The Stratagem Utilized by the Plasminogen Activator from the Snake *Trimeresurus stejnegeri* To Escape Serpins†

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ABSTRACT: The plasminogen activator isolated from the venom of the snake *Trimeresurus stejnegeri* (TSV-PA) triggers plasmin production, along with tissue-type plasminogen activators (t-PA) and urokinase (u-PA). The half-life of TSV-PA in plasma is remarkable. We unveil in this paper two of the molecular mechanisms allowing TSV-PA to escape inhibition by plasma serpins. The first involves a phenylalanine at position 193 (chymotrypsinogen numbering system). Phe[193] distinguishes TSV-PA from nearly all trypsin-like proteinases, having glycine at this position. A mutant of TSV-PA (F193G), in which Phe[193] had been replaced by a glycine, was inactivated by plasminogen activator inhibitor 1 (PAI-1) and α_2-antiplasmin 100-fold more rapidly than the wild-type enzyme. The second mechanism originates from the 37-loop of TSV-PA. Swapping the 37-loop of TSV-PA for either that of t-PA or that of u-PA also increased dramatically the rate of inactivation by PAI-1. Loop swapping and F193G mutations were additive, resulting in a rate of inactivation by PAI-1 that was 4 orders of magnitude higher than for the wild-type enzyme. The potential role of Phe[193] and of the 37-loop in the immunity of TSV-PA toward α_1-antitrypsin and antithrombin is also discussed.

Trypsin-like peptidases (belonging to the S1 family of clan SA) are characterized by a highly conserved three-dimensional structure of their catalytic domain, despite moderate sequence identity (*1*−*3*). Coagulation and fibrinolysis are among the numerous physiological processes that the S1 family fulfills (*4*). A number of snake venom components have been characterized that belong to the S1 family and interfere with hemostasis. Among them, the plasminogen activator from the *Trimeresurus stejnegeri* venom (TSV-PA)[1] activates plasminogen into plasmin by cleaving its Arg[561]—Val[562] bond (*5*), as does tissue plasminogen activator (t-PA), urokinase (u-PA), or the vampire bat plasminogen activator (*6*, *7*). Compared to t-PA and u-PA, however, the sequence of the catalytic domain of TSV-PA is shorter, in particular in the 37-loop region (*8*). Another distinctive feature of TSV-PA is phenylalanine at position 193 (i.e., two residues prior to the catalytic Ser[195] in the chymotrypsinogen numbering system)[2] in place of the almost universally conserved glycine. Mutation of Gly[193] has severe consequences in serine proteinases: for instance, factor $IX_{Eagle\ Rock}$ in which Gly[193] is replaced by valine is essentially devoid of catalytic activity (*9*). The structure of TSV-PA obtained by X-ray diffraction revealed that the aromatic ring of Phe[193] partly occludes the S_2' pocket (*8*). Nevertheless, TSV-PA is an active proteinase, and Phe[193] plays a critical role in its specificity: the F193G variant,[3] in which Phe[193] has been substituted by glycine, exhibits a 9-fold increase of the k_{cat}/K_M value for plasminogen activation and, contrary to the wild-type enzyme, is inhibited by the basic pancreatic trypsin inhibitor (BPTI) (*10*).

The activity of serine proteinases is controlled by a variety of inhibitors, a number of which belong to the serine proteinase inhibitor (serpins) family (*11*−*15*). Serpins are capable of conformational transitions: most can adopt three conformers, namely, native, latent, and cleaved. Only the native conformer is a proteinase inhibitor, forming a 1:1 complex with its target. The latent conformer remains intact in the presence of the target, whereas the cleaved serpin is released by the proteinase after consumption. An additional, intact, substrate-like conformer has been described for the plasminogen activator inhibitor 1 (PAI-1): instead of inhibiting its target, the conformer would behave as a substrate (*16*−*19*). The mechanism of action of serpins implies a reactive center loop (RCL) that is cleaved, triggering a major

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[1] Abbreviations: TSV-PA, plasminogen activator isolated from the venom of the snake *Trimeresurus stejnegeri*; t-PA, tissue-type plasminogen activator; u-PA, urokinase; PAI-1, plasminogen activator inhibitor 1; α_1-AT, α_1-antitrypsin (also called α_1-proteinase inhibitor); α_2-AP, α_2-antiplasmin; BPTI, basic pancreatic trypsin inhibitor (Kunitz type 1; also called Trasylol or aprotinin); RCL, reactive center loop; *p*NA, *p*-nitroanilide; S-2238, H-D-Phe-pip-Arg-*p*NA; S-2251, H-D-Val-Leu-Lys-*p*NA; S-2765, Z-D-Arg-Gly-Arg-*p*NA; S-2222, Bz-Ile-Glu-(γ-OR)-Gly-Arg-pNA; S-2403, pyroGlu-Phe-Lys-*p*NA; SI, apparent stoichiometry of inhibition; k_{assoc}', apparent bimolecular inactivation rate constant (not corrected for SI).

