Tryptophan 60-D in the B-Insertion Loop of Thrombin Modulates the Thrombin−Antithrombin Reaction†

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ABSTRACT: In a recent study it was demonstrated that thrombin des-PPW reacts with antithrombin (AT) very poorly. In this study it is shown that a Trp to Ala (W60A) mutant of thrombin also reacts with AT at a lower rate than thrombin. The inhibition kinetics were studied by the slow-binding kinetic approach. In both the presence and absence of heparin, the pseudo-first-order rate constant of thrombin inhibition (k_{obs}) increased linearly with AT concentration, indicating that inhibition, in the concentration range covered, conforms to a bimolecular reaction $E + I \xrightarrow{k_{assn}} E\text{-}I$. Only the second-order association rate constant (k_{assn}) for thrombin can be estimated [$6.8 \pm 2.7) \times 10^3$ M^{-1} s^{-1} in the absence of heparin and $(4.1 \pm 1.2) \times 10^6$ M^{-1} s^{-1} in the presence of heparin]. With W60A and des-PPW, the k_{obs} of inhibition increased hyperbolically as a function of AT concentration, indicating that the inhibition is a two-step process according to $E + I \overset{K_{init}}{\leftrightarrow} E{\cdot}I \xrightarrow{k_2} E\text{-}I$. The kinetic constants for W60A were estimated to be $K_{init} = 13.6 \pm 3.3$ μM and $k_2 = 0.007 \pm 0.001$ s^{-1} in the absence of heparin and $K_{init} = 13.6 \pm 3.1$ nM and $k_2 = 0.008 \pm 0.002$ s^{-1} in the presence of heparin. AT inhibited des-PPW very slowly [$k_{assn} = (2.9 \pm 0.7) \times 10^1$ M^{-1} s^{-1}], but heparin accelerated the reaction ≈20 000-fold and made it possible to demonstrate a two-step reaction mechanism for des-PPW with $K_{init} = 10.4 \pm 2.3$ nM and $k_2 = 0.006 \pm 0.001$ s^{-1}. In contrast to thrombin, an active AT-binding pentasaccharide enhanced the inhibition of des-PPW ≈15-fold. These results indicate that (1) in contrast to thrombin, the heparin-induced conformational change in AT is required for optimal inhibition of des-PPW and (1) Trp60 is essential for normal thrombin−AT reaction. On the basis of these results, a modified model for thrombin−AT interaction is proposed.

Several serine protease inhibitors (serpins) including antithrombin (AT),[1] α_1-protease inhibitor (α_1-PI), heparin cofactor II, and protease nexin I regulate the proteolytic function of thrombin in plasma (Potempa et al., 1994; Pratt & Church, 1993; Stone & Hermans, 1995). Among these serpins, AT is thought to be the most important physiological inhibitor of thrombin. AT by itself, however, inhibits thrombin at a relatively slow rate ($k_{assn} \approx 1 \times 10^4$ M^{-1} s^{-1}) (Damus et al., 1973; Olson & Shore, 1982). Heparin accelerates the reaction by forming a trimolecular complex with thrombin and AT which exhibits at least a 1000-fold increase in inhibition rate [$k_{assn} \approx (1-2) \times 10^7$ M^{-1} s^{-1}] (Olson & Shore, 1982).

The kinetics of AT inhibition of thrombin have been proposed to involve a two-step reaction mechanism consisting of an initial loose encounter complex formation with an equilibrium dissociation constant of $K_{init} = 1.4$ mM, followed by conversion to a kinetically stable complex with a high rate constant of $k_2 \approx 10$ s^{-1} (Olson & Shore, 1982; Stone & Hermans, 1995). In the case of thrombin, the primary effect of heparin is in the first step of the reaction. The template effect of heparin increases the affinity of the initial complex formation at least 1000-fold with approximately a 2-fold decrease in the inhibition rate constant of the final stable complex formation (Olson & Shore, 1982). Because AT inhibits thrombin with a high K_{init} and a high k_2, only by rapid kinetic methods in the presence of high concentrations of AT has it been possible to demonstrate the two-step reaction mechanism for thrombin−AT interaction (Olson & Shore, 1982).

Recently, the slow-binding kinetic approach (Morrison & Walsh, 1988) has been employed by a number of laboratories to demonstrate that the inhibition of other serine proteases by serpins follow a two-step reaction mechanism similar to that of thrombin−AT reaction. For instance, a two-step reaction mechanism was demonstrated for interaction of α_2-antiplasmin (α_2-AP) with plasmin and chymotrypsin (Longstaff & Gaffney, 1991), for α_1-PI with heparin-bound neutrophil elastase (Faller et al., 1993), and for cytoplasmic antiproteinase (CAP) with trypsin (Morgenstern et al., 1994).

