Role of P225 and the C136–C201 disulfide bond in tissue plasminogen activator

ALESSANDRO VINDIGNI AND ENRICO DI CERA

Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, Box 8231, St. Louis, Missouri 63110

(RECEIVED February 25, 1998; ACCEPTED May 27, 1998)

Abstract

The protease domain of tissue plasminogen activator (tPA), a key fibrinolytic enzyme, was expressed in *Escherichia coli* with a yield of 1 mg per liter of media. The recombinant protein was titrated with the *Erythrina caraffa* trypsin inhibitor (ETI) and characterized in its interaction with plasminogen and the natural inhibitor plasminogen activator inhibitor-1 (PAI-1). Analysis of the catalytic properties of tPA using a library of chromogenic substrates carrying substitutions at P1, P2, and P3 reveals a strong preference for Arg over Lys at P1, unmatched by other serine proteases like thrombin or trypsin. In contrast to these proteases and plasmin, tPA shows little or no preference for Pro over Gly at P2. A specific inhibition of tPA by Cu^{2+} was discovered. The divalent cation presumably binds to H188 near D189 in the primary specificity pocket and inhibits substrate binding in a competitive manner with a $K_d = 19 \mu\text{M}$. In an attempt to engineer Na^+ binding and enhanced catalytic activity in tPA, P225 was replaced with Tyr, the residue present in Na^+ -dependent allosteric serine proteases. The P225Y mutation did not result in cation binding, but caused a significant loss of specificity (up to 100-fold) toward chromogenic substrates and plasminogen and considerably reduced the inhibition by PAI-1 and ETI. Interestingly, the P225Y substitution enhanced the ability of Cu^{2+} to inhibit the enzyme. Elimination of the C136–C201 disulfide bond, that is absent in all Na^+ -dependent allosteric serine proteases, significantly enhanced the yield (5 mg per liter of media) of expression in *E. coli*, but caused no changes in the properties of the enzyme whether residue 225 was Pro or Tyr. These findings point out an unanticipated crucial role for residue 225 in controlling the catalytic activity of tPA, and suggest that engineering of a Na^+ -dependent allosteric enhancement of catalytic activity in this enzyme, must involve substantial changes in the region homologous to the Na^+ binding site of allosteric serine proteases.

Keywords: allostery; fibrinolysis; molecular recognition; serine proteases

Fibrinolysis is the process through which blood clots are dissolved (Collen & Lijnen, 1995). The enzyme responsible for this function is plasmin that is specifically activated from plasminogen by tPA and urokinase (Robbins et al., 1967). tPA is a serine protease of the

chymotrypsin family (Rawlings & Barrett, 1993). It has a mass of 70 kDa and is composed of a single polypeptide chain organized in a finger domain (residues 4–50), a growth factor domain (residues 50–87), two kringle domains (residues 87–176 and 176–262), and the protease domain (residues 276–527) containing the residues of the catalytic triad (Rijken & Collen, 1981; Pennica et al., 1983). Binding of tPA to fibrin via the finger and kringle two domain is required for optimal activation of plasminogen that occurs upon cleavage of a single amide bond at R560 (Madison, 1994). In this manner, tPA catalyzes the in situ activation of plasminogen on the fibrin surface where plasmin is supposed to act (Collins et al., 1997). This is clearly advantageous when compared to non-fibrin-specific thrombolytic agents, such as streptokinase and urokinase, that have no specific affinity for fibrin and therefore activate plasminogen indiscriminately in the plasma (Thorsen et al., 1972). On the fibrin surface, plasmin is also slowly inactivated by α_2 -antiplasmin, which however inhibits the protease very rapidly when free in the plasma (Holmes et al., 1987).

Effective thrombolysis should focus on the fibrin-assembled plasmin that is resistant to inactivation by α_2 -antiplasmin and produces its effects locally on the fibrin clot. This is best obtained with tPA,

Reprint requests to: Enrico Di Cera, Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, Box 8231, St. Louis, Missouri 63110; e-mail: enrico@caesar.wustl.edu.

Abbreviations: Ch, choline; DCC, N,N'-dicyclohexylcarbodiimide; DIEA, N,N-diisopropylethylamine; DMF, dimethylformamide; ETI, *Erythrina* trypsin inhibitor; FGK, H-D-Phe-Gly-Lys-p-nitroanilide; FGR, H-D-Phe-Gly-Arg-p-nitroanilide; Fmoc, fluorenylmethoxycarbonyl; FPK, H-D-Phe-Pro-Lys-p-nitroanilide; FPR, H-D-Phe-Pro-Arg-p-nitroanilide; HBTU, O-benzotriazol-1-yl-N,N,N',N'-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; HOSu, N-hydroxysuccinimide; HPLC, high performance liquid chromatography; IGR, H-D-Ile-Gly-Arg-p-nitroanilide; IPR, H-D-Ile-Pro-Arg-p-nitroanilide; MUGP, 4-methyl-umbelliferyl-p-guanidino benzoate; PAI-1, plasminogen activator inhibitor-1; PEG, poly(ethylene glycol); p-NA, paranitroaniline; RP-HPLC, reverse phase high performance liquid chromatography; TFA, trifluoroacetic acid; tPA, tissue plasminogen activator; VGK, H-D-Val-Gly-Lys-p-nitroanilide; VGR, H-D-Val-Gly-Arg-p-nitroanilide; VPK, H-D-Val-Pro-Lys-p-nitroanilide; VPR, H-D-Val-Pro-Arg-p-nitroanilide.

which has a localized and fibrin-specific action on plasminogen. For this reason, tPA is currently used in the treatment of acute myocardial infarction and stroke (Ludlam et al., 1995). However, a significant limitation of current therapeutical protocols using tPA is the failure to lyse all thrombi and the delay in restoration of blood flow. tPA is a highly selective enzyme and plasminogen is its only known physiological substrate. However, the specificity of tPA toward plasminogen is quite low and the k_{cat}/K_m constant remains well below $10^5 \text{ M}^{-1} \text{ s}^{-1}$ upon assembly of these components on the fibrin polymer (Hoylaerts et al., 1982). Engineering tPA derivatives with improved catalytic activity is therefore highly desirable (Collen & Lijnen, 1995).

The crystal structure of the catalytic domain of two-chain tPA has recently been solved (Lamba et al., 1996). Previous work has shown that, in the absence of fibrin, this domain is as active as full length tPA in the cleavage of plasminogen (Madison et al., 1995). The information emerged from the crystal structure provides little clues as to the possible origin of the low specificity of tPA. The environment of the catalytic triad shows no obvious constraints for substrate binding or catalysis. The S1 pocket contains D189 at its bottom, like trypsin and thrombin, and accounts for the preference for basic residues at P1. Like the blood clotting protease thrombin, tPA is devoid of a functional Ca^{2+} site in the 70-loop and possesses a 60-loop that is, however, shorter and does not shield the active site from the solvent. The active site of tPA is therefore fully accessible from the solvent. The S3–S4 sites are quite hydrophobic with W215 occupying a prominent position, as in thrombin and trypsin. A notable difference is residue 99 in the S3 site that in tPA is Tyr, whereas thrombin has Leu. It has been suggested that Y99 may restrict access to the active site of tPA (Lamba et al., 1996).

