The Suicide Substrate Reaction Between Plasminogen Activator Inhibitor 1 and Thrombin Is Regulated by the Cofactors Vitronectin and Heparin

By Marja van Meijer, Annelies Smilde, Guido Tans, Michael E. Nesheim, Hans Pannekoek, and Anton J.G. Horrevoets

The interaction of thrombin with plasminogen activator inhibitor 1 (PAI-1) is shown to result in the simultaneous formation of both cleaved PAI-1 and a sodium dodecyl sulfate-stable thrombin-PAI-1 complex. The kinetics of this reaction can be described by a “suicide substrate” mechanism that includes a branched reaction pathway, which terminates in either the stable inhibitor-enzyme complex or the cleaved inhibitor plus free enzyme. Because of the branched pathway, approximately three moles of PAI-1 are needed to completely inhibit one mole of thrombin. Heparin and vitronectin enhance the rate of inhibition from 9.8 × 10^7 L mol⁻¹ s⁻¹ to 6.2 × 10^8 L mol⁻¹ s⁻¹ and 2.1 × 10^8 L mol⁻¹ s⁻¹, respectively, under optimal conditions. In addition to enhancing the rate of inhibition, both cofactors increase the apparent stoichiometry of the PAI-1-thrombin interaction, with cofactor concentration dependencies similar to the inhibition reaction. Thus, at 37 ºC approximately six cleavage reactions occur per inhibition reaction. Therefore, thrombin will efficiently inactivate PAI-1 in the presence of either vitronectin or heparin, unless a sufficient excess of the inhibitor is present. These results show that physiological cofactors are able to switch a protease-serpin inhibition reaction to a substrate reaction, depending on the local concentrations of each of the components. © 1997 by The American Society of Hematology.

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KINETICS OF PAI-1:THROMBIN INTERACTION

EXPERIMENTAL PROCEDURES

Materials. Unfractionated heparin from porcine intestinal mucosa (# H-3125, grade I, Lot 29F-0314; specific activity of 178 U/mg, average Mr of 15,000 to 18,000) and hirudin were obtained from Sigma (St Louis, MO). The chromogenic substrates CH₅SO₄-, D-HHT-Gly-Arg-p-nitroanilide (Pefachrome tPA) and H-D-Phe-Pip-Arg-p-nitroanilide (where Pip is pipelic acid; S2238) were obtained from Pentapharm (Basel, Switzerland) and Chromogenix (Molndal, Sweden), respectively. The fluorescent active-site directed, reversible inhibitor of thrombin Dansylarginine N-(3-Ethyl-1,5-pentanediyl)amide (DAPA) was prepared as described. The irreversible active-site directed thrombin inhibitor Phe-Pro-Arg-Chloromethylketone (PPACK) was obtained from Calbiochem (San Diego, CA).

Proteins. Human a-thrombin and the recombinant thrombin variant, designated thrombin-VR1 (containing the VR1 motif of t-PA), were obtained as described. Thrombin and thrombin-VR1 were active site-titrated with calibrated hirudin. Vitronectin was kindly donated by Dr K.T. Preissner (Max-Planck Institute, Bad Nauheim, Germany). Two-chain Bowes melanoma t-PA (616,000 similar to the thrombin concentrations, necessitating the use of the quadratic equilibrium binding equation. The terms indicated that these incubation times were sufficient for completion of the reaction. In all cases, a control incubation containing no inhibitor was included. In some reactions, various concentrations (between 2 and 100 nmol/L) of DAPA were incubated at 37°C in HBST buffer by the PAI-1/VN complex, the concentration of which is calculated according to a model in which only the PAI-1/vitronectin complex contributes significantly to the reaction rate. In such a model the rate of thrombin inhibition can be expressed by the second-order rate equation: (3) Rate = k * [thrombin] * [PAI/VN]. The second-order rate constant for thrombin inhibition by the PAI-1/VN complex, the concentration of which is calculated according to the quadratic equilibrium binding equation: (4) [PAI/VN] = 1/2 * (P + V + kd - Sqrt((P + V + kd)² - 4 * P * V)), in which P = PAI-1, V = vitronectin, kd = dissociation constant of the PAI-1/vitronectin complex. Our experimental data were subjected to a global fit to these equations, which yields values for k and kd, by employing the nonlinear regression algorithm of Marquardt-Levenberg.