In contrast to AT inhibition of thrombin, inhibition of the target proteases by the serpins mentioned above is characterized by a low K_{init} for the initial encounter complex formation with a subsequent slow rate (k_2) for tight and stable complex formation. Because of this kinetic behavior it has been possible to determine the K_{init} and the k_2 values for these protease−serpin interactions simply by generating progress curves of inhibition of the protease in the presence of reasonably low concentrations of inhibitor and competing chromogenic substrates specific for each enzyme. Using this method and employing the slow-binding kinetic approach

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[1] Abbreviations: des-PPW, thrombin mutant in which three residues, Pro60-B, Pro60-C, and Trp60-D [in the chymotrypsin numbering system of Bode et al., (1989)] are deleted; W60A, thrombin mutant in which Trp60-D is substituted with Ala; AT, antithrombin; α_1-PI, α_1-protease inhibitor; α_2-AP, α_2-antiplasmin; CAP, cytoplasmic antiprotease; BPTI, bovine pancreatic trypsin inhibitor; PEG, poly(ethylene glycol).

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(Morrison & Walsh, 1988), it has been possible to demonstrate that α_2-AP inhibits plasmin with a K_{init} of 8 nM and a k_2 of 0.006 s^{-1} (Longstaff & Gaffney, 1991), α_1-PI inhibits heparin-bound neutrophil elastase with a K_{init} of 80 nM and k_2 of 0.15 s^{-1} (Faller et al., 1993), and CAP inhibits trypsin with a K_{init} of 2 nM and k_2 of 0.006 s^{-1} (Morgenstern et al., 1994).

Previous studies with a deletion mutant of thrombin, des-PPW, indicated that the inhibition of this mutant by bovine pancreatic trypsin inhibitor (BPTI) was improved by 3 orders of magnitude (Le Bonniec et al., 1993). But thrombin des-PPW was inhibited by AT poorly, with a second-order association rate constant that was at least 100-fold lower (Le Bonniec et al., 1993). The reason for the poor reactivity of des-PPW and AT was not studied. In this study, when a Trp60-D to Ala (W60A) substitution mutant was prepared, its inhibition by AT was also impaired, suggesting that Trp60-D contributes positively to thrombin−AT reaction. The slow-binding kinetic approach is employed to determine the role of Trp in the thrombin−AT reaction. The results indicate that Trp60-D restricts the access of AT for the initial high-affinity complex formation, but once the complex is formed it contributes to the high rate of conversion of the reversible complex to a stable and irreversible complex. In a model proposed in this paper it is hypothesized that Trp60-D plays an important role in the transition-state stabilization of the thrombin-AT reaction.

MATERIALS AND METHODS

Mutagenesis, Expression, and Purification of Recombinant Proteins. The expression and purification of thrombin des-PPW has been previously described (Le Bonniec et al., 1993). The W60A mutant of thrombin was prepared by polymerase chain reaction (PCR) mutagenesis methods. The two mutagenesis primers were 5′-TCCTGTACCCGCCCgcGGA-CAAGAACTTCA-3′ as the sense primer and 5′-TGAAGT-TCTTGTCCgcGGGCGGGTACAGGA-3′ as the antisense primer (the mutant bases are shown in lower case). All manipulations were carried out in a prethrombin-1 derivative of prothrombin cDNA and then transferred to the mammalian expression vector pNUT-PL2. This vector is constructed by the modification of pNUT-hII (Le Bonniec et al., 1991). pNUT-PL2 contains all the features of the previously described RSV-PL4 expression vector (Rezaie & Esmon, 1992), which includes the sequence of the transferrin signal peptide for secretion and the sequence of a 12-residue epitope for a Ca^{2+}-dependent monoclonal antibody, HPC4, for purification. These elements are ligated downstream from the mouse metallothionein promoter and prethrombin-1 is expressed as a fusion protein with the HPC4 epitope. This vector also contains a mutant *dhfr* gene for selection in high concentrations of methotrexate (Le Bonniec et al., 1991). The expression vector containing the prethrombin-1 cDNA fragment or its W60A mutant was transferred to baby hamster kidney (BHK) cells by Lipofectin (Gibco BRL, Gathersburg, MD), and methotrexate-resistant clones were selected and grown in a 96-well plate. Supernatants were examined for expression by Western blot using the HPC4 antibody. High-expressing clones were identified, and after expansion the cell culture supernatant were collected, concentrated, and purified by immunoaffinity chromatography using HPC4 antibody linked to Affi-Gel 10 (Bio-Rad) as described (Le Bonniec et al., 1992). Prethrombin-1 activation by the prothrombinase complex (Le Bonniec et al., 1992) and purification on an FPLC Mono S column (Pharmacia) were previously described (Le Bonniec & Esmon, 1991).

Bovine AT was prepared and its concentration was determined as described (Owen, 1975; Kurachi et al. 1976). Recombinant thrombin, W60A, and des-PPW concentrations were determined by absorbance at 280 nm, assuming a molecular weight of 36 600 and extinction coefficients $E_{cm}^{1\%}$ of 17.1 for thrombin and 15.6 for both W60A and des-PPW. The concentration of active enzymes were also determined by active-site titration with *p*-nitrophenyl *p*′-guanidinobenzoate (Chase & Shaw, 1970) which agreed within 10% with the values calculated on the basis of absorbance at 280 nm. All proteins were homogeneous as determined by polyacrylamide gel electrophoresis in sodium dodecyl sulfate.

