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FOR THE RECORD

Role of residue Y99 in tissue plasminogen activator

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Abstract: The crystal structure of the fibrinolytic enzyme tissue plasminogen activator (tPA) shows that the bulky side chain of Y99 hinders access to the active site by partially occluding the S2 site and may be responsible for the low catalytic activity of tPA toward plasminogen. We have tested the role of Y99 by replacing it with Leu, the residue found in more proficient proteases like trypsin and thrombin. The Y99L replacement results in an increase in the k_{cat}/K_m for chromogenic substrates due to enhanced diffusion into the active site. The increase is modest (threefold) for substrates specific for tPA that carry Pro or Gly at P2, but reaches 80-fold for less specific substrates carrying Arg at P2. On the other hand, the Y99L mutation has no effect on the activity of tPA toward the natural substrate plasminogen, that carries Gly at P2, and reduces more than 10-fold the inhibition of tPA by plasminogen activator inhibitor-1 (PAI-1), that carries Ala at P2. We conclude that the steric hindrance provided by Y99 in the crystal structure affects mostly nonphysiological substrates with bulky residues at P2. In addition, residue Y99 plays an active role in the recognition of PAI-1, but not plasminogen. Mutations of Y99 could therefore afford a resistance to inhibition by PAI-1 without compromising the fibrinolytic potency of tPA, a result of potential therapeutic relevance.

Keywords: kinetics; protein engineering; serine proteases

Blood clots are dissolved by plasmin that is specifically activated from plasminogen by the serine proteases tPA and urokinase (Collen & Lijnen, 1995). Unlike urokinase, tPA catalyzes the activation of plasminogen on the fibrin surface (Collins et al., 1997), where plasmin is slowly inactivated by α_2-antiplasmin (Holmes et al., 1987). This important property presently makes tPA the molecule of choice in the treatment of acute myocardial infarction and stroke (Ludlam et al., 1995). Although tPA is extremely selective, its specificity constant toward plasminogen measured as k_{cat}/K_m is below 10^6 M^{-1} s^{-1} even upon assembly of the components on the fibrin mesh (Hoylaerts et al., 1982; Higgins et al., 1990). Therapeutic applications would therefore benefit from tPA derivatives that have either improved catalytic activity toward plasminogen or decreased tendency to be inactivated by PAI-1 (Collen & Lijnen, 1995). Previous mutagenesis studies have shown that deletion of several residues in the 37-loop flanking the active site (Lamba et al., 1996) compromises inhibition by PAI-1, but has no effect on the cleavage of plasminogen (Madison et al., 1989). The same effect is obtained with charge reversal of R37a and R37b in the loop (Madison et al., 1990). However, the significant improvement of the catalytic activity of tPA toward plasminogen or even synthetic substrates remains a challenge for rational protein engineering.

tPA has been studied in considerable detail, but the exact structural determinants of its low specificity are not known. The crystal structure of the catalytic domain of two-chain tPA shows an environment for the catalytic triad without apparent constraints for substrate binding or catalysis (Lamba et al., 1996). A notable difference, however, is observed at the level of residue 99. In trypsin and thrombin this residue is Leu, whereas tPA has Tyr at this position like factor IXa and plasma kallikrein. It has been proposed that the bulky aromatic side chain of Y99 may restrict access to the S2 site, thereby impairing the catalytic efficiency of tPA (Lamba et al., 1996). In coagulation factor IXa, Y99 is in a conformation that blocks access to the S2 site and accounts for the extremely low activity of this enzyme (Hopfner et al., 1999). Reengineering the region around Y99 brings about a dramatic increase in catalytic activity in factor IXa. Mutagenesis studies carried out on thrombin have shown that the replacement of L99 with Tyr compromises sevenfold the k_{cat}/K_m toward synthetic substrates carrying Pro at P2 (Rezaie, 1998) and about fivefold the inhibition of thrombin by antithrombin III that carries Gly at P2 (Rezaie, 1997). These studies suggest that a bulkier side chain at position 99 may be deleterious to binding, even when the substrate has a small group at the P2 position. The results on factor IXa and thrombin are relevant to tPA because the physiologic substrate plasminogen and the inhibitor PAI-1 carry Gly and Ala at P2, respectively. We have therefore