Gel electrophoretic analysis of PAI-1 cleavage and complex formation. To prevent protein adsorption all experiments were performed in Eppendorf tubes or in wells of a microtiter plate (Nunc Maxisorp; GIBCO-BRL, Gaithersburg, MD) that had been pretreated according to the chromogenic substrate S2238 (thrombin or thrombin-VR1) or t-PA was determined by titration of the enzyme with stepwise increased concentrations in a fluorimeter microcuvette (Hellma Benelux, Rijswijk, the Netherlands). The inhibition reaction was started by the addition of a small volume of PAI-1 and the reaction was monitored continuously until no further change in fluorescence intensity was observed. Alternatively, the inhibition reaction was stopped after approximately 10 minutes by the addition of 0.5 μL of 3.2 mmol/L of the active-site directed irreversible thrombin inhibitor PPACK to determine the maximal change in fluorescence intensity. Initial reaction rates of thrombin inhibition were calculated from the slope of a plot of fluorescence intensity versus time. The initial reaction rate equals: T₈ *slope(F₈/F₀) where T₈ represents total thrombin concentration, F₈ is the initial fluorescence intensity at time zero, and F₀, is the fluorescence intensity at complete inhibition. Alternatively, initial reaction rates were determined by fitting inhibition curves with single exponential decay and multiplying the rate constant with T₈. To correct for the attenuation of the reaction by DAPA, rates were multiplied with: (1) 1/(DAPA,kt) when DAPA concentrations exceeded the thrombin concentration by at least 10-fold, or with (2) (1-((1/2 * (kd + T₈ + DAPA)) - Sqrt((kd + T₈ + DAPA)² - (4 * DAPA * T₈)))), when DAPA concentrations were similar to the thrombin concentrations, necessitating the use of the quadratic equilibrium binding equation. The terms DAPA, and kd, represent total DAPA concentration and the dissociation constant of thrombin for DAPA, respectively. The data for the inhibition of thrombin by PAI-1 in the presence of vitronectin were analyzed according to a model in which only the PAI-1/vitronectin complex contributes significantly to the reaction rate. In such a model the rate of thrombin inhibition can be expressed by the following rate equation: (3) Rate = k * [thrombin] * [PAI/VN]. The apparent stoichiometry of the interaction for PAI-1 with thrombin, thrombin-VR1, or t-PA was determined by titration of the enzyme with stepwise increased concentrations of PAI-1 as described in detail. These titrations were performed for various times (between 1.5 and 18 hours) at 37°C with various concentrations (between 2 and 100 mmol/L) of either thrombin, thrombin-VR1, or t-PA in a volume of 25 μL of HBST buffer. The incubation times used were determined from calculations using known inhibition rate constants. Control experiments indicated that these incubation times were sufficient for completion of the reaction. In all cases, a control incubation containing no inhibitor was included. In some reactions, various concentrations of vitronectin or heparin were included. Residual protease activity was subsequently determined at 37°C by the addition of a sample to a mixture of 150 μL HBST buffer and 25 μL of 4 mmol/L of the chromogenic substrate S2238 (thrombin or thrombin-VR1) or Pefachrome tPA (t-PA), respectively, by measuring the increase in absorbance at 405 nm in a Titertek Twinreader (Flow Laboratories, Irvine, UK).