Unfractionated heparin (porcine intestinal mucosa, sodium salt, grade II) was purchased from Sigma. Spectrozyme TH (SPTH) was purchased from American Diagnostica, Greenwich, CT. The chromogenic substrate S2238 was purchased from Kabi Pharmacia/Chromogenix, Franklin, OH. The antithrombin-binding pentasaccharide (*N*-sulfonyl-6-*O*-sulfonyl-D-glucosamine)α1→4(D-glucuronic acid)β1→4(*N*-sulfonyl-3,6-di-*O*-sulfonyl-D-glucosamine)α1→4(2-*O*-sulfonyl)-L-iduronic acid)α1→4(*N*-sulfonyl-3,6-di-*O*-sulfonyl-D-glucosamine) was a generous gift from Dr. Jeffrey Weitz.

Kinetic Methods. Inhibition of thrombin, W60A, and des-PPW by AT in the presence of heparin was measured under pseudo-first-order conditions at room temperature with at least a 10-fold excess of AT over enzymes with a V_{max} kinetics microplate reader (Molecular Devices, Menlo Park, CA). A series of inhibition progress curves for each derivative were generated by adding 0.5 nM enzyme (final concentration) to wells of a 96-well polystyrene plate containing various concentrations of AT and 1 unit/mL heparin and 250 μM competing chromogenic substrate SPTH. In the case of W60A and des-PPW, the final concentrations of AT ranged from 0 to 200 nM. With thrombin, the highest concentration of AT used was 50 nM. In the presence of heparin, AT concentrations above 50 nM yielded curves with barely detectable amplitude and unreliable values for the rate constants. With W60A and des-PPW, AT concentrations of 0−50 nM were also used for the generation of inhibition progress curves, but in this case the concentration of the competing chromogenic substrate SPTH was reduced to 50 μM. In the absence of heparin, the inhibition rates were measured under the same conditions except that the concentration of AT was 0−73 μM and S2238 was used as the competing chromogenic substrate. Similar to the case with SPTH, the concentration of S2238 had to be adjusted for each enzyme to obtain exponentials reliable for the rate measurements. The optimal concentration of S2238 was 300 μM for thrombin and 150 μM for W60A. In both cases, however, only the data with less than 10% chromogenic substrate hydrolysis were analyzed. In the absence of heparin, by this method of analysis no inhibition was observed for des-PPW. For des-PPW in the absence of heparin, only the second-order association rate constant was estimated by a discontinuous assay (see below). In all measurements the reaction buffer contained 0.02 M Tris-HCl, pH 7.5, 0.1 M NaCl, 1 mg/mL bovine serum albumin

(BSA), and 0.1% poly(ethylene glycol) 8000 (PEG 8000).

Data from each curve at different inhibitor concentrations were fitted to the integrated rate equation for slow-binding inhibition (Morrison & Walsh, 1988) by nonlinear regression analysis using the Enzfitter computer program (R. J. Leatherbarrow, Elsevier, Biosoft):

$$A = v_s t + (v_0 - v_s)(1 - e^{-k_{obs}t})/k_{obs} + A_0 \qquad (1)$$

where A is absorbance at 405 nm at time t, v_0 and v_s are initial and final steady-state velocity, k_{obs} is the apparent first-order-rate constant, and A_0 is the initial absorbance at 405 nm. Fitting estimates values for v_0, v_s, k_{obs}, and A_0 for each progress curve. These values are analyzed by different methods to obtain inhibition and reaction rate constants (see below). The K_m values for SPTH hydrolysis at room temperature in TBS containing 1 mg/mL BSA, 0.1% PEG 8000, and 1 unit/mL heparin were 6 μM for thrombin, 12 μM for W60A, and 16 μM for des-PPW. The K_m values for S2238 hydrolysis were 4 μM for thrombin, 24 μM for W60A, and 53 μM for des-PPW.

The inhibition of thrombin and the mutants in the absence or presence of the pentasaccharide were examined by a discontinuous assay method. In this case thrombin, W60A, or des-PPW (1 nM) was incubated with at least a 10-fold excess of AT (10−500 nM for thrombin and 150 nM−10 μM for W60A and des-PPW) in the absence or presence of 10 μM pentasaccharide at room temperature for 20 min in TBS buffer containing 1 mg/mL BSA and 0.1% PEG 8000. The volume of the reaction was 50 μL in 96-well polystyrene plates. After 20 min of incubation, 50 μL of SPTH was added to a final concentration of 200 μM and the residual thrombin activity was determined from the rate of chromogenic substrate hydrolysis measured with a V_{max} kinetics microplate reader. The second-order association rate constants (k_{assn}) were calculated using

$$k_{assn} = (-\ln a)/t[I] \qquad (2)$$

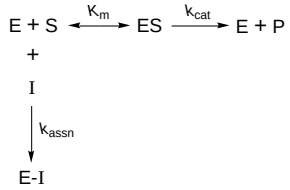
where a is residual proteinase activity, t is time, and [I] is the AT concentration.

