Elucidation of the Structural Basis for the Slow Reactivity of Thrombin with Plasminogen Activator Inhibitor-1†

Alireza R. Rezaie*

Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma 73104

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ABSTRACT: Plasminogen activator inhibitor-1 (PAI-1) is a serine protease inhibitor of the serpin superfamily which rapidly inactivates tissue plasminogen activator (tPA), but reacts with thrombin at a much slower rate. Based on the previous mutagenesis studies and the X-ray crystal structure of the thrombin E192Q−bovine pancreatic trypsin inhibitor (BPTI) complex, the structural basis for the slow reactivity of thrombin with PAI-1 is investigated in this study. In the crystal structure of the thrombin E192Q−BPTI complex, the reactive site loop of BPTI is stabilized in a canonical conformation by several productive interactions (e.g., Glu39 of thrombin is ion-paired to the P5′ Arg, and Gln192 is hydrogen-bonded to the P2 and P4 backbone carbonyls of BPTI). PAI-1 contains Glu residues at both the P4′ and P5′ positions, and previous mutagenesis studies suggest that these residues make productive interactions with Arg39 of tPA as well as with two other positively charged residues present on the 39-loop of the protease (chymotrypsin numbering). Glu39 and Glu192 of thrombin would be unable to make such productive interactions with PAI-1. Instead, their repulsive interactions with the similarly charged residues and/or the backbone carbonyls of the PAI-1 reactive site loop could restrict the reaction. To test this, the rate constants (k_2) for the PAI-1 inactivation of wild-type, E39K, E39Q, E192Q, E192M, and E39K/E192Q thrombins were determined. The inactivation rates of E39K [$k_2 = (4.3 \pm 0.2) \times 10^4$ M^{-1} s^{-1}] and E39Q [$k_2 = (1.0 \pm 0.1) \times 10^4$ M^{-1} s^{-1}] were 50- and 12-fold faster than the inactivation of wild-type thrombin [$k_2 = (8.6 \pm 0.5) \times 10^2$ M^{-1} s^{-1}], respectively. Relative to thrombin, the PAI-1 inactivation rates were improved 31-fold for E192Q [$k_2 = (2.7 \pm 0.5) \times 10^4$ M^{-1} s^{-1}] and 5-fold for E192M [$k_2 = (4.3 \pm 0.8) \times 10^3$ M^{-1} s^{-1}] thrombins. With the double mutant E39K/E192Q, the inactivation rate [$k_2 = (5.4 \pm 0.4) \times 10^5$ M^{-1} s^{-1}] was improved 628-fold over wild-type thrombin. These results suggest that repulsive interactions and/or lack of productive electrostatic interactions between PAI-1 and Glu39 and Glu192 of thrombin are responsible for the slow reaction of thrombin with this serpin.

Plasminogen activator inhibitor-1 (PAI-1)[1] is a serine protease inhibitor of the serpin superfamily which rapidly inactivates the proteinases of the fibrinolytic system, tissue-type (tPA) and urokinase-type (uPA) plasminogen activators, but reacts with thrombin at a much slower rate (*3*, *4*). The structural basis for the high reactivity of tPA with PAI-1 has been investigated by several mutagenesis studies in the past (*5*, *6*). Collectively, the results suggest that tPA contains a secondary binding site for PAI-1 on a loop in the protease domain known as variable region 1 (*7*), or the 39-loop (chymotrypsin numbering) (*1*). Relative to trypsin, this loop of tPA has an insertion of seven amino acids (residues 296−302), three of which are positively charged and contact a region of PAI-1 on the reactive site loop that contains two negatively charged Glu residues at the P4′ and P5′ sites[2] of the scissile bond (*5*, *6*). Previous mutagenesis studies demonstrated that charge reversal or deletion of the positively charged residues from the 39-loop of tPA, or substitution of Arg304 (Arg39 in chymotrypsin numbering) with Glu, results in tPA variants which are resistant to inactivation by PAI-1 (*6*, *8*). Furthermore, compensatory mutations in PAI-1 (Glu to Arg substitutions at the P4′ and P5′ positions) resulted in serpin variants which reacted effectively with the tPA substitution mutants containing negatively charged residues at the 39-loop, but very poorly with wild-type tPA (*9*). These observation suggested that the 39-loop plays a decisive role in the high reactivity of tPA with PAI-1.

