Identification of a Flexible Loop Region (297–313) of Urokinase-type Plasminogen Activator, Which Helps Determine Its Catalytic Activity*

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Pro-urokinase has a much higher intrinsic catalytic activity than other zymogens of the serine protease family. Lys300 (c143) in an apparent “flexible loop” region (297–313) was previously shown to be an important determinant of this intrinsic catalytic activity. This was related to the loop allowing the positive charge of Lys300 (c143) to transiently interact with Asp355 (c194), thereby inducing an active conformation of the protease domain (Liu, J. N., Tang, W., Sun, Z., Kung, W., Pannell, R., Sarmientos, P., and Gurewich, V. (1996) Biochemistry 35, 14070–14076). To further test this hypothesis, the charge at position 300 (c143) and the flexibility of the loop were altered using site-directed mutagenesis designed according to a computer model to affect the interaction between Lys300 (c143) and Asp355 (c194). When the charge at Lys300 (c143) but not Lys313 (c156) was reduced, a significant reduction in the intrinsic catalytic activity occurred. Similarly, when the flexibility (wobbliness) of the loop was enhanced reducing the size of side chain, the intrinsic catalytic activity was also reduced. By contrast, when the loop was made less flexible, the intrinsic catalytic activity was increased. These findings were consistent with the hypothesis. The effects of these mutations on two-chain activity were less and often discordant with the intrinsic catalytic activity, indicating that they can be modulated independently. This structure-function disparity can be exploited to create a more zymogenic pro-urokinase (lower intrinsic catalytic activity) with a high catalytic activity, as exemplified by two of the mutants. The changes in intrinsic catalytic activity and two-chain activity induced by the mutations were due to changes in $k_{cat}$ rather than $k_{on}$. Some significant structure-function differences between pro-urokinase and its highly homologous counterpart, tissue plasminogen activator, were also found.

Urokinase-type plasminogen activator (u-PA),1 which includes its single-chain zymogen, pro-urokinase (pro-UK), and two-chain enzyme, urokinase (UK), is one of two natural human plasminogen activators. The other one is tissue plasminogen activator (t-PA). Their principal function is fibrinolysis (1), although u-PA has also been implicated in other physiological functions such as embryogenesis, cell migration, tissue remodeling, ovulation, and wound healing (2–6). The structure-function relationship of t-PA has been extensively investigated, but relatively little is known about this aspect of u-PA.

u-PA is a serine protease with a high substrate specificity. Although UK is a typical enzyme of this family, pro-UK has several unusual properties that distinguish it from other serine protease zymogens. These include its high intrinsic catalytic activity (ICA) (7–9), its reversible inhibition by diisopropylfluorophosphate (10), the existence of a unique hypercatalytic transitional state against plasminogen during the conversion or pro-UK to UK (11), and its strong fibrin promotion, which gives the ICA of pro-UK an activity equivalent to the TCA of UK (12).

The structural basis of these unusual properties remains unknown. For this reason, the high ICA of pro-UK was recently investigated using a three-dimensional homology model of the protease domain calculated from the crystallographic structure of trypsinogen. A single charged residue Lys300 (c143)2 was identified that appeared to serve as a surrogate of the neo-terminal Ile300 (c143)1 residue of two-chain UK and interacts with Asp355 (c194) to induce an active conformation of the protease domain. When the positive charge in this position was neutralized by site-directed mutagenesis (Lys300 (c143) → Ala), a 40-fold reduction in the ICA occurred, consistent with the hypothesis (13).

It was noted that the Lys300 (c143) residue was located in a loop region (297–313) believed to be “flexible” because of its very high isotropic temperature factor in the homologous structures of trypsinogen and chymotrypsinogen (13). The function of this “flexible loop” in serine proteases is unknown, although it is a part of the activation domain important in trypsinogen activation (14). In the case of pro-UK, we postulated that this loop was involved in the interaction of Lys300 (c143) and Asp355 (c194). Because the ICA was believed to be determined by this interaction, altering the flexibility by mutations in the loop should result in changes in the ICA of pro-UK. The effect of mutations in this region on the TCA is unknown, but the

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The abbreviations used are: u-PA, urokinase-type plasminogen activator; pro-UK, single-chain u-PA; UK, urokinase or two-chain u-PA; t-PA, tissue plasminogen activator; ICA, the intrinsic catalytic activity of pro-UK; TCA, the two-chain catalytic activity of u-PA.

