Identification of Thrombin Residues That Modulate Its Interactions with Antithrombin III and α1-Antitrypsin†

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ABSTRACT: The role of thrombin's catalytic groove in the interaction with serpin has been investigated by comparing the association rate constant (k_{on}) of several mutated thrombins with various serpins. The results indicated that Glu[192], located three residues prior to the catalytic serine, and the major insertion in the sequence of thrombin compared with trypsin (residues Tyr[60A]−Trp[60D]) play an important role in modulating thrombin's interactions with serpins. Replacement of Glu[192] by glutamine increased by 3 orders of magnitude the k_{on} value with α1-antitrypsin (which has a P_1 methionine) but did not markedly alter the k_{on} value with serpins containing a P_1 arginine. The des-PPW thrombin mutant (lacking residues Pro[60B], Pro[60C], and Trp[60D]) exhibited a similar k_{on} value as thrombin with protease nexin-1 but a k_{on} value 2 orders of magnitude lower with antithrombin III. Thus, the 60-loop insertion of thrombin appears critical for its interaction with antithrombin III but dispensable for the formation of a complex with protease nexin-1. Heparin increased markedly the k_{on} values for antithrombin III and protease nexin-1 with all thrombin variants tested, but a more dramatic effect was observed with a thrombin mutant (des-ETW) lacking residues Glu[146], Thr[147], and Trp[148] (on the opposite side of the catalytic site relative to the 60-loop insertion). At the optimum concentration, heparin increased the k_{on} value of the des-ETW−antithrombin III interaction by nearly 5 orders of magnitude, considerably more than for thrombin, suggesting that heparin is able to compensate in part for the adverse effects of the des-ETW mutation on the structure of thrombin.

The serpins constitute a family of 400−450-residue proteins which selectively inhibit serine proteases (Huber & Carrell, 1989; Bode & Huber, 1992). The mechanism of inhibition involves an initial loose association, which subsequently evolves to yield a tight complex (Olson & Shore, 1982; Stone & Hermans, 1995). This reaction involves binding of a reactive-site loop, which connects two elements of secondary structure within the serpin (the A and C β-sheets; Bode & Huber, 1992), to the protease's catalytic groove. The reactive-site loop contains a P_1 residue which plays a major role in determining the serpin specificity and presumably occupies the primary specificity pocket of the enzyme in the complex. The exact structure of the enzyme−inhibitor complex, however, is not fully understood. In particular, it is not known if the serpin's reactive-site loop adopts, within the catalytic groove of the protease, the same canonical conformation as the reactive-site loops of the Kunitz- and Kazal-type inhibitors (Laskowski & Kato, 1980; Bode & Huber, 1992).

Antithrombin III (ATIII; Travis & Salvesen, 1983; Bode & Huber, 1992)[1] and protease nexin-1 (PN1; Baker et al., 1980; Gloor et al., 1986; Sommer et al., 1987) are two serpins which both have an arginine as the P_1 residue and inhibit thrombin very efficiently in the presence of heparin (Olson & Shore, 1981; Stone et al., 1987, 1994; Pratt et al., 1992). The serpin α1-antitrypsin (α1-$AT_{(P_1=Met)}$, also called α1-protease inhibitor; Travis & Salvesen, 1983; Huber & Carrell, 1989) has methionine as the P_1 residue and inhibits members from the three classes of serine proteases (elastase-, chymotrypsin- and trypsin-like enzymes). α1-Antichymotrypsin (α1-$AC_{(P_1=Leu)}$) with a leucine as the P_1 residue (Travis & Salvesen, 1983; Baumann et al., 1991; Wei et al., 1994) inactivates several chymotrypsin-like proteases but not trypsin. Neither α1-$AT_{(P_1=Met)}$ nor α1-$AC_{(P_1=Leu)}$ inhibit thrombin, but with both serpins, single replacement of the P_1 residue by arginine dramatically alters their specificity: α1-$AT_{(P_1=Arg)}$ and α1-$AC_{(P_1=Arg)}$ efficiently inactivate several trypsin-like proteases, including thrombin (Owen et al., 1983; Rubin et al., 1990).

In previous studies, we have identified several motifs of thrombin involved in its specificity. Substitution in the catalytic groove of Glu[192] by glutamine, three residues prior

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[1] Abbreviations: des-ETW, mutated thrombin where amino acids Glu[146], Thr[147], and Trp[148] have been deleted [The amino acid sequence numbering of thrombin, as suggested by Bode et al. (1989), is based on its three-dimensional topological identity with chymotrypsin, and insertion residues are marked by capital letters in alphabetic order.]; des-PPW, mutated thrombin where amino acids Pro[60B], Pro[60C], and Trp[60D] have been deleted; E192Q, mutated thrombin where Glu[192] has been replaced by glutamine; ATIII, antithrombin III; PN1, protease nexin-1; α1-$AT_{(P_1=Met)}$, α1-antitrypsin; α1-$AT_{(P_1=Arg)}$, Pittsburgh variant of α1-antitrypsin with arginine as the P_1 residue (The residues of the serpin's reactive-site loop are numbered from P_4 to P_4', where P_4 and P_4' refer to four residues removed from the cleavage site on the amino and carboxyl sides, respectively.); α1-$AC_{(P_1=Leu)}$, α1-antichymotrypsin; α1-$AC_{(P_1=Arg)}$, α1-antichymotrypsin mutant with arginine as the P_1 residue.

