14. A. O. Tzianabos, unpublished data.
15. H. J. Jennings, C. Lugowski, N. M. Young, *Biochemistry* **19**, 4712 (1980).
16. B. Lindberg, B. Lindqvist, J. Lonngren, D. A. Powell, *Carbohydr. Res.* **78**, 111 (1980).
17. R. E. Reeves and W. F. Goebel, *J. Biol. Chem.* **139**, 511 (1941).
18. B. Lindberg, J. Lonngrenn, D. A. Powell, *Carbohydr. Res.* **58**, 177 (1977).
19. A. K. Bhattacharjee, H. J. Jennings, C. P. Kenny, A. Martin, I. C. P. Smith, *J. Biol. Chem.* **250**, 1926 (1975).
20. H. J. Jennings, K.-G. Rosell, D. L. Kasper, *Biochemistry* **22**, 1258 (1983).
21. M. R. Wessels, V. Pozsgay, D. L. Kasper, H. J. Jennings, *J. Biol. Chem.* **262**, 8262 (1987).
22. K. Heyns and G. Kiessling, *Carbohydr. Res.* **3**, 340 (1967).
23. H. J. Jennings, R. Roy, F. Michon, *J. Immunol.* **134**, 2651 (1985).
24. A. Varki, *Glycobiology* **3**, 97 (1993).
25. L. J. Reed and H. Muench, *Am. J. Hyg.* **27**, 493 (1938).
26. A mathematical model was devised (i) to compare the biologic activities of modified and unmodified polysaccharides over a range of three doses (200, 20, and 2 μg) and (ii) to calculate AD_{50} values for each polysaccharide type. The general mathematical model, which is based on logistic regression analysis, was $\log[p/(1 - p)] = \alpha + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4$ where p is the probability of abscess formation, α is the intercept, and $x_1 = \log(\text{dose}/20)$; $x_2 = 1$ if in the first modified polysaccharide group tested, otherwise $x_2 = 0$; $x_3 = 1$ if in the second modified polysaccharide group tested, otherwise $x_3 = 0$; $x_4 = 1$ if in the third modified polysaccharide group tested, otherwise $x_4 = 0$; and β_1, β_2, β_3, and β_4 are the regression coefficients corresponding to x_1, x_2, x_3, and x_4. The AD_{50} estimates were obtained from $-\alpha/\beta_1$ if in the unmodified group, $-(\alpha + \beta_2)/\beta_1$ if in the first modified polysaccharide group tested, $-(\alpha + \beta_3)/\beta_1$ if in the second modified polysaccharide group tested, and $-(\alpha + \beta_4)/\beta_1$ if in the third modified polysaccharide group tested.
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Converting Tissue Plasminogen Activator to a Zymogen: A Regulatory Triad of Asp-His-Ser

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Unlike most serine proteases of the chymotrypsin family, tissue-type plasminogen activator (tPA) is secreted from cells as an active, single-chain enzyme with a catalytic efficiency only slightly lower than that of the proteolytically cleaved form. A zymogenic mutant of tPA has been engineered that displays a reduction in catalytic efficiency by a factor of 141 in the single-chain form while retaining full activity in the cleaved form. The residues introduced in the mutant, serine 292 and histidine 305, are proposed to form a hydrogen-bonded network with aspartate 477, similar to the aspartate 194–histidine 40–serine 32 network found to stabilize the zymogen chymotrypsinogen.

Proteases are normally synthesized and secreted as zymogens that must be proteolytically cleaved to display their full enzymatic activity. The increase in catalytic efficiency (measured by k_{cat}/K_m, where k_{cat} is the rate of catalysis and K_m is the Michaelis constant) after cleavage can be dramatic. For example, the value of k_{cat}/K_m for the prototypical serine proteases chymotrypsinogen and trypsinogen increases by a factor of 10^4 to 10^6 immediately after cleavage of a single peptide bond (*1*).