The structural basis for the slow reactivity of thrombin with PAI-1 is not known. Though, a recent mutagenesis

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* Correspondence should be addressed to this author at the Oklahoma Medical Research Foundation, Cardiovascular Biology Research, 825 N.E. 13th St., Oklahoma City, OK 73104. Phone: (405) 271-7264. Fax: (405) 271-7417. E-mail: alireza-rezaie@omrf.ouhsc.edu.

[1] Abbreviations: E39K and E39Q thrombins, thrombin mutants in which Glu39 in the chymotrypsin numbering system of Bode et al. (*1*) is substituted with Lys and Gln, respectively; E192Q and E192M thrombins, thrombin mutants in which Glu192 in the same numbering system is substituted with Gln and Met, respectively; tPA, tissue plasminogen activator; uPA, urokinase plasminogen activator; serpin, serine protease inhibitor; PAI-1, plasminogen activator inhibitor-1; BPTI, bovine pancreatic trypsin inhibitor; PEG, poly(ethylene glycol).

[2] Nomenclature of Schechter and Berger (*2*) used to describe the subsites of interaction between a protease and its substrate. Amino acid residues of the substrate are referred to as P1, P2, etc. on the N-terminal side of the substrate scissile bond and those on the C-terminal side as P1′, P2′, etc. The corresponding sites on the enzyme where substrate residues interact are designed S1, S2, ... and S1′, S2′, ..., respectively.

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study demonstrated that it may be possible to partially transfer the inhibitor specificity of tPA to thrombin by replacing the entire 39-loop of thrombin (Phe34−Leu40) with the corresponding sequence of tPA (*10*). The improvement in the reactivity of this mutant with PAI-1 was to the extent that the rate constant for its inactivation by PAI-1 was only ~10-fold lower than the rate constant for the PAI-1 inactivation of tPA. The result of this interesting study provided further evidence that the interaction of the 39-loop with the reactive site loop of PAI-1 is critical for efficient protease inactivation by PAI-1 (*10*). This study, however, did not reveal if any of the deleted native residues of the 39-loop of thrombin play a restrictive role in the reaction of thrombin with PAI-1.

Thrombin is resistant to inactivation by several other inhibitors including the Kunitz-type inhibitors, bovine pancreatic trypsin inhibitor (BPTI) and tissue factor pathway inhibitor, and the serpin, α_1-antitrypsin. It was previously demonstrated that the substitution of Glu192 of thrombin with Gln results in a mutant (E192Q) which is effectively inactivated by all three inhibitors (*11*, *12*). Recently, the crystal structure of thrombin E192Q in complex with BPTI was resolved (*13*). Structural analysis of the complex indicated that BPTI binds to the active site of thrombin E192Q in a canonical conformation identical to that observed in trypsin (*14*). Similar to trypsin, the carboxamide nitrogen atom of Gln192 of the mutant protease was found in hydrogen bonds with the backbone carbonyls of Cys14 (P2) and Gly12 (P4) of BPTI. Furthermore, in the crystal structure of the thrombin E192Q−BPTI complex, Glu39 of thrombin was ion-paired to the P5′ Arg20 of BPTI. Such productive interactions cannot occur in the reaction of thrombin with PAI-1. Instead, repulsive interactions of Glu39 and Glu192 with the similarly charged P4′ and/or P5′ residues as well as with the backbone carbonyls of PAI-1 are likely to destabilize the enzyme−inhibitor interaction.