RESULTS

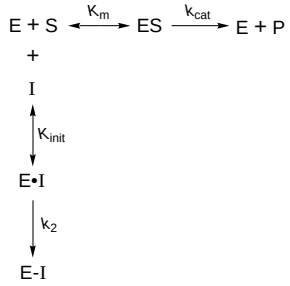
Recombinant thrombin, des-PPW, and W60A were prepared as described in Materials and Methods. It was previously demonstrated that deletion of the three residues Pro, Pro, and Trp from the B-insertion loop of thrombin does not adversely affect the charge stabilizing system and the reactivity of the catalytic triad (Le Bonniec et al., 1993). As expected, this was also true for the W60A mutant. The SPTH hydrolysis by recombinant thrombin, W60A, and des-PPW were all similar. W60A and des-PPW demonstrated higher K_m values toward SPTH, but at the saturating concentration of chromogenic substrates, the mutants hydrolyzed SPTH faster than thrombin. The kinetic constants of SPTH hydrolysis at room temperature in TBS containing 1 mg/mL BSA, 0.1% PEG 8000, and 1 unit/mL heparin were, for thrombin, $K_m = 6$ μM and $k_{cat} = 18$ s^{-1}; for W60A, $K_m = 12$ μM and $k_{cat} = 33$ s^{-1}; and for des-PPW, $K_m = 16$ μM and $k_{cat} = 36$ s^{-1}.

Slow-Binding Kinetic Analysis. Recently a number of laboratories used the slow-binding kinetic approach (Morrison & Walsh, 1988) to analyze the inhibition of proteases by several serpins (Stone & Hermans, 1995; Longstaff & Gaffney, 1991; Morgenstern et al., 1994; Longstaff et al., 1990). In these studies, the kinetic parameters of inhibition are determined in the presence of a competing chromogenic substrate using equation 1. This method of analysis can be described by Scheme 1 or 2 (Tian & Tsou, 1982), where E is enzyme, S is a chromogenic substrate specific for the enzyme, I is inhibitor, E-I is the final stable complex, and E·I is an intermediate reversible complex whose concentration is described by K_{init}.

Scheme 1



Scheme 2



Scheme 1 predicts that the k_{obs} values, as determined by eq 1, will increase linearly with the inhibitor concentration and only the second-order association rate constant (k_{assn}) for the inhibition can be estimated from eq 3. Scheme 2, on the other hand, predicts that k_{obs} values will increase hyperbolically with the inhibitor concentration, and the values of k_2 and K_{init} can be estimated using eq 4.

$$k_{obs} = k_{assn}[I]/(1 + [S]/K_m) \qquad (3)$$

$$k_{obs} = k_2[I]/([I] + K_{init}(1 + [S]/K_m)) \qquad (4)$$

These methods of kinetic analysis were used to study the inhibition of thrombin, W60A, and des-PPW by AT. Inhibition was studied in the presence and absence of heparin for thrombin and W60A and only in the presence of heparin for des-PPW. In the absence of heparin, inhibition of des-PPW by AT is markedly imparied and no inhibition rate measurement was possible in the presence of a competing chromogenic substrate. First, to determine the optimal concentration of heparin for acceleration of inhibition by AT, the inhibition of thrombin, W60A, and des-PPW by AT was performed in the presence of various concentrations of heparin. As shown in Figure 1, the optimal concentration of heparin was 0.5 unit/mL for thrombin and 1 unit/mL for both W60A and des-PPW. Then, a series of progress curves were generated for each enzyme with a range of AT and chromogenic substrate concentrations under optimized conditions described in Materials and Methods. An example of such progress curves for W60A in the presence of 1 unit/mL heparin is shown in Figure 2. Data from 3−11 independent experiments for each enzyme were fitted to eq 1 to obtain values for k_{obs}. To determine between Schemes

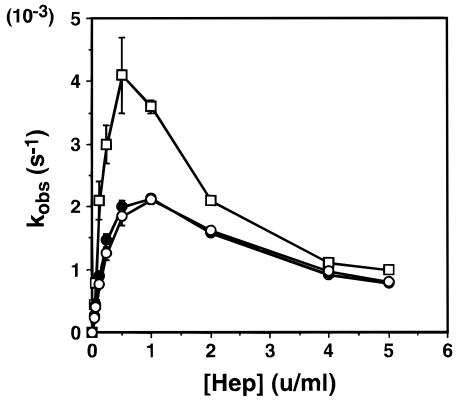


FIGURE 1: Heparin concentration dependence of thrombin, W60A, and des-PPW inhibition by AT. Thrombin (□), W60A (●), or des-PPW (○) (0.5 nM) was added to reactions containing SPTH (250 μM), AT (50 nM for thrombin and 200 nM for both W60A and des-PPW inhibition), and indicated concentrations of heparin in TBS buffer containing 1 mg/mL BSA and 0.1% PEG 8000. The inhibition reactions were carried out for 30 min at room temperature. The pseudo-first-order association rate constant (k_{obs}) for inhibition was determined by fitting the data to eq 1 as described in Materials and Methods. The k_{obs} values from an average of three independent measurements are plotted as a function of heparin concentration.