A few serine proteases are unusual in that they are secreted from cells not as inactive precursors but as single-chain polypeptides that display high levels of enzymatic activity. For example, tPA is secreted as a 527–amino acid polypeptide that efficiently converts the plasma-borne zymogen plasminogen into the active protease plasmin, which then degrades the fibrin meshwork of thrombi (*2*). Plasmin also cleaves single-chain tPA into a two-chain form whose catalytic efficiency is certainly increased relative to the single-chain form, but only by a very modest amount.

Tissue-type plasminogen activator has been widely used as a therapeutic agent for the treatment of acute myocardial infarction. However, the systemic activity of clinical doses of tPA causes a significant depletion of circulating fibrinogen in patients who receive the drug (*3*), and a small minority of patients suffer severe hemorrhagic complications. These problems might be reduced if zymogen-like variants of tPA (zymogenic tPA) were available. In such cases, the catalytic activity of the circulating single-chain form of zymogenic tPA would be greatly diminished; however, once attached to the fibrin meshwork of a thrombus, zymogenic tPA could be cleaved by plasmin generated locally at the clot by the patient's own tPA and would then display full catalytic activity. We therefore investigated whether tPA could be converted into a zymogen by replacing key amino acids with residues that stabilize the zymogen forms of other serine proteases.

The serine protease domains of tPA and chymotrypsin have a 40% sequence identity; thus, comparing the sequence of tPA with the structure of chymotrypsinogen, we identified residues of tPA that are likely to contribute to the constitutive activity of single-chain tPA. The active conformation of members of the chymotrypsin family is secured by an ion pair between the ammonium ion of the mature NH_2-terminus and the carboxylate of Asp^{194} (*4*), an invariant residue that is flanked by amino acids that form the oxyanion hole (*5*) (Fig. 1). Although the zymogens chymotrypsinogen (*4*, *6*) and trypsinogen (*4*, *7*) differ in structure, both exhibit an altered conformation in which the side-chain of Asp^{194} is stabilized by an ion pair with a buried histidine, His^{40}. His^{40} also forms a hydrogen bond with Ser^{32} (*4*), which creates a triad (Asp^{194}-His^{40}-Ser^{32}) whose members are linked by ionic bonds (Fig. 1). The geometry of this "zymogen triad" found in chymotrypsinogen is very similar to that of the familiar catalytic triad, which consists of the same three residues.

In contrast to chymotrypsinogen and trypsinogen, tPA lacks the zymogen triad. Phe^{305} and Ala^{292} in tPA occupy positions that are homologous to His^{40} and Ser^{32} of chymotrypsin. To assess whether zymogen status and activation could be conferred on tPA, we used oligonucleotide-directed, site-specific mutagenesis to construct complementary DNAs (cDNAs) encoding the enzymes tPA(F305H) and tPA(A292S,F305H) (*8*). The mutated cDNAs were ligated into the SV40-based, transient expression vector pSVT7(RI−), and the resulting constructs were used to transfect COS-1 cells by the DEAE-dextran method (*9*). Enzymes were harvested from serum-free media, and their concentration was measured by solid-phase radioimmunoassay.

The activity of both the single- and two-chain forms of tPA, tPA(F305H), and tPA(A292S,F305H) toward the synthetic substrate methylsulfonyl-D-cyclohexyltyrosylglycyl-arginine-*p*-nitroaniline acetate was measured as previously described (*10*). Data from these assays allow (Table 1) several conclusions. (i) The catalytic efficiency of the two-chain form of both tPA(F305H) and

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tPA(A292S,F305H) is similar to that of two-chain, wild-type tPA. (ii) As previously reported by our laboratory (*10*) and others (*11*), the catalytic efficiency of single-chain tPA is approximately 15% that of two-chain tPA. (iii) Single-chain tPA(A292S,F305H) possesses 0.7% the catalytic efficiency and 1.2% the k_{cat} of two-chain tPA(A292S,F305H), whereas single-chain tPA(F305H) has approximately 3.1% the catalytic efficiency and 5.3% the k_{cat} of the corresponding two-chain enzyme. (iv) The decreased activity of the single-chain form of the mutated enzymes is almost entirely due to a decreased k_{cat}. Upon activation cleavage, the k_{cat} of tPA increases by approximately 2.5-fold, whereas those of tPA(F305H) and tPA(A292S,F305H) increase by 19-fold and 81-fold, respectively.