Phage display approaches have been successful in identifying some aspects of tPA specificity that bear on the activity of the enzyme in vivo (Ding et al., 1995). Fluorescence substrates specific for tPA have been synthesized and some requirements for residues present at the P1–P3 positions have been proposed (Coombs et al., 1996; Butenas et al., 1997). Elucidation of the molecular basis of tPA specificity is a prerequisite for improving the catalytic activity of the enzyme to boost its therapeutic potency. A systematic approach for the dissection of enzyme specificity has been developed recently (Vindigni et al., 1997; Di Cera, 1998) and has been applied to thrombin, plasmin, and trypsin. This approach is extended in the present study to tPA in order to gain a more quantitative understanding of the energetic basis of its low specificity.

Improving enzyme specificity remains a challenging problem in protein engineering and current approaches in this area are largely empirical (Zhou et al., 1997). The recent discovery of the Na^+ -dependent allosteric enhancement of catalytic activity in serine proteases of the chymotrypsin family provides a promising rational strategy to improve enzyme specificity (Di Cera et al., 1997). In this family of enzymes, residue 225 determines whether the protease is subject to the Na^+ -dependent allosteric enhancement of catalytic activity (Dang & Di Cera, 1996). Proteases with Y225 like thrombin, all vitamin K-dependent factors and some complement factors, bind Na^+ and enhance their catalytic activity in the presence of this cation, whereas proteases with P225 like the digestive and fibrinolytic enzymes, including tPA, are devoid of such property. The Y225P substitution in thrombin obliterates Na^+ binding (Dang & Di Cera, 1996). Furthermore, mutations around the Na^+ site (Di Cera et al., 1995) that compromise Na^+ binding also compromise the catalytic activity of the enzyme (Dang et al., 1997a, 1997b). Some of these mutations occur naturally and lead

to phenotypes with reduced clotting activity (Miyata et al., 1992; Henriksen et al., 1998).

The role of residue 225 in Na^+ binding opens the intriguing possibility of engineering a functional Na^+ site in proteases carrying P225 by introducing the substitution P225Y. The Na^+ -dependent enhancement of catalytic activity would obviously be beneficial in the extracellular environment where the NaCl concentration is high and may lead to important pharmacological and biotechnological applications. tPA is an ideal enzyme to pursue such goal, in view of its modest catalytic activity and the enormous benefits that would derive from its improvement. Extension to other serine proteases becomes straightforward once the requirements to engineering a functional Na^+ site in any member of this class of enzymes are fully understood.

Results

tPA was expressed in *E. coli* following a protocol similar to that of Kohnert et al. (1996). The truncated construct des(Val4–Cys261)tPA comprises the first three N-terminal residues of full length tPA and the catalytic domain. The in vitro refolding procedure resulted in the production of up to 1 mg of correctly folded non-glycosylated tPA per liter of media. The properties of the catalytic domain of recombinant tPA obtained in this manner are summarized in Table 1 and compare well to those of the full length molecule, in the absence of fibrin, reported by others (Madison et al., 1990, 1995), as also demonstrated previously (Kohnert et al., 1996).

Precise determinations of the active site concentration of the enzyme and the K_i for ETI binding were obtained from a new titration method (see Materials and methods) and the results are shown in Figure 1. The value of K_i for ETI is in the nM range (Table 1) and compares well with previously reported estimates (Onesti et al., 1992). ETI behaves as a slow binding inhibitor, as

Fig. 1. tPA titration using ETI. Continuous lines were drawn according to Equation 1 in the text with the value of K_i reported in Table 1 and refer to four different enzyme concentrations (from the bottom up): 2, 4, 8, and 16 nM. Experimental conditions are: 5 mM Tris, 200 mM NaCl, 0.1% PEG, pH 8.0 at 25 °C.

Table 1. Properties of wild-type and mutant tPA molecules^a

	wt	P225Y	C136A/C201A	C136A/C201A/P225Y
FPR	48 ± 1	2.6 ± 0.2	45 ± 1	1.0 ± 0.1
k_{cat}/K_m (mM ⁻¹ s ⁻¹)				
Plasminogen	59 ± 4	0.74 ± 0.02	38 ± 1	0.29 ± 0.01
k_{cat}/K_m (mM ⁻¹ s ⁻¹)				
PAI-1	1.2 ± 0.1	0.083 ± 0.002	1.0 ± 0.1	0.065 ± 0.001
k_{on} (μM ⁻¹ s ⁻¹)				
ETI	0.17 ± 0.01	0.0050 ± 0.0002	0.24 ± 0.01	0.0045 ± 0.0002
k_{on} (μM ⁻¹ s ⁻¹)				
ETI	0.0011 ± 0.0001	0.0037 ± 0.0005	0.0017 ± 0.0002	0.0049 ± 0.0006
k_{off} (s ⁻¹)				
ETI	6.2 ± 0.2	740 ± 70	7.1 ± 0.3	1,100 ± 100
K_d (nM)				
Cu ²⁺	19 ± 4	5.5 ± 0.3	25 ± 6	2.8 ± 0.5
K_d (μM)				

^aExperimental conditions: 5 mM Tris, 200 mM NaCl, 0.1% PEG, pH 8.0, 25 °C.

demonstrated by the progress curves of substrate hydrolysis in Figure 2. The k_{on} for inhibition by PAI-1 and the k_{cat}/K_m for cleavage of plasminogen in the absence of fibrin (Table 1) determined from analysis of progress curves are in good agreement with literature data (Madison et al., 1990, 1995).

A discovery was made when the effect of Cu²⁺ was tested on tPA. Analysis of over 300 sequences in the peptidase family S1

(Rawlings & Barrett, 1993) shows that tPA is the only enzyme to carry His at position 188 near D189 at the bottom of the primary specificity pocket. H188 appears to be absolutely conserved among tPA from different species. In trypsin, where residue 188 is Lys, Briand et al. (1997) have recently made the K188H replacement and engineered a Cu²⁺ chelation site. This resulted in a drastic inhibition of catalytic activity in the presence of Cu²⁺ due to chelation at H188 and neutralization of the negative charge of D189. We therefore decided to test the effect of Cu²⁺ on the catalytic activity of tPA, which carries H188 in its wild-type sequence. Cu²⁺ has a profound inhibitory effect on the hydrolysis of chromogenic substrates, consistent with competitive inhibition of binding (Fig. 3). The K_i for Cu²⁺ binding is 19 μM and signals the presence of extremely specific binding. Similar experiments carried out with trypsin and thrombin show no appreciable effect of Cu²⁺ on the catalytic activity of these enzymes.

