The Contribution of Residues 192 and 193 to the Specificity of Snake Venom Serine Proteinases*

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Snake venom serine proteinases, which belong to the subfamily of trypsin-like serine proteinases, exhibit a high degree of sequence identity (60–66%). Their stringent macromolecular substrate specificity contrasts with that of the less specific enzyme trypsin. One of them, the plasminogen activator from Trimeresurus stejnegeri venom (TSV-PA), which shares 63% sequence identity with batroxobin, a fibrinogen clotting enzyme from Bothrops atrox venom, specifically activates plasminogen to plasmin like tissue-type plasminogen activator (t-PA), even though it exhibits only 23% sequence identity with t-PA. This study shows that TSV-PA, t-PA, and batroxobin are quite different in their specificity toward small chromogenic substrates, TSV-PA being less selective than t-PA, and batroxobin not being efficient at all. The specificity of TSV-PA, with respect to t-PA and batroxobin, was investigated further by site-directed mutagenesis in the 189–195 segment, which forms the basement of the S1 pocket of TSV-PA and presents a His at position 192 and a unique Phe at position 193. This study demonstrates that Phe193 plays a more significant role than His192 in determining substrate specificity and inhibition resistance. Interestingly, the TSV-PA variant F193G possesses a 8–9-fold increased activity for plasminogen and becomes sensitive to bovine pancreatic trypsin inhibitor.

Because thrombotic disorders remain a major cause of morbidity and mortality in many countries, studies on the fibrinolytic system, which provides a unique counterbalance to the blood coagulation cascade, have called for intense research efforts in the past (1–3). The rate-limiting step in fibrinolysis is catalyzed by tissue-type plasminogen activator (t-PA), a member of the serine proteinase family which converts plasminogen into an active proteinase plasin (3). This encouraged extensive studies on t-PA, which has now become a standard therapeutic agent for acute myocardial infarction (4, 5). The specificity of t-PA for plasminogen, which has been attributed to their simultaneous binding to fibrin through their kringle domains (6), is in fact an inherent property of its protease domain because it is maintained in the absence of fibrin (7, 8). On the other hand, recent structural investigations confirm the close similarity of the t-PA proteinase domain to that of nonspecific proteinases such as trypsin (9, 10). Nevertheless, the molecular basis for the t-PA specificity for plasminogen remains poorly understood.

Snake venoms are a rich source of proteinases which have been characterized for their activity on a large variety of substrates (11). Some of them belong to the trypsin family of serine proteinases and exhibit a high degree of sequence identity (60–66%). Nevertheless, each one is quite specific toward different macromolecular substrates, particularly from the blood coagulation cascade (12). Among these proteinases, the Trimeresurus stejnegeri venom plasminogen activator (TSV-PA) has been characterized recently (13). Like t-PA, it selectively converts plasminogen into plasmin, by cleavage of the peptide bond Arg661-Val662. However, TSV-PA shares only 23% sequence identity with the catalytic domain of t-PA, although its tridimensional structure demonstrates a close structural similarity to the latter (14). On the other hand, TSV-PA exhibits 63% amino acid sequence identity with batroxobin, a specific fibrinogen clotting enzyme from Bothrops atrox venom (15). These two snake venom serine proteinases, TSV-PA and batroxobin, appear as useful tools to investigate the molecular basis for the specificity of the catalytic domain of t-PA. Indeed, the sequence alignment of TSV-PA and batroxobin shows a perfect conservation for the 12 cysteine residues, with only 3 residues left out in batroxobin. Consequently, their overall tridimensional structures are expected to be highly similar, yet the surface loops that constrict their active site clefts may in part explain their differences in macromolecular substrate recognition (16). In this line, Zhang et al. (15) demonstrated the major role of the 99-loop for the specificity of TSV-PA toward plasminogen. The replacement in TSV-PA of this acidic edge (DDE 96a-98), which is also present in t-PA (DDD 95–97), by the corresponding region (NVI 96a-98) of batroxobin, abolished the plasminogen activation activity of mutated TSV-PA (15).