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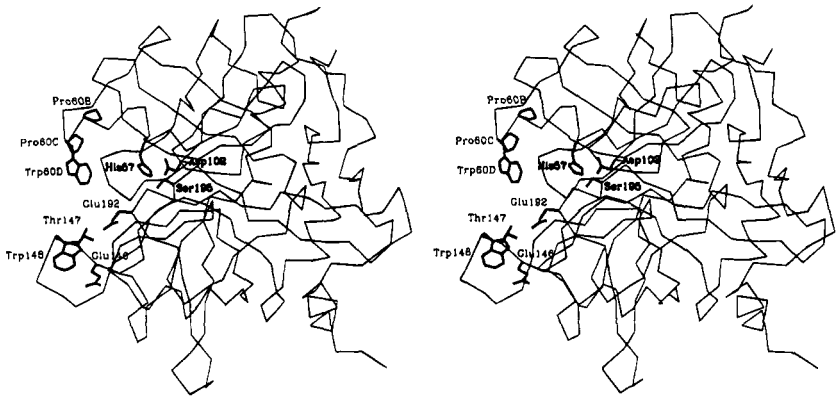


FIGURE 1: Stereodiagram of the thrombin catalytic groove. Coordinates are from Bode et al. (1989). Side chains are fully drawn only for the sites of mutation and the catalytic residues (boldface); other amino acids are represented by their α-carbon chain. Substitution of Glu^{192} for glutamine is sufficient to allow inhibition by α1-$AT_{(P_1=Met)}$. Deletion of Pro^{60B}, Pro^{60C}, and Trp^{60D} impairs interaction with ATIII but not that with PN1.

to the catalytic Ser^{195} (Figure 1), broadens the enzyme's specificity, without loss of the normal functions. Fibrinogen cleavage by this mutant (E192Q) is essentially normal, but in contrast to thrombin, E192Q activates bovine factor X (Le Bonniec et al., 1992a). Furthermore, protein C activation by E192Q is, in the absence of thrombomodulin, 20-fold faster than by thrombin (Le Bonniec & Esmon, 1991). Deletion of residues Pro^{60B}, Pro^{60C}, and Trp^{60D} from the 60-loop insertion of thrombin, to give the des-PPW mutant, also transforms thrombin's specificity, but unlike E192Q, several activities characteristic of thrombin are greatly diminished (e.g., fibrinogen cleavage; Le Bonniec et al., 1993). Interestingly, des-PPW and E192Q are both much more sensitive to bovine pancreatic trypsin inhibitor than is thrombin (Guinto et al., 1994). Finally, the des-ETW mutant (Le Bonniec et al., 1992b) consists of the deletion of Glu^{146}, Thr^{147}, and Trp^{148}, within the autolysis loop of thrombin (Bode et al., 1989). This mutation impairs most thrombin functions due to a reduced reactivity of the charge-stabilizing system, as a consequence of the relatively large conformational change induced by the deletion (Le Bonniec et al., 1992b, 1994).

Although the importance of the interaction between the P_1 residue of the serpin and the primary specificity pocket of the protease is firmly established, the role of other interactions of the serpin's reactive-site loop within the catalytic groove of the protease has been less well studied. To identify motifs in thrombin involved in serpin binding, we have compared the association rate constant (k_{on}) of ATIII, PN1, α1-$AT_{(P_1=Met)}$, α1-$AT_{(P_1=Arg)}$, α1-$AC_{(P_1=Leu)}$, and α1-$AC_{(P_1=Arg)}$ with thrombin, E192Q, des-PPW, des-ETW, and trypsin, both in the presence and in the absence of heparin. Our results indicate that thrombin's Glu^{192} impairs markedly the interaction with α1-$AT_{(P_1=Met)}$, that thrombin's 60-loop insertion ensures optimum interaction with ATIII but is not essential for PN1 binding, and that heparin compensates in part for the adverse structural damages induced in thrombin by the des-ETW mutation.

MATERIALS AND METHODS

Proteins. Plasma-derived thrombin (Stone & Hofsteenge, 1986) and the thrombin mutants E192Q (Le Bonniec & Esmon, 1991), des-ETW (Le Bonniec et al., 1992b), and des-PPW (Le Bonniec et al., 1993) were prepared and purified as described previously. Trypsin was from Worthington (Lorne Laboratories, England). ATIII was isolated from human plasma as previously described (McKay, 1981), and recombinant PN1, expressed in yeast, was a generous gift from Dr. D. Monard (Friedrich Miescher-Institut, Basel, Switzerland). Recombinant α1-$AT_{(P_1=Met)}$ and the Pittsburgh variant α1-$AT_{(P_1=Arg)}$ were expressed in *Escherichia coli* and purified as described previously (Hopkins et al., 1993). Recombinant α1-$AC_{(P_1=Leu)}$ and its mutant with an arginine in the P_1 position (α1-$AC_{(P_1=Arg)}$) were expressed in *E. coli* and purified essentially as described (Rubin et al., 1990), using the expression vector pZMS-ACT, generously provided by Dr. H. Rubin (University of Pennsylvania, Philadelphia, PA).

Reagents. The substrates H-D-Phe-pipecolyl-Arg-*p*-nitroanilide (S-2238), H-D-Val-Leu-Arg-*p*-nitroanilide (S-2266), and H-D-Pro-Phe-Arg-*p*-nitroanilide (S-2302) were purchased from Chromogenix (Mölndal, Sweden); H-D-cyclohexylglycyl-Ala-Arg-*p*-nitroanilide (Pefachrome TH) was from Pentapharm (Basel, Switzerland). The concentrations of intact substrate and of *p*-nitroaniline released after cleavage were determined spectrophotometrically by using absorption coefficients of 8270 M^{-1} cm^{-1} at 342 nm and 9230 M^{-1} cm^{-1} at 400−410 nm, respectively (Lottenberg & Jackson, 1983; Stone et al., 1991). Unfractionated porcine mucosal heparin was from Grampian Enzymes (Aberdeen, U.K.).

