Thrombin-Variable Region 1 (VR1)

EVIDENCE FOR THE DOMINANT CONTRIBUTION OF VR1 OF SERINE PROTEASES TO THEIR INTERACTION WITH PLASMINOGEN ACTIVATOR INHIBITOR 1 *

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Anton J. G. Horrevoets, Guido Tanst, Annelies E. Smilde, Anton-Jan van Zonneveld, and Hans Pannekoek
From the Department of Molecular Biology, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, 1066 CX Amsterdam, The Netherlands

The importance of a specific variable region in different serine proteases for the interaction with plasminogen activator inhibitor 1 (PAI-1) is studied. To that end, we have constructed a thrombin substitution variant, thrombin-VR1, in which the entire variable region 1 (VR1) of the protease domain (Phe-34 to Leu-40) has been replaced by the corresponding sequence (Phe-294 to Phe-305) of tissue-type plasminogen activator. The substitution resulted in a 2000-fold increase of the second-order rate constant of inhibition by PAI-1 $k_2=2.2 \times 10^8 \text{ M}^{-1} \text{s}^{-1}$ as compared to $\alpha$-thrombin $k_2=1.1 \times 10^8 \text{ M}^{-1} \text{s}^{-1}$. Inhibition of thrombin-VR1 by PAI-1 is mediated by the formation of SDS-stable, enzyme-inhibitor complexes. The substitution did not affect the rate constant of inhibition by antithrombin III, whereas clotting efficiency and the rate of inhibition by heparin cofactor II were decreased 3-fold. These results demonstrate the importance and specificity of the protease domain VR1 region for the interaction of PAI-1 with its target proteases.

The activity of plasma serine proteases is controlled by the action of a number of serine protease inhibitors that belong to a family of highly homologous proteins ("serpins") (1). Apart from the interaction between the P1 reactive site residue of the serpin and the catalytic center of the protease, it is conceivable that the specificity of the serpins for their target proteases is largely determined by other specific regions both of the protease and of the inhibitor. Both components consist of conserved regions and variable regions (VR)1 (1, 2).

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† On leave of absence from the Dept. of Biochemistry, University of Limburg, Maastricht, The Netherlands.
‡ To whom correspondence should be addressed: Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Dept. of Molecular Biology, Plesmanlaan 125, 1066 CX Amsterdam, The Netherlands. Tel.: 31-20-5123125; Fax: 31-20-5123474.

The abbreviations used are: VR, protease domain variable region; ATIII, antithrombin III; HCII, heparin cofactor II; PAI-1, plasminogen activator inhibitor 1; t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator; r-, recombinant; PAGE, polyacrylamide gel electrophoresis.

The variable regions of the proteases are mainly arranged at the protein surface and include a number of surface-exposed loops (2). These loops have been suggested to contribute to the high degree of specificity, e.g. the VR2 of $\alpha$-thrombin contains the anion-binding exosite (residues 70–80, chymotrypsin numbering) (3) and has been implicated in the specific interaction with fibrinogen (4), hirudin (6), and the thrombin receptor on platelets (6). Furthermore, the interaction of PAI-1 with the plasminogen activators t-PA and urokinase (u-PA) is strongly dependent on the presence of the variable region VR1 on the protease domain of these enzymes (7, 8). Both t-PA and u-PA contain a VR1 that is rich in positively charged amino acids, including the sequences KHRR (t-PA) and RRHR (u-PA). Deletion or charge reversal of these basic residues results in a more than 1000-fold reduction of the second-order rate constant of inhibition. Recently, we reported that $\alpha$-thrombin is yet another target enzyme for PAI-1. Interestingly, efficient thrombin-inhibition by PAI-1 is only observed in the presence of either vitronectin (9) or heparin (10). Accordingly, we speculated on the requirement for these cofactors and the differences in the corresponding VR1 regions of $\alpha$-thrombin and the plasminogen activators (11). This paper provides positive evidence for the dominant role of the VR1 region of serine proteases in the specific interaction with PAI-1. Finally, an explanation is presented for the function of the indicated cofactors in the inhibition of thrombin by PAI-1.