[2] The amino acid numbering utilized for the catalytic domain of serine proteinases refers to the chymotrypsinogen numbering system, based on the topological similarities with chymotrypsin.

[3] Mutations of TSV-PA are designed by the standard nomenclature where the first and last (capital) letters represent the amino acid in the wild and mutant protein, respectively (i.e., F193G designates a TSV-PA mutant in which Phe[193] had been replaced by glycine).

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rearrangement of the structure (*20*−*22*). Residues forming the RCL bind to the catalytic groove of the proteinase and determine in part the specificity of the serpin (*23*−*28*). Conversely, residues forming the catalytic groove govern in part the vulnerability of the target to a given serpin. For instance, thrombin resists α_1-antitrypsin (α_1-AT), but when either the P_1 methionine of α_1-AT is replaced by arginine or when Glu^{192} of thrombin is replaced by glutamine, the association rate constant increases by 3 orders of magnitude (*11*, *12*, *29*). Besides interactions between the RCL and the catalytic groove, secondary binding sites play a major role in the inhibition of their targets by serpins. The contribution of such secondary binding sites has been well characterized for several serpin−target couples. The negatively charged region in the distal part of the RCL of PAI-1 interacts with the positively charged 37-loop of t-PA or u-PA (*30*−*33*). Carboxyl-terminal lysines of α_2-antiplasmin (α_2-AP) interact with a lysine binding site located in kringle 4 of plasmin (*34*−*36*). Finally, the 60-loop of thrombin has been involved in its interaction with antithrombin (*29*).

In this paper, we unveil two of the molecular mechanisms utilized by TSV-PA to avoid serpin neutralization. A series of TSV-PA variants were prepared that included the F193G mutation and/or swapping of the 37-loop for that of t-PA, u-PA, or a polyalanine stretch. The ability of PAI-1, α_2-AP, α_1-AT, and antithrombin to inhibit the TSV-PA mutants was determined. We conclude that Phe^{193} allows TSV-PA to escape α_2-AP, PAI-1, and to a lower extent α_1-AT. Lack of the 37-loop further secures TSV-PA against PAI-1.

Table 1: Nomenclature of the TSV-PA Mutants Prepared[a]

	Loop-37	Residue
	30----------42	193
TSV-PA	SLVVLFN----------SNGFLC	CH**F**DSGGP
F193G/Wild	SLVVLFN----------SNGFLC	CH**G**DSGGP
Wild/t-PA	SLVVLFAKHRRSP----GFLC	CH**F**DSGGP
F193G/t-PA	SLVVLFAKHRRSP----GFLC	CH**G**DSGGP
Wild/u-PA	SLVVLF-RRHRGGSNGFLC	CH**F**DSGGP
F193G/u-PA	SLVVLF-RRHRGGSNGFLC	CH**G**DSGGP
Wild/PolyA	SLVVLF-AAAAGGSNGFLC	CH**F**DSGGP
F193G/PolyA	SLVVLF-AAAAGGSNGFLC	CH**G**DSGGP
t-PA	WQAAIFAKHRRSPGERFLC	CQ**G**DSGGP
u-PA	WFAAIY-RRHRGGSVTYVC	CQ**G**DSGGP

[a] The sequence surrounding the 37-loop of the TSV-PA variants (mutated residues are underlined) is given together with the residues neighboring the active Ser^{195} (residue 193 is in bold). The numbering system used is that of chymotrypsinogen. The corresponding region of t-PA and u-PA (last two rows) is also given. Abbreviations: F193G/wild, TSV-PA mutant where Gly^{193} replaces the normal phenylalanine of TSV-PA but has the normal 37-loop; wild/u-PA, wild/t-PA, and wild/polyA are TSV-PA variants with Phe in position 193 (unmutated) but carrying the 37-loop of u-PA, t-PA, or a polyalanine stretch, respectively; F193G/u-PA, F193G/t-PA, and F193G/polyA are TSV-PA variants where Gly^{193} replaces the normal phenylalanine and where the 37-loop is that of u-PA, t-PA, or a polyalanine stretch, respectively.

