Role of Exosites 1 and 2 in Thrombin Reaction with Plasminogen Activator Inhibitor-1 in the Absence and Presence of Cofactors†

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ABSTRACT: The cofactors heparin, vitronectin (VN), and thrombomodulin (TM) modulate the reactivity of α-thrombin with plasminogen activator inhibitor (PAI-1). While heparin and VN accelerate the reaction by ∼2 orders of magnitude, TM protects α-thrombin from rapid inactivation by PAI-1 in the presence of VN. To understand how these cofactors function, we studied the kinetics of PAI-1 inactivation of α-thrombin, the exosite 1 variant γ-thrombin, the exosite 2 mutant R93,97,101A thrombin, and recombinant meizothrombin in both the absence and presence of these cofactors. Heparin and VN accelerated the second-order association rate constant [$k_2 = (7.9 \pm 0.5) \times 10^2$ M^{-1} s^{-1}] of α-thrombin with PAI-1 ∼200- and ∼240-fold, respectively. The k_2 value for γ-thrombin [$(7.9 \pm 0.7) \times 10^1$ M^{-1} s^{-1}] was impaired 10-fold, but was enhanced by heparin and VN ∼280- and ∼75-fold, respectively. Similar to inactivation of γ-thrombin, PAI-1 inactivation of α-thrombin in complex with the epidermal growth factor-like domains 4−6 of TM (TM4−6) was impaired ∼10-fold. The exosite 2 mutant R93,97,101A thrombin, which was previously shown not to bind heparin, and meizothrombin, in which exosite 2 is masked, reacted with PAI-1 at similar rates in both the absence and presence of heparin [$k_2 = (1.3-1.5) \times 10^3$ M^{-1} s^{-1} for R93,97,101A thrombin and $k_2 = (3.6-5.1) \times 10^2$ M^{-1} s^{-1} for meizothrombin]. Unlike heparin, however, VN enhanced the k_2 of R93,97,101A thrombin and meizothrombin inactivation ∼80- and ∼30-fold, respectively. Continuous kinetic analysis as well as competition kinetic studies in the presence of S195A thrombin suggested that the accelerating effect of VN or heparin occurs primarily by lowering the dissociation constant (K_d) for formation of a noncovalent, Michaelis-type complex. Analysis of these results suggest that (1) heparin binds to exosite 2 of α-thrombin to accelerate the reaction by a template mechanism, (2) VN accelerates PAI-1 inactivation of α-thrombin by lowering the K_d for initial complex formation by an unknown mechanism that does not require binding to either exosite 1 or exosite 2 of α-thrombin, (3) α-thrombin may have a binding site for PAI-1 within or near exosite 1, and (4) TM occupancy of exosite 1 partially accounts for the protection of thrombin from rapid inactivation by PAI-1 in the presence of vitronectin.

Plasminogen activator inhibitor-1 (PAI-1)[1] is a serine proteinase inhibitor of the serpin superfamily which rapidly inactivates both of the serine proteinases of the fibrinolytic system, the tissue-type and urokinase-type plasminogen activators, but reacts very slowly with the serine proteinases of the coagulation system (*1, 2*). Recently, however, PAI-1 was shown to inactivate thrombin with more than a 200-fold accelerated second-order association rate constant in the presence of the cofactors vitronectin and heparin (*3*−*6*). The physiological significance of the high reactivity of PAI-1 with thrombin in the presence of vitronectin and heparin is not completely understood since the cofactors also endow the serpin with a substrate property as evidenced by an increase in the stoichiometry of inhibition in the presence of the cofactors (SI ∼ 5−7) (*4, 7*). Vitronectin is known to bind PAI-1 with high affinity and stabilize the active conformation of the serpin in plasma and in the extracellular matrix (*6, 8, 9*). On the basis of these and other observations, it has been speculated that in addition to regulation of the fibrinolytic system, PAI-1 in complex with vitronectin may also play a role in regulation of the coagulation cascade and/or other (patho)physiological processes such as degradation of extracellular matrixes (*4, 10, 11*).

Similar to most other serpins, PAI-1 inactivates thrombin by a two-step reaction mechanism whereby an enzyme−inhibitor encounter complex is initially formed which is then converted to a stable, covalent complex (*4, 5*). A previous kinetic study demonstrated that vitronectin accelerates PAI-1 inactivation of thrombin primarily by lowering the dissociation constant for initial encounter complex formation (*5*). This type of cofactor effect accounts for the accelerating effect of heparin in antithrombin inactivation of thrombin (*12*). The result of this previous study, therefore, indicated

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[1] Abbreviations: PAI-1, plasminogen activator inhibitor-1; VN, vitronectin; serpin, serine protease inhibitor; R93,97,101A and S195A thrombins, thrombin mutants in which Arg^{93}, Arg^{97}, Arg^{101} and Ser^{195} in the chymotrypsin numbering system of Bode et al. (*46*) are substituted with Ala; meizothrombin, an active prothrombin variant in which Arg^{155}, Arg^{271}, and Arg^{284} are substituted with Ala; TM, thrombomodulin; sTM, soluble TM; TM4−6, epidermal growth factor-like domains 4−6 of TM; SI, stoichiometry of inactivation; PEG, poly(ethylene glycol).

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that vitronectin may also accelerate PAI-1 inactivation of thrombin by a template mechanism (*5*). Other studies, however, have disputed such a mechanism for vitronectin since, unlike heparin, a bell-shaped cofactor concentration dependence curve for vitronectin has not been observed even if the concentration of the cofactor is increased to 300-fold in excess of PAI-1 in the reaction (*4, 13*).