In order to gain a better understanding of the energetic and structural origin of the low catalytic activity of tPA, a library of substrates analogous to that recently used for thrombin (Vindigni et al., 1997) was synthesized, and the specificity constants are reported in Table 2. tPA has a preference for Arg at P1, as previously reported (Butenas et al., 1997), but this preference over Lys is more pronounced than in other proteases. The Arg → Lys replacement at P1 causes a loss of specificity in excess of 2.5 kcal/mol, whereas the same substitution in thrombin and trypsin produces a loss of specificity of 1.4 kcal/mol (Table 3). The large loss of specificity upon the Arg → Lys replacement indicates that the environment of the S1 site in tPA is more selective than in cognate proteases. This may well be a factor in the extreme selectivity of tPA toward plasminogen. Another peculiar property of tPA is that it shows little or no preference between Pro or Gly at P2, whereas the Pro → Gly substitution severely compromises specificity in thrombin, trypsin, or plasmin. At the S2 site, the bulky side chain of Y99 may constrain the environment relative to trypsin and thrombin that carry Leu at this position. The bulkier Tyr may play a key role in determining P2-specificity and providing an environment where a rigid P2-P3 bond is inconsequential on the gain of specificity. As for thrombin, aromatic or hydrophobic residues at

Fig. 2. Progress curves for the hydrolysis of FPR by tPA in the presence of ETI. The biphasic nature of the curves demonstrates the slow inhibitory behavior of ETI. Experimental conditions are: tPA 4 nM, 5 mM Tris, 200 mM NaCl, 0.1% PEG, pH 8.0 at 25 °C. The concentration of ETI ranges from 0 (top curve) to 80 nM (bottom curve), scaled by a factor of 2. Curves were drawn according to Equation 2 in the text with the values of k_{on} and K_d reported in Table 1. A similar behavior was observed for all mutants (Table 1).

Table 2. Specificity constants $s = k_{\text{cat}}/K_m$ (in $\mu\text{M}^{-1} \text{s}^{-1}$) for tPA and other serine proteases^a

	FPR	FPK	FGR	VPR	FGK	VPK	VGR	VGK
tPA	0.048	0.00070	0.048	0.021	0.00066	0.00039	0.010	0.00013
Thrombin ^b	90	7.9	2.0	100	0.021	2.1	0.34	0.0047
Trypsin ^b	8.9	0.95	2.2	6.9	0.22	0.75	0.67	0.069
Plasmin ^b	0.031	0.047	0.0018	0.028	0.0048	0.058	0.0016	0.0037

^aErrors are $\pm 2\%$ or less. Experimental conditions: 5 mM Tris, 200 mM NaCl, 0.1% PEG, pH 8.0, 25 °C.^bData from Vindigni et al. (1997).

P3 in the D enantiomer are recognized equally well. A prominent aspect of tPA specificity is the lack of coupling between substitutions made at the P1–P3 sites of the substrate (Table 3). Specificity losses at individual sites are simply additive when multiple substitutions are introduced in the substrate. This behavior is analogous to that of trypsin and plasmin, but not thrombin, and suggests that the enzyme puts little constraints on the bound substrate in the transition state based on the interpretation of the coupling free energy values (Vindigni et al., 1997; Di Cera, 1998). This conclusion is in agreement with the wide accessibility of the active site documented by the crystal structure (Lamba et al., 1996).

As a result of the presence of P225, tPA shows no gain in catalytic activity in the presence of Na^+ . The values of k_{cat}/K_m are comparable in NaCl and ChCl up to 800 mM. To exclude directly the presence of bound Na^+ , fluorescence titrations were carried out at constant ionic strength of 800 mM. No significant change in intrinsic fluorescence could be detected, and the emission spectra in the presence of NaCl or ChCl were superimposable. The region

of tPA homologous to the Na^+ binding loop of thrombin has a similar main chain architecture, except for the expected notable shift in the position of the carbonyl O atom of residue 224 due to the presence of P225 (Fig. 4). The P225Y mutant was therefore constructed to assess the effect of relieving the constraints imposed by Pro on the main chain around residue 224. This mutant shows no appreciable difference in catalytic activity when tested in the presence of different monovalent cations. However, a most unexpected finding of this mutation is that it actually brings about a significant decrease in catalytic activity toward chromogenic substrates and plasminogen (Fig. 5; Table 1). The mutant also has a reduced affinity for the inhibitor ETI, due to a reduced k_{on} , and interacts with the natural inhibitor PAI-1 with a k_{on} of only 5% of that of the wild-type (Table 1). These changes suggest that residue 225 in tPA plays an important role in determining the specificity of the enzyme and that the P225Y substitution perturbs the environment of the specificity sites or the active site in a significant manner. Consistent with this hypothesis, binding of Cu^{2+} is increased in the P225Y mutant (Table 1).

A notable difference between allosteric and non-allosteric proteases is the absence of the C136–C201 disulfide bond in the former group. We therefore removed this disulfide bond in tPA (Fig. 6) to test the hypothesis that a more relaxed environment around the active site S195 would be required for the allosteric enhancement of catalytic activity induced by Na^+ binding, or even facilitate binding of the cation, once P225 is replaced by Tyr. Removal of this disulfide bond in tPA is inconsequential on the functional properties of the enzyme (Table 1), whereas it improves the expression yield in *E. coli* more than fivefold. When this mutation is coupled with the P225Y replacement, the resulting properties of the triple mutant are practically identical to those of the P225Y single mutant (Table 1). Na^+ binding cannot be detected by fluorescence measurements or enzymatic assays and no change in

Fig. 3. Effect of Cu^{2+} on substrate hydrolysis by tPA. Shown is the steady state velocity of FPR hydrolysis normalized by the substrate concentration ($\bullet$, 20 μM ; $\circ$, 200 μM). Experimental conditions are: 5 mM Tris, 200 mM NaCl, 0.1% PEG, pH 8.0 at 25 °C. Continuous lines were drawn according to Equation 5 in the text with the value of K_i reported in Table 1. The two data obtained at different substrate concentrations, up to the estimated value of $K_m = 200 \mu\text{M}$, yield the same value of K_i and demonstrate the competitive nature of the inhibition by Cu^{2+} .

Table 3. Free energy values (in kcal/mol) due to perturbation of the P1–P3 sites of FPR^a

	ΔG_1	ΔG_2	ΔG_3	ΔG_{12}	ΔG_{13}	ΔG_{23}	ΔG_{123}
tPA	2.5	0.0	0.5	−0.0	−0.1	0.4	0.5
Thrombin	1.4	2.3	−0.1	1.3	0.8	1.1	2.2
Trypsin	1.3	0.8	0.2	0.0	−0.0	0.6	0.6
Plasmin	−0.3	1.7	0.1	−0.3	−0.2	0.0	−0.2

^aErrors are ± 0.1 kcal/mol or less. Values were obtained from the data in Table 2 using Equations 3a–3g in the text.

Fig. 4. Comparison of the Na^+ binding loop of thrombin (green) with the homologous region of tPA (yellow). In tPA, the presence of a Pro residue in position 225 reorients the carbonyl O atom of residue 224 in a position that is incompatible with Na^+ binding.

specificity is observed toward synthetic or natural substrates and inhibitors in the presence of different cations. Removal of the C136–C201 disulfide bond is not linked to residue 225 and whatever perturbation is caused by replacing P225 is not affected by the presence of the C136–C201 disulfide bond.

Discussion

The successful expression of the protease domain of tPA in *E. coli* and the demonstration that this domain has several functional properties comparable to full-length tPA in the absence of fibrin represent an important step toward the systematic mutational analysis of this enzyme. We have developed an efficient titration method for tPA and its mutants that also yields detailed information on the interaction of tPA with ETI. The assay developed for testing plasminogen activation and the inhibition by PAI-1 are also simple and accurate and minimize the amount of reagents to be used for the determination of the kinetic constants. Of particular interest is the specific inhibition of tPA by Cu^{2+} , reported here for the first time, that occurs in the μM range. Although the physiological role of this inhibition remains to be demonstrated, Cu^{2+} binding is likely to involve H188 in the S1 specificity pocket and provides an invaluable probe for the environment of this site of the enzyme. tPA is unique in showing this inhibition and is the only serine protease with His at position 188. Mutational studies of H188 are underway to directly demonstrate that this is the binding site for the cation.