Titration and Stability of the Enzymes. All kinetic experiments were performed at 37 °C in 0.05 M Tris-HCl, pH 7.8, containing 0.1 M NaCl, 0.2% (w/v) poly(ethylene glycol) (M_r 6000), and 1 mg/mL bovine serum albumin (protease-free, Sigma). Enzyme concentrations were determined by active-site titration using H-D-Phe-Pro-Arg-CH_2-Cl (PPACK, Calbiochem; Kettner & Shaw, 1981). Enzyme stability was assessed according to the method of Selwyn (1965), by comparing the time courses of substrate hydrolysis at three enzyme concentrations, in the absence of inhibitor. With each enzyme, plots of product formation as a function of time multiplied by enzyme concentration were superimposable for the whole time course of the longest kinetic assay (Figure 2). Thus, no decay of the enzyme activity could be detected.

Titration of Serpins. The "effective" concentration of each serpin (I_t), relative to that determined from its absorbance at 280 nm (I_{A280}), was estimated by titration against the protease most rapidly inhibited. Various concentrations of

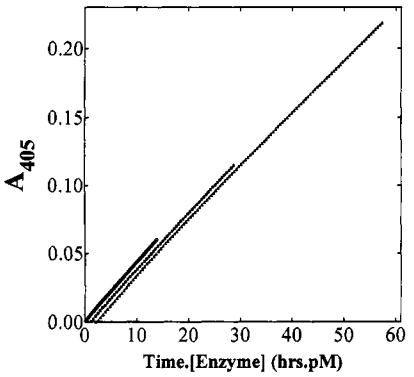


FIGURE 2: Stability of E192Q thrombin. Selwyn plot of product formation as a function of time multiplied by enzyme concentration. Each data series was obtained by following for 3 h the hydrolysis of 200 μM S-2238 at 37 °C by 5 pM (□), 10 pM (●), and 20 pM (▲) E192Q. For the purpose of clarity, only fourth data points are plotted, and data obtained at higher enzyme concentrations were shifted from the origin. For the analysis of progress curves of enzyme inhibition, only the data corresponding to <10% hydrolysis of the substrate were used.

serpin (typically 16, with I_{A280} varying from 1 nM to 1 μM) were incubated with a fixed concentration of enzyme (E_t, 10−100 nM). Inhibition reactions were carried out to completion (30 min to 3 h, depending upon the half-life of complex formation), and the residual free enzyme concentrations were determined by measuring the steady state velocities (v_s), after addition of 200 μM *p*-nitroanilide substrate. I_t was then estimated by nonlinear regression analysis of the dependence of v_s on I_{A280} using the equation for tight-binding inhibition (Cha, 1975; Williams & Morrison, 1979):

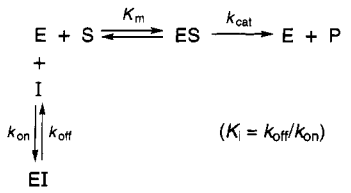
$$v_s = (v_o/2E_t)\{[(K_i + xI_{A280} - E_t)^2 + 4K_iE_t]^{1/2} - (K_i + xI_{A280} - E_t)\} \quad (1)$$

where v_o is the reaction velocity in the absence of inhibitor, K_i the inhibition constant for the stable protease−serpin complex, and x is a factor which relates I_{A280} to I_t, such that $I_t = xI_{A280}$. In most instances, E_t was much higher than K_i, so the inhibition reaction appeared essentially irreversible. The titration of ATIII by thrombin was conducted in the presence of 1 U/mL heparin. To verify that full inhibition was achieved with α1-$AT_{(P_1=Met)}$ and α1-$AC_{(P_1=Leu)}$, residual activities were compared after 3 and 6 h incubation. The results indicated that the longer incubation did not result in further enzyme inhibition. Serpins were 60−95% active (i.e. $0.60I_{A280} < I_t < 0.95I_{A280}$).

Formation of NaDodSO$_4$−Stable Complexes. Complexes between enzymes and serpins were formed by incubating 2 μM serpin with 1 μM enzyme at 25 °C for 20 min. Complexes were detected by NaDodSO$_4$ gel electrophoresis (12% in acrylamide with an acrylamide−bisacrylamide ratio of 20/1) after denaturation at 65 °C for 10 min in 0.37 M Tris-HCl, pH 8.8, containing NaDodSO$_4$ (1%, w/v) and glycerol (10%, v/v) with or without β-mercaptoethanol (5%, v/v).

Determination of Association Rate Constants by Progress-Curve Kinetics. Progress-curve kinetics were started by the addition of 10 pM to 1 nM enzyme such that v_o of *p*-nitroaniline release was about 0.2 μM min^{-1}. The reaction was followed for up to 3 h using a Hewlett-Packard diode array spectrophotometer, but only data corresponding to less than 10% substrate hydrolysis were used in the analysis.

Scheme 1



Choice and concentration of the *p*-nitroanilide substrate (100−200 μM) were dictated by the apparent half-life of complex formation: to minimize the competition introduced by the substrate in the inhibition reaction, the ratio of the substrate concentration over its K_m value for the enzyme was set to the lowest possible when k_{on} was less than 10^5 M^{-1} s^{-1}. For rapid reactions, the substrate over K_m ratio was set as high as possible, to increase the apparent half-life of complex formation. With trypsin, S-2238 (K_m = 28 μM) and S-2302 (K_m = 154 μM) were used as the strongly and weakly competing substrates, respectively. S-2238 (K_m = 18 μM) and S-2266 (K_m = 522 μM) were used with E192Q and thrombin (K_m = 3.6 and 486 μM, respectively). S-2238 (K_m = 78 μM) and pefachrome TH (K_m = 189 μM) were used with des-PPW, whereas only S-2238 (K_m = 523 μM) was used with des-ETW. The above K_m values were determined by standard steady state kinetics under the same experimental conditions as the inhibition reactions.