In contrast to vitronectin, the accelerating effect of heparin in thrombin inactivation by PAI-1 is known to exhibit a bell-shaped dependence on the heparin concentration (*4, 11*). Moreover, heparin chains greater than 14 saccharide units in length are shown to be required for acceleration of thrombin inactivation by PAI-1 (*11*). These observations have indicated that the cofactor function of heparin in PAI-1 inactivation of thrombin is mediated by a template mechanism, the same role heparin plays in antithrombin inactivation of thrombin (*12*). The template role of heparin in acceleration of thrombin inactivation by antithrombin is established by several mutagenesis studies utilizing exosite 2 mutants of thrombin (*14*−*16*). In the case of PAI-1, however, a similar mutagenesis study has not been conducted.

When thrombin binds to the endothelial cell receptor thrombomodulin (TM), its macromolecular substrate specificity changes so that thrombin no longer functions as a procoagulant enzyme. Instead, thrombin functions as an anticoagulant enzyme by rapidly activating protein C (*17*). In switching the specificity of thrombin, epidermal growth factor-like domains 4−6 of TM (TM4−6) bind to exosite 1, and the chondroitin sulfate moiety of TM binds to exosite 2 of thrombin (*18*−*21*). A previous study, which examined the reactivity of thrombin with PAI-1 in the endothelial cell matrix, demonstrated that only the procoagulant form of thrombin interacts with PAI-1. This was evidenced by the observation that the addition of rabbit TM to the subendothelial matrix prevented the formation of sodium dodecyl sulfate (SDS)-stable thrombin−PAI-1 complexes and/or cleavage of the serpin by thrombin (*10*). It is not known how TM attenuates the reactivity of thrombin with PAI-1.

To understand how these cofactors modulate the reactivity of thrombin with PAI-1, we studied the kinetics of PAI-1 inactivation of the wild-type α-thrombin, the exosite 1 variant γ-thrombin, and the exosite 2 mutant R93,97,101A thrombin and recombinant meizothrombin, which was prepared by substitution of Arg^{155}, Arg^{271}, and Arg^{284} of prothrombin with Ala followed by activation of the mutant zymogen by *Echis carinatus* venom. In meizothrombin, the noncatalytic fragment 1.2 of prothrombin remains associated with the protease domain (*22, 23*). Fragment 2 is known to bind exosite 2 of this derivative, thereby preventing the interaction of this site with heparin or other related ligands (*24, 25*). In addition, we generated an active-site mutant, S195A thrombin, and examined its ability to compete with α-thrombin for binding to the cofactor−PAI-1 complexes by competition kinetic studies. Our results suggest that heparin binds to exosite 2 of thrombin to accelerate PAI-1 inactivation of thrombin by a template mechanism. Vitronectin, similar to heparin, accelerates PAI-1 inactivation of thrombin by lowering the dissociation constant for initial enzyme−inhibitor complex formation; however, unlike heparin, binding of the cofactor to exosite 2 may not be essential for acceleration. Our results further suggest that thrombin may have a binding site for PAI-1 within or near exosite 1, and that TM occupancy of this site partially protects the anticoagulant form of thrombin from rapid inactivation by PAI-1 in the presence of vitronectin.

MATERIALS AND METHODS

Proteins and Reagents. Methodologies for the expression, purification, and activation of the prethrombin 1 forms of the wild-type, Arg93,97,101→Ala (R93,97,101A), and Ser→Ala (S195A) mutants of thrombin were previously described (*14, 26*). Recombinant human TM4−6 (*27*) and soluble full-length TM containing chondroitin sulfate (*21*) were expressed in mammalian cells and characterized as previously described. Recombinant meizothrombin was prepared by *Echis carinatus* venom (Sigma) activation of a prothrombin mutant in which Arg^{155}, Arg^{271}, and Arg^{284} of prothrombin were substituted with Ala by standard polymerase chain reaction (PCR) mutagenesis methods (*22, 28*). Human γ-thrombin, in which $Arg^{70/73}$ and Arg^{154} in the B-chain of thrombin are cleaved (product HCGT-0021), was purchased from Haematologic Technologies Inc. (Essex Junction, VT). Recombinant human active PAI-1 (product PAI-A, 95 ± 5% active as titrated by uPA) and plasma-derived human native vitronectin (product HVN) were purchased from Molecular Innovations, Inc. (Royal Oak, MI). SDS−PAGE analysis confirmed the homogeneity of all proteins used in this study. Polybrene and unfractionated heparin (porcine intestinal mucosa, sodium salt, grade II) were purchased from Sigma Chemical Co. (St. Louis, MO). The chromogenic substrate Spectrozyme PCa (SpPCa) and hirudin synthetic C-terminal dodecapeptide (*N*-acetyl-Hir-53′-64′-OH with sulfato-Tyr63′, product 5343) were purchased from American Diagnostica (Greenwich, CT). The fluorogenic substrate *N*-*p*-tosyl-Gly-Pro-Arg-7-amido-4-methylcoumarin (Tosyl-GPA-amc) was purchased from Sigma. The fluorogenic substrate was dissolved in distilled water and passed through a 0.22 μm filter, and its concentration was determined in 0.1 M sodium acetate using an absorption coefficient of 2×10^4 M^{-1} cm^{-1} at 325 nm as described (*29*).