Thus, simultaneous replacement of two residues—F305H and A292S—decreases the catalytic efficiency of single-chain tPA on synthetic substrates without altering the properties of the two-chain form of the enzyme. The net effect is a significant improvement (approximately 20-fold) in the zymogenicity of tPA (defined as the ratio of the catalytic efficiencies of the two-chain mature enzyme and of the single-chain proenzyme). Replacement of both residues is required to achieve maximal reduction in the catalytic activity of the single-chain enzyme: substitution of Phe^{305} by His alone improves the zymogenicity of the enzyme by only fivefold. We conclude that the introduction of His^{40} contributes directly to the zymogenicity of tPA and that Ser^{32}, well removed from the catalytic center, further stabilizes the zymogen conformation of the single-chain enzyme.

To examine whether the two-chain forms of tPA(F305H) and tPA(A292S,F305H) are also fully active on the natural substrate plasminogen, we performed standard indirect chromogenic assays of tPA activity (Table 2). When assayed in the presence of saturating concentrations of both the cofactor, DE-SAFIB, and the substrate, Lys-plasminogen, the specific activities of the two-chain forms of tPA(F305H) and tPA(A292S,F305H) were 97 and 87%, respectively, that of two-chain tPA (*12*). A kinetic characterization of the two-chain forms of these three enzymes in the indirect assay is summarized in Table 2. The close correspondence between values of both K_m and k_{cat} for all three enzymes demonstrates that, as with small, synthetic substrates, the two-chain forms of tPA(F305H) and tPA(A292S,F305H) are fully active on the protein substrate Lys-plasminogen.