Fluorometric assay for thrombin inhibition. The reaction between thrombin and PAI-1 was monitored by including the fluorocent, reversible thrombin inhibitor DAPA in the reaction solution as described. Traditional progress curve analysis could not be performed, since PAI-1 is rapidly inactivated by a number of chromogenic substrates (Van Meijer M. and Horrevoets A.J.G., unpublished observations, March 1993, and ref 31). Fluorescence intensity was measured at 37°C with a Perkin-Elmer LS-50 fluorimeter (Norwalk, CT). The excitation wavelength was 280 nm with a band pass of 10 nm, the emission wavelength was 565 nm with a 15-nm band pass, using a 430-nm cut-off filter in the emission beam. The dissociation constant (kd) for DAPA of the thrombin used in this study was determined to be 63.5 nmol/L. Briefly, a solution of thrombin (final concentration of 18.6 mmol/L or 36.6 mmol/L) was prewarmed in HBST buffer in the presence of DAPA (370.4 mmol/L or 182.9 mmol/L) and vitronectin or heparin at various concentrations in a fluorimeter microcuvette (Hellma Benelux, Rijswijk, the Netherlands). The inhibition reaction was started by the addition of a small volume of PAI-1 and the reaction was monitored continuously until no further change in fluorescence intensity was observed. Alternatively, the inhibition reaction was stopped after approximately 10 minutes by the addition of 0.5 μL of 3.2 mmol/L of the active-site directed irreversible thrombin inhibitor PPACK to determine the maximal change in fluorescence intensity. Initial reaction rates of thrombin inhibition were calculated from the slope of a plot of fluorescence intensity versus time. The initial reaction rate equals: T₈ *slope(F₈/F₀) where T₈ represents total thrombin concentration, F₈ is the initial fluorescence intensity at time zero, and F₀, is the fluorescence intensity at complete inhibition. Alternatively, initial reaction rates were determined by fitting inhibition curves with single exponential decay and multiplying the rate constant with T₈. To correct for the attenuation of the reaction by DAPA, rates were multiplied with: (1) 1/(DAPA,kt) when DAPA concentrations exceeded the thrombin concentration by at least 10-fold, or with (2) (1-((1/2 * (kd + T₈ + DAPA)) - Sqrt((kd + T₈ + DAPA)² - (4 * DAPA * T₈)))), when DAPA concentrations were similar to the thrombin concentrations, necessitating the use of the quadratic equilibrium binding equation. The terms DAPA, and kd, represent total DAPA concentration and the dissociation constant of thrombin for DAPA, respectively. The data for the inhibition of thrombin by PAI-1 in the presence of vitronectin were analyzed according to a model in which only the PAI-1/vitronectin complex contributes significantly to the reaction rate. In such a model the rate of thrombin inhibition can be expressed by the following rate equation: (3) Rate = k * [thrombin] * [PAI/VN]. The apparent stoichiometry of the interaction for PAI-1 with thrombin, thrombin-VR1, or t-PA was determined by titration of the enzyme with stepwise increased concentrations of PAI-1 as described in detail. These titrations were performed for various times (between 1.5 and 18 hours) at 37°C with various concentrations (between 2 and 100 mmol/L) of either thrombin, thrombin-VR1, or t-PA in a volume of 25 μL of HBST buffer. The incubation times used were determined from calculations using known inhibition rate constants. Control experiments indicated that these incubation times were sufficient for completion of the reaction. In all cases, a control incubation containing no inhibitor was included. In some reactions, various concentrations of vitronectin or heparin were included. Residual protease activity was subsequently determined at 37°C by the addition of a sample to a mixture of 150 μL HBST buffer and 25 μL of 4 mmol/L of the chromogenic substrate S2238 (thrombin or thrombin-VR1) or Pefachrome tPA (t-PA), respectively, by measuring the increase in absorbance at 405 nm in a Titertek Twinreader. Results were plotted as the fraction of residual enzyme activity versus the initial inhibitor concentration. The intercept on the abscissa is the apparent stoichiometry of the reaction, being equal to 1 + the partition ratio (r). This partition ratio (kₚ/kₐ, see Fig 1) represents the number of catalytic turnovers per inactivation event.