For many of the progress curves studied, inhibition appeared reversible; a finite steady state velocity (v_s) was observed and a first-order decay equation (assuming v_s = 0) could not adequately describe the experimental data. Although there is evidence that the formation of the stable thrombin−serpin complex involves two steps (Olson & Shore, 1982; Stone & Hermans, 1995), a significant concentration of the initial complex did not form at the serpin concentrations used. When a significant concentration of an initial complex forms, the initial velocity of the progress curve varies with the concentration of inhibitor (Williams & Morrison, 1979). Thus, the mechanism presented in Scheme 1 was found to describe the data adequately. In most instances, depletion of the free serpin remained negligible for the whole time course of the kinetic experiment. However, during the reaction of PN1 with E192Q, depletion of the inhibitor due to stable complex formation could not be ignored, and proper analysis required the use of an equation for tight-binding inhibition. Thus, the apparent inhibition constant for the stable complex (K_i') and the apparent association rate constant (k_{on}') were estimated by global nonlinear regression analysis of the dependence on time of the absorbance at 400−410 nm (A_{405}), using the equation for slow, tight-binding inhibition (Cha, 1975, 1976; Williams & Morrison, 1979):

$$A_{405} = v_st + (v_o - v_s)(1 - d)/(dk') \ln\{[1 - d\exp(-k't)]/(1 - d)\} \quad (2)$$

where A_{405} is directly proportional to the amount of *p*-nitroaniline released at time t and v_s, the steady state velocity, is defined by eq 1. The parameters d and k' are functions of the two parameters F_1 and F_2 such that $d = (F_1 - F_2)/(F_1 + F_2)$ and $k' = k_{on}'F_2$, where $F_1 = K_i' + I_t + E_t$ and $F_2 = (F_1^2 - 4E_tI_t)^{1/2}$. This equation simplifies to the one for slow-binding inhibition when $I_t \gg E_t$. The true k_{on} and K_i values were calculated by correcting for the concentration of the

substrate (S) using the relationships given in eq (Hermans et al., 1994):

$$K_i = K_i'/(1 + S/K_m) \quad (3a)$$

$$k_{on} = k_{on}'(1 + S/K_m) \quad (3b)$$

Typically, k_{on} and K_i values were determined from a series of inhibition reactions obtained with six different I_t's in the presence of the same amount of enzyme. The influence of heparin was studied by performing the inhibition kinetics in the presence of increasing concentrations of this cofactor (0.25−8 U/mL).

For interactions exhibiting very low values of k_{on} ($<10^2$ M^{-1} s^{-1}), estimates of k_{on} were obtained from kinetic experiments performed in microplates under pseudo-first-order conditions. In these experiments, rate constants of inhibition were estimated by nonlinear regression analysis of the residual activities versus time (first-order decay plot) and k_{on} values deduced from the linear plot of the rate constants against the inhibitor concentrations, as previously described (Le Bonniec et al., 1991, 1994).

RESULTS

Inhibition of E192Q Thrombin by Serpins. The serpin α1-$AT_{(P_1=Met)}$ not only rapidly inactivates neutrophil elastase (k_{on} = 6.8 × 10^7 M^{-1} s^{-1}) and bovine chymotrypsin (k_{on} = 5.9 × 10^6 M^{-1} s^{-1}) but also inhibits trypsin (k_{on} = 2.2 × 10^5 M^{-1} s^{-1}), suggesting that the nature of the P_1 residue does not absolutely govern the specificity of this serpin (Beatty et al., 1980; Potempa et al., 1994). While α1-$AT_{(P_1=Met)}$ inhibits members of the three classes of serine proteases, thrombin and protein C are both resistant to inhibition by this serpin with a P_1 methionine (k_{on} < 10^2 M^{-1} s^{-1}, Table 1; Hermans & Stone, 1993). In chymotrypsin, residue 192 is a methionine, whereas in elastase- and trypsin-like enzymes the most common residue at this position is glutamine. Thrombin and protein C, with glutamate at position 192, constitute two notable exceptions among 60 serine proteases. It has been reported that the E192Q mutation in activated protein C increases by 280-fold the k_{on} value of α1-$AT_{(P_1=Met)}$ for the protease (Rezaie & Esmon, 1993). To investigate the possible contribution of thrombin's Glu^{192} in restricting the inhibitory potential of α1-$AT_{(P_1=Met)}$, we have compared the k_{on} values for trypsin, thrombin, and E192Q (Table 1). E192Q was inactivated by α1-$AT_{(P_1=Met)}$ with a k_{on} value only 5-fold lower than that for trypsin (i.e., 3 orders of magnitude greater than the value for thrombin, Figure 3). Thus, for thrombin and protein C, two trypsin-like enzymes, presence of a glutamate in position 192 (instead of the glutamine found in trypsin) dramatically impairs the inhibitory potential of α1-$AT_{(P_1=Met)}$.

The effect of the E192Q mutation on the inhibition by α1-$AT_{(P_1=Met)}$ was not due to a general increase in the mutant's sensitivity to serpins. When the P_1 residue was Leu, as in α1-$AC_{(P_1=Leu)}$, the E192Q mutation increased the k_{on} value only 2-fold relative to that of thrombin (Table 1). The E192Q mutation did not affect the association with ATIII (Table 1; Le Bonniec & Esmon, 1991), and the influence of the E192Q mutation on the inhibition kinetics with other serpins containing a P_1 arginine residue was also quite limited. Although this mutation increased slightly the k_{on} value with α1-$AC_{(P_1=Arg)}$ and PN1 (5.7- and 3.7-fold, respectively), the k_{on} value with α1-$AT_{(P_1=Arg)}$ was actually somewhat lower (2-fold) for E192Q than for thrombin (Table 1). Therefore, replacement in α1-$AT_{(P_1=Met)}$ of the P_1

