal P14 and P15 residues of the reactive site loop of AT are inserted into the A-β-sheet of the molecule in the native conformation of the serpin (6, 8). This unique structural feature, which is only observed in AT renders the serpin inactive toward reaction with FXa (8), but it is not influential in reaction with thrombin (10). Recent crystal structure determination of AT in complex with H5 suggests that the binding of H5 on the β-helix of the serpin results in expulsion of the two N-terminal P14 and P15 residues of the reactive site loop of AT (22), which is accompanied by a 200–300-fold enhancement in the reactivity of AT with FXa with no significant effect on its reaction with thrombin (10). The molecular basis for differential reactivity of FXa and thrombin with the native and activated conformations of AT is not known. Relative to α1-antitrypsin, the reactive site loop of AT has three additional residues, Arg$^{299}$, Val$^{300}$, and Thr$^{301}$ at the C-terminal P' end of the loop (Fig. 1). Since the conformation of the reactive site loop in AT is mobile and linked to the heparin-binding β-helix of the serpin (3, 8), it was hypothesized in this study that there might be a size limitation for the length of the loop between the A and C sheets, and any excess in its length causes a constraint on the loop, leading to insertion of two N-terminal residues into β-sheet A and thus preventing the reactive site loop from accepting an optimal conformation to fit into the active site.
The values in the presence of optimal concentration of H70 (absence, but not in the presence of H5. These results suggest that the native equilibrium fraction of the des-VT conformational state (either preinserted or exposed). It follows, therefore, that the native equilibrium hypothesis of AT and for the first time provides an explanation for why fXa and thrombin differ in their ability to react with the native and heparin-activated conformations of the serpin.

The observation that a longer reactive site loop of AT is primarily responsible for the slow reactivity of the serpin with fXa, but is also required for AT to regulate the proteolytic activity of thrombin in the native conformation, has important implications for the physiological function of AT in circulation. AT, by virtue of its unique reactive site loop, can inhibit thrombin in the native conformation, but requires activation before it can effectively inhibit fXa. It follows, therefore, that fXa in vivo can be inhibited primarily by the small fraction of AT that is bound to the heparin-like molecules on the vasculature (6, 26). Otherwise, in the presence of a high concentration of circulating active AT (2.3 μM), maintenance of normal hemostasis would have been compromised due to rapid inactivation of fXa, which is generated at a minute concentration (at picomolar range) during the propagation phase of the coagulation cascade (27). On the other hand thrombin may be generated at very high concentrations (up to several hundred nM) under various (patho)physiological conditions (28). If AT were inactive toward thrombin in its native conformation, then thrombosis would be a common problem. The unique reactive site loop of AT therefore enables the serpin to effectively regulate the activity of several clotting enzymes without compromising hemostasis.

Finally, the results of this study suggest that, unlike the mutagenesis of the P site reactive site loop of AT, which interferes with the mechanics of loop insertion, thereby converting all highly reactive mutants to efficient substrates (9), the mutagenesis of the P residues of the C-terminal end of the reactive site loop of AT does not influence the substrate pathway of the reaction. Rather, it constitutively activates the mutant serpin. Such a mutagenesis approach holds a great promise for development of recombinant AT derivatives capable of specifically inhibiting fXa independent of polysaccharide cofactors.

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**Table I**

| Cofactor (× 10^5) | H5 (× 10^5) | H70 (× 10^5) | Fold catalytic effect |
|------------------|------------|-------------|----------------------|
| pAT | 2.6 ± 0.1 | 5.9 ± 0.4 | 17.7 ± 1.2 | 227 | 68,077 |
| rAT | 2.3 ± 0.1 | 7.3 ± 0.4 | 13.8 ± 0.7 | 317 | 60,000 |
| Des-R | 1.6 ± 0.1 | 2.6 ± 0.1 | 6.7 ± 0.2 | 163 | 41,875 |
| Des-V | 5.0 ± 0.5 | 2.7 ± 0.3 | 3.5 ± 0.2 | 54 | 7,000 |
| Des-T | 5.7 ± 0.6 | 3.5 ± 0.4 | 10.0 ± 0.4 | 61 | 17,544 |
| Des-VT | 27.0 ± 0.4 | 9.6 ± 0.9 | 12.8 ± 0.6 | 36 | 4,741 |

The second-order rate constants (k2) in both the absence and presence of H5 were determined by incubation of 1 nM fXa with 5–500 nM AT derivatives in TBS containing 0.1 mg/ml BSA, 0.1% PEG 8000, and 2.5 mM Ca^{2+} for 0.5–30 min as described under “Experimental Procedures.” The values of optimal concentration of H5 (50 nM) were determined by the same procedure except that 0.2 nM fXa was incubated with AT derivatives for 10–20 s. All values are averages of at least three independent measurements ± S.D.

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**Table II**

| Cofactor (× 10^5) | H5 (× 10^5) | H70 (× 10^5) | Fold catalytic effect |
|------------------|------------|-------------|----------------------|
| pAT | 7.3 ± 0.4 | 2.0 ± 0.1 | 14.7 ± 1.7 | 3 | 29,137 |
| rAT | 5.8 ± 0.3 | 1.7 ± 0.1 | 14.4 ± 0.5 | 3 | 24,828 |
| Des-R | 0.4 ± 0.1 | 0.4 ± 0.04 | 2.8 ± 0.4 | 10 | 70,000 |
| Des-V | 0.8 ± 0.5 | 0.6 ± 0.03 | 3.5 ± 0.3 | 8 | 43,750 |
| Des-T | 1.3 ± 0.2 | 0.9 ± 0.02 | 12.4 ± 1.7 | 7 | 95,385 |
| Des-VT | 0.5 ± 0.1 | 2.9 ± 0.1 | 13.8 ± 1.4 | 46 | 276,000 |

The second-order rate constants (k2) in both the absence and presence of H5 were determined by incubation of 1 nM thrombin with 50–500 nM AT derivatives in TBS containing 0.1 mg/ml BSA, 0.1% PEG 8000, and 2.5 mM Ca^{2+} for 2–60 min as described under “Experimental Procedures.” The values in the presence of optimal concentration of H5 (50 nM) were determined by the same procedure except that 0.2 nM enzyme was incubated with AT derivatives for 10–20 s. All values are averages of at least three independent measurements ± S.D.
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