Analysis of the energetic basis of enzyme specificity using a library of chromogenic substrates carrying substitutions at P1, P2,

Fig. 5. Progress curves for the hydrolysis of VGK by plasmin, following activation by tPA or the mutant P225Y (used at a concentration fivefold that of wt). Dotted curves were drawn from the integrated rate Equations 4a–4c in the text, with the best-fit parameter values reported in Tables 1 and 2. Experimental conditions are: 5 mM Tris, 200 mM NaCl, 0.1% PEG, pH 8.0 at 25 °C.

and P3 has proved invaluable in understanding substrate recognition by thrombin, trypsin, and plasmin (Vindigni et al., 1997). Extension of this analysis to tPA has revealed new and important aspects of the molecular basis of tPA specificity. This enzyme is unique in its strong preference for Arg over Lys at P1, combined with the lack of discrimination between Pro and Gly at P2. Interactions among the substitutions at the P1–P3 sites of the substrate are also absent in tPA, as in trypsin and plasmin. It is likely that the substrate is not highly constrained in the transition state, as in thrombin, consistent with the wide open access of the active site in tPA (Lamba et al., 1996). However, access to the S1 pocket must have stringent requirements and becomes problematic with the shorter side chain of Lys compared to Arg. The bulky side chain of Y99 may exacerbate these requirements and, at the same time, provide steric hindrance at the S2 site that makes a rigid P2–P3 backbone in the substrate no longer beneficial for binding. Mutation of Y99 to Leu, the residue found in thrombin that discriminates quite well between Pro and Gly, will be of obvious interest. It is quite possible that the Y99L may also enhance the catalytic activity of tPA by removing steric hindrance in the S2 cavity.

The disulfide bonds highly conserved among all serine proteases are C42–C58, C168–C182, and C191–C220, the last one being absent only in cathepsin G and the granzymes (Rawlings & Barrett, 1993). These disulfide bonds secure strands that shape the environment of the specificity site S1, the entrance to the active site and the essential catalytic residues H57 and S195 (Fig. 6). Recent work on factor VIIa has shown that the C168–C182 disulfide bond is important for recognition of tissue factor (Higashi et al., 1997), whereas removal of the C191–C220 disulfide bond in trypsin affects significantly the amidolytic activity of the enzyme (Wang et al., 1997). The role of the less conserved C136–C201 disulfide bond has not been tested before. This disulfide bond is

Fig. 6. Ribbon representation of tPA from the crystal structure determined by Lamba et al. (1996). The molecule has the typical fold of the serine proteases of the chymotrypsin family, or peptidase family S1 (Rawlings & Barrett, 1993). Two six-stranded β -barrels are arranged asymmetrically with the residues of the catalytic triad (shown in green), H57, D102, and S195, occupying the interface between the domains. Also shown are D189 at the bottom of the primary specificity pocket and the four disulfide bonds that stabilize the fold. Note the disorder in the 184 loop that is only partially defined in the crystal structure.

absent in all allosteric serine proteases and its presence correlates with Pro at position 225 in many enzymes of the chymotrypsin family (Rawlings & Barrett, 1993). We have found that removal of the C136–C201 disulfide bond is inconsequential on the properties of tPA, whether residue 225 is Pro or Tyr, but it improves the yield of expression in *E. coli* fivefold. This finding is of significant practical relevance because the availability of large quantities of recombinant tPA facilitates structural studies and the production of this enzyme for pharmacological purposes.

The results obtained with the P225Y mutant suggest that engineering Na^+ binding in non-allosteric serine proteases may require more than the simple replacement of P225 with Tyr, which appears to be necessary but not sufficient. Although residue 225 is a dominant factor to determine whether a serine protease may bind Na^+ , other structural determinants influence Na^+ binding significantly. Natural and engineered mutation of thrombin that disrupt ion pairs around the Na^+ site severely decrease (Miyata et al., 1992; Gibbs

et al., 1995; Tsiang et al., 1995; Dang & Di Cera, 1996; Henriksen et al., 1998), or even suppress (Di Cera et al., 1995) monovalent cation sensitivity. Mutation of residues in the allosteric core of thrombin as much as 17 Å away from the Na^+ site can compromise Na^+ binding (Guinto et al., 1995; Guinto & Di Cera, 1997). Another important structural determinant of Na^+ affinity is the 184 loop that shapes the Na^+ binding environment from behind (Di Cera et al., 1995; Krem & Di Cera, 1998). In tPA, this loop is longer (Table 4) and highly disordered (Fig. 6), with the densities of several residues missing in the refined crystal structure (Lamba et al., 1996). This region of tPA seems to play a role in the activation of plasminogen in the absence of fibrin (Paoni et al., 1993). In thrombin, the 184 loop is well defined and contributes to the stabilization of the cluster of water molecules ligating the Na^+ (Krem & Di Cera, 1998). It is likely that engineering Na^+ binding in tPA and other non-allosteric proteases may require modifications of the 184 loop and the region homologous to the Na^+ binding loop in thrombin spanning residues 220–225.

Materials and methods

Expression, refolding, and purification of tPA

The cDNA of human tPA was obtained from ATCC (Rockville, Maryland). The expression vector pET11c and the Nova Blue, BL21(DE3), BL21(DE3)pLysS cells were from Novagen (Madison, Wisconsin). ETI was from Tecnism (Johannesburg, South Africa). Two chain tPA from human melanoma cell culture and plasmin from porcine blood were from Sigma. CNBr-activated Sepharose and the XK 26/20 column were from Pharmacia Biotech.

We expressed a truncated form of tPA, tPA_{des}(Val4–Cys261), that includes the first three N-terminal residues and the catalytic domain of the enzyme. The sequence coding for the catalytic domain was constructed by PCR and the plasmid pETPA-1 was made by insertion of the cDNA into the expression vector pET11c using the *Bam*HI and *Nde*I restriction sites. The protein was expressed in *E. coli* BL21(DE3)pLysS cells. Cells were grown overnight at 37 °C in 50 mL of Luria broth containing 100 mg/mL of ampicillin. Cultures were diluted 1/80 into 3.2 L of Luria broth containing 100 mg/mL of ampicillin and grown at 37 °C until they reached an $\text{OD}_{600} = 0.4$ – 0.7 . Expression was induced with 1.0 mM isopropyl-1-thio- β -D-galactopyranoside and growth was continued for additional 3 h. Cells were harvested by centrifugation at 4,500 rpm for 30 min at 4 °C. The cell pellet was resuspended in 60 mL of 50 mM Tris, 2 mM EDTA, 0.1% Triton X-100, pH 8.0, incubated for 30 min at room temperature and then sonicated on ice. After centrifugation at 12,000 g for 20 min at 4 °C, the pellet was washed by resuspension in 60 mL of 50 mM Tris, 2 mM EDTA, pH 8.0 containing 2.5%, 0.5%, and 0% Triton X-100. The presence of tPA in the inclusion bodies was checked by SDS-PAGE gel electrophoresis.