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Abbreviations: DRR, H-D-Asp-Arg-Arg-*p*-nitroanilide; ETI, *Erythrina caraffa* trypsin inhibitor; FGR, H-D-Phe-Gly-Arg-*p*-nitroanilide; FPR, H-D-Phe-Pro-Arg-*p*-nitroanilide; IGR, H-D-Ile-Gly-Arg-*p*-nitroanilide; IPR, H-D-Ile-Pro-Arg-*p*-nitroanilide; LDPR, H-Leu-Asp-Pro-Arg-*p*-nitroanilide; PEG, poly(ethylene glycol); pEPR, pyroGlu-Pro-Arg-*p*-nitroanilide; *p*-NA, paranitroaniline; VGR, H-D-Val-Gly-Arg-*p*-nitroanilide; VLR, H-D-Val-Leu-Arg-*p*-nitroanilide; VPR, H-D-Val-Pro-Arg-*p*-nitroanilide; WRR, H-D-Trp-Arg-Arg-*p*-nitroanilide.

decided to test directly the role of Y99 in tPA by site-directed mutagenesis.

Results and discussion: Compared to wild-type, the Y99L mutant shows a significant increase in the specificity constant k_{cat}/K_m for chromogenic substrates (Fig. 1), due entirely to improved binding and lower K_m. In the case of the most specific substrates (IPR, FPR, and FGR), the increase is up to fivefold. Inspection of Figure 1 reveals a consistently higher increase for substrates carrying Pro at P2 over Gly, indicating that the Y99L substitution may facilitate docking of a bulkier hydrophobic group at P2. However, this trend is broken in the VLR substrate that experiences a smaller increase in specificity compared to both VGR and VPR. The increase in specificity of FPR was investigated further by measuring the k_{cat}/K_m ratio as a function of temperature (Fig. 2) to obtain the second-order rate constant for diffusion of the substrate into the active site k_1 as first demonstrated for the thrombin–fibrinogen interaction (Vindigni & Di Cera, 1996). The value of k_1 increases about threefold in the Y99L mutant relative to wild-type (Table 1), demonstrating that the S2 site has become more accessible with replacement of the Tyr. No other significant difference is displayed by the Y99L mutant in the hydrolysis of FPR, with the activation energies for substrate binding, dissociation, and acylation, as well as the stickiness parameter, being similar to those of the wild-type (Table 1).

In the case of the least specific substrates (DRR and WRR), the increase in specificity constant is significantly more pronounced and goes up to 80-fold (Fig. 1). The resulting value of k_{cat}/K_m for DRR and WRR is only 10-fold lower than that of the more specific substrates FPR and IPR, indicating that the Y99L mutant has acquired specificity for the XRR sequence. This finding is intriguing