EXPERIMENTAL PROCEDURES

Proteins and Reagents. Human thrombin and antithrombin were kindly provided by Dr. Jandrot-Perrus (Laboratoire de recherche sur l'hémostase et la thrombose, Hôpital Bichat, Paris, France). PAI-1 was purchased from Molecular Innovation (Royal Oak, MI); bovine trypsin (tosylphenylalanine chloromethyl ketone treated), human t-PA (two-chain, 70 kDa), α_1-AT, and α_2-AP were from Sigma (St. Louis, MO). The *p*-nitroanilide (*p*NA) substrates, H-D-Phe-pip-Arg-*p*NA (S-2238), H-D-Val-Leu-Lys-*p*NA (S-2251), Z-D-Arg-Gly-Arg-*p*NA (S-2765), Bz-Ile-Glu(γ-OR)-Gly-Arg-*p*NA (S-2222), and pyroGlu-Phe-Lys-*p*NA (S-2403) were obtained from Chromogenix (Mölndal, Sweden). Oligonucleotides were synthesized by Genset Laboratory (Paris, France).

Preparation and Characterization of the TSV-PA Variants. Polymerase chain reaction (PCR) was used for mutagenesis of plasmid pET(tsvpa)15 expressing TSV-PA, as previously described (*37*). Expression, purification, refolding, and titration of the variants were also performed as previously described (*37*). Wild-type and mutated TSV-PA were at least 95% active in comparison to the total protein concentration determined by the Bio-Rad protein assay kit (Marnes la Coquette, France). The various mutants prepared and their nomenclature are summarized in Table 1. The amidolytic activity of the TSV-PA variants was examined with S-2238 as substrate. Assays were performed in 20 mM Tris-HCl, pH 8.0, containing 0.01% (v/v) Tween-80 (buffer A). The enzyme concentration varied between 0.4 and 10 nM and that of the substrate between 10 and 400 μM. The values of K_M and k_{cat} were estimated by nonlinear regression analysis of the dependence of the initial velocity of hydrolysis upon the substrate concentration using the Michaelis−Menten equation. The ability of the TSV-PA variants to activate Lys-plasminogen (Sigma) was measured by an indirect chromogenic assay as previously described (*10*).

Titration of Serpins and Determination of the Apparent Stoichiometry of Inhibition. The effective concentration of PAI-1, α_2-AP, α_1-AT, and antithrombin was determined by titration against t-PA, plasmin, trypsin, and thrombin, respectively. Briefly, the active site concentrations of t-PA, plasmin, trypsin, and thrombin were initially determined by titration with 4-methylumbelliferyl *p*-guanidinobenzoate. Various concentrations of the serpin (0.35−790 nM, according to the A_{280}) were incubated at 25 °C with a fix concentration of enzyme (7−25 nM, depending upon the proteinase), and the residual free enzyme concentration was evaluated from the slope of hydrolysis of 100 μM S-2765, S-2251, S-2222, or S-2238 (for t-PA, plasmin, trypsin, or thrombin, respectively). Residual enzyme activities were compared after 3 h and up to 8 h incubation to verify that maximum inhibition was achieved. The effective concentrations of serpins were then estimated by regression analysis of the dependence of the residual enzyme concentration upon the concentration of added serpin (according to its A_{280}). The concentration of antithrombin determined by titration with thrombin did not differ significantly from that estimated according to its A_{280}. The concentrations of α_2-AP and α_1-AT determined by titration represented at least 62% of that estimated from their A_{280} but, probably due to spontaneous transition to the latent conformer, the concentration of "active" PAI-1 represented only about 40% of the theoretical maximum calculated from the A_{280}. Apparent stoichiometries of inhibition (SI) with TSV-PA or its mutants were deter-

mined by comparing the active concentration of the serpin (determined as above) with the minimum concentration needed to fully inhibit the target. SI values reported correspond to the ratio of the active over minimum concentrations. A ratio higher than 1 means that the number of moles of serpin required to inhibit 1 mol of proteinase is higher than 1 (i.e., a number of serpin molecules are cleaved by the target and/or binding is too weak for productive interaction).

Kinetics of Inhibition of the TSV-PA Variants by PAI-1, α_2-AP, α_1-AT, or Antithrombin. Values of the apparent bimolecular inactivation rate constant (k_{assoc}') of the TSV-PA variants were estimated under pseudo-first-order conditions at 25 °C in buffer A containing 0.2% (w/v) poly(ethylene glycol) 6000. Values of k_{assoc}' lower than 10^4 M^{-1} s^{-1} were estimated from kinetics performed in microplates. Inhibition of 3−15 nM enzyme (depending upon the TSV-PA variant) by 0.3−5 μM serpin (such that the molar excess was at least 10-fold the product of the enzyme by SI; *23*) was followed for up to 2 h. Residual activities were recorded at timed intervals by measuring the rate of 100 μM S-2238 hydrolysis after the inhibition reaction was quenched by dilution. Rates of inhibition were estimated by nonlinear regression analysis of the residual activities versus time (using a first-order decay equation), and k_{assoc}' values were deduced from the linear plot of the pseudo-first-order rate constant versus the inhibitor concentration. When the interaction of proteinase−serpin exhibited k_{assoc}' higher than 10^4 M^{-1} s^{-1}, values were estimated by progress curve kinetics as described (*24*). The concentration of serpin (2.5−200 nM) was chosen such that a significant inhibition would be observed over the time of the experiment (2 h). The reaction was started by the addition of the enzyme (0.2−1.6 nM) adjusted such that, in the absence of inhibitor, less than 10% of the substrate (S-2238) was hydrolyzed within 30 min. For most proteinase−serpin interactions studied, the progress curve exhibited a small final steady-state velocity, and fitting to a single exponential function with zero end point was not reliable. Therefore, data were analyzed by nonlinear regression analysis to the general equation for slow binding inhibition (*38*−*40*):