The S1 pocket of trypsin-like serine proteinases is highly conserved with an Asp at position 189 which forms a canonical ion pair interaction with the positively charged side chain of the P1 residue of the substrate molecule (10, 17). The segment 189–195, located at the bottom of the active site cleft, contributes to determining the specificity of the coagulation serine proteinases (17–21). The preference of t-PA for Arg over Lys at P1 is enhanced by the presence of Ala residue at position 190 (22). In addition, several studies have demonstrated the pivotal role of 245-247 residues on t-PA specificity (23, 24).

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‡ The abbreviations used are: t-PA, tissue-type plasminogen activator; TSV-PA, T. stejnegeri venom plasminogen activator; BPTI, bovine pancreatic trypsin inhibitor; pNA, p-nitroanilide.

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1 The standard chymotrypsin numbering is used throughout this article (30). DDE 96a-98 and HF 192–193 correspond to DDE 81–83 and HF 178–179 in the TSV-PA numbering (13). H192G and H192Q indicate TSV-PA in which His192 is replaced with Gly and Asn, respectively. F193G and F193R indicate TSV-PA in which Phe193 is replaced with Gly and Arg, respectively.
role of residue 192 for substrate and inhibitor specificity in blood coagulation serum proteinases like thrombin, protein C, and factor Xa (19–21). TSV-PA and batroxobin show differences in the amino acid composition of segment 189–195. In particular, Gly193 of batroxobin, which is highly conserved in all coagulation serum proteinases, is replaced by a Phe in TSV-PA (13).

Furthermore, a common characteristic of t-PA with snake venom serum proteinases, in particular TSV-PA and batroxobin, is their resistance to Kunitz-type inhibitors such as bovine pancreatic trypsin inhibitor (BPTI). In t-PA this is caused by the presence of the phenolic side chain of Tyr151, which restricts the accessibility of the S2′ pocket (23). The resistance of TSV-PA to inhibition by BPTI might be attributed to the presence of the bulky residue Phe at position 193, which could restrict in a similar fashion the accessibility of the S2′ pocket (14), as it has been reported for Arg193 in the case of mesotrypsinogen, a natural mutant of trypsin resistant to BPTI (24).

In this study, we examine the functional significance of His192 and Phe193 of TSV-PA. We use site-directed mutagenesis to replace these residues with the corresponding ones of t-PA (Gln192 and Gly193) and of batroxobin (Gly192 and Gly193). The specificity of wild type recombinant TSV-PA and of TSV-PA variants toward small chromogenic substrates, plasminogen and fibrinogen, and their sensitivity to BPTI inhibition were then compared with those of t-PA and batroxobin.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human Lys-plasminogen, plasmin, bovine fibrinogen, BPTI, and t-PA (two-chain form, molecular mass 70 kDa) were obtained from Sigma (St. Louis, MO, USA). p-Nitroanilide (pNA) chromogenic substrates, H-D-Phe-Pip-Arg-pNA (S-2238), H-D-Val-Leu-Lys-pNA (S-2251), H-D-Val-Leu-Arg-pNA (S-2266), H-D-Val-Gly-Lys-pNA (S-2232) pyroGlu-Pro-Arg-pNA (S-2366), pyroGlu-Gly-Arg-pNA (S-2444), and Z-D-Arg-Gly-Arg-pNA (S-2765) were obtained from Chromogenix (Molndal, Sweden). Bovine factor Xa was from Enzyme Research Laboratories (South Bend, IN, USA). Batroxobin was from Ortho Clinical Diagnostic (Greenwich, CT, USA). Oligonucleotide primers were synthesized either by Unité de Chimie organique (Institut Pasteur, Paris, France) or by Genentech Laboratory (Paris).

**Construction of TSV-PA Variants by Site-directed Mutagenesis**—Polymerase chain reaction was used to construct TSV-PA variants. The expression plasmid, pET(svpa)15 (15), was used as template. One fragment was amplified between the sense direction primer, containing the sequence of the desired mutation, and the antisense T7 terminal primer (5′-GTTCCTAGGGC-3′), located downstream of the T7 promoter region (TAATACGACTCACTATAGGG). The two fragments were gel purified using a Jetrock kit from Genomed (Research Triangle Park, NC, USA) and combined in a new polymerase chain reaction amplification between the T7 promoter and terminal primers, where the overlapping ends annealed. The polymerase chain reaction product was subcloned into a pGEM vector (Promega, Madison, WI, USA), and DNA sequencing was performed to ensure that the coding sequence of the TSV-PA variant was correct. After digestion with BamHI and EcoRI, the DNA variant was gel purified and ligated into pET(17b) vector at BamHI and EcoRI sites to generate an expression plasmid.