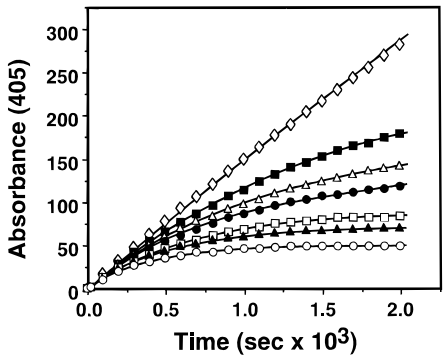


FIGURE 2: Typical progress curves for inhibition of W60A by AT in the presence of 1 unit/mL heparin. W60A (0.5 nM) was added to reactions containing 250 μM SPTH and varying concentrations of AT in complex with 1 unit/mL heparin in TBS buffer containing 1 mg/mL BSA and 0.1% PEG 8000. The concentration of AT in reactions were 0 (◇), 31 nM (■), 50 nM (△), 62 nM (●), 100 nM (□), 150 nM (▲), and 200 nM (○). The pseudo-first-order association rate constant (k_{obs}) for inhibition was determined by fitting the data to eq 1 as described in Materials and Methods. Only values for every 100 s are plotted.

1 and 2, the k_{obs} values were plotted as a function of AT concentrations. With thrombin, in both the presence (Figure 3A) and absence (Fig. 3B) of heparin the k_{obs} values increased linearly with the AT concentration. In this case only the values for k_{assn} were obtained for thrombin from equation 3 and they are shown in Table 1. In contrast to thrombin, the k_{obs} values of W60A both in the presence (Fig. 4A) and in the absence of heparin (Fig. 4B) and those of des-PPW (Fig. 4A), measured only in the presence of heparin, increased hyperbolically with AT concentration, suggesting that the inhibition is a two-step process as described by Scheme 2 and eq 4. Nonlinear regression analysis of data fitted to eq 4 estimates $K_{init} = 13.6 \pm 3.3$ μM, $k_2 = 0.0007 \pm 0.001$ s^{-1} ($n = 3$, ± SD) in the absence of heparin and $K_{init} = 13.6 \pm 3.1$ nM and $k_2 = 0.008 \pm 0.002$ s^{-1} ($n = 6$, ± SD) in the presence of heparin for W60A. These values for des-PPW in the presence of heparin were $K_{init} = 10.4 \pm 2.3$ nM and $k_2 = 0.006 \pm 0.001$ s^{-1} ($n = 11$, ± SD). The values for the second-order association rate constant were calculated from

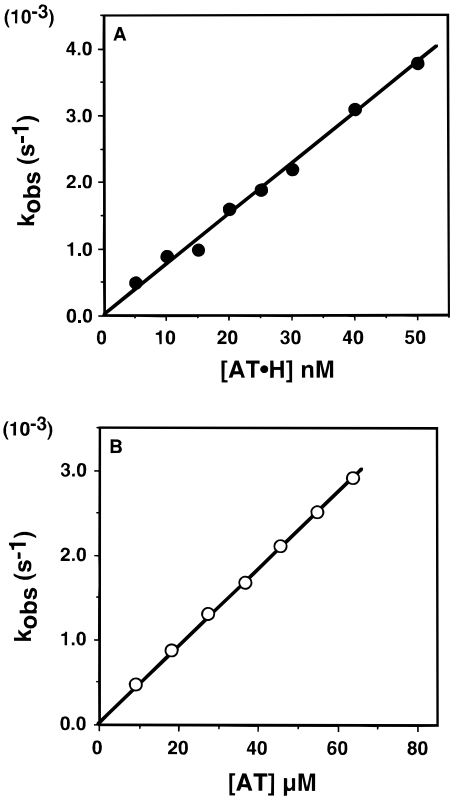


FIGURE 3: Dependence of k_{obs} on AT concentration with thrombin in the presence and absence of heparin. (A) The k_{obs} values for thrombin inhibition at different concentration of AT in the presence of heparin were calculated from a series of progress curves similar to those in Fig. 2. The slope of the straight line represents the second-order association rate constant (k_{assn}) of thrombin inhibition according to eq 3. (B) Same as panel A except that the k_{obs} values for inhibition is determined in the absence of heparin.