EXPERIMENTAL PROCEDURES

Materials—Monkey kidney CV1 cells were from Flow. Chromogenic substrates D-Phe-pipocetyl-Arg-p-nitroanilide (S2238) and D-Ile-Pro-Arg-p-nitroanilide (S2258) were obtained from Chromogenix (Mülndal, Sweden). Heparin (from porcine intestinal mucosa), hirudin (from leeches), and vitamin K, were from Sigma. Heparin-Sepharose and Q-Sepharose Fast Flow were from Pharmacia. Plasmid pCDNA was purchased from Invitrogen. The vaccinia virus expression vector pATA-18 was a kind gift of Dr. H. Stumpe (EMBL, Heidelberg, Germany).

Proteins—Echis carinatus crude venom and human ATIII were from Sigma. Purified human HCII was a kind gift from Dr. R. Bertina (State University, Leiden, The Netherlands). Bowes melanoma t-PA (two-chain) was obtained from Biopool. Purified, active site-titrated human $\alpha$-thrombin was from Dr. K. Mertens (this institute). Vitronectin was donated by Dr. K. T. Preissner (Bad Nauheim, Germany). Purification of recombinant PAI-1 from Escherichia coli lysates, activation by guanidine-HCl, and titration of active inhibitor on active site-titrated t-PA have been described (9, 11).

Construction of Prothrombin Variant VR1—The isolation of full-length prothrombin cDNA from a human liver cDNA bank, employing standard polymerase chain reaction amplification with simultaneous introduction of an unique EcoRI site upstream and an XbaI site downstream of the prothrombin coding sequence, will be described elsewhere. The resulting cDNA was inserted as an EcoRI-XbaI fragment into the polylinker of the plasmid pCDNA1 and entirely sequenced. The sequence was identical to the one previously reported (12). Mutagenesis was performed by the polymerase chain reaction overlap extension technique (13), using two, partially overlapping oligonucleotides with a 33-base pair mutated sequence (underlined) and a 50-base pair annealing sequence at the 3' end (5'-GCC AAC CAC CGG CGG AGT CCC GGG GAG CCC TCT TTC TCT GGG GCC AGC AGC CTC ATC AGT GAC GAC-3' and 5'-GAA GCG CTC CCC GGG ACT GCC CCG GTC CTT CTT GCA AAG CAT CAC AGT GCA AGG GAC GAC-3'). This procedure results in the replacement of the amino acid sequence RKSfiNEL (prothrombin residues 340–346) by AKHRRSPGERF (t-PA residues 295–305). The mutated fragment was used to substitute the "wild-type" Ssl-BglII.
fragment of thrombin in peDNA. The absence of undesired mutations was verified by DNA sequencing of the entire fragment.

Expression and Partial Purification of Recombinant Prothrombin Using the Recombinant Vaccinia Virus Expression System—Prothrombin and thrombin-VR1 were expressed in a recombinant vaccinia virus expression system, essentially as described before (14). Prothrombin cDNA was inserted as an EcoRI-XbaI fragment into the vaccinia expression vector pATA-18, and thymidine kinase-negative recombinant virus was produced and selected as described (15).

Purified recombinant virus stocks were assayed for prothrombin production by incubation of conditioned medium with E. carinatus venom. Subsequently, the amidolytic activity was determined using the chromogenic substrate S2238. CV-1 cells were grown to confluence and growth medium (Iscove's modified Dulbecco medium, 10% fetal bovine serum) was supplemented with 10 μg/ml vitamin K, one day prior to infection. Cells were washed with serum-free medium and infected with 200 μl recombinant vaccinia virus at a multiplicity of infection of 5, after which the cells were washed twice with serum-free medium. Production was continued for 36 h in serum-free medium, supplemented with 10 μg/ml vitamin K. Conditioned medium was harvested and diluted with an equal volume of 10 mM Tris-HCl (pH 8.3), 30 mM EDTA, 5 mM benzamidine, and 0.1% (v/v) Triton X-100. Subsequently, diluted medium was applied to a Q-Sepharose Fast Flow column, equilibrated with 25 mM Tris-HCl (pH 8.3). The column was washed with 10 volumes of 25 mM Tris-HCl (pH 7.5), 100 mM NaCl and, finally, recombinant (r-)prothrombin was eluted in one volume of 25 mM Tris-HCl (pH 7.5), 500 mM NaCl. Analysis of the purified protein was performed by SDS-PAGE followed by silver staining, showed r-prothrombin to be the major band displaying a mobility equal to plasma thrombin.