**Expression and Purification of Recombinant TSV-PA and TSV-PA Variants**—The procedure described by Zhang et al. (15) for expression and purification of recombinant TSV-PA was used for the preparation of TSV-PA variants. Briefly, inclusion bodies were obtained in Escherichia coli strain BL21(DE3) after induction with isopropyl-1-thio-β-D-galactopyranoside. They were dissolved in urea and then dialyzed against 50 mM Tris-HCl, pH 8.0, 0.25 M NaCl, 2 mM EDTA for digestion of the fusion protein by bovine factor Xa. Denaturation was achieved in 8 M urea, 50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 50 mM β-mercaptoethanol, and refolding was performed by dilution in 50 mM Tris-HCl, pH 8.0, 2 mM EDTA. It was controlled by measuring the amidolytic activity of recombinant TSV-PA or variants with chromogenic substrate S-2238. Purification was performed by fast performance liquid chromatography in a Mono Q (HR 5/5) column with Amersham Pharmacia Biotech equipment (Uppsala, Sweden). The homogeneity of the preparation was checked by polyacrylamide gel electrophoresis performed in native conditions and in the presence of SDS.

**Measurement of Enzyme Concentration**—The concentration of TSV-PA and TSV-PA variants was determined as described previously (15). Briefly, enzyme concentration was determined by Bio-Rad protein assay, and the molar concentration of active sites was determined by active site titration with 4-methylumbelliferyl p-guanidinozoate (25). The same molar ratio of active site per molecule was obtained for recombinant TSV-PA and TSV-PA variants compared with that of natural TSV-PA within 10% limits.

**Chromogenic Assays and Kinetic Analysis**—The amidolytic activity of TSV-PA, TSV-PA variants, t-PA, and batroxobin was measured with a Kontron spectrophotometer in a 1-cm path length plastic cuvette. Assays were performed in 20 mM Tris-HCl, pH 8.0, 0.01% Tween 80 in a total volume of 500 μl. The final concentrations of enzymes varied from 0.4 to 80 nM and that of the chromogenic substrates from 0.01 to 0.6 mM. The kinetic parameters Km and kcat plasminogen activation being blocked by addition of 50 μM of acetic acid 100%. The formation of pNA was monitored at 405 nm as a function of time. The initial rate was plotted against substrate concentration in a Lineweaver-Burk representation.

**Plasminogen Activation Assay**—Plasminogenolytic activation was measured with a Kontron spectrophotometer. Assays were performed in 20 mM Tris-HCl, pH 8.0, 0.01% Tween 80, and the appropriate substrate concentration, in a total volume of 500 μl which contained 0.5 mM S-2403. The concentration of plasminogen varied from 10 to 100 nM, and the final concentration of t-PA was 200 nM except for TSV-PA variants F193G (1.35 nM), H192G/F193G (15 nM), and H192Q/F193G (10 nM), and for batroxobin (800 nM) and t-PA (0.01 nM). The end point technique was used to determine the kinetic parameters Km and kcat, plasminogen activation being blocked by addition of 50 μM of acetic acid 100%. The formation of pNA was monitored at 405 nm as a function of time. The initial rate was plotted against substrate concentration in a Lineweaver-Burk representation.

**Fibrinogenolytic Assay**—Tests were performed in enzyme-linked immunosorbent assay microplates (Nunc, Roskilde, Denmark). The concentration of batroxobin, t-PA, TSV-PA, and TSV-PA variants was 200 nM in the first well, and 1/2 serial dilutions were made in 10 mM Tris, pH 7.4, in a final volume of 25 μl, then 25 μl of fibrinogen (4 mg/ml) in 10 mM Tris, pH 7.4, and 50 μl were added. The microplate was incubated at 37 °C, and clotting was monitored every hour with a microplate reader MR 5000 (Dynatech Laboratories, Denkendorf, Germany), by the increase of turbidity at 405 nm and 630 nm. The absorbance was plotted against time, and the fibrinogenolytic activity was determined by the slope.