Refolding of tPA was carried out using a procedure analogous to that described by Rudolph et al. (United States Patent No. 5,543,363). Inclusion bodies were solubilized in 10 mL of 0.1 M Tris, 1 mM EDTA, 6 M GdnHCl, 150 mM DTE, pH 8.6, and incubated at room temperature for 3 h. Insoluble material (e.g., cell wall fragments) was eliminated by centrifugation at 35,000–50,000 g, 4 °C, for 30 min. The supernatant was dialyzed overnight against 3 M GdnHCl, pH 2.5. The thiol groups of the reduced protein were derivatized with oxidized glutathione by incubation in 10 mL of 0.05 M Tris, 6 M GdnHCl,

Table 4. Sequence comparison in the Na^+ binding loop (residues 220–226) and the 184-loop (residues 182–191)^a

	182–191	220–226
Chymotrypsin	CAG-AS-----GVSSC	CS-TSTPG
Trypsin	CAGYLEG-----GKDSC	CAQKNKPG
Thrombin	CAGYKPDEGK---RGDAC	CDRDGKYG
tPA	CAGDTRSGGPQANLHDAC	CGQKDVPG

^aNumbering refers to the chymotrypsin sequence.

0.1 M GSSG, pH 9.3 for 3.5 h at 25 °C. The mixed disulfide was then dialyzed against 3 M GdnHCl, pH 8.0. Refolding was achieved by stepwise addition of the mixed disulfide solution to 400 mL of refolding buffer containing 0.7 M L-Arg-HCl, 2 mM GSH, 1 mM EDTA, pH 8.6, and leaving the solution under gentle stirring for 24 h at room temperature.

Correctly folded tPA was purified by affinity chromatography on an ETI-Sepharose column (Heussen et al., 1984). ETI-agarose was prepared by coupling 200 mg of the inhibitor with 40 mL of swollen CNBr-activated Sepharose. The resulting gel was resuspended in the equilibration buffer A (0.01 M Na₂HPO₄, 0.4 M NaCl, pH 7.4) and packed onto a XK 26/20 column. Purification of tPA was then carried out by FPLC as described (Heussen et al., 1984). The solution containing the protein was loaded onto the column equilibrated with buffer A at a flow rate of 1.5 mL/min. The column was washed with approximately 200 mL of equilibration buffer and then correctly folded tPA was eluted with 1.6 M KSCN dissolved in buffer B (0.01 M Na₂HPO₄, 0.4 M NaCl, 1.6 M KSCN, pH 7.4). The sample was concentrated for desalting using Centrprep-10 concentrators and reconstituted to the original volume with 0.1 M Tris, pH 8.1. The solution was concentrated again to a final volume of 3 mL. Finally, the concentrated sample was passed through an Econo-Pac PD10 desalting column equilibrated with 0.1 M Tris, pH 8.1. The concentration of the correctly folded catalytic domain of tPA was determined spectrophotometrically using an extinction coefficient $\epsilon_{280} = 1.386 \text{ mg}^{-1} \text{ cm}^2$. The final yield was 1–5 mg/L of cell culture. The identity and purity of the protein were confirmed by SDS-gel electrophoresis and electrospray mass spectrometry.

Single chain tPA was converted to the two chain form tPA using a plasmin-Sepharose resin prepared by coupling 10 mg of porcine plasmin with 2 mL of swollen CNBr-activated Sepharose. The activated resin was stored at 4 °C in an equal volume of 0.1 M Tris, 1 M NaCl, pH 8.0 at 25 °C. Activation was carried out by adding 100 mL of the gel slurry to 1 mL of the solution containing single chain tPA and incubating for 2 h at room temperature. The resin was then removed by spinning down at 10,000 g for 5 min and filtering the supernatant with a 0.22 mm filter unit. The absence of single chain tPA was confirmed by SDS-gel electrophoresis and mass spectrometry.

Active site titration

The precise determination of the active site concentration is of crucial importance in mutagenesis studies of enzymes. A widely used procedure to titrate the active site of tPA is based on the binding of a fluorogenic suicide inhibitor (Ding et al., 1995). The active site concentration derived from this method is correct only if the acyl intermediate accumulates, i.e., when deacylation is slow compared to acylation. Although this has been proved to be the case for wild-type tPA (Urano et al., 1988), there is no guarantee that the condition is met by mutant molecules. We have therefore developed an alternative titration method analogous to that used for thrombin (Dang & Di Cera, 1994). In this method, the tPA inhibitor ETI is preincubated with the enzyme and the assay is started with the addition of a chromogenic substrate (FPR). The initial rate of substrate hydrolysis is measured as an increase in absorbance at 405 nm due to the release of the *p*-NA for 1–5 min. Because ETI is a slow binding inhibitor of tPA (see below), the initial velocity of substrate hydrolysis, v , obeys the following equation (Dang & Di Cera, 1994):

$$v = v_0 \frac{(e_T - i_T - K_i) + \sqrt{(e_T - i_T - K_i)^2 + 4K_i e_T}}{2} \quad (1)$$

where v_0 is the velocity in the absence of inhibitor, i_T is the known total concentration of functional ETI determined by titration of a reference sample of tPA titrated with MUGP as described (Urano et al., 1988). K_i is the dissociation constant for the interaction of tPA with ETI and e_T is the total concentration of the active site to be determined. Measurements taken as a function of i_T and e_T enable an accurate resolution of K_i and e_T (Fig. 1). When the affinity of the inhibitor decreases, as in the case of the P225Y mutant, higher concentrations of enzyme are required for titration. In the titration of the wild-type, the enzyme concentrations ranged from 2 to 16 nM, whereas the ETI concentration ranged from 0 to 240 nM. Titration of the P225Y mutant required enzyme concentrations from 50 to 150 nM and inhibitor concentrations up to 10 μ M. Measurements were performed in the presence of 200 mM NaCl, 5 mM Tris, 0.1% PEG, pH 8.0 at 25 °C. The substrate concentration was 20 μ M in all cases. The fraction of active enzyme found with this titration method ranged from 70% to 95%. Wild-type and mutant enzymes were also titrated with MUGB as described (Urano et al., 1988). The values of active site concentration obtained with this method were practically identical to those determined from titration with ETI.

ETI was found to obey as a slow binding inhibitor, as demonstrated by the typical biphasic behavior of progress curves of substrate hydrolysis started by addition of enzyme (Fig. 2). This behavior was also observed for the P225Y mutant that has a reduced binding affinity, and enabled the complete characterization of the kinetic rate constants for binding and dissociation of the inhibitor (Table 1). The analytical expression for the analysis of the data in Figure 2 is (Ayala & Di Cera, 1994)

$$[P] = v_\infty t + \frac{(v_0 - v_\infty)(1 - \gamma)}{\lambda \gamma} \ln \frac{1 - \gamma \exp(-\lambda t)}{1 - \gamma} \quad (2)$$

where $[P]$ is the concentration of *p*-NA released upon cleavage of FPR, v_0 the initial velocity, v_∞ the steady state velocity attained after complete equilibration of tPA with substrate and inhibitor, and γ and λ are two parameters to be determined from experimental data. The expressions for the various terms in Equation 2 are given elsewhere (Equations 2–4 in Ayala & Di Cera, 1994) and contain the k_{on} and K_d of the inhibitor that can be resolved directly from the data, the concentration and K_m of the substrate, as well as the total concentration of enzyme and inhibitor determined by titration (see above).