Table 1: Association Rate Constants for Serpins and Influence of Heparin[a]

		k_{on} (M^{-1} s^{-1} ± standard error, %)[b]	
serpin	enzyme	no heparin	with heparin
α1-$AT_{(P_1=Arg)}$	trypsin	6.7 × 10^6 (±2.1)	4.9 × 10^6 (±2.2)
	E192Q	6.4 × 10^5 (±0.6)	3.3 × 10^5 (±1.4)
	thrombin	1.2 × 10^6 (±3.5)	5.9 × 10^5 (±2.2)
	des-PPW	2.2 × 10^4 (±2.1)	1.3 × 10^4 (±1.9)
	des-ETW	2.0 × 10^4 (±1.7)	9.9 × 10^3 (±4.5)
α1-$AT_{(P_1=Met)}$	trypsin	2.2 × 10^5 (±3.4)	1.4 × 10^5 (±3.2)
	E192Q	4.5 × 10^4 (±1.2)	1.9 × 10^4 (±2.1)
	thrombin	3.9 × 10^1 (±9.8)	1.5 × 10^1 (±5.4)
	des-PPW	<10	<10
	des-ETW	<10	<10
α1-$AC_{(P_1=Arg)}$	trypsin	7.6 × 10^5 (±7.5)	3.5 × 10^5 (±2.7)
	E192Q	2.4 × 10^4 (±3.7)	4.9 × 10^4 (±2.1)
	thrombin	4.2 × 10^3 (±0.4)	2.5 × 10^4 (±0.9)
	des-PPW	1.2 × 10^2 (±1.8)	1.1 × 10^3 (±1.5)
	des-ETW	1.1 × 10^1 (±8.3)	4.1 × 10^2 (±7.9)
α1-$AC_{(P_1=Leu)}$	trypsin	<10	2.1 × 10^1 (±7.5)
	E192Q	2.2 × 10^1 (±9.6)	1.3 × 10^2 (±6.6)
	thrombin	1.1 × 10^1 (±5.6)	3.4 × 10^1 (±13.2)
	des-PPW	<10	<10
	des-ETW	<10	<10
ATIII	trypsin	4.8 × 10^4 (±2.3)	2.4 × 10^5 (±3.1)
	E192Q	1.7 × 10^4 (±2.3)	1.4 × 10^8 (±1.0)
	thrombin	1.3 × 10^4 (±1.8)	1.2 × 10^8 (±2.3)
	des-PPW	6.1 × 10^1 (±5.6)	4.9 × 10^6 (±3.7)
	des-ETW	3.7 × 10^1 (±4.9)	2.1 × 10^6 (±2.6)
PN1	trypsin	4.8 × 10^6 (±6.0)	1.7 × 10^7 (±3.7)
	E192Q	5.6 × 10^6 (±2.2)	2.1 × 10^9 (±4.5)
	thrombin	1.5 × 10^6 (±3.4)	7.0 × 10^8 (±1.7)
	des-PPW	8.7 × 10^5 (±2.1)	1.1 × 10^8 (±2.6)
	des-ETW	1.3 × 10^3 (±2.9)	1.1 × 10^6 (±1.7)

[a] The apparent k_{on} values were derived by nonlinear regression analysis using eq 2 of data obtained in the absence and in the presence of the optimum concentration of heparin and the true k_{on} values calculated after correction for the substrate concentration according to the relationships given in eq 3; k_{on} values $<10^2$ M^{-1} s^{-1} were estimated using a first-order decay equation. Abbreviations: α1-$AT_{(P_1=Met)}$ and α1-$AT_{(P_1=Arg)}$, α1-antitrypsin (methionine and arginine as the P_1 residue, respectively); α1-$AC_{(P_1=Leu)}$ and α1-$AC_{(P_1=Arg)}$, α1-antichymotrypsin (leucine and arginine as the P_1 residue, respectively); ATIII, antithrombin III; PN1, protease nexin-1. [b] The values in parentheses represent the standard error expressed as a percentage of the determined k_{on} value.

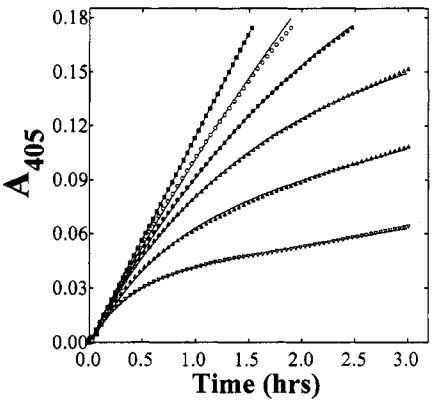


FIGURE 3: Progress curves of E192Q thrombin inhibition by α1-$AT_{(P_1=Met)}$. Lines represent the result of the global nonlinear curve fitting according to eq 2. Each data series was obtained by following for 3 h at 37 °C the hydrolysis of 200 μM S-2238 by 30 pM E192Q in the presence of α1-$AT_{(P_1=Met)}$ at 0 nM (■), 8 nM (○), 16 nM (●), 32 nM (△), 64 nM (▲) and 128 nM (▼). For the purpose of clarity, only each fourth data point is plotted.

methionine residue by arginine resulted in a $3 \cdot 10^4$-fold increase of the k_{on} value for thrombin interaction but only a 14-fold increase with E192Q. The mutation of Glu^{192} in thrombin to glutamine resulted in a protease that was remarkably similar to trypsin in its interactions with serpins; the greatest difference in the k_{on} values between E192Q and trypsin was 5-fold (Table 1).

$NaDodSO_4$-stable complexes between the serpins tested and E192Q or thrombin were always detectable. Even α1-$AT_{(P_1=Met)}$ and α1-$AC_{(P_1=Leu)}$, with very low k_{on} values, produced discernible $NaDodSO_4$-stable complexes after incubation with thrombin.