To elucidate the contribution of these negatively charged residues to the slow reactivity of thrombin with PAI-1, the second-order rate constant for the PAI-1 inactivation of wild-type thrombin was determined and compared to the corresponding values obtained from the PAI-1 inactivation of E39K, E39Q, E192Q, E192M, and E39K/E192Q thrombins. The reactivities of all mutants with PAI-1 were improved at varying degrees. However, the improvements in the reactivities were the highest for the mutants that contained a positively charged Lys at position 39 and a Gln at position 192. Relative to wild-type thrombin, the PAI-1 inactivation of the double mutant E39K/E192Q was enhanced 628-fold. These results suggest that both Glu39 and Glu192 of thrombin are responsible for the slow reactivity of thrombin with PAI-1. Comparison of the PAI-1 inactivation rates of E39K and E192Q thrombins with those of E39Q and E192M thrombins suggests that the repulsive negative−negative charge interactions and/or lack of productive electrostatic interactions between these residues and those of the reactive site loop of PAI-1 may account for the poor reactivity of thrombin with this serpin.

MATERIALS AND METHODS

Proteins and Reagents. The prethrombin 1 forms of the wild-type and mutant proteins were expressed in mammalian cells, purified to homogeneity, activated to thrombin, and active-site-titrated as previously described (*12*, *15*). Recombinant active human PAI-1 (product PAI-A, 95 ± 5% active as titrated by uPA) was obtained from Molecular Innovations, Inc. (Royal Oak, MI). The plasmid used to produce active PAI-1 in bacteria has been described (*16*, *17*). The second-order rate constant for the tPA and uPA inactivation by this preparation of PAI-1 is $\sim 1 \times 10^7$ M^{-1} s^{-1}. Spectrozyme PCa (SpPCa) was purchased from American Diagnostica, Greenwich, CT. Polybrene and unfractionated heparin (porcine intestinal mucosa, sodium salt, grade II) were purchased from Sigma.

Table 1: Alignment of the Amino Acid Sequences from Positions 28−42 and 189−198 of tPA, Thrombin, and Trypsin[a]

	296	304	475
tPA	PWQAAIFAKHRRSPGERFLC		DACQGDSGGP
Trypsin	PYQVSLNS . . . . . GYHFC		DSCQGDSGGP
Thrombin	PWQVMLFRK . . . . SPQELLC		DACEGDSGGP
	36	39	192

[a] The residue numbers listed above the sequences are in tPA, and those at the bottom are in chymotrypsin numbering systems. Residues F34−L40 of thrombin (F294−F305 of tPA) are referred to as the 39-loop (*1*) or variable region 1 (*7*).

Kinetic Methods. The PAI-1 inactivation rates of wild-type and mutant thrombins were measured under pseudo-first-order rate conditions by a discontinuous assay method as described previously (*18*, *19*). Briefly, 1 nM wild-type or mutant thrombins were incubated at room temperature with 25−200 nM PAI-1 in 50 μL reactions in Tris-HCl (pH 7.5), 0.1 M NaCl buffer (TBS) containing 1 mg/mL BSA and 0.1% PEG 8000. At the end of the incubation times (20 s to 45 min, depending on the reaction rates), 50 μL of SpPCa was added to a final concentration of 0.2 mM. The remaining activities of thrombins were measured from the rate of chromogenic substrate hydrolysis using a V_{max} Kinetics Microplate Reader (Molecular Devices, Menlo Park, CA). The pseudo-first-order inactivation rate constants (k') were calculated by fitting the time-dependent change of the thrombin activity to a first-order rate equation using the Enzfitter computer program (R. J. Leatherbarrow, Elsevier, Biosoft). The second-order inactivation rate constants (k_2) were calculated from the slope of the linear plot of the k' values vs PAI-1 concentrations. All reactions contained 100 μg/mL Polybrene to eliminate any possible trace contamination of PAI-1 with heparin. In all assays, less than 10% chromogenic substrate was utilized.