Kinetic Methods. The rate of inactivation of thrombin and the variants in the absence of cofactors was measured under pseudo-first-order rate conditions by a discontinuous assay method as previously described (*30*). Briefly, 0.5 nM samples of wild-type or variant thrombins were incubated at room temperature with 50−1000 nM PAI-1 in 50 μL reactions in Tris-HCl (pH 7.5), 0.1 M NaCl buffer (TBS) containing 0.1 mg/mL bovine serum albumin (BSA) and 0.1% poly(ethylene glycol) 8000 (PEG 8000). In inactivation of meizothrombin, 2.5 mM $CaCl_2$ was also included in the buffer. This was done to ensure that the Gla-domain of the variant protease is folded properly. At the end of the incubation time (10−45 min), 50 μL of SpPCa was added to a final concentration of 0.25 mM. The remaining activities of uninhibited thrombins were measured from the rate of chromogenic substrate hydrolysis using a *Vmax* kinetics microplate reader (Molecular Devices, Menlo Park, CA). The pseudo-first-order rate constant of inactivation was calculated by fitting the time-dependent change of thrombin activity to a first-order rate equation as previously described (*30, 31*). The second-order inactivation rate constants (k_2) were calculated from the slope of the linear plot of the pseudo-first-order rate constants vs PAI-1 concentrations.

The heparin concentration dependence of PAI-1 inactivation of different thrombin derivatives was studied by incubating a 0.5 nM sample of each enzyme with 10−50 nM PAI-1 and 0−50 μM heparin at room temperature in 50 μL reactions in the same buffer system described above. At the end of the incubation time (1−5 min), 50 μL of 0.5 mM SpPCa in TBS buffer containing 1 mg/mL Polybrene (to block heparin function immediately) was added. Incubation was continued for another 10−15 min so that ~5% of the chromogenic substrate was hydrolyzed. The reactions were then terminated by the addition of 50 μL of 1.5 M acetic acid to each well. The Polybrene−heparin precipitates were separated by centrifugation, the absorbance was read at 405 nm, and the inactivation rate constants were calculated from the residual thrombin activities as described above.

Inactivation kinetic studies in the presence of S195A thrombin were carried out by incubating 1 nM α-thrombin for 5−10 min at room temperature with 200 nM PAI-1 and either 100 nM vitronectin or 10 nM heparin in the presence of 0−2 μM S195A thrombin in TBS buffer containing 0.1 mg/mL BSA and 0.1% PEG 8000. PAI-1 was incubated with the cofactors for 5 min at room temperature before the reaction was initiated by the addition of α-thrombin together with different concentrations of S195A thrombin. Under these conditions, ~90% of the activity of thrombin was inhibited in the absence of S195A thrombin. The residual thrombin activity was determined, and the competitive effect of S195A thrombin was calculated from the ratio of % PAI-1 inactivation of α-thrombin in the presence of S195A to % PAI-1 inactivation in the absence of S195A thrombin. Data were normalized and plotted as a function of different concentrations of S195A thrombin.

A detailed kinetic analysis in the presence of vitronectin was carried out by a continuous assay method using the fluorogenic substrate Tosyl-GPA-amc in a *fmax* fluorescence microplate reader (Molecular Devices). The excitation and emission wavelengths were set to 355 and 460 nm, respectively. In this assay, a series of inhibition progress curves were generated by adding 5 pM of each thrombin derivative to the wells of a 96-well polystyrene plate containing 0−400 nM PAI-1, 500 nM vitronectin, and a 20 μM aliquot of the competing fluorogenic substrate Tosyl-GPA-amc in the TBS buffer system described above. Data from each curve at different inhibitor concentrations in the presence of the cofactors were fitted to the following integrated rate equation as previously described (*32*):

$$\mathrm{RFU} = v_s t + (v_o - v_s)(1 - e^{-k^{obs}t})/k_{obs} + \mathrm{RFU}_o \quad (1)$$

where RFU at time t is a relative fluorescence unit, v_o and v_s are the initial and final steady-state velocities, respectively, k_{obs} is the apparent first-order-rate constant, and RFU_o is the initial fluorescence. Fitting gives estimates for the values of v_o, v_s, k_{obs}, and RFU_o for each progress curve. These values are analyzed according to the method of Tian and Tsou (*33*) to obtain inactivation and reaction rate constants as described below (Results). Control experiments using excess thrombin indicated that complete hydrolysis of 1.0 μM Tosyl-GPA-amc results in an RFU value of 0.9−1.0 at the excitation and emission wavelengths mentioned above. This was done to ensure that in all experiments less than 10% of the fluorogenic substrate was hydrolyzed. The K_m values for hydrolysis of Tosyl-GPA-amc at room temperature in the TBS buffer containing 0.1 mg/mL BSA, 0.1% PEG 8000, and 500 nM vitronectin were 4.2 ± 0.7 and 3.2 ± 0.1 μM for α-thrombin and R93,97,101A thrombin, respectively.

To ensure that both types of measurements give comparable results, the discontinuous assays, which were carried out with the chromogenic substrate SpPCa, were also carried out with the fluorogenic substrate Tosyl-GPA-amc under identical conditions, with the exception that the thrombin concentration in the assays was reduced to 10 pM. Since a very low concentration of thrombin was needed to monitor the enzyme activity, this assay was also used to reliably determine the inactivation rate constants in the presence of the cofactors under pseudo-first-order rate conditions (PAI-1 concentration ≫ 10 × SI values). SI values of 5−7 are reported for PAI-1 inactivation of thrombin in the presence of the cofactors, heparin and vitronectin (*4*). In this assay, 10 pM thrombin was incubated with 10−400 nM PAI-1 in the presence of 500−1000 nM vitronectin or 50−200 nM heparin for 5−30 min at room temperature in the buffer system described above. At the end of the incubation times, 50 μL of 300 μM Tosyl-GPA-amc was added to each reaction, and the observed rate constants were determined from the residual thrombin activities measured at the excitation and emission wavelengths of 355 and 460 nm, as described above.