Table 1: Kinetic Constants for Inhibition of Thrombin, W60A, and des-PPW by AT

enzyme	heparin	K_{init}	k_2 (s^{-1})	k_{assn} (M^{-1} s^{-1})
thrombin[a]	−			$(6.8 \pm 2.7) \times 10^3$
	+			$(4.1 \pm 1.2) \times 10^6$
W60A[b]	−	13.6 ± 3.3 μM	0.007 ± 0.001	$(5.2 \pm 1.0) \times 10^2$
	+	13.6 ± 3.1 nM	0.008 ± 0.002	$(5.9 \pm 1.3) \times 10^5$
des-PPW[c]	−			$(2.9 \pm 0.7) \times 10^1$
	+	10.4 ± 2.3 nM	0.006 ± 0.001	$(5.8 \pm 1.8) \times 10^5$

[a] By the slow-binding kinetic approach using SPTH as the competing chromogenic substrate, no estimates of K_{init} or k_2 were obtained for thrombin and only the k_{assn} values were calculated using eq 3 as described in Results. [b] By the slow-binding kinetic approach a two-step inhibition mechanism was observed for W60A in both the presence and absence of heparin. The k_{assn} values are calculated from the relationship $k_{assn} = k_2/K_{init}$. [c] Only in the presence of heparin was a two-step inhibition mechanism observed for des-PPW. The k_{assn} value in the absence of heparin was estimated by a discontinuous assay method using eq 2 as described in Materials and Methods. All values are the average of 3−11 independent measurements ± SD.

the relationship $k_{assn} = k_2/K_{init}$ (Stone & Hermans, 1995). These values are all presented in Table 1.

To ensure that the inhibition kinetic behavior observed with W60A and des-PPW is not due to higher concentrations of AT in the reactions (200 nM), the inhibition in the presence of 1 unit/mL heparin was also studied with AT concentrations of 0−50 nM, the same concentration used for thrombin inhibition. In this case, however, the concentration of SPTH was reduced to 50 μM and the concentration of enzymes was reduced to ≈0.2 nM to assure that the

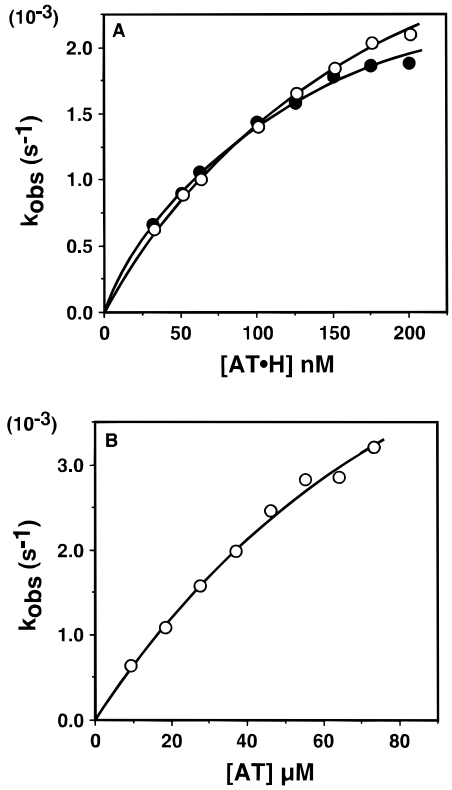


FIGURE 4: Dependence of k_{obs} on AT concentration with W60A and des-PPW in the presence of heparin and with W60A in the absence of heparin. (A) The k_{obs} values for W60A (○) and des-PPW (●) are calculated from progress curves similar to those shown in Fig. 2 using eq 1 as described in Materials and Methods. The data were fitted to eq 4, a hyperbolic relationship that describes the dependence of k_{obs} as a function of inhibitor concentration for a two-step reaction mechanism described by Scheme 2 in Results. (B) Same as panel A except that the k_{obs} values for inhition for only W60A is determined in the absence of heparin.

chromogenic substrate hydrolysis was linear for the duration of the experiment. Under this condition a two-step reaction mechanism for both W60A and des-PPW was again observed (data not shown). The inhibition reactions were also carried out with S2238 as the chromogenic substrate and similar results were observed (data not shown). In all experiments only the data with less than 10% chromogenic substrate hydrolysis have been analyzed.

As noted, by this kinetic method only a single-step bimolecular association mechanism is demonstrated for the thrombin−AT interaction according to Scheme 1. This observation is not at odds with previous studies where a two-step reaction mechanism for the thrombin−AT interaction has been demonstrated (Olson & Shore, 1982). But it implies that due to a high K_{init} value, under the experimental conditions, it is not possible to saturate thrombin with AT to observe a limiting rate for the k_2 estimation and the resolution of these reaction steps. A simple calculation indicates that with 50 nM AT and 250 μM SPTH (K_m = 6 μM) the highest K_{init} value which can be determined for thrombin under this experimental conditions from the equation [I]/(1 + [S]/K_m), is only ≈ 1 nM, which is much lower than the K_{init} value of 120 nM determined before (Olson & Shore, 1982).