Inhibition of Des-ETW and Des-PPW by Serpins. The k_{on} value of ATIII with the des-ETW thrombin mutant was 350-fold lower than that for thrombin (Table 1; Le Bonniec et al., 1992b). A dramatic influence of the des-ETW mutation on the rate of association with PN1 and α1-$AC_{(P_1=Arg)}$ was also observed (1150- and 380-fold decreases, respectively), but the effect of the mutation on the k_{on} for the interaction with α1-$AT_{(P_1=Arg)}$ was more limited (60-fold decrease). Similarly, the des-PPW mutation induced a notable decrease of the k_{on} value with ATIII (210-fold lower than with thrombin) and a comparatively limited reduction of that with α1-$AT_{(P_1=Arg)}$ (50-fold). However, in contrast to the des-ETW mutation, the PPW deletion induced only a 35-fold decrease of the k_{on} value with α1-$AC_{(P_1=Arg)}$ and, surprisingly, did not affect the interaction with PN1 (k_{on} value reduced less than 2-fold). The exact contribution of the P_1 residue on des-PPW and des-ETW inhibition was difficult to ascertain because inactivation by α1-$AC_{(P_1=Leu)}$ and α1-$AT_{(P_1=Met)}$ was not detectable (even after 3 h incubation in the presence of 5 μM serpin). Nevertheless, it can be concluded that arginine is undoubtedly preferred in the P_1 position of the reactive-site loop for these two thrombin mutants. With the P_1 arginine serpins studied, both des-ETW and des-PPW produced $NaDodSO_4$-stable complexes. Thus, while the des-ETW mutation affected the interactions with all serpins assayed, the PPW deletion appears more selective; the interaction with PN1 was not affected.

Effect of Heparin on Protease−Serpin Interactions. Heparin is a cofactor which increases k_{on} values of particular serpin-protease associations, by interacting with the inhibitor, the enzyme, or both (Olson et al., 1992). When binding occurs to both, heparin often acts through a template mechanism, as in thrombin inhibition by ATIII (Griffith, 1982; Nesheim, 1983). Thus the effect of heparin can be extremely variable, and in fact, alteration of the k_{on} values ranged from a 2-fold decrease to a nearly 10^5-fold increase. Compared with thrombin, the magnitude of the changes upon addition of the cofactor were roughly the same with E192Q, but this was not the case with des-ETW and des-PPW (Table 1). Heparin had no discernible influence on the ability of the proteases to form $NaDodSO_4$-stable complexes. With or without heparin, complexes were either detectable or not detectable (α1-$AC_{(P_1=Leu)}$ and α1-$AT_{(P_1=Met)}$ with des-ETW or des-PPW).

As previously reported for α1-$AT_{(P_1=Arg)}$ and α1-$AT_{(P1=Met)}$ (Pixley & Danishefsky, 1983), when the concentration of heparin was increased, the k_{on} value with thrombin decreased slightly. This effect of heparin was observed with all proteases assayed; the maximum decrease in the k_{on} value was 2-fold for heparin concentrations above 4 U/mL. With

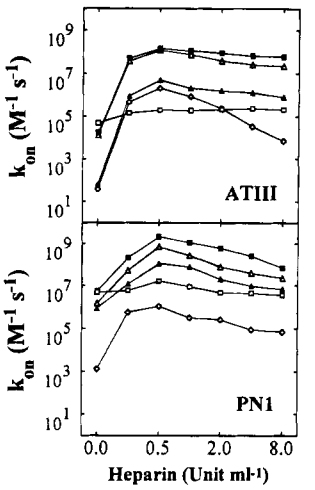


FIGURE 4: Influence of heparin on the k_{on} value for ATIII (upper panel) and PN1 (lower panel) of trypsin (□), thrombin (△), E192Q thrombin mutant (■), des-PPW thrombin mutant (▲), and des-ETW thrombin mutant (◇). Heparin had little influence on trypsin inhibition, and the des-ETW mutant displayed the greatest acceleration by heparin.

α1-$AC_{(P_1=Arg)}$ and α1-$AC_{(P_1=Leu)}$, there was also a hyperbolic dependence of the k_{on} value on the concentration of heparin, but the effect was variable. With saturating amounts, the k_{on} value for α1-$AC_{(P_1=Arg)}$ decreased about 2-fold with trypsin but increased slightly with E192Q, thrombin, and des-PPW (2-, 6-, and 9-fold, respectively, Table 1). Similarly, when inhibition by α1-$AC_{(P_1=Leu)}$ was detectable (E192Q and thrombin), heparin caused a slight increase in the k_{on} value. Interestingly, heparin had a relatively large effect on the interaction of α1-$AC_{(P_1=Arg)}$ with des-ETW (37-fold increase of the k_{on} value).

For the heparin-binding serpins, PN1 and ATIII, heparin caused only a slight acceleration of the reaction with trypsin but a marked acceleration with the thrombin mutants (Table 1). In addition, the k_{on} values displayed a bell-shaped dependence on the concentration of heparin, with the maximum effect between 0.5 and 2.0 U/mL, suggesting that the cofactor acts through a template mechanism (Figure 4). The most dramatic effect was observed for des-PPW and des-ETW inhibition by ATIII (8×10^4- and 5.7×10^4-fold increase of the k_{on} value, respectively). Thus, heparin accelerated inhibition of des-ETW and des-PPW 5−10 times more than it accelerated inactivation of thrombin or E192Q. Heparin not only improved the ability of des-ETW and des-PPW to interact with ATIII but also reduced the effect of these mutations; the differences between the k_{on} values of thrombin and des-ETW or des-PPW were smaller in the presence of heparin.

DISCUSSION

Previous studies have indicated that there are at least three factors that govern the ability of a serpin to neutralize its target protease: (1) productive binding of the serpin's reactive-site loop to the catalytic groove of the protease, (2) possible additional interactions between the serpin and the protease at sites outside of the catalytic groove, and (3) the presence of cofactors, such as heparin, that accelerate the reaction. The present study provides new insights into these three determinants of thrombin inhibition: (1) within the catalytic groove of thrombin, Glu^{192} selectively restricts the

enzyme's ability to accept methionine as the P_1 residue; (2) on the rim of the catalytic groove, the 60-loop insertion is required for efficient inhibition by ATIII but is not essential for interaction with PN1; and (3) heparin reduces the detrimental effects of the des-PPW and des-ETW mutations with respect to inhibition by ATIII.