Substrate hydrolysis

Progress curves of the release of *p*-NA following the hydrolysis of chromogenic substrates by mutant and wild-type tPA molecules were measured under experimental conditions of 5 mM Tris, 200 mM NaCl, 0.1% PEG, pH 8.0 at 25 °C. The effects of different monovalent cations were studied at a concentration of 200 mM CHCl, KCl, or LiCl. Progress curves were analyzed using KINSIM and FITSIM (Dang & Frieden, 1997). The site-specific analysis of tPA specificity was carried out as described (Vindigni et al., 1997; Di Cera, 1998) using the library of chromogenic substrates in Table 2. For each substrate, the value of the specificity constant

$s = k_{cat}/K_m$ was measured from progress curves and the site-specific free energies were calculated according to the expressions

$$\Delta G_1 = -RT \ln(s_{FPK}/s_{FPR}) \quad (3a)$$

$$\Delta G_2 = -RT \ln(s_{FGR}/s_{FPR}) \quad (3b)$$

$$\Delta G_3 = -RT \ln(s_{VPR}/s_{FPR}) \quad (3c)$$

$$\Delta G_{12} = -RT \ln(s_{FGK} s_{FPR}/s_{FPK} s_{FGR}) \quad (3d)$$

$$\Delta G_{13} = -RT \ln(s_{VPK} s_{FPR}/s_{FPK} s_{VPR}) \quad (3e)$$

$$\Delta G_{23} = -RT \ln(s_{VGR} s_{FPR}/s_{FGR} s_{VPR}) \quad (3f)$$

$$\Delta G_{123} = -RT \ln(s_{VGK} s_{FPR}^2/s_{FPK} s_{FGR} s_{VPR}) \quad (3g)$$

where the indices refer to the various substrates. ΔG_1 , ΔG_2 , and ΔG_3 are the changes in specificity due to the single-site substitutions at P1, P2, and P3 of the substrate. ΔG_{12} , ΔG_{13} , and ΔG_{23} are the second-order coupling free energies for substitutions made at the three possible pairs of sites, and ΔG_{123} is the third-order coupling free energy for the triple substitution. These terms reflect interactions between substitutions made at different sites that may reduce ($\Delta G > 0$) or enhance ($\Delta G < 0$) specificity beyond simple additivity. Attempts made to accurately measure K_m and k_{cat} separately were unsuccessful because K_m was found to be in the mM range for most substrates. The only case where these parameters could be determined was for the wild-type in the presence of 200 mM NaCl. The values found were $K_m = 200 \pm 20 \mu\text{M}$ and $k_{cat} = 8.8 \pm 0.1 \text{ s}^{-1}$ in agreement with the value of $k_{cat}/K_m = 48 \pm 1 \text{ mM}^{-1} \text{ s}^{-1}$ determined directly from analysis of progress curves. Product inhibition was carefully checked by analysis of progress curves run with different substrate concentrations and found to be negligible in all cases.

Inhibition by PAI-1

PAI-1 was obtained from American Diagnostica (Greenwich, Connecticut). The apparent second-order rate constant (k_{on}) for the inhibition of tPA by PAI-1 was measured by progress curves from the effect of the inhibitor on the hydrolysis of FPR under experimental conditions of 5 mM Tris, 200 M NaCl or ChCl, 0.1% PEG, pH 8.0 at 25 °C. Assays were started by addition of enzyme to the cuvette containing FPR and PAI-1. Data were collected at different concentrations of PAI-1, ranging from onefold to threefold the enzyme concentration, and keeping the concentration of the enzyme (16–200 nM depending on the mutant) and substrate (20 μM) constant. Analysis was carried out using KINSIM and FITSIM assuming an irreversible reaction between the enzyme and PAI-1.

Plasminogen activation

Lys-plasminogen was obtained from American Diagnostica. The rate of Lys-plasminogen activation by tPA was measured by continuous progress curves, using a strategy developed for the analysis of protein C activation by thrombin (Dang et al., 1995). In this

assay, the general reaction scheme for the catalytic conversion of plasminogen (Plg) to plasmin (Pl) is

where S is a chromogenic substrate specific for plasmin (VGK). The product P is the *p*-NA released upon cleavage of S by plasmin activated by tPA and provides the signal followed at 405 nm as a function of time. The release of P depends on three reactions: (1) Cleavage of S by plasmin (reaction 4b), (2) activation of plasminogen to plasmin by tPA (reaction 4a), and (3) cleavage of S by tPA (reaction 4c). The reactions 4b and 4c were studied directly in separate experiments carried out under identical solution conditions. The kinetic parameters pertaining to reaction 4a, which is the one of interest, were decoupled from analysis of the progress curve started by adding tPA to a solution containing plasminogen and S, using the information derived independently from reactions 4b and 4c. Consistent with the results obtained in the analogous assay for protein C activation by thrombin (Dang et al., 1995, 1997a), this assay yielded accurate determinations of the hydrolysis of plasminogen by tPA, because VGK is far more specific for plasmin than tPA and simplifies the analysis of progress curves in terms of Equations 4a–4c. This assay is very informative because of the constraints imposed by the parallel reactions 4a–4c on the fitting function and the ability of FITSIM to handle the kinetic mechanism of hydrolysis through direct integration of the rate equations, without any approximation (Dang & Frieden, 1997). Measurements were carried out under experimental conditions of 5 mM Tris, 200 M NaCl or ChCl, 0.1% PEG, pH 8.0 at 25 °C. The tPA concentration ranged from 4 to 40 nM and plasminogen was used at concentrations from 100 to 800 nM, well below the estimated K_m in the μM range. Under these conditions, the progress curve of VGK hydrolysis was very sensitive to the specificity constant k_{cat}/K_m for plasminogen cleavage by tPA. The concentration of VGK was 150 μM . Product inhibition was checked as discussed in the section of substrate hydrolysis and found to be negligible.

Inhibition by Cu^{2+}

tPA was found to be inhibited specifically and competitively by Cu^{2+} , presumably due to the presence of H188 next to D189 in the S1 pocket (see Results). The dissociation constant $K_d = K_i$ for Cu^{2+} binding to tPA was determined by measuring the initial rate of substrate hydrolysis, v , as a function of $[\text{Cu}^{2+}]$ that was found to obey the expression for competitive inhibition

$$v = v_0 \frac{1 + \frac{[S]}{K_m}}{1 + \frac{[I]}{K_i} + \frac{[S]}{K_m}} \quad (5)$$

where v_0 is the velocity in the absence of inhibitor, K_m is the Michaelis constant for substrate binding, and $[I]$ the concentration of free inhibitor. The concentration of Cu^{2+} ranged from 0 to

400 μ M. The enzyme concentration ranged from 4 to 50 nM and the substrate concentration ranged from 20 to 200 μ M.