Activation of r-Prothrombin to α-Thrombin—r-Prothrombin was activated for 30 min with E. carinatus venom (16), in 25 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 10 mM CaCl2, 0.1% (v/v) Tween-80 at 37 °C, and the reaction was terminated by adding EDTA to a final concentration of 15 mM. The mixture was applied to a heparin-Sepharose column, washed with 25 mM Tris-HCl (pH 7.5), 0.25 mM NaCl, 0.1% (v/v) Tween-80 and α-thrombin was eluted in one step with the same buffer, containing 0.6 M NaCl. Both under non-reduced and reduced conditions, we detected the same mobility by SDS-PAGE for r-thrombin, thrombin-VR1, and plasma α-thrombin. The concentration of active r-thrombin was determined by titration with hirudin (17), the concentration of which was first calibrated with active site-titrated a-thrombin.

Kinetic Data Analysis—Second-order rate constants of inhibition of thrombin by various serpins were determined at 37 °C in HST buffer (20 mM HEPES (pH 7.5), 150 mM NaCl, 0.1% (v/v) Tween-80 in polyurethane tubes that had been pretreated with 1% (w/v) polyethylene glycol 20,000). The inhibitors were preincubated at 37 °C in 300 µl of HST and, the reaction was started by the addition of 100 µl of thrombin saturated with the chromogenic substrate 1 nM thrombin and varying inhibitor concentrations and, when appropriate, 1 unit/ml heparin or 20 nM vitronectin. At various time intervals, 25-µl aliquots were withdrawn and assayed for residual amidolytic activity using 1 mM S2238, at 405 nm in a Titertek Twinreader. Progress curves were straight up to 30 min, indicating that the inhibition reaction was sufficiently quenched by this procedure. Control experiments without inhibitor indicated that no loss of thrombin activity occurred throughout the experiments. The second-order rate constants of inhibition (k2) were determined as follows: rate constants less than 104 M⁻¹ s⁻¹ were determined under first-order conditions, whereas initial inhibitor concentrations (L) of 50–250 times higher than enzyme concentration (Eo) from a linear plot of pseudo first-order rate constant of inhibition versus L. To determine rate constants larger than 104 M⁻¹ s⁻¹, enzyme and inhibitor were incubated at equimolar concentrations (Eo = Lo), in which case kinetics are second order according to the equation: 1/Ef  =  1/E0 - k2 t (18). Thus, k2 is obtained from a linear plot of 1/Ef  =  1/E0 versus time. At intermediate rate constants, standard second-order kinetics was used with a 5-fold excess of inhibitor over enzyme, according to the equation: k2 t = 1/(L0 - E0) - In(1 + ((E0/L0) - E0)) as previously described (8).

SDS-stable Complex Formation of Thrombin and Thrombin-VR1 with Various Serpins—2.5 nM thrombin or thrombin-VR1 (19) labeled by the IODGEN method) were incubated for 30 min at 37 °C with 10 μl equimolar excess of active serpins in HST, supplemented with 0.05% (w/v) bovine serum albumin. In addition, particular reactions contained 1 unit/ml heparin. Subsequently, an equal volume of sample buffer (5% (w/v) SDS, 45% (v/v) glycerol, 0.05 M Tris-HCl (pH 6.8), and 0.05% (w/v) bromphenol blue) was added and the mixtures were briefly heated at 95 °C to quench the reaction. SDS-PAGE was performed on 7.5% (w/v) polyacrylamide gels (19), and the radiolabeled material was visualized by autoradiography.

**RESULTS**

Expression, Activation, and Partial Characterization of r-Prothrombin and Prothrombin-VR1—A variant of human recombinant prothrombin was constructed, denoted prothrombin-VR1, in which the entire VR1 (Phe-34 to Leu-40, chymotrypsin numbering; Ref. 12) has been replaced by the corresponding VR1 of t-PA (Phe-294 to Phe-305; Ref. 20) (Table I). Recombinant prothrombin and prothrombin-VR1 were expressed upon infection of monkey kidney CV-1 cells with recombinant vaccinia virus. Both proteins were produced at a level of approximately 4 μg/10⁶ cells/day. Purification from the conditioned medium yielded proteins with a molecular weight identical to that of plasma prothrombin as judged by SDS-PAGE followed by immunoblotting (data not shown). Upon activation with E. carinatus venom these proteins were converted to meizothrombin and, similar to plasma thrombin, subsequently to α-thrombin (16). Titration of the recombinant thrombins with hirudin showed no apparent differences in the dissociation constant as compared to plasma α-thrombin. Hence, hirudin was used as an active site titrant to calibrate the concentrations of the recombinant proteins to α-thrombin. Based on this calibration, the recombinant thrombins had similar amidolytic activities toward the chromogenic substrate S2238. However, the clotting activity showed a 3-fold decrease for r-thrombin-VR1 (50 NIH units/nmol) as compared to r-thrombin and plasma thrombin (both 150 NIH units/nmol).