2 "(cnnn)" next to the position numbers of residues indicates chymotrypsin numbering.
recently published x-ray structure of low molecular weight UK (15) suggested that the flexible loop mutations could also influence the TCA of UK by modulating the charge interaction between Asp55(c194) and Ile6(c166).

In this study, the function of the flexible loop on the ICA and TCA of u-PA was examined by characterizing selected u-PA mutants in which the charge at position300(c143) or the flexibility of the loop region was altered.

**EXPERIMENTAL PROCEDURES**

**Materials**

The synthetic chromogenic substrate for UK (S2444) was obtained from Kabi (Franklin, OH). Lys plasmin was obtained from American Diagnostica Inc. (Greenwich, CT). Enzymes and reagents for gene manipulation and expression of pro-UK were purchased from New England BioLabs (Beverly, MA) and Boehringer Mannheim. S-Sepharose, Sephadex G-25, benzamidine-Sepharose, aprotinin, and apotinin-Sepharose were from Sigma.

**Methods**

**Proposed Mutations for Testing the Hypothesis**—Based on the three-dimensional structural model of the pro-UK catalytic domain (Fig. 1), the following residues within the flexible loop were selected for site-directed mutagenesis to test the influence of charge at position300(c143) or flexibility of the loop on the ICA and TCA of u-PA (see Table I).

The mutant Tyr306(c149) → Gly was designed to increase the flexibility of the loop because its much smaller size allows the main peptide chain to move with less restraint and thereby destabilizes the interaction between Lys300(c143) and Asp55(c194) and increasing the ICA. However, the effect of Glu301(c144) → His could also be related to a charge change in this position, because Glu is negatively charged and His is partially positively charged. To evaluate this possibility, Glu301(c144) → Asp and Glu301(c144) → Ala were made. Asp has the same negative charge as Glu, and Ala is neutral. Because a Lys300(c143) → Ala mutation abolished the charge and reduced the ICA 40-fold (13), a Lys300(c143) → His mutation was made to reduce rather than abolish the charge at this position. This was expected to induce a more modest reduction in the ICA. However, His has a large side chain that could also reduce the flexibility of the loop and increase the ICA by this mechanism. To determine whether the reduced charge or reduced loop flexibility was involved, a Lys300(c143) → Trp mutant was made. Trp has a larger side chain but no charge.

A Lys313(c156) → Ala mutant was made because Lys313(c156) is the only other positively charged residue in the flexible loop. The comparable residue in t-PA (Lys429(c156)) was found to be important to its single-chain activity (16, 17).

Finally, the mutation Ser303(c146) → Glu was made to serve as a control to show that mutations within the loop region do not necessarily induce changes in the catalytic properties of pro-UK or UK.

**Site-directed Mutagenesis and Production of pro-UK Mutations in the Flexible Loop (297–313)—** As described previously (13), site-directed mutagenesis was used to create the desired mutants of pro-UK. Briefly, the cDNA of the pro-UK mutant was obtained by site-directed mutagenesis after subcloning the HindIII-BamHI restriction fragment from pFC16 plasmid containing the full-length cDNA of pro-UK (18) into an M13 vector (mp18). A specific synthetic oligonucleotide coding for the designed site-mutant was first hybridized to the recombinant M13 vector and then extended with T4 DNA polymerase. Following ligation and transformation of bacterial cells, positive clones were selected for vector and then extended with T4 DNA polymerase. Following ligation and transformation of bacterial cells, positive clones were selected for the desired site-directed mutation. Tyr306(c149) was shown to be critical for the ICA by interacting with Lys300(c143); its effect on the ICA by charge or side chain was investigated by mutagenesis. Tyr306(c149) is a large side chain residue positioned at the tip β-turn of the loop. Replacement with glycine would be expected to destabilize the β-turn structure and increase the flexibility of the loop. Lys313(c156) is the only other positively charged residue in the flexible loop and probably has no effect on the ICA based on the model.

**ICA Assay**—For hydrolysis of S2444, the single-chain forms (1.0–20.0 μM) were incubated with 0.6 nM of S2444 in 0.05 M sodium phosphate, 0.10 M NaCl, 0.2% bovine serum albumin, and 0.01% Tween 80 (pH 7.4) at room temperature. The reaction rate was measured by linear optical density increase over time at 410 nm against a reference wavelength of 490 nm (410/490 nm) on a microtitre plate reader. 1.0–10.0 nM of the UK International Standard was used for the standard curve of S2444 activity of UK. The kinetics of S2444 amidolysis were determined using a range of concentrations (0.03–2.4 mM) of S2444, as described previously (29).