Synthesis of chromogenic substrates

All chromogenic substrates were synthesized by liquid phase using Fmoc-derivatized amino acids. Amino acids were from Bachem (Torrance, California) with the exception of Fmoc-Gly that was obtained from Peptide International. *p*-NA was from Aldrich (Milwaukee, Wisconsin). Arg-*p*-nitroanilide was obtained from Bachem, while Lys-*p*-nitroanilide was prepared as described below. The mixture of 0.45 HBTU/HOBT was used for activation of the carboxylic group of each amino acid to be coupled. For the coupling of the first amino acid (e.g., Pro) to the Arg-*p*-nitroanilide, 1.1 mmol of Arg-*p*-nitroanilide was dissolved in 2 mL of DMF and 420 mL of DIEA was added to the solution. At the same time, 1.0 mmol of Fmoc-Pro was dissolved in 2.3 mL of 0.45 HBTU/HOBT and the solution was left standing for 10 min. The solution of Fmoc-Pro was then added dropwise to that containing Arg-*p*-nitroanilide. The coupling reaction was carried out overnight and at the end the product was extracted in benzene. The organic layer was then washed with solutions of saturated NaCl and NaHCO₃. Water retained in the organic phase was eventually removed by leaving the solution in a flask with Na₂SO₄. After removing the sodium sulfate by filtration, the solution was dried in vacuo and the peptide was resuspended in 6–9 mL of CH₃CN. The solution was sonicated to favor complete dissolution of the peptide and 3 mL of piperidine was added to the flask. The reaction was left under gentle stirring for 15–30 min and subsequently CH₃CN was removed in vacuo. The dried peptide was dissolved in 5 mL of TFA and the new solution was added dropwise to a 50 mL Falcon tube containing 45 mL of ethyl ether. The peptide precipitated in ether and was separated by centrifugation. The product was washed twice with ether. Finally, the peptide was lyophilized and used for the next coupling step.

All coupling steps were monitored with a MALDI-TOF mass spectrometer. Purity of the peptide was checked by RP-HPLC using a C-18 Vydac column (4.6 $\times$ 150 mm) and a linear gradient of CH₃CN from 0 to 60% of CH₃CN, 0.1% TFA in 30 min. The flow rate was 1 mL/min. The absorbance was read at the peak of absorption of *p*-NA at 256 nm. A second run was performed by reading at 215 nm. The peptide was finally purified using a C4 column, or a C18 column when a higher degree of resolution was required.

The coupling of Fmoc-Gly to Arg-*p*-nitroanilide was performed with a different procedure because Gly does not have problems of racemization. In this procedure, DCC was used for the activation step in place of HBTU/HOBT. Initially, 1 mmol Fmoc-Gly was dissolved into 1 mmol of HOSu in 20 mL of dioxane and 1 mmol of DCC was dissolved in 5 mL of the same solvent. The two solutions were mixed under the hood at 0°C and left for 1 h under stirring. The solution was then left overnight under stirring at 4°C, filtered and washed with 2–3 mL of dioxane. After evaporation, the product was washed with ether, left standing for 2–3 h and then filtered. Addition of ether induced the formation of crystals of Fmoc-Gly-Osu. The product was dried in a desiccator. For the coupling reaction, 1 mmol of Arg-*p*-nitroanilide was dissolved in 2 mL of DMF and then 350 mL of DIEA was added to the solution. At the same time, 1 mmol of Fmoc-Gly-Osu was dissolved in 1 mL of DMF and the resulting mixture was added dropwise to the Arg-*p*-nitroanilide solution. The kinetics of the reaction was followed by mass spectrometry. The protecting group Fmoc was re-

moved with the procedure described above and the product was purified by RP-HPLC.

For the synthesis of Lys-*p*-nitroanilide, 10 mmol of t-Boc-Lys and 10 mmol of *p*-NA were dissolved in the presence of 30 mL of dry pyridine and the yellowish mixture was cooled at (–15°C under argon. When the two reagents were completely dissolved, 1.036 mL of POCl₃ were added dropwise under vigorous stirring and the reaction was completed in about 30 min. The reaction was quenched with 100 mL of crushed ice and water. The nitroanilide was extracted into EtOAc and the EtOAc layer was washed with saturated solutions of NaHCO₃ and NaCl. The EtOAc layer was not dried to prevent premature crystallization and was directly evaporated in vacuo. The oily product was co-evaporated with 50 mL of toluene and 100 mL of 1:1 EtOAc:MeOH to remove residual pyridine. The dry product was then suspended in diethyl ether and filtered to remove eventual unreacted *p*-NA. The purity of t-Boc-Lys-*p*-nitroanilide were checked by measuring the melting temperature and by RP-HPLC. The final yield was typically 82%. The t-Boc protecting group was removed by dissolving 2 mmol of t-Boc-Lys-*p*-nitroanilide in 10 mL of solution containing 4 M HCl in 1,4-dioxane. The reaction was left under stirring for 1 h and the product was washed several times with ether. The identity and purity of Lys-*p*-nitroanilide were checked by mass spectrometry and RP-HPLC.

Acknowledgments

This work was supported by NIH Research Grants HL49413 and HL58141 and by Grants from the American Heart Association and Monsanto-Searle. E.D.C. is an Established Investigator of the American Heart Association and Genentech. A.V. is a Research Fellow of the American Heart Association, Missouri Affiliate.

References

- Ayala YM, Di Cera E. 1994. Molecular recognition by thrombin: Role of the slow $\rightarrow$ fast transition, site-specific ion binding energetics and thermodynamic mapping of structural components. *J Mol Biol* 235:733–746.
- Briand L, Chobert JM, Tauzin J, Declercq N, Leonil J, Molle D, Tran V, Haertle T. 1997. Regulation of trypsin activity by Cu²⁺ chelation of the substrate binding site. *Protein Eng* 10:551–560.
- Butenas S, Kalafatis M, Mann KG. 1997. Analysis of tissue plasminogen activator using peptidyl fluorogenic substrates. *Biochemistry* 36:2123–2131.
- Collen D, Lijnen HR. 1995. Molecular basis of fibrinolysis, as relevant for thrombolytic therapy. *Thromb Hemost* 74:167–171.
- Collins R, Peto R, Baigent C, Sleight P. 1997. Aspirin, heparin, and fibrinolytic therapy in suspected acute myocardial infarction. *Drug Therapy* 336:847–860.
- Coombs GS, Dang AT, Madison EL, Corey DR. 1996. Distinct mechanisms contribute to stringent substrate specificity of tissue-type plasminogen activator. *J Biol Chem* 271:4461–4467.
- Dang QD, Di Cera E. 1994. A simple activity assay for thrombin and hirudin. *J Protein Chem* 13:367–373.
- Dang QD, Di Cera E. 1996. Residue 225 determines the Na⁺-induced enhancement in catalytic activity of serine proteases. *Proc Natl Acad Sci USA* 93:10653–10656.
- Dang QD, Frieden C. 1997. New PC versions of the kinetic simulation and fitting programs, KINSIM and FITSIM. *Trends Biochem Sci* 22:317.
- Dang QD, Guinto ER, Di Cera E. 1997a. Rational engineering of catalytic activity and specificity in a serine protease. *Nature Biotechnol* 15:146–149.
- Dang QD, Sabetta M, Di Cera E. 1997b. Selective loss of fibrinogen clotting in a loop-less thrombin. *J Biol Chem* 272:19649–19651.
- Dang QD, Vindigni A, Di Cera E. 1995. An allosteric switch controls the pro-coagulant and anti-coagulant activities of thrombin. *Proc Natl Acad Sci USA* 92:5977–5981.
- Di Cera E. 1998. Site-specific analysis of mutational effects in proteins. *Adv Protein Chem* 51:59–119.
- Di Cera E, Dang QD, Ayala YM. 1997. Molecular mechanisms of thrombin function. *Cell Mol Life Sci* 15:701–730.