Determination of Inhibition Stoichiometry. The SI values for PAI-1 inactivation of thrombin derivatives were determined by titration of 10−100 nM thrombin in the presence of 200−750 nM vitronectin or heparin with increasing concentrations of PAI-1 corresponding to PAI-1/thrombin molar ratios of 0 to 12, in TBS containing 0.1 mg/mL BSA and 0.1% PEG 8000. The residual amidolytic activities of thrombin and the variant enzymes were monitored for up to 24 h at room temperature by the hydrolysis of SpPCa as described above. After completion of the inhibition reactions, the PAI-1/thrombin ratios were plotted versus the residual activities of enzymes, and the SI values were determined from the *x*-intercept of the linear regression fit of the inhibition data.

Data Analysis. The ENZFITTER computer program (R. J. Leatherbarrow, Elsevier, Biosoft) was used to analyze data using appropriate equations. All values are an average of at least 3−5 independent measurements ±SD.

RESULTS

The second-order association rate constants (k_2) for PAI-1 inactivation of different thrombin derivatives are presented in Table 1. These values were determined by a discontinuous assay method using either the chromogenic or the fluorogenic substrate as described under Materials and Methods. As expected, essentially identical results were obtained with both types of substrates. The k_2 value for PAI-1 inactivation of the exosite 1 variant γ-thrombin was impaired ~10-fold in the absence of the cofactors, suggesting that an intact exosite 1 is required for normal reactivity of thrombin with PAI-1 (see below). The reactivity of PAI-1 with meizothrombin, a variant in which exosite 2 is masked, was also impaired ~2-fold. Under the same conditions, however, k_2 for R93,97,-101A thrombin was improved ~2-fold (Table 1). The reason for minor changes in the reactivities of the latter two variants of thrombin with PAI-1 was not studied. However, thrombin is a complex allosteric enzyme, and differences in the

Table 1: Second-Order Association Rate Constants (k_2) for PAI-1 Inactivation of Different Thrombin Derivatives in the Absence and Presence of Heparin or Vitronectin[d]

	−cofactor[a]	+heparin[b]	+vitronectin[c]
α-thrombin	$(7.9 \pm 0.5) \times 10^2$	$(1.6 \pm 0.2) \times 10^5$	$(1.9 \pm 0.3) \times 10^5$
R93,97,101A	$(1.5 \pm 0.1) \times 10^3$	$(1.3 \pm 0.2) \times 10^3$	$(1.2 \pm 0.1) \times 10^5$
γ-thrombin	$(7.9 \pm 0.7) \times 10^1$	$(2.2 \pm 0.3) \times 10^4$	$(5.9 \pm 2.1) \times 10^3$
meizothrombin	$(4.6 \pm 0.3) \times 10^2$	$(3.6 \pm 0.1) \times 10^2$	$(1.4 \pm 0.2) \times 10^4$
thrombin + TM4−6	$(6.7 \pm 0.5) \times 10^1$	$(1.9 \pm 0.4) \times 10^4$	$(8.3 \pm 1.6) \times 10^3$
thrombin + sTM	$(1.1 \pm 0.3) \times 10^2$	ND[e]	$(1.0 \pm 0.2) \times 10^3$

[a] The k_2 values (M^{-1} s^{-1}) were determined by a discontinuous assay method using either the chromogenic substrate Spectrozyme PCa or the fluorogenic substrate Tosyl-GPA-amc as described under Materials and Methods. [b] The k_2 values were determined in the presence of optimal concentration of heparin (50−200 nM) by a discontinuous assay method as described above and under Materials and Methods. [c] The k_2 values were determined in the presence of 500−1000 nM vitronectin by a discontinuous assay method as described above and under Materials and Methods. [d] Around 40−80% of enzyme activity was inhibited for calculation of the inactivation rate constants. Prolonged incubation and/or incubation at high concentrations of PAI-1 resulted in complete inhibition of all thrombin variants. All values are an average of at least three independent measurements ±SD. [e] ND, not determined.

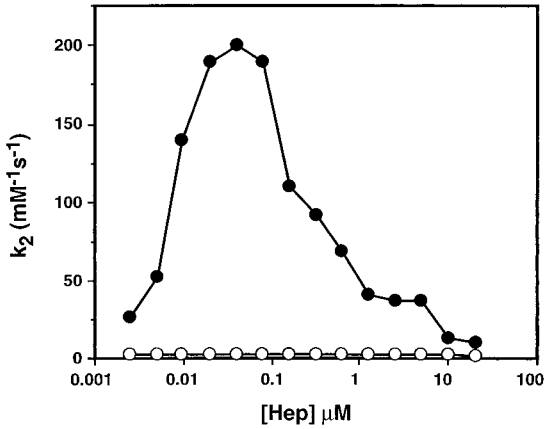

FIGURE 1: Heparin concentration dependence of the inactivation of α-thrombin and R93,97,101A thrombin by PAI-1. The k_2 values for wild-type (●) or R93,97,101A thrombin (○) were determined in the presence of the indicated concentrations of heparin in TBS buffer containing 0.1 mg/mL BSA and 0.1% PEG 8000 at room temperature as described under Materials and Methods.

conformation of exosites 1, 2 and/or the active site pocket of the variants can account for these observations.

Cofactor Effect of Heparin. To determine the k_2 values for PAI-1 inactivation of thrombin in the presence of heparin, the rate constants were initially determined as a function of increasing concentrations of the cofactor. With both α- and γ-thrombin, the accelerating effect of unfractionated heparin showed a bell-shaped dependence on the polysaccharide concentration, which was optimal at ∼50 nM (∼0.8 μg/mL) heparin for α-thrombin and 100−200 nM heparin for γ-thrombin (Figure 1, shown for α-thrombin only). In contrast to a ∼200-fold acceleration of α-thrombin and ∼280-fold acceleration of γ-thrombin inactivation at optimal concentrations of heparin (Table 1), PAI-1 inactivation of R93,97,101A thrombin was insensitive to the presence of heparin in the reaction (Figure 1). Similarly, heparin did not influence the PAI-1 inactivation of meizothrombin. PAI-1 inactivated each of the latter two variants with similar k_2 values in both the absence and presence of heparin (Table 1). These results suggest that heparin binding to exosite 2 of thrombin is required for the catalysis of thrombin inactivation by PAI-1 by a template mechanism.