**TCA Assay**—For hydrolysis of S2444, the two-chain forms (5.0–20.0 nM) were incubated with 0.6 nM S2444 in 0.05 M sodium phosphate, 0.10 M NaCl, 0.2% bovine serum albumin, and 0.01% Tween 80 (pH 7.4) at room temperature. The reaction rate and kinetics were measured as described above.

**Plasmin-resistant pro-UK Double Mutants**—To avoid interference previously (7). Plasmin was removed with aprotinin-Sepharose.
from two-chain activity generated during the plasminogen activation assay of the single-chain forms, the following mutants were made to be plasmin-resistant by additionally mutating Lys300(c143) → Ala pro-UK and the mutants Lys300(c143) → Ala, Lys300(c143) → Ala, Glu301(c144) → Ala, and Lys300(c143) → His. Plasminogen (2.0 μM) activation by the two-chain forms (0.1 mM) and their single-chain plasmin-resistant counterparts (10 nM) was measured using the S2251 (1.5 mM) indirect assay as described previously (20).

RESULTS

The Effect of Mutagenesis on the ICA of pro-UK against Synthetic Substrate—Using the synthetic substrate S2444 (0.6 mM), the ICA of wild-type pro-UK was determined to be 0.2% of its TCA, consistent with previous reports (7–10, 12, 20). As shown in Fig. 2A, the mutations changed the ICA from 40-fold lower to 4.7-fold higher than wild-type pro-UK.

The mutant Tyr306(c149) → Gly, which greatly reduced the size at this position, reduced the ICA 11-fold. This finding was consistent with the hypothesis that increasing the flexibility of the loop by allowing it to move with less restraint would interfere with the stability of the interaction between Lys300(c143) and Asp305(c194).

The opposite effect on the ICA was induced by the mutant Glu301(c144) → His, which was designed to reduce the flexibility of the loop by adding a large side chain (Fig. 2A). This mutation increased the ICA 4.7-fold, consistent with the hypothesis. Because His is a modest positive charge, a Glu301(c144) → Ala was also tested. Ala is neutral and has a small side chain. This mutation induced a 12-fold reduction in the ICA compared with the wild-type pro-UK or induced a 55-fold reduction compared with the Glu301(c144) → His mutant. Therefore, the promoting effect of His301(c144) on the ICA was mainly related to its large side chain reducing the flexibility of the loop. A Glu301(c144) → Asp mutant induced a 6.9-fold reduction of the ICA (Fig. 2A). Because Asp is similar to Glu in charge but smaller than Glu in size, this observation supports the concept that flexibility rather than charge was responsible for the changes in the ICA induced by mutations at position 301(c144).

When Lys300(c143) was mutated to a less positively charged amino acid, histidine, the ICA was reduced by 5-fold. In contrast, the mutant Lys300(c143) → Ala, which eliminated the positive charge all together, induced a 40-fold reduction of the ICA (13). Therefore, the present findings are consistent with the concept that the charge interaction between position 300(c143) and Asp305(c194) is largely responsible for the relatively high ICA of pro-UK. The effect of the large side chain of histidine on the ICA was tested by the mutant Lys300(c143) → Trp. Tryptophan has a larger side chain but is neutral. This mutant reduced the ICA 38-fold, indicating that at this position in the loop, a positive charge was the major determinant of the ICA rather than flexibility. By contrast, at other positions in the loop, flexibility was the more important determinant of the ICA.

By contrast, Lys313(c156), the only other positively charged residue in the flexible loop, when mutated to neutral Ala had no effect on the ICA. The comparable residue in t-PA (Lys228(c156)) has a major role in its single-chain activity (16, 17), illustrating one of the structure-function differences in the protease domains of the two activators.

The Ser306(c149) → Gly mutant had an ICA equivalent to that of the wild-type pro-UK, indicating that all mutations within the flexible loop do not affect function.

These structural changes and their effects on the ICA are summarized in Table I.