The second-order association rate constant of des-PPW with AT in the absence of heparin was obtained by a discontinuous assay method and using equation 2 as described in Materials and Methods. As shown in Table 1, AT inhibited des-PPW ≈234-fold more slowly [k_{assn} = (2.9 ± 0.7) × 10^1] than thrombin [k_{assn} = (6.8 ± 2.7) × 10^3]. But AT inhibited des-PPW in the presence of heparin with a second-order association constant that was only ≈10-fold lower than that for thrombin [k_{assn} = (4.1 ± 1.2) × 10^6 M^{-1} s^{-1} for thrombin and (5.8 ± 1.8) × 10^5 M^{-1} s^{-1} for des-PPW]. Heparin enhanced AT inhibition of W60A ≈1100-fold [k_{assn} = (5.2 ± 1.0) × 10^2 M^{-1} s^{-1} in the absence and (5.9 ± 1.3) × 10^5 M^{-1} s^{-1} in the presence of heparin] and that of des-PPW 20 000-fold (Table 1).

To understand the reason for the lower reactivity of des-PPW with AT in the absence of heparin, the inhibition was also studied by the discontinuous assay method in the presence of an active AT-binding pentasaccharide (Olson & Shore, 1986; Ye et al., 1994). The active pentasaccharide is known to enhance the inhibition rate of thrombin less than 2-fold (Craig et al. 1989). In this study, the pentasaccharide enhanced inhibition rate of thrombin ≈2-fold, W60A ≈3-fold, and des-PPW ≈15-fold (data not shown). These results suggest that the heparin conformer of AT fits better to the active site of des-PPW.

To ensure that des-PPW and W60A form kinetically pseudoirreversible complexes with AT, dilution experiments were performed where the mutant enzymes were totally inhibited by AT + heparin and then diluted into buffer containing no heparin, and the dissociation of the complex was measured by the reappearance of the amidolytic activity monitored by the hydrolysis of Spectrozyme TH. Similar to thrombin less than 1% of the amidolytic activity was recovered for both mutants during 8 h of measurements, suggesting that the inhibitor−protease complex is essentially irreversible (data not shown). Furthermore, SDS−PAGE analysis of the enzyme−AT reactions indicated that AT is not proteolytically cleaved by any of the mutant enzymes (data not shown).

DISCUSSION

Recently, the slow-binding kinetic approach (Morrison & Walsh, 1988) has been used to study the inhibition mechanisms of several serine proteases by their serpin inhibitors (Longstaff & Gaffney, 1991; Longstaff et al., 1990; Faller et al., 1993; Morgenstern et al., 1994; Stone & Hermans, 1995). These kinetic studies suggest that most serpin−protease interactions take place by a two-step reaction mechanism: an initial loose Michaelis complex similar to that of natural substrate−protease interaction followed by isomerization to a tight complex. In this kinetic process the first step is described by an equilibrium dissociation constant K_{init} and the second step is a rate constant k_2 for the conversion of a reversible complex to a kinetically stable complex. With serpin−protease reactions in which the K_{init} of initial complex formation is in the nanomolar range and the rate constant k_2 is also low (i.e., α_2-AP inhibition of plasmin; Longstaff & Gaffney, 1991), it has been possible to resolve these two kinetic steps in the presence of competing chromogenic substrates and low concentrations of inhibitors using conventional kinetic instruments. In the case of the thrombin−AT reaction, however, where the two-step kinetic mechanism is characterized by a high K_{init} and high k_2, only by rapid kinetic measurements has it been possible to resolve these two steps (Olson & Shore, 1982).

In this study, in the presence of competing chromogenic substrate it was possible to demonstrate that deletion of three residues, Pro, Pro, and Trp, from the B-insertion loop or substitution of only Trp with Ala result in thrombin mutants, which, in contrast to AT inhibition of thrombin but similar to α_2-AP inhibition of plasmin (Longstaff & Gaffney, 1991), are inhibited by AT with a kinetic mechanism characterized by a low K_{init} and low k_2. These results suggest that Trp in this insertion loop may restrict the access of AT to the active-site pocket of thrombin for the initial weak complex formation. This is consistent with the observation that the indole moiety of Trp60-D is fully exposed to solvent in the crystal structure of thrombin, which is hypothesized to contribute to the narrow specificity of thrombin by restricting the access of the macromolecular substrates and inhibitors to the active-site pocket of thrombin (Bode et al., 1989). This hypothesis is also supported by the previous observation that BPTI inhibits des-PPW with an equilibrium constant that is over 3 orders of magnitude tighter than thrombin (Le Bonniec et al., 1993). In the case of AT inhibition, however, the improvement in the initial complex formation is more than compensated by a dramatic decrease in the rate of stable complex formation. Therefore, Trp60-D appears to play a dual role in thrombin−AT interaction: it slows the reaction by blocking the active-site pocket which increases the K_{init} for the initial complex formation, it enhances the reaction by trapping the inhibitor−enzyme in a stable complex, probably through the transition-state stabilization.