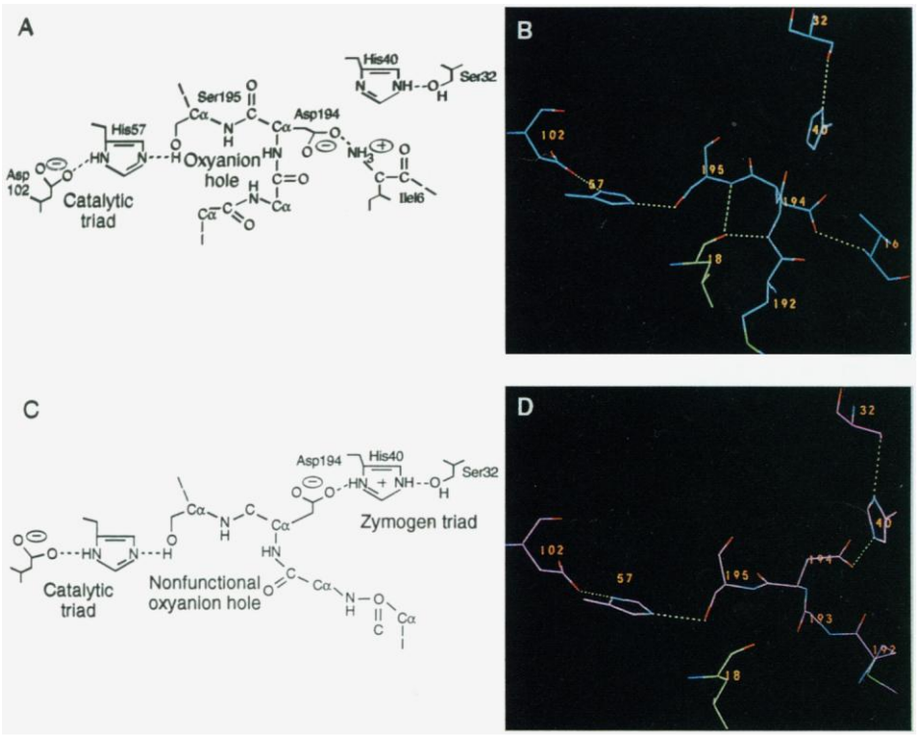

Fig. 1. Interactions in the active site of chymotrypsin and chymotrypsinogen. (**A**) Schematic presentation and (**B**) atomic coordinates from Protein Data Bank file 1cho (*17*) of the active site of chymotrypsin. (**C**) Schematic presentation and (**D**) atomic coordinates from Protein Data Bank file 2cga (*6*) of the active site of chymotrypsinogen. Shown are the catalytic triad Asp^{102}-His^{57}-Ser^{195}, residues 193 to 195 of the oxyanion hole, the NH_2-terminus Ile^{16} (which forms an ion pair with Asp^{194} in the mature enzyme only), His^{40} and Ser^{32}, and Leu^{18} of the ovomucoid inhibitor, which binds to the oxyanion hole in the mature enzyme. The triad of interactions (Asp^{194}-His^{40}-Ser^{32}) is present only in the zymogen and may be present in the single-chain form of tPA(A292S,F305H). Leu^{18} of the inhibitor is displayed in (D) to show that the backbone amides of the oxyanion hole (residues 193 and 195) are too far away to form hydrogen bonds.

Table 1. Kinetic constants for cleavage of the chromogenic substrate methylsulfonyl-D-cyclohexyltyrosylglycyl-arginine-*p*-anitroaniline acetate (Spectrozyme tPA, American Diagnostica, Greenwich, Connecticut) by both the single- and the two-chain forms of tPA, tPA(F305H), and tPA(A292S,F305H) (*25*). Reaction conditions were as recommended by the manufacturer.

Form of tPA	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	$\Delta\Delta G\dagger$ (kcal/mol)	Zymogenicity
		Two-chain			
Wild-type tPA	0.3	77	2.5×10^5		
tPA(F305H)	0.5	89	1.8×10^5		
tPA(A292S,F305H)	0.7	89	1.3×10^5		
		Single-chain			
Wild-type tPA	0.8	30	3.9×10^4		
tPA(F305H)	0.8	4.6	5.7×10^3	1.2	
tPA(A292S,F305H)	1.2	1.1	9.2×10^2	2.3	
		Two-chain–single-chain			
Wild-type tPA	0.4	2.5			6.7
tPA(F305H)	0.6	19			32
tPA(A292S,F305H)	0.6	81			141

The single-chain form of the zymogen-like variants of tPA might be expected to display reduced reactivity toward not only synthetic and natural substrates but also specific inhibitors (*13*). The most important inhibitor of tPA in vivo is endothelial cell plasminogen activator inhibitor type 1 (PAI-1), a 50-kD member of the serpin (serine protease inhibitor) gene superfamily (*14*). To measure the rate of interaction between PAI-1 and the single-chain forms of tPA, tPA(F305H), and tPA(A292S,F305H), we performed a series of kinetic experiments, and the corresponding second-order rate constants were calculated. As shown in Table 3, single-chain tPA-(A292S,F305H) is inhibited by PAI-1 at approximately 4% the rate of single-chain tPA.

These data demonstrate that the substitution of His for Phe at position 305 selectively reduces the reactivity of the single-chain tPA toward both substrates and specific inhibitors. It appears likely that this reduced activity is a consequence of a hydrogen bond formed between the side chains of His^{305} and Asp^{477}, as

Table 2. Kinetic constants for the cleavage of Lys-plasminogen by the two-chain forms of tPA, tPA(F305H), and tPA(A292S,F305H). Reaction conditions were as described (*12*).