$$A_{405} = v_s t + (v_i - v_s)[1 - \exp(-k't)]/k' + A_0 \quad (1)$$

where A_{405} is directly proportional to the amount of *p*NA released at time *t*, k' is the apparent pseudo-first-order rate constant, v_i is the initial velocity (in the absence of inhibitor), v_s is the steady-state (final) velocity, and A_0 the initial A_{405} (before addition of the enzyme). The value of k_{assoc}' was deduced from k' using eq 2 to account for the competition introduced by the substrate:

$$k_{assoc}' = k'(1 + S/K_M)/I \quad (2)$$

where *S* is the concentration of the *p*NA substrate, K_M is its Michaelis constant for the proteinase, and *I* is the concentration of added serpin as determined by titration.

The ability of each TSV-PA variant (3 μM) to form stable complexes with the serpins (3 μM) was examined after a 2 h incubation at 25 °C in buffer A. Samples were denatured by a 10 min boiling and analyzed by SDS−polyacrylamide gel electrophoresis (gradient 8−25% acrylamide).

Table 2: Kinetic Parameters of PAI-1, α_2-AP, and α_1-AT Interaction with TSV-PA and Its Variants[a]

	PAI-1		α_2-AP		α_1-AT	
	k_{assoc}'	SI	k_{assoc}'	SI	k_{assoc}'	SI
TSV-PA	1.0×10^2	>100	<10	ND	<10	ND
wild/polyA	2.0×10^2	85	<10	ND	<10	ND
wild/t-PA	2.5×10^3	80	<10	ND	<10	ND
wild/u-PA	1.0×10^4	45	73	ND	<10	ND
F193G/wild	1.7×10^4	11	7.5×10^3	18	2.1×10^2	>500
F193G/polyA	3.0×10^5	7	7.6×10^3	15	50	ND
F193G/t-PA	2.5×10^6	2	1.1×10^4	9	86	ND
F193G/u-PA	6.0×10^6	1	2.3×10^4	4	57	ND

[a] Assays were performed and data were analyzed as described in Experimental Procedures to yield estimates of k_{assoc}' (in M^{-1} s^{-1}) and of the apparent stoichiometry of inhibition (SI value is in moles of inhibitor per mole of protease). It was not possible to obtain a reliable estimate of SI when the k_{assoc}' value was less than 10^2 M^{-1} s^{-1} (ND). Reported values are the weighted mean of at least two determinations with a resulting standard error of ±12% or less. Antithrombin was unable to inhibit any of the TSV-PA variant. Nomenclature of the variants is given in Table 1.

RESULTS

Phe^{193} of TSV-PA Prevents Its Inhibition by PAI-1 and α_2-AP. In a previous study (*10*) we reported that when Phe^{193} of TSV-PA is replaced by glycine, the k_{cat}/K_M values for S2238 hydrolysis and plasminogen activation increase 8- and 9-fold, respectively. We also reported that mutation dramatically modifies the sensitivity of TSV-PA to BPTI: whereas TSV-PA inhibition cannot be detected with concentrations as high as 10 μM BPTI, the F193G mutant is neutralized with less than 0.1 μM. To investigate if this mechanism also prevents TSV-PA inhibition by plasma serpins, we evaluated the k_{assoc}' and SI values of PAI-1, α_2-AP, α_1-AT, and antithrombin for TSV-PA and its F193G mutant (Table 2). Only PAI-1 exhibited a weak, yet detectable, potential to inhibit TSV-PA ($k_{assoc}' = 10^2$ M^{-1} s^{-1}). In contrast, only antithrombin lacked detectable ability to neutralize F193G. The most dramatic outcome of the mutation was for inhibition by α_2-AP (k_{assoc}' value of 7.5×10^3 versus less than 10 M^{-1} s^{-1}), but the effect for inhibition by PAI-1 and α_1-AT was also remarkable (k_{assoc}' values increased 170-fold and over 20-fold, respectively). In addition, the SI value for PAI-1 interaction decreased dramatically following the F193G mutation (from over a 100 to 11; Figure 1). When detectable inhibition occurred, complexes between the proteinase and the serpin were visible after SDS−polyacrylamide gel electrophoresis, but the pattern also included cleaved serpins (Figure 2). Therefore, it is reasonable to assume that Phe^{193} of TSV-PA is critical for its immunity to α_2-AP and participates in its resistance to PAI-1 and α_1-AT.