RESULTS

Time course of PAI-1 cleavage by thrombin and of thrombin/PAI-1 complex formation. Gel electrophoretic analysis
of the time course of inhibition of thrombin by PAI-1 shows
the appearance of cleaved inhibitor, in addition to stable
complex formation (Fig 1A). This observation can be ex-
plained by two different reaction pathways: (1) after for-
mation of the SDS-stable complex, hydrolysis of the complex
results in release of active enzyme and cleaved inhibitor,
and (2) the serpin can act as a suicide substrate. This means
that after the formation of the reversible complex, two path-
ways can be followed: one leading to the formation of the
SDS-stable complex (inhibition pathway) and one lead-
ing to cleavage of the inhibitor and release of the active enzyme
(substrate pathway) (Fig 2). To discriminate between these
possibilities, the progress of the reaction between thrombin
and PAI-1 and the appearance of the different species was
followed for a time period much longer than the time needed
to inhibit all thrombin present. The reaction products seen
on the gel were quantified using laser densitometry (Fig 1A
and B). Complex formation and cleavage of PAI-1 clearly
occur simultaneously during the reaction as depicted by the
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on the gel were quantified using laser densitometry (Fig 1A
and B). Complex formation and cleavage of PAI-1 clearly
occur simultaneously during the reaction as depicted by the
fitted first-order reaction curve for the appearance of both
species. The rate of disappearance of the thrombin band
agrees with a second-order rate constant of 9.8 × 10^2 L mol^{-1} s^{-1}, which is consistent with published numbers. As
a control, the amidolytic activity of thrombin was determined
at similar time points and was shown to decrease with an
identical second-order rate constant (data not shown). Fur-

Fig 2. The branched pathway of a suicide substrate mechanism. The inhibitor (I) forms a reversible complex (EI) with its target serine
protease (E), characterized by the bimolecular rate constant $k_1$ and the dissociation rate constant $k_{-1}$. Subsequently, an intermediate complex
(EI') is formed, which can convert with a rate constant $k_2$ into the SDS-stable complex E-I for it can react according to a substrate mechanism,
resulting in free enzyme and cleaved inhibitor (I*) with the corresponding rate constant $k_3$. The partition ratio ($r = k_3/k_4$) represents the number
of catalytic turnovers per inactivation event, $1 + r$ is the apparent stoichiometry. Finally, the stable bimolecular complex (E-I*) can dissociate
with a rate constant $k_5$ into the free active enzyme (E) and cleaved, inactive inhibitor (I*).
Table 1. Apparent Stoichiometry for Protease Inhibition by PAI-1

<table>
<thead>
<tr>
<th>Protease</th>
<th>1.5 h</th>
<th>2.5 h</th>
<th>4.3 h</th>
<th>18 h</th>
<th>+Heparin</th>
<th>+Vitronectin</th>
</tr>
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<tbody>
<tr>
<td>Thrombin</td>
<td>3.2</td>
<td>3.1</td>
<td>2.8</td>
<td>3.0</td>
<td>7.1</td>
<td>5.1</td>
</tr>
<tr>
<td>Thrombin-VR1</td>
<td>2.3</td>
<td>2.9</td>
<td>2.3</td>
<td>2.3</td>
<td>6.6</td>
<td>7.4</td>
</tr>
<tr>
<td>t-PA</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

The apparent stoichiometries were determined by titration at 37°C at least in duplicate as described under “Experimental Procedures.” Indicated are results obtained at different time points and, when indicated, in the presence of either an optimal heparin concentration (ie, 1 U/mL in case of thrombin (502.5 nmol/L) inhibition, and 0.5 U/mL in case of thrombin-VR1 (20 nmol/L) inhibition), or 200 nmol/L vitronectin. Furthermore, the concentration of cleaved inhibitor remained stable during a further 19 hours incubation without reappearance of free active thrombin and little degradation of the complex was observed. The apparent stoichiometry determined within the experimental limits of gel quantitation was about 3, which is in good agreement with the more accurate numbers as determined by titration (Table 1). Control experiments performed with latent PAI-1, which is unable to complex with thrombin, revealed no cleavage of PAI-1 (data not shown).