- Di Cera E, Guinto ER, Vindigni A, Dang QD, Ayala YM, Wuyi M, Tulinsky A. 1995. The Na⁺ binding site of thrombin. *J Biol Chem* 270:22089–22092.
- Ding L, Coombs GS, Strandberg L, Navre M, Corey DR, Madison EL. 1995. Origins of the specificity of tissue-type plasminogen activator. *Proc Natl Acad Sci USA* 92:7627–7631.
- Gibbs CS, Coutre SE, Tsiang M, Li W-X, Jain AK, Dunn KE, Law VS, Mao CT, Matsumura SY, Mejza SJ, Paborsky LR, Leung LLK. 1995. Conversion of thrombin into an anticoagulant by protein engineering. *Nature* 378:413–416.
- Guinto ER, Di Cera E. 1997. Critical role of W60d in thrombin allostery. *Biophys Chem* 64:103–109.
- Guinto ER, Vindigni A, Ayala YM, Dang QD, Di Cera E. 1995. Identification of residues linked to the slow → fast transition of thrombin. *Proc Natl Acad Sci USA* 92:11185–11189.
- Henriksen RA, Dunham CK, Miller LD, Casey JT, Menke JB, Knupp CL, Usala SJ. 1998. Prothrombin Greenville, Arg⁵¹⁷ → Gln, identified in an individual heterozygous for dysprothrombinemia. *Blood* 91:2026–2031.
- Heussen C, Joubert F, Dowdla EB. 1984. Purification of human tissue plasminogen activator with Erythrina trypsin inhibitor. *J Biol Chem* 259:11635–11638.
- Higashi S, Matsumoto N, Iwanaga S. 1997. Conformation of factor VIIa stabilized by a labile disulfide bond (Cys310–Cys329) in the protease domain is essential for the interaction with tissue factor. *J Biol Chem* 272:25724–25730.
- Holmes WE, Nelles L, Lijnen HR, Collen D. 1987. Primary structure of human α₂-antiplasmin, a serine proteases inhibitor (serpin). *J Biol Chem* 262:1659–1664.
- Hoyleaerts M, Rijken DC, Lijnen HR, Collen D. 1982. Kinetics of the activation of plasminogen by human tissue plasminogen activator. Role of fibrin. *J Biol Chem* 257:2912–2919.
- Kohnert U, Hellerbrand K, Martin U, Stern A, Popp F, Fischer S. 1996. The recombinant *E. coli*-derived protease-domain of tissue-type plasminogen activator is a potent and fibrin specific fibrinolytic agent. *Fibrinolysis* 10:93–102.
- Krem MM, Di Cera E. 1998. Conserved water molecules in the specificity pocket of serine proteases and the mechanism of Na⁺ binding. *Proteins Struct Funct Genet* 30:34–42.
- Lamba D, Bauer M, Huber R, Fischer S, Rudolph R, Kohnert U, Bode W. 1996. The 2.3 Å crystal structure of the catalytic domain of recombinant two-chain human tissue-type plasminogen activator. *J Mol Biol* 258:117–135.
- Ludlam CA, Bennett B, Fox KAA, Lowe GDO, Reid AW. 1995. Guidelines for the use of thrombolytic therapy. *Blood Coagul Fibrinol* 6:273–285.
- Madison EL. 1994. Probing structure-function relationships of tissue-type plasminogen activator by site-specific mutagenesis. *Fibrinolysis* 8:221–236.
- Madison EL, Coombs GS, Corey DR. 1995. Substrate specificity of tissue plasminogen activator. *J Biol Chem* 270:7558–7562.
- Madison EL, Goldsmith EJ, Gerard RD, Gething MJH, Sambrook JF, Bassel-Duby RS. 1990. Amino acid residues that affect interaction of tissue-type plasminogen activator with plasminogen activator inhibitor I. *Proc Nat Acad Sci USA* 87:3530–3533.
- Miyata T, Aruga R, Umeyama H, Bezeaud A, Guillin M-C, Iwanaga S. 1992. Prothrombin Salakta: Substitution of glutamic acid-466 by alanine reduces the fibrinogen clotting activity and the esterase activity. *Biochemistry* 31:7457–7462.
- Onesti S, Matthews DJ, Aducci P, Amiconi G, Bolognesi M, Menegatti E, Ascenzi P. 1992. Binding of the Kunitz-type trypsin inhibitor DE-3 from *Erythrina caraffa* seeds to serine proteinases: A comparative study. *J Mol Recogn* 5:105–114.
- Paoni NF, Chow AM, Pena LC, Keyt BA, Zoller MJ, Bennett WF. 1993. Making tissue-type plasminogen activator more fibrin specific. *Protein Eng* 6:529–534.
- Pennica D, Holmes WE, Kohr WJ, Harkins RN, Vehar GA, Ward CA, Bennett WF, Yelverton E, Seeburg PH, Heyneker HL, Goeddel DV, Collen D. 1983. Cloning and expression of human tissue-type plasminogen activator cDNA in *E. coli*. *Nature* 301:214–221.
- Rawlings RD, Barrett AJ. 1993. Evolutionary families of peptidases. *Biochem J* 290:205–218.
- Rijken DC, Collen D. 1981. Purification and characterization of human vascular plasminogen activator secreted by human melanoma cell culture. *J Biol Chem* 256:7035–7041.
- Robbins KC, Summaria L, Hsieh B, Shah RJ. 1967. The peptide chain of human plasmin. Mechanism of activation of human plasminogen to plasmin. *J Biol Chem* 242:2333–2342.
- Schechter I, Berger A. 1967. On the size of the active site in proteases. I. Papain. *Biochem Biophys Res Commun* 27:157–162.
- Thorsen S, Glas-Greenwalt P, Astrup T. 1972. Differences in the binding to fibrin of urokinase and tissue plasminogen activator. *Thromb Diath Haemorrh* 28:65–74.
- Tsiang M, Jain AK, Dunn KE, Rojas ME, Leung LLK, Gibbs CS. 1995. Functional mapping of the surface residues of human thrombin. *J Biol Chem* 270:16854–16863.
- Urano T, Urano S, Castellano FS. 1988. Reaction of tissue-type plasminogen activator with 4-methylumbelliferyl-p-guanidinobenzoate hydrochloride. *Biochem Biophys Res Commun* 150:45–51.
- Vindigni A, Dang QD, Di Cera E. 1997. Site-specific dissection of substrate recognition by thrombin. *Nature Biotechnol* 15:891–895.
- Wang ECW, Hung SH, Cahoon M, Hedstrom L. 1997. The role of the Cys191–Cys220 disulfide bond in trypsin: New targets for engineering substrate specificity. *Protein Eng* 10:405–411.
- Zhou HX, Wong KY, Vijayakumar M. 1997. Design of fast enzymes by optimizing interaction potential in active site. *Proc Natl Acad Sci USA* 94:12372–12377.