RESULTS

Table 1 shows the alignment of the amino acid sequences of the 39-loop, and the amino acid sequences from position 189 to position 198 of thrombin, tPA, and trypsin. Relative to trypsin, this loop of tPA contains an insertion of seven amino acids, three of which are basic Lys and Arg residues. All three proteases contain a variant residue at position 39. This residue is Arg in tPA, Glu in thrombin, and Tyr in trypsin. In the crystal structure of the trypsin−BPTI complex, Tyr39 of trypsin makes a van der Waals contact with the P4′ Ile of the inhibitor (*14*, *20*). Using the structure of this complex as a model, Madison et al. (*5*) demonstrated that two of the positively charged insertion residues as well

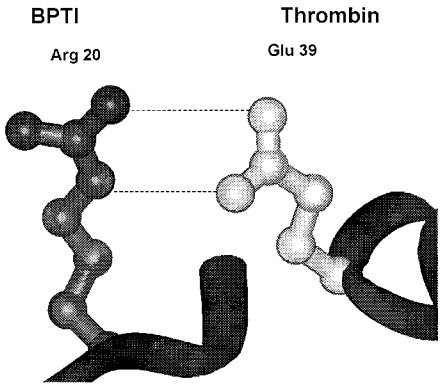

FIGURE 1: Ball and stick representation of the side chains of Arg20 of BPTI and Glu39 of thrombin E192Q with the salt-bridges shown as dashed lines. The coordinates are from the Brookhaven Protein Data Bank (Accession No. IBTH).

as Arg39 of tPA interact with the P4′ and/or P5′ Glu residues of PAI-1, resulting in rapid tPA inactivation. Thrombin also differs from tPA and trypsin in that it has a Glu, instead of Gln, at position 192 (Table 1). Previous mutagenesis studies suggest that residue 192 plays a critical role in the macromolecular substrate and inhibitor specificity of thrombin and other coagulation proteases (*12*). The substitution of Glu192 of thrombin with Gln results in a mutant, E192Q, which, in contrast to wild-type thrombin, is effectively inactivated by α_1-antitrypsin, BPTI, and tissue factor pathway inhibitor (*11*, *12*). Recently, the crystal structure of the thrombin−BPTI complex was resolved (*13*). Similar to trypsin, the carboxamide nitrogen atom of Gln192 of the thrombin mutant hydrogen bonds to the backbone carbonyls of the P2 and P4 residues of BPTI. The other interesting observation is that Glu39 of thrombin forms an ion pair with the P5′ Arg20 of BPTI (Figure 1). Using this structure as a model and the observations mentioned above, it was hypothesized in this study that both Glu39 and Glu192 would restrict the specificity of thrombin in reaction with PAI-1.

To test this hypothesis, the pseudo-first-order rate constants (k') for the PAI-1 inactivation of wild type as well as the residue 39 and the residue 192 substitution mutants of thrombin were determined at several concentrations of PAI-1 as described under Materials and Methods. A straight line was observed when the k' values were plotted vs PAI-1 concentrations (Figure 2A, shown for wild-type, E39K, and E192Q thrombins; and Figure 2B, shown for E39K/E192Q thrombin). The second-order inactivation rate constants (k_2) of the thrombin derivatives were determined from the slope of these plots which are presented in Table 2. PAI-1 inactivated the thrombin mutants considerably faster than wild-type thrombin. The k_2 values for the inactivation of thrombin E39K and E192Q were 50- and 31-fold faster than the k_2 value for the inactivation of wild-type thrombin, respectively (Table 2). Relative to thrombin, the k_2 value for the double mutant, E39K/E192Q, was improved 628-fold. The concentration dependence of the k' values remained linear with all thrombin derivatives even if the concentration of PAI-1 was increased to 2 μM, suggesting that the apparent dissociation constant (K_d) of the thrombin−PAI-1 complex was higher than 2 μM for all thrombin derivatives (data not shown). This is consistent with the previous results for the thrombin−PAI-1 reaction reported by other investigators (*21*).