Kinetic analysis of cofactor influence on the inhibition pathway. Previous data from our laboratory,32 suggested that heparin might accelerate the inhibition of thrombin by PAI-1 by acting as a template. In Fig 3 we have now elaborated on these observations by studying this reaction in more detail. This was done by studying these kinetics by means of displacement of the thrombin specific fluorescent inhibitor DAPA from the active site by PAI-1. Traditional progress curve analysis could not be performed, since we observed that PAI-1 is rapidly inactivated by a number of chromogenic substrates tested (data not shown) as has been reported during the progress of these studies by others.31 Central to the template mechanism is that inhibitor and enzyme are concentrated on the template in a way that favors the interaction. At any fixed concentration of inhibitor and enzyme, an optimum in the inhibition rate with increasing concentrations of heparin is observed. Furthermore, the reaction rate saturates...
in both proteins at a fixed concentration of heparin.\textsuperscript{33} Figure 3A illustrates that increased concentrations of heparin, in the presence of fixed concentrations of thrombin and PAI-1, result in increased initial rates of thrombin inhibition up to a maximum, followed by a decrease in rates. Furthermore, with increasing concentrations of PAI-1, the rate obtained at the peak of the bell-shaped curves, and the heparin concentration required to obtain the maximum, increases. The maximal rate of inhibition accords to a second-order rate constant for inhibition of $6.2 \times 10^5$ L mol$^{-1}$ s$^{-1}$, representing a 124-fold increase over the rate in the absence of heparin. As shown in Fig 3B, the thrombin inhibition rate saturates at high PAI-1 concentrations, reflecting saturation of the binding sites on heparin with PAI-1. The existence of saturation implies that PAI-1 does not effectively compete with functionally significant bound thrombin. In contrast, when the rate of thrombin inhibition was determined at various thrombin concentrations at fixed concentrations of PAI-1, heparin, and DAPA, the observed rate of inhibition increases up to a maximum and then decreases (Fig 3C). This decrease in the inhibition rate may indicate that thrombin can bind to functionally significant PAI-1 binding sites on heparin.\textsuperscript{33} The linear increase in the double-logarithmic plot of rate versus thrombin exhibits a slope of 1.0, indicating that the reaction is first order with respect to thrombin. The inset of Fig 3C shows the relation between fractional lifetime ($t_\text{1/2}$) as a function of thrombin concentration.\textsuperscript{30} The inflection point indicates a transition from first to zero-order kinetics with respect to the thrombin concentration upon saturation of the heparin template. This occurs when approximately two molecules of thrombin are bound to one heparin molecule. Figure 4 shows the effect of increasing concentrations of vitronectin on the rate of thrombin inhibition by PAI-1. Increasing concentrations of vitronectin result in increased rates for inhibition up to a maximum. Within the experimentally feasible range of concentrations, however, no decrease is observed at higher concentrations of this cofactor. Apparently, at each of the PAI-1 concentrations tested, rates are maximal when all the PAI-1 is complexed to vitronectin. In agreement with this finding, the maximum rate of thrombin inhibition observed was proportional to the concentration of PAI-1 present. Furthermore, the rates as a function of the bimolecular PAI-1/vitronectin complex at each of the PAI-1 concentrations could be well fitted with a $kd$ of approximately 50 nmol/L for the formation of the PAI-1/vitronectin complex and a second-order rate constant for thrombin inhibition by this complex of $2.1 \times 10^{12}$ L mol$^{-1}$ s$^{-1}$ (solid lines, Fig 4).