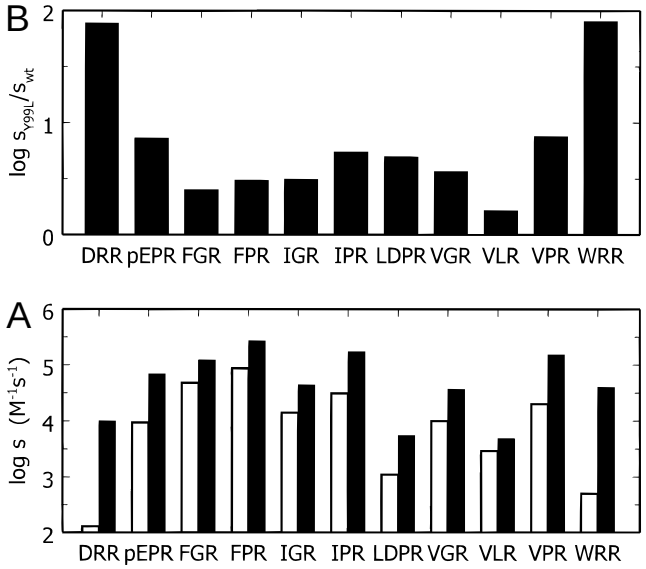

Fig. 1. Effect of the Y99L substitution on the hydrolysis of chromogenic substrates by tPA. **A:** The data are reported as specificity constants $s = k_{cat}/K_m$ for the wild-type (white bars) and the Y99L mutant (black bars). **B:** The increase in specificity of the mutant relative to wild-type. Replacement of Y99 enhances specificity in all cases. The increase reaches 80-fold for DRR and WRR. Experimental conditions are 5 mM Tris, 200 mM NaCl, 0.1% PEG, pH 8.0 at 25 °C.

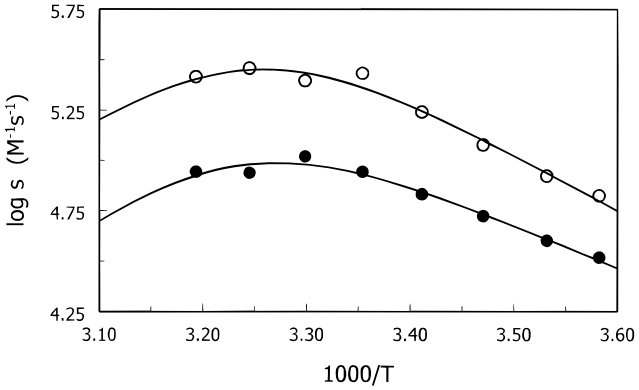

Fig. 2. Arrhenius plot of the specificity constant $s = k_{cat}/K_m$ for the cleavage of FPR by wild-type tPA (●) and the Y99L mutant (○). Experimental conditions are 5 mM Tris, 200 mM NaCl, 0.1% PEG, pH 8.0. Continuous lines were drawn according to Equation 1 in the text, with best-fit parameter values listed in Table 1. The Y99L mutant has higher specificity than wild-type due to a slightly improved diffusion of substrate into the active site.

because XRR is the consensus sequence present in coagulation factor Va at one of the sites cleaved by the anticoagulant enzyme activated protein C (Egan et al., 1997; Kalafatis et al., 1994). In fact, both DRR and WRR were originally synthesized as substrates selective for activated protein C (Dang & Di Cera, 1997). We therefore tested the possibility that the Y99L mutant of tPA could have acquired specificity toward coagulation factor Va. In the presence of activated protein C and phospholipid vesicles, factor Va was inactivated within 10 min of incubation and displayed the expected cleavage products (Egan et al., 1997; Kalafatis et al., 1994), as determined by western blot analysis and N-terminal sequencing. On the other hand, the activity of the factor Xa-factor Va complex toward prothrombin, with factor Va treated for 90 min with wild-type tPA or the mutant Y99L, was identical to control without treatment. Results from both the western blot analysis and the N-terminal sequencing showed no difference between factor Va treated with wild-type and Y99L mutant tPA and untreated factor Va. Hence, despite its dramatic increase in specificity for DRR and WRR, no evidence was found that the Y99L mutant of tPA could cleave and inactivate factor Va at R506, following the amino acid sequence DRR.