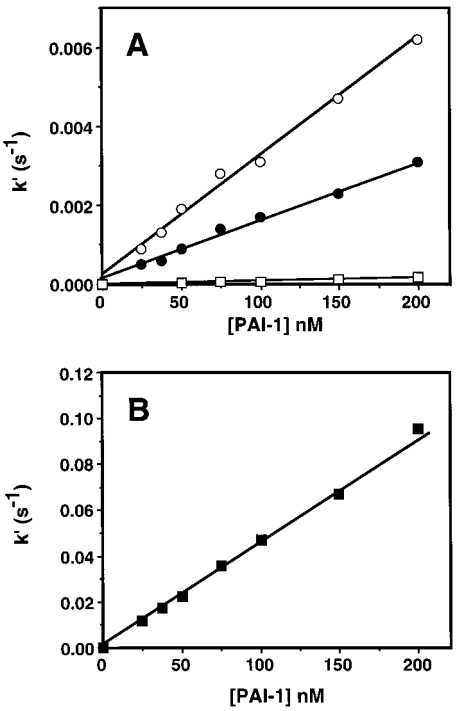

FIGURE 2: Dependence of the pseudo-first-order (k') rate constants on PAI-1 concentration. (A) The k' values for the inactivation of wild-type (□), E39K (○), and E192Q (●) thrombins (1 nM) were determined at varying concentrations of PAI-1 (25−200 nM) and plotted vs PAI-1 concentrations. The k_2 values were calculated from the slopes of the straight lines which are presented in Table 2. (B) The same as panel A except that the k' values for the inactivation of thrombin E39K/E192Q (■) are plotted vs PAI-1 concentrations.

Table 2: Second-Order Rate Constants for the PAI-1 Inactivation of Wild-Type and Mutant Thrombins

	k_2 (M^{-1} s^{-1})[a]	relative rate[b]
thrombin	$(8.6 \pm 0.5) \times 10^2$	1
E39K	$(4.3 \pm 0.2) \times 10^4$	50
E39Q	$(1.0 \pm 0.1) \times 10^4$	12
E192Q	$(2.7 \pm 0.5) \times 10^4$	31
E192M	$(4.3 \pm 0.8) \times 10^3$	5
E39K/E192Q	$(5.4 \pm 0.4) \times 10^5$	628
des-PPW	$(6.8 \pm 0.2) \times 10^2$	0.8
des-YPPW	$(4.0 \pm 1.7) \times 10^2$	0.5

[a] The second-order inactivation rate constants were determined from the linear slope of the plot of the k' values vs PAI-1 concentrations as shown in Figure 2, and described under Materials and Methods. [b] k_2 of mutant/k_2 of wild-type thrombin. All values are the average of at least three independent measurements ± SD.

To determine whether the high reactivities of E39K and E192Q thrombins with PAI-1 are only due to the elimination of repulsive negative−negative charge interactions or if there are additional productive electrostatic interactions that may also contribute to the accelerated rates of the reactions, the PAI-1 inactivation rates of E39Q and E192M thrombin were studied. The rationale was that, unlike Glu39, Gln39 would not be repulsive for interaction with the negatively charged P4′ and/or P5′ Glu residues of PAI-1, and unlike Lys, it would not be capable of forming potentially stabilizing ion-pairs with the reactive site loop of the serpin. Similarly, Met192 is not likely to create repulsive interactions with the PAI-1 reactive site loop, and unlike Gln, it cannot form potentially productive hydrogen bonds with the backbone carbonyls of the serpin. The results in Table 2 indicate that

although the PAI-1 inactivation rates were significantly improved with both E39Q and E192M thrombins, the inactivation rates, nevertheless, with both mutants were still 4−6-fold lower than the inactivation rates of the corresponding mutants with a Lys39 or Gln192 at the indicated positions. These results suggest that the productive electrostatic interactions of Lys39 and Gln192 in thrombin mutants, and most likely in tPA, contribute to the stabilization of the protease−serpin complexes.

It was previously demonstrated that the 60-D insertion loop of thrombin is critical for the reaction of thrombin with its specific serpin inhibitor, antithrombin (*22*). Deletion of three residues, Pro, Pro, Trp (des-PPW) (*22*), or four residues, Tyr, Pro, Pro, Trp (des-YPPW) (*23*), from the insertion loop of thrombin impaired the reactivities of the mutants with antithrombin 500−1000-fold over wild-type thrombin. To determine whether the 60-D loop plays a similar role in the reaction of thrombin with PAI-1, the k_2 values for the PAI-1 inactivation of these mutants were studied. As shown in Table 2, PAI-1 inactivated des-PPW thrombin with a rate constant that was similar to that of wild-type thrombin, and with des-YPPW thrombin, the inactivation rate was impaired only ∼2-fold. These results suggest that in contrast to the reaction of thrombin with antithrombin, the 60-D insertion loop is not as critical for the thrombin−PAI-1 reaction.