**Distribution of PAI-1 over substrate and inhibition pathways.** In the previous section we have shown that PAI-1 acts as a suicide substrate (Fig 1). Furthermore, we have shown that the cofactors heparin and vitronectin increase the rate of thrombin inhibition by at least two orders of magnitude (Figs 3 and 4). Initially, we hypothesized that the increased inhibition rates in the presence of the cofactors might be the result of protection against PAI-1 cleavage. Therefore, we monitored the thrombin and, as a control, the t-PA inhibition reaction under several conditions. In addition, we used a thrombin variant, denoted thrombin-VR1, that is inhibited three orders of magnitude faster by PAI-1 than native thrombin, even in the absence of cofactors.\textsuperscript{13} First, we determined that the apparent stoichiometry of the PAI-1-protease reaction is stable in time at 37°C for all proteases (Table 1) and is independent of protease concentrations (data not shown). Second, we determined the cofactor concentration dependence of the cleavage pathway by determining the amount of PAI-1 required to inhibit 1 nmol/L of thrombin-VR1. This thrombin variant was used for this study, since the inhibition rate with native thrombin is too low to perform these experiments with experimentally feasible concentrations of the proteins. As shown in Fig 5A, similar to the cofactor dependence observed for the rate of thrombin inhibition (Fig 3A), the stoichiometry showed an optimum with respect to the heparin concentration. Moreover, the optimal heparin concentrations are similar, and both pathways show a bell-shaped profile (Fig 3A and 5A). In contrast, as shown in Fig 5B, in the case of PAI-1 and vitronectin there is a maximum PAI-1 cleavage at high vitronectin concentrations. Figure 5B is an endpoint titration in which the components are incubated for one hour. Therefore, the stoichiometry of PAI-1 to vitronectin is not necessarily 1:1, since vitronectin can be recycled for another interaction with uncomplexed PAI-1. This result is comparable with the relationship of increasing vitronectin concentrations and maximum initial rate of inhibition (Fig 4). Next, we determined the apparent stoichiometry of native thrombin inhibition in the presence of optimal concentrations of the cofactors (Table 1). Both cofactors increase the apparent stoichi-
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ometry approximately twofold to threefold not only for native thrombin but also for thrombin-VR1. So although cofactors hardly influence the inhibition rate for this variant, they do have the same effect on the partition ratio. In contrast, t-PA inhibition by PAI-1 did not show an increased stoichiometry in the presence of heparin and vitronectin (Table 1). This demonstrates that the effect of the cofactors on product distribution is specific for thrombin.

DISCUSSION

The data presented in this report show that the inhibition of thrombin by PAI-1 can be described in terms of a suicide substrate mechanism (Fig 2) in which after formation of the bimolecular complex, the enzyme can either be trapped in a SDS-stable complex or can cleave the inhibitor and thereby become available for another catalytic turnover. In agreement with such a mechanism it was found that the kinetics of complex formation and PAI-1 cleavage are virtually identical (Fig 1), and prolonged incubation of thrombin and PAI-1 (19.5 hours) did not result in detectable release of active thrombin, excluding progressive cleavage of the SDS-stable thrombin/PAI-1 complexes within the experimental time. Thus the value of rate constant $k_5$ (Fig 2) is negligible. The product distribution of the reaction between thrombin and PAI-1 can be deduced from the apparent stoichiometry of the reaction (Table 1). Notably, the variant thrombin-VR1, which is inhibited three orders of magnitude faster than thrombin, shows a similar apparent stoichiometry. In contrast, t-PA inhibition by PAI-1 in a purified system shows a 1:1 stoichiometry at 37°C, excluding the presence of a “substrate-form” of PAI-1 in our preparation.