**Inhibition by BPTI**—TSV-PA, TSV-PA variants, t-PA, batroxbin, and trypsin used as a positive control were incubated at 37 °C with BPTI (4 nM) in 450 μl of 20 mM Tris-HCl, pH 8.0. After incubation, a final concentration (200 μM) of S-2238 (TSV-PA, TSV-PA variants, and batroxobin), of S-2765 (t-PA), or of S-2222 (trypsin) was added, and the residual activity was measured immediately in less than 2 min recording absorbance at 405 nm. For all enzymes and variants, which were inhibited by BPTI, the equilibrium was reached after 1 h of incubation. Therefore, the inhibition with variable concentrations of BPTI (from 0 to 5,000 nM) was tested in the same conditions after 2 h of incubation at 37 °C, in order to be certain that the equilibrium was reached in all experimental conditions. Furthermore, the same experiment was performed with two other substrate concentrations (i.e. 100 and 300 μM instead of 200 μM), and similar relative values (expressed as percent of the control enzymatic activity without BPTI) were obtained, within the experimental errors (less than 10%).

**Molecular Modeling**—The coordinates from the x-ray structure of TSV-PA (14) provided a starting point for modeling studies. Phenylalanine 193 of TSV-PA was mutated to Gly, and the resulting geometry of the model was optimized using the program TURBO-FRODO. The structure of the porcine kallikrein-BPTI complex (26) was superposed to the structure of wild type and F193G-TSV-PA, yielding an optimal docking of BPTI into the active site of both proteinases.

**RESULTS**

**Amidolytic Activity of Recombinant TSV-PA and TSV-PA Variants Compared with t-PA and Batroxobin**—A series of synthetic chromogenic substrates was used first to compare the specific amidolytic activity of TSV-PA with that of t-PA and batroxobin. As shown in Table I, the seven chromogenic substrates tested were hydrolyzed with different efficiencies by TSV-PA, five of them being also hydrolyzed by t-PA and only
two by batroxobin. As reported previously (13), TSV-PA showed a preference for substrates with Arg over Lys at their P$_1$ position. Table I further shows that substrates with a hydrophobic amino acid residue such as Leu at the P$_2$ position are well hydrolyzed by TSV-PA, whereas they are poor substrates for t-PA, which hydrolyzes only substrates with amino acid residues with small side chain at position P$_2$ (23). This is in agreement with the crystal structures that show that the S$_2$ pocket of TSV-PA is less restricted than that of t-PA. Moreover, an amino acid with a small side chain (such as Gly in S-2444 or Pro in S-2366) has the same effect for TSV-PA and t-PA. Although TSV-PA does not discriminate substrates with pyroGlu (S-2444) or Z-D-Arg (S-2765) residues at P$_2$ position, t-PA shows a preference for substrates with a Z-D-Arg at this position. In contrast to TSV-PA, t-PA has a 10-fold higher preference for S-2765 over S-2444, implying that the S$_2$/S$_1$ pocket of TSV-PA differs from that of t-PA. Moreover, a hydrophobic aliphatic residue at P$_3$ (as D-Val) increases the efficiency of TSV-PA by 5–10-fold, and that of t-PA remains unchanged. Batroxobin hydrolyzes S-2238 and S-2266 with a 100-fold lower efficiency than TSV-PA. Paradoxically, batroxobin cleaves substrates with hydrophobic bulky residues at the P$_3$ position (S-2238 and S-2266), but it does not hydrolyze substrates with small side chain residues (S-2444, S-2765, S-2366, and S-2322) (Table I).