The significant enhancement of catalytic activity experienced by the Y99L mutant toward chromogenic substrates is not seen toward

Table 1. *Properties of wild-type and the Y99L mutant of tPA*

	wt	Y99L	R^a
Plasminogen k_{cat}/K_m (mM^{-1} s^{-1})	59 ± 4	54 ± 2	0.9 ± 0.1
PAI-1 k_{on} (μM^{-1} s^{-1})	1.2 ± 0.1	0.11 ± 0.01	0.09 ± 0.01
ETI K_d (nM)	6.2 ± 0.2	99 ± 9	16 ± 2
Cu^{2+} K_d (μM)	19 ± 4	22 ± 3	1.2 ± 0.3
FPR $k_{1,0}$ (mM^{-1} s^{-1})	100 ± 20	280 ± 80	2.8 ± 0.9
E_1 (kcal/mol)	10 ± 2	13 ± 3	1.3 ± 0.4
α_0	6 ± 3	5 ± 3	0.8 ± 0.6
E_α (kcal/mol)	30 ± 10	30 ± 10	1.0 ± 0.5

[a]Change of the parameter in the Y99L mutant relative to wild-type.

the natural substrate plasminogen (Table 1). Plasminogen carries Gly at P2 and perhaps the absence of a side chain at this position makes the steric hindrance provided by Y99 inconsequential on binding. This conclusion is consistent with a comparative structural analysis of tPA, trypsin, and thrombin (Renatus et al., 1998; Hopfner et al., 1999), where the constraints imposed by the S2 site on synthetic and natural substrates have been discussed. It is also consistent with the data shown in Figure 1, where the presence of Gly at P2 results only in a small increase in the k_{cat}/K_m value.

The Y99L substitution is also inconsequential on the affinity for Cu^{2+} binding (Table 1). Recent mutagenesis studies of tPA show that Cu^{2+} binds near H188 in the S1 cavity (Cantwell & Di Cera, unpubl. results), a domain that is separate enough from the S2 site and apparently not energetically linked to it. The lack of linkage between the S1 and S2 sites is also consistent with previous data on the site-specific dissection of substrate binding to tPA (Vindigni & Di Cera, 1998).

The foregoing results suggest a predominantly mechanical role for Y99 that would restrict access to the active site of tPA to substrates carrying bulky side chains at P2. However, Y99 must also play an active role in the recognition of ETI and PAI-1 (Table 1). Inhibition of tPA by ETI is significantly compromised in the Y99L mutant, with the value of K_d increasing 16-fold relative to wild-type. Furthermore, the inhibitor no longer behaves as slow-binding, as for wild-type (Vindigni & Di Cera, 1998), at least under the conditions that enable accurate measurements of K_d for ETI binding. The structure of the tPA-ETI complex is not known, but most likely Y99 participates in favorable van der Waals or polar contacts with residues of the inhibitor and removal of these contacts overrides possible favorable contributions to binding coming from a less constrained S2 environment. As for ETI, binding of PAI-1 is reduced 10-fold upon the Y99L substitution, supporting an important role for Y99 in the recognition of the physiological inhibitor of tPA. PAI-1 carries Ala in P2 and is difficult to envision how the Tyr → Leu substitution at position 99 may reduce the k_{on} for the inhibitor. Any van der Waals contact of the P2 residue with Y99 would be preserved for the most part in the Y99L mutant. It is more likely that the hydroxyl group of Y99 makes a polar interaction with other residues of PAI-1 that stabilize the enzyme-inhibitor complex.

The mutagenesis data reported in this study show that Y99 in the S2 specificity site of tPA is more important for recognition of PAI-1 than plasminogen. Hence, binding of the physiological substrate and inhibitor can be dissociated by targeting either the 37-loop (Madison et al., 1989, 1990), or the region around Y99. In thrombin, mutations in and around the S2 site that compromise binding of the natural substrate fibrinogen also compromise the inhibition of the enzyme by antithrombin III (Rezaie, 1997; Di Cera, 1998), and the two interactions can only be dissociated by compromising binding of the cofactor heparin (Tsiang et al., 1997). The effect observed on PAI-1 is of pharmacological interest provided it can be reproduced in vivo. As for the mutants in the 37-loop (Madison et al., 1989, 1990), the Y99L mutant may offer an advantage over the wild-type in the treatment of acute myocardial infarction and stroke because the increased resistance to PAI-1 inactivation, coupled to normal fibrinolytic activity, would prolong the half-life and therapeutic effect of tPA in the blood.