Role of the 37-Loop in the Interaction of TSV-PA with PAI-1. The value obtained for F193G inhibition by PAI-1 (1.7×10^4 M^{-1} s^{-1}) was still much lower than that for t-PA inhibition (2.3×10^7 M^{-1} s^{-1}; *41*), suggesting that at least another determinant other than Gly^{193} protected TSV-PA. It is well established that the positive charges carried by the 37-loop of t-PA and u-PA are critical for their inhibition by PAI-1 (*30*−*33*). The 37-loop of TSV-PA is shorter than that of t-PA or u-PA and is devoid of charged residues (Table 1). Thus, we anticipated that swapping the 37-loop would improve the potency of PAI-1 to inhibit TSV-PA. Chimeras

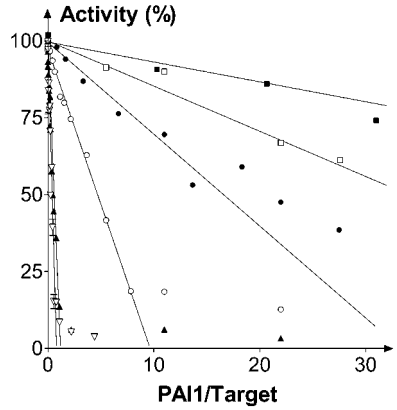

FIGURE 1: Apparent stoichiometry of inhibition of selected TSV-PA variants by PAI-1. Plot of the remaining activity (in percent) as a function of the ratio of PAI-1 over its target: filled squares, TSV-PA; open squares, wild/polyA; filled circles, wild/u-PA, open circles, F193G/polyA; filled triangles, F193G/t-PA; open triangles, F193G/u-PA. The nomenclature of the TSV-PA mutants is given in Table 1. The effective concentration of PAI-1, determined by titration against t-PA, was used to calculate the ratio of inhibitor to proteinase (*X* axis). Apparent stoichiometry higher than 1 implies that part of the inhibitor is cleaved rather than forming a stable complex and/or that binding is too weak for productive interaction. Lines were obtained by linear regression analysis of the data representing less than 75% inhibition.

of TSV-PA, carrying the 37-loop of t-PA, that of u-PA, or an alanine stretch of the same length, were prepared and characterized. With respect to S-2238 hydrolysis, chimeras exhibited 2−6-fold lower K_M values than TSV-PA, as well as 2−3-fold lower k_{cat} values (Table 3). Surprisingly, the TSV-PA chimeras carrying the 37-loop of t-PA or u-PA were in essence unable to activate plasminogen. It is unlikely that this loss of function originated from a steric hindrance due to the length of the 37-loop, because the chimera carrying a polyalanine stretch was able to trigger plasmin formation, albeit at a lower rate than TSV-PA (Table 3). The ability of PAI-1, α_2-AP, α_1-AT, and antithrombin to neutralize these chimeras was also examined. Compared with TSV-PA, the variants carrying the 37-loop of u-PA or t-PA were neutralized by PAI-1 with higher k_{assoc}' values (100- and 24-fold, respectively; Table 2). In contrast, little change was observed for the inhibition by PAI-1 of the chimera carrying a stretch of alanine. Consistent with the hypothesis that the 37-loop is only important for inhibition by PAI-1, none of the loop swaps improved the ability of α_2-AP, α_1-AT, or antithrombin to inhibit TSV-PA, although inhibition by α_2-AP was

Table 3: Kinetic Constants for the Hydrolysis of S-2238 and Lys-plasminogen by TSV-PA and Its Variants[a]

	S-2238			Lys-plasminogen		
	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1}\ s^{-1}$)	K_M (nM)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1}\ s^{-1}$)
wild/wild	26.0	9.5	3.6×10^5	55	0.015	2.7×10^5
F193G/wild	11.0	30.0	2.7×10^6	65	0.160	2.4×10^6
wild/u-PA	4.6	3.3	7.1×10^5	ND	ND	ND
wild/t-PA	7.4	3.2	4.3×10^5	ND	ND	ND
wild/polyA	10.0	4.9	4.9×10^5	36	0.006	1.7×10^5
F193G/u-PA	7.6	5.8	7.6×10^5	200	0.032	1.6×10^5
F193G/t-PA	16.7	6.3	3.8×10^5	408	0.021	5.1×10^4
F193G/polyA	21.2	17.8	8.4×10^5	182	0.032	1.7×10^5