The Effect of Mutagenesis on the ICA against Plasminogen—The ICAs against plasminogen were measured using plasmin-resistant (Lys158(c15) → Ala) mutants to eliminate the plasmin-mediated generation of two-chain enzymes. In the presence of 2.0 μM plasminogen and 1.5 mM S2251, the ICA of plasmin-resistant pro-UK was determined as 0.04 nM plasmin generated per min per nM activator. The ICAs of plasmin-resistant mutants Lys300(c143) → Ala, Glu301(c144) → Ala, and Lys300(c143) → His were about 40-, 9-, and 4-fold lower, respectively, than the ICA of pro-UK. These results were similar to the ICAs obtained with these mutants against the synthetic substrate, S2444.

The Effect of Mutagenesis on the TCA of UK against Synthetic Substrate—Using synthetic substrate S2444 (0.6 mM), the TCA of wild-type UK was determined to be 1.6 × 10⁻³/
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| Mutation   | Functional change | TCA |
|------------|-------------------|-----|
| Tyr300(c143) → Gly | Reduction of side chain to increase flexibility | 11-fold less |
| Glu301(c144) → His | Enhancement of side chain to reduce flexibility | 4.7-fold more |
| Glu301(c144) → Ala | Reduction of side chain to increase flexibility | 12-fold less |
| Glu301(c144) → Asp | Reduction of side chain to increase flexibility | 6.9-fold less |
| Lys300(c143) → Ala | Elimination of positive charge | 40-fold less |
| Lys300(c143) → His | Reduction of positive charge and enlargement of side chain | 5-fold less |
| Lys313(c156) → Ala | Elimination of positive charge and enlargement of side chain | 38-fold less |
| Ser303(c146) → Glu | (Positive charge eliminated) No change |

| Mutation   | Functional change | ICA |
|------------|-------------------|-----|
| Tyr300(c143) → Gly | Reduction of side chain to increase flexibility | 11-fold less |
| Glu301(c144) → His | Enhancement of side chain to reduce flexibility | 4.7-fold more |
| Glu301(c144) → Ala | Reduction of side chain to increase flexibility | 12-fold less |
| Glu301(c144) → Asp | Reduction of side chain to increase flexibility | 6.9-fold less |
| Lys300(c143) → Ala | Elimination of positive charge | 40-fold less |
| Lys300(c143) → His | Reduction of positive charge and enlargement of side chain | 5-fold less |
| Lys313(c156) → Ala | Elimination of positive charge and enlargement of side chain | 38-fold less |
| Ser303(c146) → Glu | (Positive charge eliminated) No change |

*Charge changes in positions other than 300(c143) were found not to affect the ICA or TCA and have been omitted from the table.*

The Effect of Mutagenesis on the Zymogenicity of pro-UK—Zymogenicity was defined as the ratio of the TCA to the ICA. As shown in Fig. 2C, the zymogenetics of mutations Lys300(c143) → Ala, Lys300(c143) → His, Lys300(c143) → Trp, Glu301(c144) → Ala, and Glu301(c144) → Asp were higher than wild-type pro-UK by 13.2-, 9.9-, 5.8-, 12.8-, and 10.3-fold respectively. In contrast, mutant Glu301(c144) → His was 10.4-fold less zymogenic than pro-UK.

The Changes in ICA or TCA Were Due to Changes in Catalytic Reactivity—A kinetic study with a range of concentrations of the synthetic substrate was done for both the single-chain and two-chain mutants. It was found that the parameters of \( k_{cat} \) (catalytic reactivity) varied among the mutants, whereas \( K_m \) remained unchanged, being 50–60 and 75–90 \( \mu \)m for single-chain and two-chain mutants, respectively (Table II). These kinetic constants were similar to those of wild-type u-PA (13, 20), indicating that changes in the ICA or TCA induced by the mutations were mainly due to changes in \( k_{cat} \). This finding was consistent with previous results that two-chain mutant Lys300(c143) → Ala had the same \( K_m \) but 3-fold lower \( k_{cat} \) than the wild-type UK against S2444 (13).

**DISCUSSION**

In this study, mutations in the flexible loop of u-PA were found to alter its ICA substantially and the TCA more modestly by a direct effect on catalytic reactivity (\( k_{cat} \)) rather than on substrate binding (\( K_m \)). The catalytic reactivity of a two-chain serine protease is primarily determined by the spatial arrangement of the active site residues (His215(c57), Asp260(c102), and Ser275(c105)), formed by a charge interaction between Asp355(c194) and the newly released N-terminal Ile156(c16). We have recently provided evidence that in single-chain u-PA or pro-UK, this Asp355(c194) interacts with Lys300(c143), which acts as a surrogate of Ile156(c16) and that this charge interaction explains the relatively high ICA of pro-UK (13).