Materials and methods: All the procedures for expression, purification, titration, and functional characterization of the Y99L mutant employed in this study are described in detail elsewhere (Vindigni & Di Cera, 1998). The Y99L mutant was expressed in *Escherichia coli* as a truncated form of tPA, tPAdes(Val4–Cys261). Refolding and purification of the mutant by affinity chromatography on an ETI-Sepharose column were carried out to a final yield >2 mg/L of media. Single chain tPA was converted to the two-chain tPA using a plasmin-Sepharose resin. Titration of the active site with a reference solution of ETI revealed a 98% degree of activity of the mutant. The titration also yielded the K_d for ETI binding to tPA (Table 1). The identity and purity of the protein were confirmed by SDS-gel electrophoresis and electrospray mass spectrometry.

All measurements were carried out under experimental conditions of 5 mM Tris, 200 M NaCl, 0.1% PEG, pH 8.0 at 25 °C. Chromogenic substrates were synthesized and purified to homogeneity as described (Vindigni & Di Cera, 1998). Progress curves of the release of *p*-NA following the hydrolysis of chromogenic substrates were analyzed using KINSIM and FITSIM (Dang & Frieden, 1997) to extract the value of the specificity constant k_{cat}/K_m properly corrected for product inhibition, if necessary.

The value of k_{cat}/K_m for the hydrolysis of FPR by tPA was also studied under experimental conditions of 5 mM Tris, 200 mM NaCl, 0.1% PEG, pH 8.0, over the temperature range from 5 to 45 °C. The pH was precisely adjusted at room temperature to obtain the value of 8.0 at the desired temperature. Tris buffer has a $pK_a = 8.06$ at 25 °C and a temperature coefficient of $\Delta pK_a/\Delta T = -0.027$. These properties ensured buffering over the entire temperature range examined. It has been shown that the temperature dependence of k_{cat}/K_m for the hydrolysis of amide substrates by serine proteases obeys the equation (Vindigni & Di Cera, 1996)

$$\frac{k_{cat}}{K_m} = k_{1,0}\frac{\alpha_0 \exp\left\{\frac{E_\alpha - E_1}{R}\left(\frac{1}{T} - \frac{1}{T_0}\right)\right\}}{1 + \alpha_0 \exp\left\{\frac{E_\alpha}{R}\left(\frac{1}{T} - \frac{1}{T_0}\right)\right\}} \quad (1)$$

where R is the gas constant, T the absolute temperature, E_1 the activation energy for substrate binding, E_α the difference in the activation energies for substrate dissociation and acylation, $k_{1,0}$ the value of the second-order rate constant for substrate binding k_1 at the reference temperature $T_0 = 298.15$ K and α_0 the parameter of stickiness defined as the ratio k_2/k_{-1} (k_2, rate of acylation; k_{-1}, rate of substrate dissociation) at the reference temperature. Details on the properties of Equation 1 are given elsewhere (Vindigni & Di Cera, 1996).

The apparent second-order rate constant (k_{on}) for the inhibition of tPA by PAI-1 (American Diagnostica, Greenwich, Connecticut) was measured by progress curves from the effect of the inhibitor on the hydrolysis of FPR and were analyzed using KINSIM and FITSIM assuming an irreversible reaction between the enzyme and inhibitor. For each concentration of enzyme and substrate, different concentrations of PAI-1 were used to derive the value of k_{on} from a global analysis of the kinetic traces. The rate of Lys-plasminogen (American Diagnostica) activation by tPA was measured by continuous progress curves analyzed with KINSIM and FITSIM as described (Vindigni & Di Cera, 1998). Different concentrations of plasminogen were used in this assay to ensure accurate determination of the k_{cat}/K_m value. The equilibrium dissociation constant for Cu^{2+} binding to tPA was determined by

measuring the initial rate of substrate hydrolysis as a function of $[Cu^{2+}]$ and analyzing the data for competitive inhibition.

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