Two-chain tPA	Substrate kinetics	
	K_m (μM)	k_{cat} (s^{-1})
Wild-type tPA	0.018	0.22
tPA(F305H)	0.020	0.21
tPA(A292S,F305H)	0.018	0.19

Table 3. Second-order rate constants for the inhibition of the single-chain forms of tPA, tPA(F305H), and tPA(A292S,F305H) by PAI-1. Reaction conditions were as described (*26*).

Single-chain tPA	Second-order rate constant (M^{-1} s^{-1})
Wild-type tPA	1.4×10^6
tPA(F305H)	1.8×10^5
tPA(A292S,F305H)	5.5×10^4

is found between the corresponding residues in chymotrypsinogen (Fig. 1) to stabilize a nonfunctional oxyanion hole (Fig. 1B). Consistent with this model, the zymogenicity of the mutant tPA(A292S,F305H) results primarily from a reduction in k_{cat} of the single-chain enzyme. The catalytic efficiency of tPA(A292S,F305H) is 2.4% that of wild-type tPA. This corresponds to a change in activation energy ($\Delta\Delta G\dagger$) of 2.3 kcal/mole (Table 1), which is equivalent to the loss of a single hydrogen bond [for example, in transfer RNA synthetase (*15*)], but less than that reported for modification of residues in the catalytic triad of trypsin (*16*) or subtilisin (*17*).

Transition-state destabilization, observed as decreases in k_{cat}, is not the only source of zymogenicity in chymotrypsin family proenzymes. In terms of catalytic efficiency toward good peptide substrates, trypsinogen and chymotrypsinogen have values for k_{cat}/K_m that are 10^{-5} to 10^{-6} those of trypsin and chymotrypsin (*1*). Both k_{cat} and K_m are reduced by two to three orders of magnitude (*18*), and the zymogenicity is thought to include contributions from both substrate binding (*1, 6*) and transition-state stabilization (*1, 18*). By contrast, with weakly bound substrates such as alyl or glycyl nitrophenyl esters K_m remains unchanged and a reduction by a factor of 100 is observed in k_{cat} in the zymogen (*18*). Thus, single-chain tPA(A292S,F305H) reproduces the k_{cat} effects exhibited by trypsinogen and chymotrypsinogen toward weakly bound substrates but exhibits none of the lowered K_m effects of the strongest zymogens.

The geometry of the Asp^{194}-His^{40}-Ser^{32} zymogen triad is similar to that of the catalytic triad of Asp^{102}-His^{57}-Ser^{195}; the two triads in chymotrypsinogen [Protein Data Bank (Brookhaven National Laboratories, Upton, New York) files 1cho (*19*) and 2cga (*6*)] superimpose with a root mean square deviation of only 1.3 Å. However, because there is no similarity of the surrounding backbone structure, it appears that these triads evolved separately and are not the result of a gene duplication. The catalytic triad has also evolved several times and has been observed in the active site of subtilisins (*5*), phospholipases (*20*) and a plant serine carboxypeptidase (*21*). It is clear, then, that the Asp-His-Ser triad has been adapted to catalyze different hydrolytic reactions. The evidence presented here, however, demonstrates that this triad has also been adapted to perform a regulatory role.

The magnitude of activation by proteolytic cleavage-activation, or zymogenicity, varies among enzymes of the chymotrypsin family. Zymogens such as chymotrypsinogen, trypsinogen, proelastase, and plasminogen are essentially inactive in the uncleaved state, with measured zymogenicities of 10^4 to 10^6. Other proteases of the chymotrypsin family exhibit an intermediate zymogenicity. For example, the activity of two-chain tPA is 6.7-fold greater than that of single-chain tPA (*10, 11*), the activity of urokinase is 250-fold greater than that of prourokinase (*22*), and the activity of factor XIIa is 4000-fold greater than that of factor XII (*23*). The reasons for this wide range of zymogenicities are unknown. Although the best of these zymogens contain both His^{40} and Ser^{32}, our results show that the presence of these two residues is not sufficient to endow tPA with full zymogenicity. In the case of chymotrypsinogen and trypsinogen, changes in both the specificity pocket and the oxyanion hole could contribute to the differences in k_{cat} and K_m of the zymogens (*16*). Other parts of the tPA structure may also be involved, because Petersen *et al.* (*24*) have shown that the single- and two-chain forms of the mutant enzyme tPA(K416L) (*8*) are lower in catalytic activity than the corresponding wild-type enzymes.