DISCUSSION

The structural and kinetic basis for the slow reactivity of thrombin with PAI-1 was investigated in this study. Based on the structure of the thrombin E192Q−BPTI complex as a model, and the previous mutagenesis studies, three structural elements were identified which appear to be responsible for the inability of thrombin to react efficiently with PAI-1. First, the substitution of Glu39 of thrombin with Lys or Gln markedly improved the reactivity of thrombin with PAI-1. Since PAI-1 has Glu residues at both the P4′ (Glu350) and P5′ (Glu351) positions of the reactive site loop, it appears that repulsive negative−negative charge interactions and/or lack of electrostatic interactions between Glu39 of thrombin with either one of these residues restrict the reactivity of thrombin with PAI-1. This is consistent with the previous mutagenesis studies of Madison et al., who also demonstrated that substitution of Arg39 with Glu in tPA results in a 61-fold reduction in the reactivity of this mutant with PAI-1 (*6*). Further support for the productive interaction of Arg39 of tPA with the negatively charged residues of PAI-1 came from the observation that compensatory mutations in PAI-1 either improved (Glu351→Arg) or completely restored (Glu350→Arg) the reactivities with the mutant tPA, but exhibited much diminished reactivities with the wild-type tPA (*9*). Second, relative to trypsin, tPA has an insertion of seven amino acids at the 39-loop, three of which are positively charged (Table 1). Previous mutagenesis studies suggested that two of these positively charged residues (Arg298 and Arg299) are critical for the rapid reaction of tPA with PAI-1 (*6*). The charge reversal or deletion of these residues resulted in dramatic decrease in the reactivity of tPA with PAI-1, suggesting that these residues form as yet another binding site for PAI-1 that is critical for the high reactivity of tPA with PAI-1 (*5*, *6*, *8*). In contrast to tPA, thrombin has an insertion of three residues in this loop, only one of which is positively charged and conserved at the identical positions of both enzymes (Lys36 = Lys296 in tPA). As demonstrated by the mutagenesis studies, however, this residue does not play a role in the reaction of tPA with PAI-1. This appears to be also true for thrombin, since the side chain of this residue in the crystal structure of thrombin points away from the active site pocket of thrombin, making it unlikely to contact the reactive site loop of PAI-1 (*1*). Third, similar to trypsin, tPA has a Gln at position 192, but this residue is a Glu in thrombin. The observation that thrombin E192Q reacted with PAI-1 much better than thrombin suggests that Glu192 is another residue that restricts the specificity of thrombin in reaction with PAI-1. This is consistent with the conclusion of the previous studies which suggested that Glu192 restricts the specificity of thrombin in reaction with several other macromolecular substrates and inhibitors (*11*, *12*, *24*).

The results discussed above suggest that the structural differences between tPA and thrombin in the residues of the 39-loop, and residue192 are primarily responsible for the inefficient reactivity of thrombin with PAI-1. This is consistent with the observation that thrombin E39K/E192Q reacted with PAI-1 with a second-order inactivation rate constant that was 628-fold higher than the inactivation rate of wild-type thrombin. In yet another study, Horrevoets et al. replaced the entire variable region 1 (Phe34−Leu40) of thrombin with the corresponding region of tPA, and demonstrated a 2000-fold higher reactivity for this mutant with PAI-1 (*10*). However, the PAI-1 inactivation rate constant of the thrombin mutant (2.2×10^6 M^{-1} s^{-1}) was still ∼10-fold lower than the inactivation rate constant of tPA (2.6×10^7 M^{-1} s^{-1}) reported by the authors in their previous studies under identical experimental conditions (*25*, *26*). Based on the current results, it is proposed that the lower reactivity of the latter mutant with PAI-1 is due to the absence of a tPA-specific residue (Gln) at position 192 of the mutant thrombin. Similarly, the lower reactivity of thrombin E39K/E192Q with PAI-1 appears to be due to the absence of tPA-specific Arg residues (Arg298 and Arg299) at the variable region 1 (39-loop) of this mutant. Taken together, these results suggest that by grafting all three tPA-specific structural elements on thrombin, it may be possible to completely transfer the PAI-1 reaction specificity of tPA to thrombin. This raises an interesting question: What is the role of the 60-D insertion loop in the thrombin−PAI-1 reaction? This is important because the previous mutagenesis studies designated a critical role for this loop of thrombin in reaction with the natural macromolecular substrates and inhibitors (*22*). In two separate studies, des-PPW (*22*) and des-YPPW thrombins (*23*) reacted with antithrombin 500−1000-fold slower than did wild-type thrombin, suggesting a crucial role for the 60-D loop of thrombin in reaction with this serpin. By contrast, the inactivation rate of des-PPW by PAI-1 was nearly normal, and the inactivation of des-YPPW was impaired only ∼2-fold. These results suggest that the nature of interactions of the extended binding pocket residues of thrombin with antithrombin and PAI-1 may be different and that the 60-D insertion loop is not as critical for the thrombin−PAI-1 reaction as it is for the thrombin−antithrombin reaction.