Glu^{192} Modulates the P_1 Specificity of Thrombin in Its Interaction with Serpins. The P_1 residue of a serpin reactive-site loop unquestionably participates in the serpin's specificity. Replacement of the P_1 methionine in α1-$AT_{(P_1=Met)}$ for arginine markedly increases the rate of reaction with thrombin (Owen et al, 1983). Similar results were observed with the arginine for leucine substitution in α1-$AC_{(P_1=Leu)}$ (Rubin et al., 1990). Conversely, replacement of the P_1 arginine in other serpins that normally inhibit arginine-specific proteases results in loss of inhibitory potency with respect to these proteases as documented by the methionine for arginine substitution in plasminogen activator inhibitor 1 (Keijer et al., 1991) and protein C inhibitor (Phillips et al., 1994). Arginine in the P_1 position is not, however, a prerequisite for efficient inhibition of a trypsin-like enzyme; in spite of its P_1 methionine residue, α1-$AT_{(P_1=Met)}$ inhibits trypsin. Although replacement of the P_1 leucine residue with arginine increases the k_{on} value of heparin cofactor II for thrombin (Derechin et al., 1990), the wild-type serpin is, in the presence of heparin, a potent inhibitor of thrombin (k_{on} = 5.0 × 10^6 M^{-1} s^{-1}; Church et al., 1985; Rogers et al., 1992). While surprising at first, it is conceivable that leucine and methionine could be accommodated in the primary specificity pocket of trypsin and thrombin. Leucine and methionine are not bulkier than arginine and should fit within the pocket. Thrombin also appears to accommodate histidine as a P_1 residue in the complex with the Kazal-type inhibitor rhodniin (K_i = 203 fM; Friedrich et al., 1993). While thrombin's primary specificity pocket might be roomy enough for histidine, leucine, and methionine, interactions within the S_1 subsite are not optimal with these residues. Indeed, substitution in ATIII of the P_1 arginine by histidine dramatically impairs its function (Lane et al., 1993). The astonishing affinity of thrombin for heparin cofactor II (leucine in P_1) and rhodniin (histidine in P_1) most certainly results from interactions remote from the catalytic groove. The NH_2-terminal hirudin-like sequence of heparin cofactor II and the interdomain acidic sequence of rhodniin most likely bind to thrombin's anion-binding exosite I, and these exosite interactions compensate for the suboptimal binding within the primary specificity pocket. In the absence of such exosite interactions, robust binding to the primary specificity pocket seems to be necessary for efficient inhibition of thrombin. The great increase in the susceptibility of E192Q to inhibition by α1-$AT_{(P_1=Met)}$, however, indicates that an interaction with a glutamine at position 192 can substitute at least partially for interactions with the primary specificity pocket. The effects of the E192Q mutation may be due to a modification of the primary specificity pocket such that it interacts more favorably with a methionine residue, but the primary specificity pockets of thrombin (Glu^{192}) and trypsin (Gln^{192}) are very similar (Bode et al., 1989). Another possible explanation is that interactions between Gln^{192} and the reactive-site loop of α1-$AT_{(P_1=Met)}$ compensate for the suboptimal binding of methionine in the primary specificity pocket. However, such an interaction can not fully explain the favorable interaction of E192Q and trypsin with α1-$AT_{(P_1=Met)}$ because plasmin is a trypsin-like enzyme with glutamine at position 192 which is only poorly inhibited by α1-$AT_{(P_1=Met)}$ (k_{on} = 1.9 × 10^2 M^{-1} s^{-1}; Beatty et al., 1980).

Table 2: Comparison of Residues P_3–P_3' of the Serpins Assayed[a]

serpin	P_3	P_2	P_1	P_1'	P_2'	P_3'
α1-$AT_{(P_1=Arg)}$	Ile	Pro	Arg	Ser	Ile	Pro
α1-$AT_{(P_1=Met)}$	Ile	Pro	Met	Ser	Ile	Pro
α1-$AC_{(P_1=Arg)}$	Thr	Leu	Arg	Ser	Ala	Leu
α1-$AC_{(P_1=Leu)}$	Thr	Leu	Leu	Ser	Ala	Leu
ATIII	Ala	Gly	Arg	Ser	Leu	Asn
PN1	Ile	Ala	Arg	Ser	Ser	Pro

[a] Sequence alignment of the reactive-site loop P_3–P_3' residues of the various serpins assayed. Abbreviations are as in Table 1.

Interactions with the Other P_3–P_3' Residues of Serpins. The serpins utilized in this study differ at other positions than P_1 within the P_3–P_3' residues of their reactive-site loop (Table 2). Several of the corresponding subsites in thrombin, and in serine proteases in general, have a major influence on catalysis (Le Bonniec & Esmon, 1991; Le Bonniec et al., 1991, 1992b; Stubbs et al., 1992; Qiu et al., 1992; Schellenberger et al., 1994). Thus, differences in the P_3–P_3' sequences of the serpins probably contribute to the differences observed in the k_{on} values with thrombin. Proline and leucine in position P_1' have been found to restrict dramatically the ability of ATIII to inhibit thrombin, whereas serine, threonine, and glycine are more favorable (Stephens et al., 1988; Theunissen et al., 1993). ATIII, PN1, α1-$AT_{(P_1=Arg)}$, and α1-$AC_{(P_1=Arg)}$ all have the optimal serine as the P_1' residue. With peptide substrates, aspartate residues in P_3 and/or P_3' dramatically reduce the cleavage rate by thrombin (Le Bonniec & Esmon, 1991; Le Bonniec et al., 1991). Similarly, aspartate in the P_3 and/or P_3' position of the reactive-site loop of ATIII markedly reduces its inhibitory potency toward thrombin (Theunissen et al., 1993). The influence of P_3 residues such as isoleucine (α1-$AT_{(P_1=Arg)}$ and PN1), threonine (α1-$AC_{(P_1=Arg)}$), or alanine (ATIII) and of P_3' residues such as proline (α1-$AT_{(P_1=Arg)}$ and PN1), leucine (α1-$AC_{(P_1=Arg)}$), or asparagine (ATIII) remains largely unexplored. It is noteworthy, however, that the two serpins that reacted most rapidly with thrombin (α1-$AT_{(P_1=Arg)}$ and PN1) have the same P_3 and P_3' residues (isoleucine and proline, respectively). Little is also known about the potential contribution of the P_2' position, but with ATIII several residues can be accommodated (Theunissen et al., 1993). Finally, the results obtained with E192Q and thrombin suggest that it may be possible to predict the P_2 preferences for serpins from those observed with synthetic substrates. The k_{on} value for α1-$AT_{(P_1=Arg)}$ (P_2 = proline) was slightly lower with E192Q than with thrombin, whereas the opposite was true with ATIII (P_2 = glycine). Similar results were obtain when the k_{cat}/K_m values of *p*-nitroanilide substrates with thrombin and E192Q were compared (Lottenberg et al., 1983; Le Bonniec & Esmon, 1991).