To investigate further the functional role of His and Phe residues of TSV-PA at positions 192 and 193, respectively, we used site-directed mutagenesis to replace these two residues by the corresponding ones in t-PA (Gln$^{192}$ and Gly$^{193}$) and batroxobin (Gly$^{192}$ and Gly$^{193}$) (Fig. 1), as well as in mesotrypsinogen (Arg$^{193}$). The various TSV-PA variants show the same catalytic parameters as wild type TSV-PA toward the chromogenic substrates that we tested (data not shown) except for S-2238 (H-D-Phe-Pip-Arg-pNA) and S-2266 (H-D-Val-Leu-Arg-pNA), as shown in Table II. Compared with wild type TSV-PA, the catalytic efficiency ($k_{cat}/K_m$) toward S-2238 increased 3-, 7-, 8-, 13-, and 24-fold for the TSV-PA variants H192G/F193G, F193G, H192G/F193G, H192Q, and F193R, respectively. The effects are less marked in the case of S-2266, for which a 3- or 6-fold decrease in the catalytic efficiency of H192G/F193G and H192G and a 3-fold increase in that of F193R were determined (Table II). As noted for S-2238, the double mutants follow the tendency of the less effective single variants, and no cumulative effect compared with the single mutation is observed. In fact, the double mutants H192G/F193C and H192Q/F193G do not present the very low efficiency of batroxobin and t-PA toward S-2238 and S-2266, although they mimic, respectively, their sequence at positions 192 and 193. Therefore, it appears that mutations of residues 192 and 193 in TSV-PA do not substantially modify the enzymatic activity of TSV-PA toward small chromogenic substrates, in agreement with the findings for position 192 of t-PA by Zhang et al. (27).

**Table I**

| Substrate | TSV-PA | t-PA | Batroxobin |
|-----------|--------|------|-----------|
|           | $K_m$ $k_{cat}$ $k_{cat}/K_m$ | $K_m$ $k_{cat}$ $k_{cat}/K_m$ | $K_m$ $k_{cat}$ $k_{cat}/K_m$ |
| S2251 (H-D-Val-Leu-Lys-pNA) | 263 | 501 | 6.4 | 26 | 501 | ND |
| S2266 (H-D-Val-Leu-Arg-pNA) | 35 | 342 | 9.6 | 35 | 342 | ND |
| S2322 (H-D-Val-Gly-Arg-pNA) | 134 | 722 | 5.3 | 134 | 722 | ND |
| S2444 (pyroGlu-Gly-Arg-pNA) | 810 | 376 | 0.5 | 810 | 376 | ND |
| S2765 (Z-D-Arg-Gly-Arg-pNA) | 92 | 1085 | 0.9 | 92 | 1085 | ND |
| S2366 (pyroGlu-Pro-Arg-pNA) | 620 | 720 | 1.1 | 620 | 720 | ND |
| S2238 (H-D-Phe-Pip-Arg-pNA) | 26 | 570 | 22 | 26 | 570 | ND |

$^a$ ND, not detectable.

Plasminogenolytic and Fibrinogenolytic Activities of TSV-PA and TSV-PA Variants Compared with Those of t-PA and of Batroxobin—TSV-PA specifically cleaves plasminogen; however, its catalytic efficiency is 100-fold lower than that of t-PA (Table III). Furthermore, t-PA is inactive toward fibrinogen, yet TSV-PA possesses a very low but significant fibrinogenolytic activity (Table III). On the other hand, batroxobin has the ability to cleave plasminogen *in vitro* but with a 200-fold lower efficiency compared with TSV-PA (Table III). For most TSV-PA variants, $K_m$ and $k_{cat}$ toward plasminogen remained very similar; however, two exceptions were noticed. The first exception is TSV-PA variant F193G characterized by a 10-fold increase in $k_{cat}$ compared with wild type TSV-PA and no modification in the $K_m$. The plasminogenolytic activity of this TSV-PA variant ($k_{cat}/K_m$) therefore increased 10-fold compared with wild type TSV-PA and is thus only 10-fold lower than the corresponding value on t-PA (Table III). Unfortunately, for the second TSV-PA variant H192Q/F193G, which mimics the two positions of t-PA in TSV-PA, the $k_{cat}$ increased only 6-fold but the $K_m$ value also increased 4-fold, resulting in a reduction of the plasminogenolytic activity of this TSV-PA variant to that of the wild type TSV-PA (Table III). Under the conditions used in this study, the fibrinogenolytic activity of TSV-PA ($1.4 \times 10^5$ s$^{-1}$mol$^{-1}$) is 4,000-fold lower compared with batroxobin ($5.7 \times 10^5$ s$^{-1}$mol$^{-1}$), whereas t-PA had no fibrinogenolytic activity (Table III). No change in specificity toward fibrinogen was observed for any of the TSV-PA variants, except for H192G which showed no fibrinogenolytic activity, but this effect was lost in the double mutant H192Q/F193G (Table III).