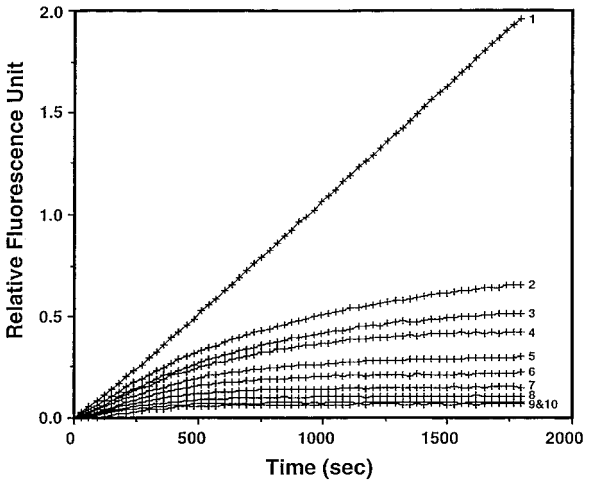

FIGURE 2: Progress curves for inactivation of α-thrombin by PAI-1 in the presence of vitronectin. α-Thrombin (5 pM) was added to reactions containing 20 μM Tosyl-GPA-amc and varying concentrations of PAI-1 in complex with 500 nM vitronectin in TBS buffer containing 0.1 mg/mL BSA and 0.1% PEG 8000. PAI-1 concentrations were: 0 (1), 25 nM (2), 37.5 nM (3), 50 nM (4), 75 nM (5), 100 nM (6), 150 nM (7), 200 nM (8), 300 nM (9), and 400 nM (10). The pseudo-first-order rate constants (k_{obs}) at each inhibitor concentration were determined from nonlinear regression fit of data, collected every 30 s, to eq 1 as described under Materials and Methods.

Cofactor Effect of Vitronectin. In contrast to heparin, vitronectin accelerated the PAI-1 inactivation of all thrombin derivatives in a saturable manner. With 100 nM PAI-1, saturation was observed at ∼500 nM vitronectin, and increasing concentrations of the cofactor up to 2.5 μM did not result in any appreciable decline in the second-order association rate constant with any thrombin derivative (data not shown). This is consistent with previous results in the literature (*4*, *13*). In one such study, no inhibition of the reaction was observed even if the concentration of vitronectin in the reaction was increased to orders of magnitude in excess of PAI-1 (*13*) whereas only slight inhibition was observed in another study (*5*). At a saturating concentration of vitronectin (500−1000 nM in all reactions), vitronectin accelerated PAI-1 inactivation of thrombin ∼240-fold. The accelerating effect of vitronectin was ∼75-fold with γ-thrombin, ∼80-fold with R93,97,101A thrombin, and ∼30-fold with meizothrombin (Table 1). Previous studies suggest that the SI of the thrombin−PAI-1 reaction is elevated in the presence of cofactors (*4*). We determined the SI values for the wild-type and variant thrombins in the presence of both heparin and vitronectin. An SI value of ∼4 for PAI-1 inactivation of both α- and γ-thrombin was observed in the presence of heparin. In the presence of vitronectin, SI values of ∼4−6 were obtained for all thrombin derivatives. These results suggest that an impaired but still substantial cofactor effect of vitronectin remains in the reaction with the thrombin variants. Thus, unlike the accelerating effect of heparin, which is dependent on the binding of the cofactor to exosite 2, much of the cofactor effect of vitronectin in PAI-1 inactivation of thrombin is mediated independent of either exosite 1 or exosite 2 of thrombin.

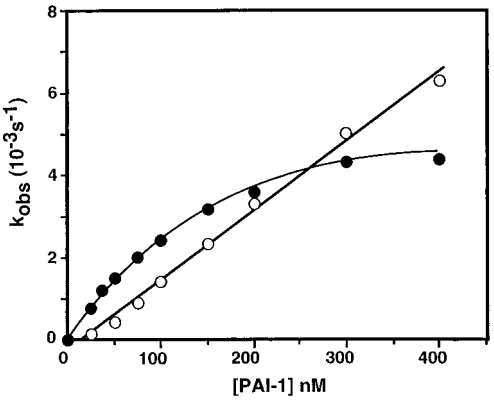

FIGURE 3: Dependence of the k_{obs} values on PAI-1 concentration with α-thrombin and R93,97,101A thrombin in the presence of vitronectin. The k_{obs} values for α-thrombin (●) inactivation at different concentrations of PAI-1 in the presence of vitronectin were calculated from the progress curves shown in Figure 2. The k_{obs} values for R93,97,101A thrombin (○) inactivation were calculated from a series of similar progress curves under identical conditions. The data for α-thrombin were fitted to eq 2, 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 1 under Results. Data for R93,97,101A thrombin were fitted to eq 3, describing a straight line, the slope of which represents the second-order association rate constant (k_2).