[a] Assays were performed and data analyzed as described in Experimental Procedures to yield estimates of K_M and k_{cat}. These values were used to calculate k_{cat}/K_M. It was not possible to obtain reliable estimates for plasminogen activation with the chimeras of TSV-PA carrying the 37-loop of t-PA or u-PA (ND). Standard errors were ±7% or less for the kinetics of S-2238 hydrolysis. Reported values for Lys-plasminogen activation are the weighted mean of at least three determinations (standard error was ±25% or less). The abbreviations used are given in the legend of Table 1.

detectable for the chimera carrying the 37-loop of u-PA. As above, analysis by polyacrylamide gel electrophoresis of the chimera−serpin interaction revealed that when detectable inhibition occurred, complexes of proteinase−serpin were visible as well as cleaved serpins. Overall, a positively charged 37-loop appeared important, but only for the interaction with PAI-1, suggesting that the F193G and the loop swapping could have independent outcome.

The F193G and Loop Swap Mutations Are Independent and Additive. The F193G mutation and substitution of the 37-loop for that of u-PA each increased over 100-fold the k_{assoc}' value for the interaction of PAI-1 with TSV-PA. Thus, if mutations were additive, the gain could be as much as 10^4-fold and the k_{assoc}' value could approach that for the PAI-1/t-PA interaction. To test this hypothesis, variants of F193G were prepared, each carrying either the 37-loop of t-PA, that of u-PA, or a polyalanine stretch. With respect to S-2238 hydrolysis, the double mutants still exhibited K_M values lower than for TSV-PA, whereas k_{cat} values were lower than for the F193G variant, the resulting k_{cat}/K_M values being somewhat comparable to that of the wild-type enzyme (Table 3). The F193G mutation restored in part the ability of the chimeras carrying the 37-loop of t-PA or u-PA to activate plasminogen, but rates remained lower than by either TSV-PA or the single F193G variant. Consistent with the

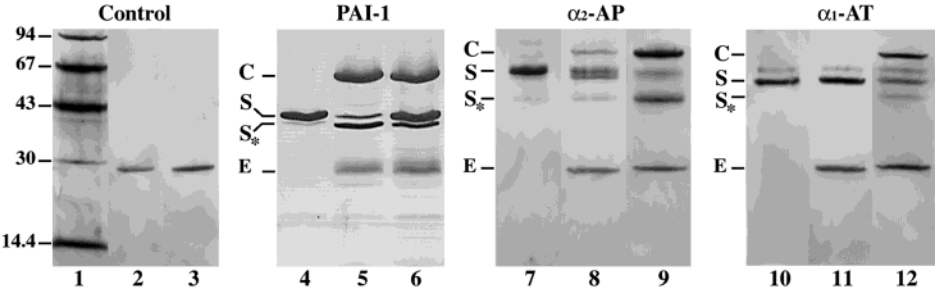

FIGURE 2: Complex formation between TSV-PA or F193G and PAI-1, α_2-AP, or α_1-AT (as indicated) analyzed by polyacrylamide gel electrophoresis (8−25% gradient acrylamide in the presence of SDS and 2-mercaptoethanol). In the control panel, lane 1 includes molecular mass markers (94, 67, 43, 30, and 14.4 kDa); lane 2, TSV-PA alone; and lane 3, F193G mutant alone (0.3 μg each). In the other panels, the left lane was obtained with the serpin alone (lanes 4, 7, and 10; 0.8 μg), the middle lane after 2 h incubation with TSV-PA (lanes 5, 8, and 11; 3 μM each), and the right lane after 2 h incubation with F193G (lanes 6, 9, and 12; 3 μM each). The resulting pattern included SDS-stable complexes (C), native serpin (S), free enzyme (E), and cleaved serpin (S*).

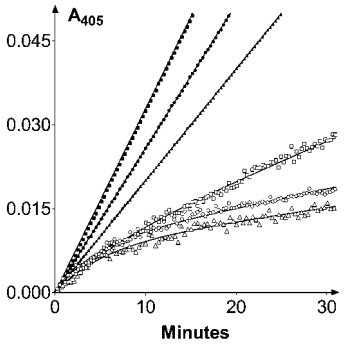

FIGURE 3: Progress curve of S2238 hydrolysis by F193G/polyA (squares), F193G/u-PA (circles), and F193G/t-PA (triangles). Filled symbols represent progress curves in the absence of inhibitor; open symbols represent kinetics in the presence of PAI-1. Comparable inhibition kinetics were obtained when 0.2 nM F193G/polyA was incubated with 200 nM PAI-1, 0.2 nM F193G/t-PA with 20 nM PAI-1, or 0.2 nM F193G/u-PA with 10 nM PAI-1. Curves were obtained by linear regression analysis in the absence of inhibitor and by nonlinear regression analysis according to eq 1 in the presence of inhibitor. The values of k_{assoc}' obtained for the F193G/u-PA chimera approach that for the interaction of t-PA with PAI-1.