It has been proposed that similar to other serpins, PAI-1 inactivates thrombin by a two-step reaction mechanism where a reversible Michaelis-type complex is formed in the first step, which is then converted to a stable, covalent complex

in the second step of the reaction (*27*, *28*). The resolution of the two-step reaction of thrombin with PAI-1 was not possible for any thrombin derivative, because the reaction rates remained linear for up to 2 μM PAI-1 concentration. It is therefore not known whether the improvement in the reactivities of the mutants with PAI-1 is due to an effect on the first step or the second step of the reaction. However, previous kinetic studies have indicated that E192Q and E39K thrombins activate protein C, the natural substrate of thrombin in the anticoagulant pathway, 22- and 2-fold faster than wild-type thrombin, respectively (*15*, *24*). In both cases, the improvements in the activation rates were localized to the second step of the reaction, i.e., in the k_{cat} of the protein C activation (*15*, *24*). These previous studies proposed a crucial role for Glu192 and Glu39 in determination of the S3 and S3′ binding specificity of thrombin, respectively (*22*, *24*, *29*). Serpins are pseudosubstrates that react with the proteases by a mechanism similar to true substrates; it is likely that the energy of interactions of Gln192 and Lys39 of the mutant thrombins with the PAI-1 reactive site loop is also utilized to stabilize the transition state of the protease−serpin reactions. In several other previous studies, a similar role for S1′−P1′ (*30*, *31*) and S2−P2 (*32*) interactions was proposed in the reaction of thrombin with antithrombin, suggesting that transition state stabilization via S−P and S′−P′ interactions may be a general feature of the protease−serpin interactions. Resolution of the two-step reaction of the wild-type and mutant thrombins with PAI-1, however, will be required to confirm this hypothesis.