The kinetics of the stimulation of the rate of thrombin inhibition by PAI-1 by the cofactor heparin are fully consistent with a template mechanism as suggested previously by studies from our laboratory.

In that study it was shown that the enzyme does not interfere with antithrombin III binding to heparin. It should be noted that in that study the heparin was fractionated on immobilized antithrombin III, to select the high affinity subfraction. However, we find competition when two thrombin binding sites are saturated, probably caused by the almost 10-fold lower affinity of PAI-1 for unfractionated heparin compared to antithrombin III.

In contrast to the results for heparin, increasing concentrations of vitronectin result in increased rates of inhibition up to a maximum without a decrease at higher concentrations of this cofactor. Thus, within our experimental limits, we did not find evidence that vitronectin would accelerate the rate of the reaction by acting as a template. This is in agreement with our previous data obtained at nanomolar concentrations of the proteins, where it was shown that a 300-fold excess of vitronectin over PAI-1 did not result in a decreased rate. Naski et al, who used a similar range of vitronectin concentrations as in the present study, suggested such a template mechanism based on indirect evidence for binding of thrombin to vitronectin.

These authors also hypothesized the existence of additional accelerating effects other than a template mechanism. Our data show optimal rates of thrombin inhibition by PAI-1 in the presence of increasing amounts of heparin (A), or vitronectin (B) were determined as described under Experimental Procedures. These titrations were performed at concentrations of thrombin-VR1 of 20 nmol/L (A) or 4 nmol/L (B) with an increasing concentration of PAI-1 (from 0 nmol/L to 50 nmol/L) to determine the apparent stoichiometry. This apparent stoichiometry represents $1 + \text{partition ratio } (k_5/k_4)$, as depicted in Fig 2.

![Fig 5. Influence of cofactors on the distribution of PAI-1 over the substrate and inhibition pathway. The apparent stoichiometry for the inhibition of thrombin-VR1 by PAI-1 in the presence of increasing amounts of heparin (A), or vitronectin (B) were determined as described under Experimental Procedures. These titrations were performed at concentrations of thrombin-VR1 of 20 nmol/L (A) or 4 nmol/L (B) with an increasing concentration of PAI-1 (from 0 nmol/L to 50 nmol/L) to determine the apparent stoichiometry. This apparent stoichiometry represents $1 + \text{partition ratio } (k_5/k_4)$, as depicted in Fig 2.](image-url)
bin inhibition at vitronectin concentrations that are equal to or exceed the PAI-1 concentration, indicating that the increased rate is a reflection of an increased concentration of the bimolecular PAI-1/vitronectin complex, which is maximized when all PAI-1 is complexed. Apparently, the PAI-1/vitronectin complex acts as a functionally different inhibitor species than uncomplexed PAI-1, as discussed in more detail below. A global fit of our data to this reaction mechanism yields a $k_d$ of approximately 50 nmol/L for the formation of the vitronectin/PAI-1 complex. This value inferred by kinetics is comparable with that for the low-affinity PAI-1 binding site on vitronectin ($k_d = 55$ nmol/L), found by equilibrium binding studies. 

Previously it has been shown that addition of high concentrations of thrombin and heparin to preformed PAI-1/thrombin complexes results in the appearance of cleaved forms of the complex and PAI-1. However, the molecular weight of the cleaved forms of PAI-1 ($<31$ kD) were not consistent with cleavage within the reactive center loop. We show for the first time that the cofactors vitronectin and heparin increase the amount of reactive center loop-cleaved, inactive PAI-1 that is formed during the interaction of thrombin with PAI-1 in addition to their accelerating effect on the formation of the inactive PAI-1/thrombin complex. Again, appearance of reactive center loop-cleaved PAI-1 (MW 39 kD; SDS-PAGE, data not shown) occurs simultaneous to but not after complex formation, consistent with a suicide substrate mechanism. Apparently, these cofactors have a profound effect on more than one of the individual rate constants that constitute the suicide substrate mechanism of thrombin/PAI-1 interaction (Fig 2), since the overall rate of thrombin inhibition is increased by two orders of magnitude, and cleavage is increased twofold to threefold, as reflected by the increased apparent stoichiometry of the reaction (Table 1).