hypothesis that the effects of the F193G mutation and of the loop swapping were independent and additive, the chimera of F193G carrying the 37-loop of u-PA was neutralized by PAI-1 with a k_{assoc}' value 58000-fold higher than that for TSV-PA (i.e., 350-fold higher than TSV-PA with the F193G mutation only and 600-fold higher than the variant with the 37-loop substitution only; Figure 3). Furthermore, the SI value was reduced to unity (Figure 1). Similarly, the chimera of F193G carrying the 37-loop of t-PA was neutralized with k_{assoc}' values 25000-, 150-, and 1000-fold higher than those for TSV-PA, F193G, and the 37-loop mutant, respectively. In comparison, the gain in sensitivity to PAI-1 of the F193G mutant carrying a polyalanine stretch for 37-loop was modest (k_{assoc}' value increased 17-fold only). Consistent with the above observation that loop swapping had limited impact on the inhibition by α_2-AP, the chimeras derived from F193G were inhibited by α_2-AP with k_{assoc}' values close to those observed with the single F193G mutant (i.e., the increase of k_{assoc}' relative to TSV-PA could be entirely attributed to the F193G mutation). A detectable inhibition of the chimeras derived from the F193G mutant occurred in the presence of α_1-AT, but the k_{assoc}' values remained 2−4-fold lower than for the single F193G mutant. Finally, none of the chimeras seemed affected by antithrombin. Overall, the effects of the F193G mutation and the loop swapping were independent and additive; the highest k_{assoc}' value obtained (6×10^6 M^{-1} s^{-1}) favorably compared with that for the interaction of t-PA with PAI-1.

DISCUSSION

The ability of a serpin to neutralize a given target appears to depend on both the sequence of its RCL and the contribution of one (or more) secondary binding site(s) (*23*−*31*, *42*, *43*). The RCL provides some selectivity to serpin, but the restriction is not as effective as for substrates: this is exemplified by α_1-AT, which inhibits efficiently trypsin-, chymotrypsin-, and elastase-type proteinases despite having a methionine in the P_1 position. On the other hand, to take advantage of a secondary binding site, the serpin needs the target to provide a complementary motif. In this study, we unveil that TSV-PA takes advantage of both mechanisms to escape several plasma serpins. Phe[193] allows TSV-PA to escape PAI-1 and α_2-AP, and the lack of a complementary binding site further warrants it to resist PAI-1. Neither mechanism, however, explained how TSV-PA escapes antithrombin.

Table 4: Comparison of the P_6−P_5' Residues of the RCL of the Serpins Examined in This Study[a]

	reactive center loop										
	P_6	P_5	P_4	P_3	P_2	**P_1**	P_1'	P_2'	P_3'	P_4'	P_5'
PAI-1	V	I	V	S	A	**R**	M	A	P	E	E
α_2-AP	S	I	A	M	S	**R**	M	S	S	S	F
α_1-AT	L	E	A	I	P	**M**	S	I	P	P	E
antithrombin	V	V	I	A	G	**R**	S	L	N	P	N
plasminogen	K	K	C	P	G	**R**	V	V	G	G	C

[a] The residues of the RCL of plasminogen activator inhibitor 1 (PAI-1), α_2-antiplasmin (α_2-AP), α_1-antitrypsin (α_1-AT), and antithrombin are given together with the sequence of the activating site in plasminogen (P_1 residue is in bold). The sequence of the RCL of PAI-1 cannot explain alone the lack of interaction with TSV-PA (see text for details).