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REFERENCES

1. Bode, W., Mayr, I., Baumann, U., Huber, R., Stone, S. R., and Hofsteenge, J. (1989) *EMBO J. 8*, 3467−3475.
2. Schechter, I., and Berger, A. (1967) *Biochem. Biophys. Res. Commun. 27*, 157−162.
3. Loskutoff, D. J. (1986) in *Vascular Endothelium in Hemostasis and Thrombosis* (Gimbrone, M. A., Ed.) pp 120−141, Churchill Livingstone, Edinburgh.
4. van Mourik, J. A., Lawrence, D. A., and Loskutoff, D. J. (1984) *J. Biol. Chem. 259*, 14914−14921.
5. Madison, E. L., Goldsmith, E. J., Gerard, R. D., Gething, M.-J. H., and Sambrook, J. F. (1989) *Nature 339*, 721−724.
6. Madison, E. L., Goldsmith, E. J., Gerard, R. D., Gething, M.-J. H., Sambrook, J. F., and Bassel-Duby, R. S. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 3530−3533.
7. Furie, B., Bing, D. H., Feldman, R. J., Robison, D. J., Burnier, J. F., and Furie, B. C. (1982) *J. Biol. Chem. 257*, 3875−3882.
8. Tachias, K., and Madison, E. L. (1997) *J. Biol. Chem. 272*, 14580−14585.
9. Madison, E. L., Goldsmith, E. J., Gething, M.-J. H., Sambrook, J., and Gerard, R. D. (1990) *J. Biol. Chem. 265*, 21423-21426.
10. Horrevoets, A. J. G., Tans, G., Smilde, A. E., van Zonneveld, A.-J., and Pannekoek, H. (1993) *J. Biol. Chem. 268*, 779−782.
11. Guinto, E. R., Ye, J., Le Bonniec, B. F., and Esmon, C. T. (1994) *J. Biol. Chem. 269*, 18395−18400.
12. Rezaie, A. R., and Esmon, C. T. (1996) *Eur. J. Biochem. 242*, 477−484.
13. van de Locht, A., Bode, W., Huber, R., Le Bonniec, B. F., Stone, S. R., Esmon, C. T., and Stubbs, M. T. (1997) *EMBO J. 16*, 2977−2984.
14. Huber, R., Kukla, D., Bode, W., Schwager, P., Bartels, K., Deisenhofer, J., and Steigemann, W. (1974) *J. Mol. Biol. 89*, 73−101.
15. Le Bonniec, B. F., MacGillivray, R. T. A., and Esmon, C. T. (1991) *J. Biol. Chem. 266*, 13796−13803.
16. Sherman, P. M., Lawrence, D. A., Yang, A. Y., Vandenberg, E. T., Paielli, D., Olson, S. T., Shore, J. D., and Ginsburg, D. (1992) *J. Biol. Chem. 267*, 7588−7595.
17. Shore, J. D., Day, D. E., Francis-Chmura, A. M., Verhamme, I., Kvassman, J., Lawrence, D. A., and Ginsburg, D. (1995) *J. Biol. Chem. 270*, 5395−5398.
18. Rezaie, A. R., Cooper, S. T., Church, F. C., and Esmon, C. T. (1995) *J. Biol. Chem. 270*, 25336−25339.
19. Rezaie, A. R. (1996) *Biochemistry 35*, 1918−1924.
20. Ruhlmann, A., Kukla, D., Schwager, P., Bartels, K., and Huber, R. (1973) *J. Mol. Biol. 77*, 417−436.
21. Naski, M. C., Lawrence, D. A., Mosher, D. F., Podor, T. J., and Ginsburg, D. (1993) *J. Biol. Chem. 268*, 12367−12372.
22. Le Bonniec, B. F., Guinto, E. R., MacGillivray, R. T. A., Stone, S. R., and Esmon, C. T. (1993) *J. Biol. Chem. 268*, 19055−19061.
23. Rezaie, A. R. (1998) *Protein Sci. 7*, 349−357.
24. Le Bonniec, B. F., and Esmon, C. T. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 7371−7375.
25. Keijer, J., Ehrlich, H. J., Linders, M., Preissner, K. T., and Pannekoek, H. (1991) *J. Biol. Chem. 266*, 10700−10707.
26. Keijer, J., Linders, M., Wegman, J. J., Ehrlich, H. J., Mertens, K., and Pannekoek, H. (1991) *Blood 78*, 1254−1261.
27. van Meijer, M., Smilde, A., Tans, G., Nesheim, M., Pannekoek, H., and Horrevoets, A. J. G. (1997) *Blood 90*, 1874−1882.
28. Gettins, P. G. W., Patston, P. A., and Olson, S. T. (1996) in *Serpins: Structure, Function and Biology*, pp 33−63, R. G. Lands Co., Austin, TX.
29. Esmon, C. T. (1993) *Thromb. Haemostasis 70*, 1−5.
30. Olson, S. T., Stephens, A. W., Hirs, C. H. W., Bock, P. E., and Björk, I. (1995) *J. Biol. Chem. 270*, 9717−9724.
31. Rezaie, A. R., and Olson, S. T. (1997) *Biochemistry 36*, 1026−1033.
32. Rezaie, A. R. (1997) *Biochemistry 36*, 7437−7446.

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