Scheme 1

$$\alpha\text{-thrombin} + \text{PAI-1}\bullet\text{VN} \underset{K_d=(27.2\pm2.9)\ \text{nM}}{\leftrightarrow} \alpha\text{-thrombin} \bullet \text{PAI-1} \bullet\text{VN} \xrightarrow{k=(6.3\pm0.3)\times 10^{-3}\ s^{-1}} \alpha\text{-thrombin-PAI-1} + \text{VN}$$

Continuous Kinetic Analysis. Since a template curve for vitronectin was not observed, we decided to further analyze the PAI-1 inactivation of thrombin in the presence of vitronectin by the slow-binding kinetic approach as previously described (*26*, *32*). In this case, a series of progress curves at several concentrations of PAI-1 in the presence of a fixed concentration (500 nM) were generated (Figure 2). Under these conditions, all PAI-1 is expected to bind vitronectin and as in previous studies can be equated with the PAI-1−vitronectin binary complex concentration (*5*). The pseudo-first-order inactivation rate constant (k_{obs}) at each concentration of PAI-1 was determined using eq 1 as described under Materials and Methods. A saturable dependence of k_{obs} on the PAI-1 concentration was observed for inhibition of α-thrombin (closed circles) in the presence of vitronectin (Figure 3). Nonlinear regression analysis of the k_{obs} values according to eq 2 showed that the data conform to a hyperbolic saturation curve which yielded the values for the ternary complex dissociation constant, K_d, and the rate constant, k, for stable complex formation as shown in Scheme 1.

$$k_{obs} = k[\text{PAI-1}]/\{[\text{PAI-1}] + K_d(1 + [\text{S}]/K_m)\} \quad (2)$$

Continuous kinetic assay was also used to study the PAI-1 inactivation of R93,97,101A thrombin in the presence of vitronectin. In this case, the individual kinetic values were not determined since the k_{obs} values remained linear under these experimental conditions (Figure 3, open circles). In this case, only the k_2 value could be estimated from eq 3:

$$k_{obs} = k_2[\text{PAI-1}]/(1 + [\text{S}]/K_m) \quad (3)$$

The second-order association constants, calculated from the ratio of the rate constant, k, to the K_d value in Scheme 1 for α-thrombin (2.3×10^5 M^{-1} s^{-1}) or from the slope of k_{obs} values in Figure 3 according to eq 3 for R93,97,101A thrombin (1.0×10^5 M^{-1} s^{-1}), are in agreement with the values determined by the discontinuous assay (Table 1), confirming the reliability of this method of kinetic analysis. It should also be noted that these results are consistent with previous kinetic studies, which used a similar approach to analyze the kinetics of the α-thrombin−PAI-1 reaction in the presence of vitronectin (*5*). The previous study determined a K_d of 75 ± 9 nM for α-thrombin, that is ∼2−3-fold higher than the K_d determined in this study. This may partly be due to differences observed in the K_m of the competing fluorogenic substrate Tosyl-GPA-amc for thrombin in these two studies. Due to a high K_d of thrombin for PAI-1, this type of kinetic analysis was not feasible in the absence of vitronectin (*5*).

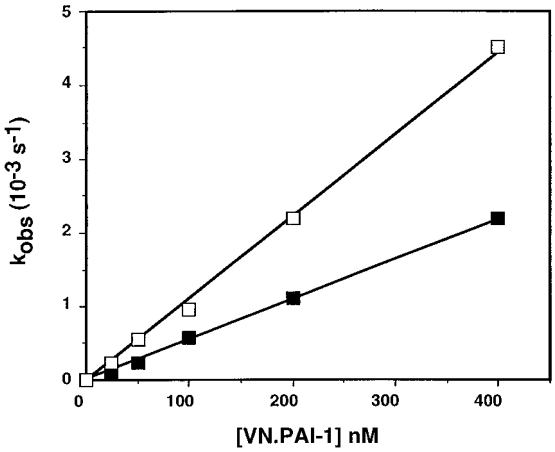

FIGURE 4: Dependence of the k_{obs} values on PAI-1 concentration with γ-thrombin and meizothrombin in the presence of vitronectin. The k_{obs} values for PAI-1 inactivation of γ-thrombin (■) or meizothrombin (□) were determined in the presence of vitronectin (1 μM) and varying concentrations of PAI-1 (25−400 nM) by a discontinuous assay and plotted vs PAI-1 concentration. The k_2 values were calculated from the slopes of the straight lines which are presented in Table 1.

In the case of the exosite 1 variant γ-thrombin as well as meizothrombin, the k_{obs} values in the presence of vitronectin could be determined by the discontinuous assay method described under Materials and Methods. As shown in Figure 4, the plot of k_{obs} as a function of increasing PAI-1 concentrations remained linear up to 400 nM PAI-1, suggesting that the K_d for noncovalent protease−serpin complexes for both variants is impaired in the presence of the cofactor, as was the case with the R93,97,101A thrombin mutant. However, it is also evident from Figures 3 and 4 that the k_{obs} values reached for the variant thrombins at the highest vitronectin−PAI-1 concentrations approached or exceeded the limiting k_{obs} value for α-thrombin (k) while showing no evidence for saturation. This implied that the k values for the variants must substantially exceed the k value determined for α-thrombin and partially compensate for the effect of the increased K_d values on k_2.