It is widely accepted that the mechanism of action of serpins involves multiple steps, the first being formation of an initial Michaelis-type complex with the target (*41*, *44*−*47*). Accordingly, the catalytic groove of the proteinase must accommodate the RCL of the serpin in a substrate-like manner, at least during the early stage of the inhibition process. Specificity of a serpin will therefore depend in part upon the sequence of its RCL, which has to tackle with the preferences of the proteinase. Little is known about the P_2−P_2' preferences of TSV-PA. The X-ray structure suggests that Glu[98] and Val[99] constrain S_2 (*8*), but in a substrate, TSV-PA prefers P_2 Leu over Gly or Pro (*10*). The P_2 residues of PAI-1, α_1-AT, and antithrombin are alanine, proline, and glycine, respectively; that of the activation site of plasminogen is glycine (Table 4). Thus, it is unlikely that TSV-PA uses constraint in S_2 to escape PAI-1, α_1-AT, and antithrombin. The P_2 residue in α_2-AP is serine, but little data are available that would allow appraisal of whether P_2 Ser is detrimental. TSV-PA has a clear preference for Arg over Lys in P_1 (*37*): the P_1 residue is arginine in PAI-1, α_2-AP, antithrombin, and in the activation site of plasminogen. The P_1 residue of α_1-AT is methionine, and it is probable that, as for thrombin, protein C, or factor Xa (*29*, *48*, *49*), this methionine prevents in part the interaction with TSV-PA. Concerning P_1', PAI-1 and α_2-AP have methionine and α_1-AT and antithrombin have serine, whereas the activation site of plasminogen carries a valine (Table 4). The X-ray structure suggests that S_1' of TSV-PA is little restricted (*8*). Finally, the crystal structure suggests that S_2' of TSV-PA is occluded by Phe[193] and therefore that TSV-PA should prefer P_2' residues with small side chains. The increase of the k_{assoc}' for inhibition of the F193G mutant by α_1-AT could be attributed to the Ile in P_2': its side chain would collide with Phe[193] in the wild-type enzyme. Similarly, P_2' Arg of BPTI rationalizes well the immunity of TSV-PA (*10*). However, to activate plasminogen, TSV-PA accommodates valine in S_2'; therefore, the alanine of PAI-1 should also fit. In addition, freeing S_2' of TSV-PA did not allow detectable inhibition by antithrombin, which has leucine in P_2'. Overall, the subsite preferences of TSV-PA do not explain its immunity to PAI-1, suggesting that the remarkable outcome

of the F193G mutation may originate from a catalytic step in the course of inhibition rather than a steric hindrance precluding binding.

A number of studies have highlighted the major role of secondary binding sites in the interaction of serpin with proteinase. These secondary sites generally involve surface loops of the serpin and the proteinase that reorient approaching molecules and/or provide complementary surfaces for binding. No information is available concerning secondary binding sites in α_1-AT, but such sites have been well characterized for the interaction of PAI-1, α_2-AP, and antithrombin with several of their targets. The positively charged 37-loop in t-PA is important for the interaction with the negatively charged residues distal to the RCL of PAI-1. Indeed, replacement of the positively charged amino acids in the 37-loop of t-PA by alanine, or by negatively charged residues, results in mutants that resist PAI-1 (*33*). The 37-loop of TSV-PA is shorter than that of t-PA and has no charged residues. Introducing the 37-loop of t-PA in TSV-PA increased 25-fold the k_{assoc}' value with PAI-1; conversely, introducing instead the 37-loop of u-PA (also positively charged) increased 100-fold the k_{assoc}' value. Consistent with our observation, swapping the 37-loop of thrombin for that of t-PA results in a chimera inhibited by PAI-1 with a k_{assoc}' value 1000-fold higher than with the wild-type proteinase (*33*). Similarly, introducing the RRHR motif of human u-PA into chicken u-PA overthrows its relative resistance to human PAI-1 (*50*). Our TSV-PA chimeras no longer activated plasminogen. This observation was surprising, because the same 37-loop is evidently adequate for plasminogen activation in the context of t-PA or u-PA, albeit dispensable (*30*, *31*). At least another region of t-PA (within the autolysis loop) is critical for plasminogen activation while not involved in its inhibition by PAI-1 (*51*). The corresponding region of TSV-PA profoundly differs from that of t-PA (*37*). Whether swapping the 37-loops introduced an unexpected steric hindrance in TSV-PA that would be counterbalanced in t-PA by the autolysis loop remains to be explored. Neither TSV-PA nor its 37-loop chimeras were inhibited by α_2-AP. The 37-loop of plasmin had not been implicated in its interaction with α_2-AP: binding rather involves C-terminal lysines of α_2-AP, which interact with a lysine binding site in kringle 4 of plasmin (*34*). TSV-PA is devoid of kringle, prohibiting α_2-AP from employing this strategy. Finally, none of the TSV-PA variants studied were inactivated by antithrombin. The sequence of the RCL of antithrombin does not appear restrictive enough to explain by itself the lack of interaction (*27*). To inhibit thrombin, antithrombin uses the YPPW motif in the 60-loop insertion of its target (*29*). Absence of such motif in TSV-PA could explain the lack of interaction with antithrombin. To test this hypothesis, we attempted to construct a TSV-PA chimera carrying the LYPPW motif inserted in the region topologically equivalent to the 60-loop of thrombin. However, the variant had no detectable catalytic activity, precluding further evaluation of this hypothesis.

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