Interaction of r-Thrombin and r-Thrombin-VR1 with PAI-1—The serine proteases r-thrombin, thrombin-VR1, and t-PA were incubated at 37 °C with increasing concentrations of PAI-1 (Fig. 1). Under these conditions (no cofactors), r-thrombin was not inhibited by PAI-1. In contrast, thrombin-VR1 was inhibited almost to the same extent as t-PA. Prolonged incubation did not alter the thrombin-VR1 profile, indicating that equilibrium had been reached. The difference with the t-PA profile could indicate a higher dissociation constant of the thrombin-VR1/PAI-1 inhibitory complex, since 1:1 complexes are formed as shown below. Alternatively, some cleavage of PAI-1 might occur, as previously reported for α-thrombin (22), resulting in an increased apparent stoichiometry.

Second-order Rate Constants of Inhibition by Various Serpins—The second-order rate constants of inhibition of r-thrombin and thrombin-VR1 by the serpins PAI-1, ATIII, and HCII were determined both in the presence and in the absence of heparin or vitronectin (Table II). The rate constants for r-thrombin are in good agreement with the published amino acid sequences of the variable region 1 (VR1) and the surrounding constant regions (CR1 and CR2) of the prothrombin (t-PA, 20, thrombin-12, thrombin-VR1, and u-PA 21) are compared. Basic residues are indicated in boldface type.

| Protease | CR1 | VR1 | CR2 |
|----------|-----|-----|-----|
| t-PA     | PWKAAI FAKHRRSPGERF LCGG  |
| Thrombin  | PWQVM/MM F-....RKSPQE-L LCGA  |
| Thrombin-VR1 | PWQVM/MM FAKHRRSPGERF LCGA  |
| u-PA     | PWFAAL I-Y-RHRRGSVTV YVCG  |
Role of Protease-VR1 Domain in Inhibition by PAI-1

The pseudo-irreversible inhibition of serine proteases by their cognate serine protease inhibitors depends on the reaction site ("bait") region of the inhibitor (1). The interaction between this region and the active site of the protease results in a serpin-specific complex that is resistant to denaturants like SDS (1). At present, the exact nature of this complex under native conditions is still a matter of debate, being either of the reversible Michaelis-type or a covalent complex in which the P1-P1' bond is cleaved. Previously, we have shown that variations in the bait region (P3-P3') hardly affect the protease specificity of PAI-1 (9, 11). Therefore, other molecular interaction sites are likely to play an essential role in the specific serpin-serine protease interactions and might control the rate of the reaction. In the case of thrombin, the anion-binding site, located as a surface-exposed loop in VR2 (21), has been implicated in the highly specific interactions with fibrinogen (4), the receptor on platelets (6), and the serpin HCII (24). It has been shown for the plasminogen activators t-PA and u-PA that the first variable region of these proteins (VR1) is essential for the high rate of inhibition by PAI-1 (7, 8). An inventory of the target proteases for PAI-1 indeed showed a remarkable coincidence between basic VR1-regions and susceptibility toward inhibition by PAI-1 (11). In this report, we present positive evidence that the interaction of PAI-1 with its target proteases is largely governed by the interaction of the serpin with the VR1 region of these proteases as exemplified by a 2000-fold increase of the second-order inhibition rate due to the replacement of the VR1 region of thrombin by the corresponding region of t-PA.