Lys300(c143) is a positively charged residue situated within a loop region postulated to be highly flexible according to homologous structures in trypsinogen and chymotrypsinogen. Therefore, it was proposed that alterations in the flexibility of this loop or the charge at position300(c143) would affect the ICA by modulating the charge interaction with Asp355(c194) and Lys300(c143) in the computer model (Fig. 3).

The mutant Ser303(c146) → Glu had no effect on the TCA, showing again that mutations in the flexible loop do not necessarily interfere with catalytic activity (Table I).

The Effect of Mutagenesis on the TCA against Plasminogen—The activities of the two-chain forms (0.1 nM) against Glu plasminogen (2.0 \( \mu \)M) showed that the activating rate of UK was 1.0 nM plasmin generated per min per nM activator. The TCA of the mutants against plasminogen, in comparison with wild-type UK, were similar to that against the synthetic substrate, S2444.
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Fig. 3. The structure of low molecular weight UK adopted from its x-ray structure (14). The neo N-terminal peptide (Ile159(c16)-Gly161(c18)-Asp355(c194)-Ser356(c195) is shown in yellow, the flexible loop (297–313) is shown in blue, and the active site peptide (Ser351(c144)-Glu352(c145)-Gly353(c146)-Asp355(c194)-Ser356(c195)) is shown in red. Lys300(c143) was shown to be involved in maintenance of the hydrophobic environment around the neo N-terminal Ile159(c16), which forms a salt bridge with Asp355(c194) in UK and generates the interactions between Lys300(c143) and Asp355(c194).

The other residues (Glu301(c144), Ser303(c146), and Lys313(c156)) had less effect on the interaction between Asp355(c194) and Ile159(c16).

The neo N-terminal peptide (Ile159(c16)-Ile160(c17)-Gly161(c18)-Gly162(c19)-Glu163(c20)) is shown in pink, and the active site peptide (Ser351(c144)-Glu352(c145)-Gln353(c192)-Asp355(c194)-Ser356(c195)) is shown in red. Lys300(c143) was shown to be involved in maintenance of the hydrophobic environment around the neo N-terminal Ile159(c16), which forms a salt bridge with Asp355(c194) in UK and generates the interactions between Lys300(c143) and Asp355(c194).

Those that decreased flexibility by increasing side chains stabilizing the interaction between Lys300(c143) and Asp355(c194) according to the computer model and were shown to increase the ICA. The reverse effect on the ICA was induced by mutations that made the loop more wobbly by reducing the size of side chains. Reduction of the positive charge of the flexible loop at Lys300(c143) had a major effect on the ICA and a modest effect on the TCA, as previously found (13). In contrast, elimination of the charge at Lys313(c156) had no effect on either the ICA or TCA. The findings provide strong support for the validity of the computer model and support the hypothesis that the ICA of pro-UK is related to a charge interaction between Lys300(c143) and Asp355(c194), which is significantly influenced by the flexibility of the loop region (297–313).

Although u-PA and t-PA are the two most homologous members of the serine protease family, they have several fundamental functional differences. For example, pro-UK is azymogen, albeit with a relatively high ICA (0.2% of its TCA) (22), whereas single-chain t-PA is an enzyme that has a single-chain activity equivalent to 15–25% of its TCA (21, 23, 24) and forms covalent complexes with plasma inhibitors (25). Second, although plasminogen activation by both t-PA and pro-UK is strongly promoted by fibrin, t-PA mediates this by fibrin affinity, whereas pro-UK does not. Pro-urokinase selectively activates plasminogen bound to C-terminal lysines of the fibrin E-domain (12, 26, 27), whereas t-PA selectively activates plasminogen bound to an internal lysine in the fibrin D-domain (26, 28).