The results of this study suggest that Trp60-D destabilizes the initial thrombin−AT complex formation, a role that is also plays in thrombin−BPTI complex formation. In the thrombin−AT reaction, however, once the initial thrombin−AT complex is formed, Trp60-D clamps on the inhibitor, increasing the rate constant of conversion of the initial complex to a more stable and irreversible complex. It appears, therefore, that the stability of the final thrombin−AT complex, in contrast to the case with BPTI, is determined by the kinetic rather thermodynamic stabilization, and overall, Trp60-D makes a positive contribution to the thrombin−AT reaction.

It has been suggested that serpins by insertion of the reactive site loops into the A β-sheet, trap their target proteases in a stable complex either in a tetrahedral intermediate or at an acyl-intermediate stage of the reactive bond cleavage (Potempa et al., 1994). Whatever the intermediate stage, one hypothesis is that insertion of the reactive-site loop into the A β-sheet creates a canonical-type conformation similar to that of low molecular weight protease inhibitors such as BPTI (Carrell et al., 1991; Bode & Huber, 1992). Recently, the result of a study by Olson et al., (1995) with Ser-294 → Leu mutant of AT at the P1′ position of the reactive site loop (AT-Denver) argues against this canonical hypothesis of the serpin−protease interaction. Instead, Olson et al., favor a model in which the stable complex is the result of a kinetic rather than thermodynamic trapping mechanism of thrombin−AT interaction (Olson et al., 1995). Olson et al., propose that, following initial complex formation, the active-site serine of thrombin attacks the P1 residue of AT on the reactive-site loop, which induces a conformational change in the inhibitor promoting the transition-state stabilization leading to formation of a tetrahedral intermediate. In this model, it is the formation of a tetrahedral intermediate which leads to the insertion of the reactive-site loop into the A β-sheet of the inhibitor, which results in trapping of enzyme at the intermediate stage.

The results of this study support the model proposed by Olson et al., (1995), where the attack by the catalytic serine on the P1 residue induces a conformational change in the inhibitor which is complementary to the active-site pocket of thrombin. The crystal structure of thrombin suggests that Trp60-D is part of the S2 specificity pocket (Bode et al., 1989); Stubbs et al., 1992). In the model proposed here, the altered conformation of the inhibitor is stabilized by Trp60-D and in the absence of this residue the final stable complex forms at a very slow rate. It is therefore hypothesized that Trp60-D plays a key role in transition-state stabilization of the thrombin−AT complex. In this model, similar to that of Olson et al., the insertion of the reactive-site loop into the A β-sheet of the inhibitor for the final stable complex formation occurs after the tetrahedral intermediate stage of the thrombin−AT reaction.

The result of a previous study indicated that protein C activation and fibrinopeptide A release were both impaired with des-PPW (Le Bonniec et al., 1993). The kinetic analysis indicated that in both cases the k_{cat} values for the reactions were impaired with minimal change in the K_m values for the substrates. Trp60-D in thrombin probably plays a similar role in interaction with both macromolecular substrates and inhibitors, the stabilization of the transition state in both reactions. It should, however, be noted that with the synthetic chromogenic substrates SPTH and S2238, the K_m and the k_{cat} values for both mutants are increased. The reason for the opposite kinetic mechanism with the chromogenic substrates is not known. But, in addition to lacking a P′ residue, the chromogenic substrates have also an unusual P3 residue that is in the D-configuration which is linked with other bulky chemical groups. It is possible that these differences in the structure orient the synthetic substrates to a different location in the specificity pocket of thrombin.

It has been demonstrated in the past that the heparin acceleration mode of AT inhibition of factor Xa is not the same as that of thrombin (Craig et al., 1989). With thrombin inhibition, the primary function of heparin is to increase the affinity of the initial complex by a template mechanism through trimolecular complex formation (Olson & Shore, 1982). In the case of factor Xa, it is thought that the accelerating effect of heparin is not by a template mechanism since, in contrast to thrombin, an active AT-binding pentasaccharide, not long enough for trimolecular complex formation, enhances factor Xa inhibition (Craig et al., 1989). In acceleration of factor Xa inhibition, the primary effect of herapin is the activation of AT by a conformational change in the reactive-site loop. In contrast to thrombin, factor Xa preferentially recognizes the heparin-stabilized conformer of AT (Craig et al., 1989). The pentasaccharide acceleration of AT inhibition of des-PPW suggests that the PPW loop may also restrict the docking of the active conformer of AT into the active site of thrombin for optimal inhibition.

In conclusion, the result of this study indicates that the kinetic mechanism of AT inhibition of des-PPW and W60A resembles that of α_2-antiplasmin inhibition of plasmin, a reaction characterized by a low initial K_{init} but also a low k_2 for conversion to a stable complex. Trp in the B-insertion loop appears to prevent rapid, high-affinity association between thrombin and AT. Thus it is intriguing to hypothesize that the B-insertion loop is an evolutionary adaptation

that may slow thrombin inhibition in solution to allow the enzyme to perform its function.

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