Competition Kinetics. To confirm that vitronectin lowered the K_d for the formation of the thrombin−PAI-1 encounter complex, kinetic studies were also carried out in the presence of an active-site mutant, S195A thrombin, by a discontinuous assay. First, we ensured that the reaction of S195A thrombin with PAI-1 did not proceed beyond the first reaction step in

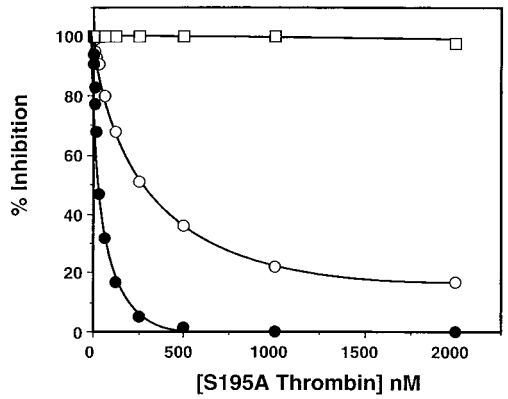

FIGURE 5: Competition of S195A thrombin with α-thrombin for binding to the vitronectin−PAI-1 or heparin−PAI-1 complexes. α-Thrombin (1 nM) was incubated with 200 nM PAI-1 alone (□) or in complex with 100 nM vitronectin (●) or 10 nM heparin (○) in the presence of the indicated concentrations of S195A thrombin. The competitive effect of S195A thrombin was determined from the ratio of % PAI-1 inactivation of α-thrombin in the presence of S195A to % PAI-1 inactivation in the absence of S195A thrombin as described under Materials and Methods. The K_d values of inhibition (30.9 ± 2.5 nM for vitronectin−PAI-1 and 234.9 ± 9.9 nM for heparin−PAI-1) were calculated from nonlinear regression analyses of data (solid lines).

both the absence and presence of vitronectin. This was evidenced from the SDS−PAGE analysis in which S195A thrombin, unlike α-thrombin, neither formed a stable complex with PAI-1 in the inhibitory pathway nor cleaved the serpin in the substrate pathway of the reaction (data not shown). These results are consistent with previous observations that serine proteinases require an intact Ser^{195} to form stable, covalent complexes with their target serpins (*34*−*36*). In the competition kinetic study shown in Figure 5, S195A thrombin inhibited the cofactor effect of vitronectin and heparin in a concentration-dependent manner. This was a cofactor-specific effect since S195A thrombin in the absence of the cofactors exhibited a negligible inhibitory effect (~3%) only at the highest concentration of the mutant thrombin (2 μM). K_d values of 30.9 ± 2.5 nM ($n = 5$, ±SE) for S195A thrombin inhibition of the vitronectin−PAI-1 complex and 234.9 ± 9.9 nM ($n = 3$, ±SE) for S195A thrombin inhibition of the heparin−PAI-1 complex were calculated by this method of analysis. Since we did not determine the K_d values for various possible binary complexes in this study, we chose experimental conditions in competition kinetic studies in which the cofactor (10−100 nM) was saturated with excess PAI-1 (200 nM). Under these conditions, the contribution of potentially inhibitory vitronectin−thrombin or heparin−thrombin binary complexes to the final ternary complex is expected to be negligible. Therefore, the K_d value that was determined for PAI-1 inactivation of thrombin in the presence of vitronectin (Scheme 1) represents the final ternary complex K_d. These results provide further evidence that both heparin and vitronectin facilitate the formation of noncovalent, Michaelis-type thrombin−PAI-1 complexes in the inactivation reactions.

PAI-1 Inactivation of the Thrombin−Thrombomodulin Complex. When thrombin binds to the endothelial cell receptor thrombomodulin (TM), the macromolecular substrate specificity of thrombin changes from a fibrinogen clotting enzyme in the procoagulant pathway to a protein C activating enzyme in the anticoagulant pathway (*17*). To determine whether TM influences the reactivity of PAI-1 with anticoagulant thrombin under different conditions, the inactivation studies were also carried out in the presence of TM4−6. It was previously demonstrated that binding of this fragment to exosite 1 switches the specificity of thrombin from a coagulant to an anticoagulant enzyme (*17, 18*). TM4−6 decreased the k_2 value of PAI-1 in a concentration-dependent manner with a K_d value that was in close agreement with its expected K_d value for binding to exosite 1 of thrombin (5−10 nM). At a saturating concentration of TM4−6 (200 nM), k_2 for PAI-1 inactivation of thrombin ($6.7 \pm 0.5 \times 10^1$) was impaired ~10-fold. However, the cofactor effect of vitronectin (~120-fold) in PAI-1 inactivation of the α-thrombin−TM4−6 complex was only impaired ~2-fold (Table 1). A similar result was observed for the vitronectin−PAI-1 inactivation of R93,97,101A thrombin−TM4−6 complex (data not shown). PAI-1 inactivation of γ-thrombin was impaired ~10-fold and was insensitive to the presence of TM4−6 under all experimental conditions. These results appear to suggest that thrombin may harbor a binding site for PAI-1 within or near exosite 1. However, the possibility that the observed effect is due to a conformational change that follows a later step in the kinetic pathway cannot be ruled out. PAI-1 inactivation of α-thrombin was also studied in the presence of increasing concentrations of hirudin C-terminal dodecapeptide, which is known to bind exosite 1 of thrombin (*37*). Similar to TM4−6, the hirudin peptide inhibited PAI-1 inactivation of thrombin in a dose-dependent manner (data not shown). Based on these results, it is expected that the occupancy of this site by other exosite 1 specific ligands will attenuate PAI-1 inactivation of thrombin ~10-fold.

To determine whether the chondroitin sulfate moiety of TM influences the reactivity of PAI-1 with the anticoagulant thrombin, the inactivation studies were also carried out in the presence of a recombinant full-length soluble TM (sTM) which contains a chondroitin sulfate moiety as previously characterized (*21*). In the presence of a saturating concentration of sTM (50 nM), PAI-1 inactivation of α-thrombin was impaired ~7-fold (Table 1). Interestingly, the cofactor effect of vitronectin in PAI-1 inactivation of the α-thrombin−sTM complex was dramatically impaired as the cofactor accelerated the reaction only ~9-fold (Table 1).