**Inhibition by BPTI**—As indicated under “Experimental Procedures,” the inhibition of TSV-PA, TSV-PA variants, t-PA, batroxobin, and trypsin by BPTI was performed at equilibrium, and the inhibition, when observed, did not change significantly when the substrate concentration varied from 100 to 300 μM. Under these experimental conditions TSV-PA, t-PA, and batroxobin were not inhibited by a 1,000-fold excess BPTI (Fig. 2). Very interestingly, the replacement of the Phe residue at position 193 by a Gly in TSV-PA makes the variants F193G and H192Q/F193G insensitive to inhibition by BPTI with an apparent IC$_{50}$ of 15 nM, very similar to the control plasminogenase-BPTI complexes was also estimated. A large concentration of TSV-PA variants (F193G, H192Q/F193G, and H192Q/F193G) or trypsin (60 nM) was preincubated at 37 °C with an excess of BPTI (80 nM) for 2 h to reach a complete inhibition. Then the mixtures were diluted 15-fold, and the enzymatic activity was measured at regular time intervals by adding the
substrate S-2238 (200 μM). Whereas trypsin remained inhibited by BPTI during at least 24 h, the inhibition of TSV-PA variants decreased during the 1st h after dilution to reach the equilibrium value determined in Fig. 2 (data not shown). This indicates that the affinities of TSV-PA variants for BPTI are lower than that of trypsin, although the same IC50 values were determined in the experimental conditions of Fig. 2.

The Phe residue at position 193 of TSV-PA is rather unique among serine proteinases that normally have a Gly residue at this position. We wished then to examine the role of residue 193 in more detail using the coordinates from the x-ray structure of TSV-PA (14). To do that, we built a three-dimensional model of the TSV-PA variant F193G. We then used the coordinates of BPTI from the porcine kallikrein-BPTI complex (26) to optimize the docking of the BPTI molecule onto the superimposed structures of wild type TSV-PA and its F193G variant (Fig. 3).

DISCUSSION

TSV-PA and t-PA both specifically activate plasminogen but share only 23% sequence identity. Although the affinity (i.e. $K_m$) is almost identical, the catalytic efficiency ($k_{cat}/K_m$) of TSV-PA is 100-fold lower compared with that of full-length t-PA (Table III) and 30-fold lower than that of the serine protease domain of t-PA (28). Furthermore, the behavior of TSV-PA and t-PA toward small chromogenic substrates is different. The results in Table I suggest that the S2 pocket of TSV-PA, which accepts a Leu residue at position P 2 is wider than the S 2 pocket of t-PA. Indeed, the x-ray structures show that Tyr99 of t-PA restricts the S 2 pocket more than Val 99 of TSV-PA (23).

Although they share 63% overall sequence identity, TSV-PA and batroxobin prefer different macromolecular substrates, i.e. plasminogen and fibrinogen, respectively. They also behave differently toward small chromogenic substrates (Table I). In fact, sequence differences between TSV-PA and batroxobin in the loops forming the S 2/S 4 pocket might explain in part their
Among serine proteinases, TSV-PA is unique in that it has a Phe at position 193, which restricts the S$_2$ pocket (Fig. 3). This position is occupied in the majority of trypsin-like serine proteinases by a Gly residue. Phe$_{193}$ of TSV-PA does not collide with residues of the conformationally restricted plasminogen activation loop but does so with the large P$_{\alpha}$-Arg present in BPTI and other “canonical” inhibitors, which could explain the resistance to inhibition of TSV-PA (Fig. 3). Indeed, the replacement of Phe$_{193}$ by a Gly in TSV-PA makes the variant sensitive to BPTI. In this line, the TSV-PA variant F193R, constructed with reference to the natural trypsin mutant insensitive to BPTI, mesotrypsinogen (24), proves also to be resistant to this inhibitor because the bulky side chain of Arg$_{193}$ replaces the phenolic group of Phe$_{193}$. On the other hand, the resistance of t-PA to BPTI is caused by a Tyr$_{151}$, which restricts the S$_{\prime}$$_2$ pocket (23). TSV-PA also possesses a Tyr at position 151; however, this phenolic side chain does not extend into the S$_{\prime}$$_2$ pocket (23), even more so when Phe$_{193}$ is mutated to Gly. Moreover, the Pro$_{146}$ of the “autolysis” loop of TSV-PA could contribute to make rigid and stabilize this loop in a particular conformation, disallowing Tyr$_{151}$ to restrict the S$_{\prime}$$_2$ pocket in the F193R TSV-PA variant. The resistance of batroxobin to inhibition by BPTI could be explained as in the case of t-PA. Residue 193 of batroxobin is a Gly and residue 151 a Tyr. The latter could extend into the S$_{\prime}$$_2$ pocket as in t-PA. It is likely that the 148-loop of batroxobin adopts a conformation similar to that seen in t-PA. The absence of Pro$_{146}$ in batroxobin (as opposed to TSV-PA) should allow this to happen.