Interactions of Serpins with Regions beyond the S_3–S_3' Subsites. In addition to the S_3–S_3' subsites, other binding sites have been implicated in several protease–serpin interactions. The role of the interaction between the hirudin-like NH_2-terminal sequence of heparin cofactor II and the anion-binding exosite I of thrombin has been discussed above. In tissue plasminogen activator, an insertion of basic amino acids, between residues 37 and 41, is critical for plasminogen activator inhibitor 1 binding (Madison et al.,

1989, 1990). Introduction of this basic sequence into thrombin dramatically increases its sensitivity to plasminogen activator inhibitor 1, whereas the interaction with ATIII is little affected (Horrevoets et al., 1993). In fact, replacement of thrombin Glu^{39} for lysine is sufficient to render thrombin much more sensitive to plasminogen activator inhibitor 1,[2] whereas the mutation has limited influence on the rate of inhibition by ATIII (Le Bonniec et al., 1991). Therefore, the 37−41 region of proteases appears critical for plasminogen activator inhibitor 1 binding, while it is dispensable for ATIII interaction. Our data suggest that the PPW motif constitutes another such specific area which, in thrombin, is critical for ATIII binding but dispensable for interaction with PN1. In previous data, we have shown that the des-ETW mutation disrupts the S_1 binding pocket of thrombin (Le Bonniec et al., 1994), while the PPW deletion leaves it unperturbed (Le Bonniec et al., 1993). Both mutations however disrupt the P_2 binding site of thrombin, formed in part by the PPW motif of the 60-loop insertion (Bode et al., 1989; Le Bonniec et al., 1993, 1994). Thus, the defect of the primary binding pocket rationalizes in part the low reactivity of des-ETW with ATIII and PN1, but impaired interactions with the P_2 residue of the serpin's reactive-site loop probably also account for the poor inhibition of des-PPW and des-ETW by ATIII. Yet, in spite of the disrupted S_2 subsite, PN1 inhibited des-PPW almost as efficiently as thrombin. Thus, independently from its role in the formation of the P_2 binding pocket, thrombin's 60-loop insertion appears to contribute to the interaction with ATIII, whereas this faculty is not required for PN1 binding, suggesting that the PPW motif interacts directly with ATIII in the complex. Such hypothesis supports modeling studies which proposed that ATIII packs against the 60-loop insertion of thrombin (Bode et al., 1992). In contrast to the proposed role for the 60-loop insertion in the binding of ATIII, it is unlikely that the autolysis loop of thrombin, which contains the ETW motif, interacts directly with either ATIII or PN1. Disruption of this loop by proteolysis does not markedly affect the k_{on} for thrombin of ATIII and PN1 (Bezeaud et al., 1985; Kawabata et al., 1985; Brower et al., 1987; Wallace et al., 1989; Brezniak et al., 1990).

Heparin Ameliorates the Effects of the Des-ETW and Des-PPW Mutations. Heparin greatly accelerates the inhibition of thrombin by ATIII and PN1. In the case of ATIII, this acceleration is primarily due to the cofactor increasing the tightness of the initial complex by approximating the serpin and its target through a template mechanism (Olson & Shore, 1982; Nesheim, 1983). The cofactor links thrombin's anion-binding exosite II (Sheehan & Sadler, 1994; Gan et al., 1994) with a heparin-binding site present on the α-helix D of ATIII and PN1 (Carrell et al., 1994). In agreement with this model, heparin had a very limited effect on the inhibition of trypsin by any of the serpins because there is no equivalent anion-binding exosite II in trypsin. In addition, heparin did not accelerate the inhibition of any proteases by α1-$AT_{(P_1=Met)}$ or α1-$AT_{(P_1=Arg)}$ because these serpins do not bear a heparin-binding site. Although there is no typical heparin-binding site in α1-$AC_{(P_1=Arg)}$, heparin slightly increased the rate of thrombin and E192Q inhibition and caused a pronounced acceleration of des-ETW inhibition. It has been proposed that heparin binds to α1-AC via its DNA-binding site (Hermans & Stone, 1993). With ATIII and PN1, which both have a characteristic binding site, heparin had a major influence on the inhibition of all thrombins. Interestingly, heparin increased the value of k_{on} considerably more with the des-PPW and des-ETW mutants than it did with thrombin or E192Q. Thus, heparin partially restored the sensitivity to ATIII of des-PPW and des-ETW. It is conceivable that in des-PPW, the link between anion-binding exosite II and the serpin's heparin-binding site substitutes in part for the missing 60-loop insertion. Similarly, this link may overcome the effects of the misfolded 60-loop insertion in des-ETW (Le Bonniec et al., 1994).

[2] B. F. Le Bonniec and H. J. Ehrlich, unpublished observation.

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