The function of the VR1/PAI-1 interaction might be explained by comparison with the results obtained on thrombin/hirudin interactions (25). The association rate for thrombin/hirudin is determined by a fast, ionic interaction between the acidic carboxyl-terminal region of hirudin and the basic anion-binding exosite of thrombin. Subsequently, a substantially slower, but very stable, interaction occurs between the aminoterminal region of hirudin and the active site region of thrombin, resulting in the formation of complexes with an extremely low dissociation constant (25). A similar ionic interaction may control the initial interaction between VR1 and PAI-1, yielding an optimal spatial orientation of the two proteins to facilitate the actual inhibitory interaction, mediated by the catalytic center of the protease and the bait region of the serpin. Spatially, an ionic alignment interaction that involves VR1 is feasible, since the two insertion-loops VR1 and VR2 (anion-binding exosite) are located near the active site of α-thrombin and constitute the two opposite walls of the fibrinogen binding cleft (3, 5). Steric hindrance at this site by the longer VR1 loop of thrombin-VR1 compared to thrombin may explain the observed 3-fold decrease in rate of the interactions.

TABLE II

Second-order inhibition rate constants of r-thrombin and thrombin-VR1 for different serpins

| Serpin, cofactor | r-Thrombin | Thrombin-VR1 | Ratio |
|-----------------|------------|--------------|-------|
| PAI-1, heparin | 1.1 x 10^6 | 2.2 x 10^6 | 2000 |
| PAI-1, vitronectin | 2.3 x 10^5 | 4.3 x 10^5 | 19 |
| ATIII, heparin | 4.0 x 10^5 | 5.2 x 10^5 | 1.3 |
| ATIII, heparin | 1.6 x 10^5 | 1.6 x 10^5 | 1.2 |
| HCII, heparin | 9.6 x 10^5 | 7.8 x 10^5 | 0.8 |
| HCII, heparin | 3.9 x 10^5 | 1.4 x 10^5 | 0.36 |


dlished data for plasma thrombin (10, 11, 23). In the absence of cofactors, a 2000-fold increase of the inhibition rate was observed for thrombin-VR1 as compared to r-thrombin. This rate constant of 2.2 x 10^6 M^-1 s^-1 is of the same magnitude as observed for the inhibition of α-thrombin by the other inhibitors in the presence of heparin. Clearly, replacement of the VR1 region of thrombin by that of t-PA does not affect the inhibition rate of thrombin by ATIII both in the absence and in the presence of heparin. For HCII, a 3-fold decrease in inhibition rate is observed in the presence of heparin. Furthermore, the effect of cofactors is markedly reduced for thrombin-VR1 as compared to α-thrombin; vitronectin enhancement is reduced from 200- to 2-fold, whereas the effect of heparin is reduced from 100- to 6-fold.

Serpin-specific Complex Formation—The interaction of serine proteases with serpins typically results in the formation of equimolar, SDS-stable complexes. In the absence of heparin and a 2.2-fold excess of active PAI-1, virtually no complex formation was observed for r-thrombin, whereas thrombin-VR1 was fully complexed (Fig. 2, lanes 4 and 6). In the presence of heparin, about half of r-thrombin was encountered in SDS-stable complexes (Fig. 2, lane 5). Complex formation of PAI-1 with thrombin-VR1 was not influenced by the presence of cofactors (Fig. 2, lanes 7 and 8). Furthermore, thrombin-VR1 is still fully capable of forming SDS-stable complexes with ATIII and HCII.
with fibrinogen and HCII, which both interact with the anion-binding exosite.

The function of the cofactors in this scheme is fairly obvious. They provide a template at which both protease and serpin align to facilitate the catalytic site/bait region interaction as exemplified by the well described template mechanism for ATIII/heparin (26). In accordance with this view, the stimulating effect of the cofactors vitronectin and heparin on thrombin-VR1 inhibition by PAI-1 is considerably reduced on replacing the P1 leucine of HCII by arginine, resulting in a 100-fold increase in the inhibition rate of thrombin-VR1 as compared to the 2 orders of magnitude enhancement observed for α-thrombin. This observation is reminiscent of the effect of replacing the P1 leucine of HCII by arginine, resulting in a 100-fold increase in the inhibition rate of thrombin and a simultaneous decrease of the cofactor function of heparin (27).

We propose that non-optimal molecular contacts between proteases and serpins enable cofactors to efficiently regulate this interaction. In conclusion, we provide additional, positive evidence that surface-exposed VR1 of target proteases determines the rate of interaction with PAI-1.

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