Table II

Kinetics of S2444 (0.03–2.4 mM) amidolysis by u-PA and mutants

|                        | Single-chain activators (1.0–20.0 μM) | Two-chain activators (5.0–20.0 nM) |
|------------------------|--------------------------------------|----------------------------------|
|                        | kcat/min | K_M | kcat/K_M | kcat/min | K_M | kcat/K_M |
| Wild-type u-PA         | 0.32 ± 0.15 | 51 ± 9 | 0.00627 | 180 ± 130 | 78 ± 15 | 2.31 |
| Ly300(c143) → Ala      | 0.977 ± 0.0052 | 54 ± 10 | 0.00014 | 60 ± 24 | 77 ± 12 | 0.78 |
| Ly300(c143) → His      | 0.065 ± 0.030 | 52 ± 12 | 0.00125 | 350 ± 110 | 75 ± 18 | 4.67 |
| Ly300(c143) → Trp      | 0.0085 ± 0.0068 | 59 ± 15 | 0.00014 | 27 ± 18 | 74 ± 14 | 0.36 |
| Lys313(c156) → Ala     | 0.33 ± 0.18 | 54 ± 6 | 0.00611 | 190 ± 30 | 80 ± 11 | 2.38 |
| Glu301(c144) → Ala     | 0.027 ± 0.014 | 51 ± 13 | 0.00053 | 200 ± 150 | 87 ± 22 | 2.30 |
| Glu301(c144) → Asp     | 0.047 ± 0.035 | 60 ± 16 | 0.00078 | 270 ± 90 | 82 ± 12 | 3.29 |
| Glu301(c144) → His     | 1.5 ± 0.8 | 55 ± 27 | 0.0273 | 83 ± 37 | 89 ± 16 | 0.93 |
| Ser303(c146) → Gly     | 0.32 ± 0.07 | 53 ± 11 | 0.00604 | 180 ± 50 | 77 ± 10 | 2.33 |
| Tyr306(c149) → Gly     | 0.029 ± 0.017 | 58 ± 9 | 0.00050 | 22 ± 14 | 85 ± 18 | 0.26 |
In the present study, a number of structure-function differences between pro-UK and t-PA were identified that may help explain why some of their properties are different. Whereas the ICA of pro-UK was related predominantly to the positive charge of Lys8300(c143) and was influenced by the flexibility of the loop (297–313), the activity of single-chain t-PA has been related mostly to a loss of the zymogenic triad (23) and to certain individual residues such as Lys8416(c143), His417(c144), and Lys429(c156) (16, 17). The Lys429(c156) was suggested by the x-ray structure of t-PA to be a major determinant (16) of its single-chain activity, whereas the comparable residue in pro-UK (Lys8313(c156)) had no effect on the ICA.

The function of the amino acid residue at position c144 provides an especially interesting comparison between pro-UK and t-PA. t-PA mutants, His417(c144) to Asp and His417(c144) to Glu reduced the single-chain activity by 4-6- and 6-14-fold against S2888 and plasminogen, respectively (29). In pro-UK, the comparable position 301(c144) is already Glu, and a Glu301(c144) to His mutation resulted in a 4.7-fold increase in ICA against S2444, whereas mutating it to Ala induced a 12-fold reduction. This suggests that the negative charge at position 301(c144) of pro-UK does not barricade the interaction between the Asp355(c194) and Lys313(c156), in contrast to what was recently proposed (29). Therefore, the structure-function relationship of one plasminogen activator cannot be assumed to be applicable to the other.

The finding that the ICA and TCA of u-PA can be manipulated separately makes it possible to create a mutant u-PA with a lower ICA and a higher TCA as illustrated by the Lys8300 → His mutant in which the ICA was 5-fold lower and TCA 2-fold higher than wild-type u-PA (Table I and Fig. 2). This could improve its therapeutic properties because a more zymogenic form of pro-UK would be more stable in plasma at pharmacological concentrations (13, 22), whereas a higher TCA should promote its fibrinolytic properties on the clot surface (27, 30).

In conclusion, a previous study suggesting that the relatively high ICA of pro-UK is related to a charge interaction between Lys8300(c143) and Asp355(c194) is supported by the present study. This interaction was shown to be significantly influenced by certain neighboring mutations within a loop region (297–313) whose flexibility permits the interaction with Asp355(c194) to occur. Mutations designed, according to the computer model, to increase flexibility or wobbliness of the loop reduced the ICA by interfering with the interaction, whereas those that reduced flexibility increased the ICA by stabilizing the interaction. Reduction of the charge at position8300(c143) also directly reduced the ICA. The TCA of u-PA was shown to be less influenced by changes in the flexibility of the loop, probably because the charge interaction between Asp355(c194) and Ile159(c16) is stronger than that with Lys8300(c143). The ICA and TCA of u-PA could be modulated independently, a phenomenon that may be exploited for therapeutic purposes. A number of structure-function relationships of the ICA of pro-UK were found to be significantly different from that of single-chain t-PA.

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