Recently, time-resolved fluorescence spectroscopy has indicated that binding of the cofactors heparin and vitronectin to PAI-1 induces a conformational change in the reactive center of PAI-1. Apparently, the different architecture of the exposed loop upon binding of PAI-1 to one of its cofactors makes these amino acid residues more accessible for the thrombin catalytic center. Our results imply that this altered conformation of the reactive site loop results in both increased substrate and inhibition reactions in the case of thrombin. The reaction between t-PA with PAI-1 in a purified system differs from thrombin by the appearance of cleaved inhibitor in the latter case. Apparently, the $k_s$ of t-PA exceeds the $k_s$ to a great extent, since no cleavage of PAI-1 is observed, unless the reaction is performed in the ECM. An explanation for the difference in the partition ratio between t-PA and thrombin might be that thrombin is a more potent catalyst than t-PA, both for the hydrolysis of chromogenic substrates, as reflected by more than 10-fold greater $k_{cat}$ values, and for the proteolysis of plasmogen and fibrinogen, which are the natural substrates for t-PA and thrombin, respectively.

The notion that a serpin inhibits its target proteases via a suicide substrate mechanism has profound implications on our understanding and appreciation of this reaction in vivo. Especially so in the case of the interaction between thrombin and PAI-1, since in this case the outcome of the reaction can be locally influenced by the cofactors vitronectin and heparin, both of which increase the thrombin inhibition rate by PAI-1 at the expense of increased PAI-1 consumption through the cleavage pathway. It can be speculated that cleavage of PAI-1 rather than inhibition of thrombin will be the main event occurring in vivo, since the PAI-1 consumption exceeds the amount of complexes formed under most conditions tested. Thus, thrombin will efficiently inactivate PAI-1 in the presence of either vitronectin or heparin, provided thrombin is present in sufficient amounts to overcome the minor contribution of the inhibition reaction (1 in 6 catalytic events). This situation is likely to occur in plasma, where during coagulation high local concentrations of thrombin will be generated. This is particularly relevant in the setting of platelet-rich plasma clots, which are rich in granule PAI-1 and thereby thrombolysis resistant. Under these circumstances, high concentrations of thrombin will act prothrombotic by inactivating PAI-1, the inhibitor of the fibrinolytic enzyme t-PA. Therefore, the direct and efficient inhibition of thrombin during thrombolytic therapy as adjuvant therapy to prevent reocclusion, might in fact adversely affect thrombolysis, a phenomenon that needs further study.

These findings also have implications for the processes that occur in the vessel wall under (patho)physiological conditions. A vast number of studies have shown that the proteins described in this study are synthesized in the vessel wall under inflammatory conditions. Specifically, such conditions induce the synthesis and surface exposure of tissue factor by smooth muscle cells, which may ultimately result in the generation of thrombin. Recently, it has been shown that smooth muscle cells express the specific thrombin receptor, permitting effects of thrombin on smooth muscle cell specific gene expression. Furthermore, several laboratories have reported on the induction of PAI-1 synthesis and secretion by smooth muscle cells by thrombin. Finally, a natural constituent of this cell matrix, namely vitronectin, has recently been shown to promote the clearance of active thrombin in a PAI-1 dependent manner by low density lipoprotein receptor-related protein-expressing cells. Collectively, those observations suggest a potential ‘feed-back’ regulatory mechanism for thrombin activity. The data reported in this paper provide a mechanistic concept for the interactions between thrombin, PAI-1 and specific cofactors. Therefore, the relative amounts of active PAI-1 and thrombin at this site, which might be determined by the outcome of the suicide substrate mechanism, will have important implications for local cellular processes.

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