DISCUSSION

In this study, the kinetics of PAI-1 inactivation of wild-type and three different variants of thrombin were studied to understand how the cofactors, heparin and vitronectin, accelerate and thrombomodulin inhibits the reactivity of thrombin with PAI-1. The results suggest that (1) heparin binds to exosite 2 of thrombin to lower the K_d for noncovalent complex formation by a template mechanism, (2) vitronectin accelerates PAI-1 inactivation of thrombin by lowering the K_d for noncovalent complex formation similar to heparin (however, the cofactor does not appear to bind either exosite 1 or exosite 2 of thrombin), (3) there may be a cofactor-independent binding site for PAI-1 within or near exosite 1, and (4) TM containing chondroitin sulfate eliminates most of the cofactor effect of vitronectin in PAI-1 inactivation of thrombin.

In agreement with previous reports, our results suggest that heparin enhances PAI-1 inactivation of thrombin by a template mechanism (*4*, *38*). The cofactor function of heparin was minimally affected with γ-thrombin, but was completely abolished with the exosite 2 mutant R93,97,101A thrombin as well as with meizothrombin in which exosite 2 is masked by the fragment 2 domain of prothrombin. Previous mutagenesis of certain basic residues of the D-helix of PAI-1 also eliminated heparin catalysis of thrombin inactivation (*39*). These and the present mutagenesis studies firmly establish that heparin binds to the D-helix of PAI-1 and exosite 2 of thrombin to accelerate the reaction by a template mechanism. Unlike heparin, a template curve for vitronectin-mediated PAI-1 inactivation of thrombin was not observed. Moreover, most of the accelerating effect of vitronectin was mediated independent of exosite 1 or exosite 2 of thrombin. This was evidenced by the observation that the accelerating effect of vitronectin in PAI-1 inactivation of different exosite variants or meizothrombin was significant and impaired only ∼3−8-fold. These results suggest that although both heparin and vitronectin accelerate PAI-1 inactivation of thrombin by facilitating the formation of noncovalent complexes, unlike heparin, the cofactor effect of vitronectin may not at all or only to a limited extent be mediated through a similar bridging mechanism. A lack of a bell-shaped cofactor concentration dependence curve is consistent with this hypothesis. However, a slight inhibition of the vitronectin cofactor effect at high vitronectin concentrations observed in one study (*5*) would also be consistent with a bridging mechanism making a small contribution to the cofactor effect and explain why the cofactor effect was reduced with exosite 2 variants of thrombin. Nevertheless, our results support the suggestion that the bulk of the cofactor effect of vitronectin on the thrombin−PAI-1 reaction does not involve bridging but rather is due to an allosteric activation of PAI-1 (*4*, *5*).

The other interesting observation of this study is that the reactivity of PAI-1 with γ-thrombin or the α-thrombin−TM4−6 complex was impaired ∼10-fold, suggesting that the protease may harbor a binding site for the serpin within or near exosite 1. These results suggest that TM occupancy of exosite 1 can protect thrombin from rapid inactivation by PAI-1 in the presence of vitronectin. Therefore, the significant decrease in k_2 of the exosite 1 variant γ-thrombin inactivation in the presence of vitronectin (Table 1) does not involve significant impairment of the cofactor function, but rather is due to the possible inability of PAI-1 to interact with exosite 1 of thrombin independent of the cofactor. TM occupancy of exosite 1 did not account for all of the protective effect of TM in vitronectin−PAI-1 inactivation of thrombin. This is derived from the observation that only sTM which contained the chondroitin sulfate moiety could efficiently protect thrombin from rapid inactivation by PAI-1 in the presence of vitronectin. Since the chondroitin sulfate moiety of TM binds to exosite 2 of thrombin, these results may be due one or more of the following possibilities: (1) vitronectin and chondroitin sulfate bind to the same site on the protease, (2) chondroitin sulfate induces additional conformational change in the catalytic center of thrombin, or (3) chondroitin sulfate and the region of TM containing this polysaccharide sterically hinder binding of the virtronectin−PAI-1 binary complex to the active-site pocket of thrombin.

Finally, the observation that TM4−6 can protect thrombin from inactivation by PAI-1 is another example of how thrombin utilizes various cofactors to specifically interact with diverse macromolecular substrates and inhibitors. In recent years, it has become clear that thrombin can do this by having unique structural features in the residues of the active center, as well as in sites remote from the active center, which allow the enzyme to function by a complex allosteric mechanism (*17*, *40*, *41*). As an example relevant to our discussion, we previously demonstrated that repulsive interactions between the negatively charged residues in the reactive site loop of PAI-1 and the negatively charged Glu^{39} and Glu^{192} of thrombin are responsible for the slow reactivity of thrombin with this serpin (*31*). The same residues are suspected of being responsible for the poor reactivity of thrombin with protein C in the absence of TM (*42*, *43*). Previous substitution of these residues with a Lys and a Gln resulted in a thrombin mutant, E39K/E192Q, which was inactivated by PAI-1 with a 628-fold enhanced k_2 value (*31*). In the presence of the cofactors heparin and vitronectin, these inhibitory interactions are compensated for by the cofactors' ability to lower the K_d for initial complex formation, leading to rapid inactivation of thrombin and/or cleavage of the serpin (*4*). As demonstrated above, this system may be efficiently down-regulated when thrombin binds to its high-affinity receptor TM to function in the anticoagulant pathway by activating protein C. Yet, when thrombin binds to TM, the resulting anticoagulant enzyme complex becomes susceptible to inactivation by another serpin, protein C inhibitor (*30*). In this case, the TM4−6-induced conformational change in the active center of thrombin appears to largely alleviate an inhibitory S2−P2 interaction (*30*, *44*, *45*). These are a few examples, which provide yet another clue as to how the proteolytic activity of thrombin may be regulated in the intravascular and extravascular environments depending on the availability of different macromolecules under various (patho)physiological conditions.

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