As far as the catalytic activity of TSV-PA is concerned, replacement of Phe$_{193}$ by a Gly turns TSV-PA into a more efficient plasminogen activator. In fact, the TSV-PA variant F193G has an improved reactivity toward the seven chromogenic substrates tested (data not shown) and a 10-fold higher plasminogenolytic activity compared with wild type TSV-PA (Table II). On a three-dimensional model of this TSV-PA mutant, the formation of a hydrogen bond between Asn$_{38}$ and Arg$_{193}$ could be considered (not shown). This interaction could have as a consequence the widening of the S$_1$ pocket, allowing either a better accessibility to small substrates or a more rapid liberation of the product. However, the plasminogenolytic activity of F193R does not increase; in fact it is reduced 3-fold compared with wild type TSV-PA (Table III). Residue 193 of TSV-PA therefore seems to play a crucial role in substrate and inhibitor recognition. This is confirmed by the observation that the catalytic activity of variant F193R toward small chromogenic substrates is more efficient than that of TSV-PA (Table III). On a three-dimensional model of this TSV-PA mutant, the formation of a hydrogen bond between Asn$_{38}$ and Arg$_{193}$ could be considered (not shown).

The specificity of serine proteinases has been actively studied (10) and in particular the role of position 192 (19, 20). This residue is not conserved among trypsin-like serine proteinases, suggesting that it plays a role in the discrimination for macromolecular substrates or inhibitors. However, mutations of His$_{192}$ to Gln and Gly of TSV-PA do not modify dramatically the specificity and reactivity of the enzyme toward small chromogenic (Table II) and macromolecular substrates (Table III). Nevertheless, the presence of a Gly at position 192 decreases the reactivity of the variant toward small and macromolecular substrates, probably because of the loss of conformation of the loop. Thus, the role of position 192 in determining the substrate specificity or inhibition pattern does not seem to be important in TSV-PA. Indeed, a recent study on t-PA variants at position 192 (27) supports our findings on the “poor” role of this position for determining serine proteinase specificity in the fibrinolytic system.

**Fig. 3. Model of the interaction of BPTI (green) with TSV-PA (yellow) (panel A) and the TSV-PA variant F193G (yellow) (panel B).**

Although the different specificities of TSV-PA, t-PA, and batroxobin are probably a result of different interactions at subsites around the active site, differences in the efficiency of the cleavage probably originate from subsites closer to the catalytic triad. In fact, no improvement is observed in the reactivity of TSV-PA toward fibrinogen when the molecule is mutated to mimic the S$_{\prime}$$_2$ of batroxobin (Table III). This indicates that positions 192 and 193 do not play a major role in determining the cleavage of fibrinogen. Perhaps sites more distant from the active site are involved in fibrinogen recognition by batroxobin. Interestingly, the comparison among TSV-PA, batroxobin, and t-PA in this work raises the possibility that secondary sites may be involved in macromolecular substrate recognition in addition to the role played by particular amino acids in determining the specificity and reactivity of these enzymes.
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Contribution of Residues 192 and 193 to Proteinase Specificity