Finally, tPA(F305H) and tPA(A292S, F305H) display a combination of properties that might extend their therapeutic range. Because the single-chain form of the mutant enzymes is less active than wild-type tPA and is more resistant to inhibition by PAI-1, it should be possible either to increase the dose of the enzyme without increasing the risk of hemorrhage or to reduce the dose of enzyme without compromising the rate or extent of dissolution of obstructive thrombi. Both of these courses of action would have advantages in different clinical settings.

REFERENCES AND NOTES

1. A. Gertler, K. A. Walsh, H. Neurath, *Biochemistry* **13**, 1302 (1974); N. C. Robinson, H. Neurath, K. A. Walsh, *ibid.* **12**, 420 (1973).
2. D. Collen, *J. Cell. Biochem.* **33**, 77 (1987); R. D. Gerard and R. S. Meidell, *Annu. Rev. Physiol.* **51**, 245 (1989); K. Dano *et al.*, *Adv. Cancer Res.* **44**, 139 (1985).
3. D. Collen *et al.*, *Circulation* **73**, 511 (1986); K. Rao, *J. Am. Coll. Cardiol.* **11**, 1 (1988); R. M. Califf *et al.*, *Am. J. Med.* **85**, 353 (1988).
4. S. T. Freer *et al.*, *Biochemistry* **9**, 1997 (1970); H. Fehlhammer, W. Bode, R. Huber, *J. Mol. Biol.* **111**, 415 (1977).
5. J. D. Robertus, J. Kraut, R. A. Alden, J. J. Birktoft, *Biochemistry* **11**, 4293 (1972).
6. D. Wang, W. Bode, R. Huber, *J. Mol. Biol.* **185**, 595 (1985).
7. A. A. Kossiakoff, J. L. Chambers, L. M. Kay, R. M. Stroud, *Biochemistry* **16**, 654 (1977).
8. Mutations are indicated with the single-letter amino acid code. Thus, Phe^{305}→His is indicated by F305H. Abbreviations for the amino acid residues are: A, Ala; F, Phe; H, His; K, Lys; L, Leu; and S, Ser.
9. J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, ed. 2, 1989).
10. J. A. Boose *et al.*, *Biochemistry* **28**, 635 (1989).
11. L. C. Peterson *et al.*, *Biochim. Biophys. Acta* **952**, 245 (1993).
12. E. L. Madison, E. J. Goldsmith, R. D. Gerard, M.-J. Gething, J. F. Sambrook, *Nature* **339**, 721 (1989); E. L. Madison *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 3530 (1990).
13. E. Antonini *et al.*, *J. Mol. Biol.* **165**, 543 (1983).
14. R. W. Carrell and D. R. Boswell, in *Proteinase Inhibitors*, A. J. Barrett and G. S. Salvesen, Eds. (Elsevier, Amsterdam, 1986), pp. 403–420; H. Pannekock, *EMBO J.* **5**, 2539 (1986); D. Ginsberg, *J. Clin. Invest.* **78**, 1673 (1986).
15. T. N. C. Wells and A. R. Fersht, *Nature* **316**, 656 (1985).
16. D. R. Corey and C. S. Craik, *J. Am. Chem. Soc.* **114**, 1784 (1992).
17. P. Carter, L. Abrahms, J. A. Wells, *Biochemistry* **30**, 6142 (1991).
18. M. A. Kerr, K. A. Walsh, H. Neurath, *ibid.* **15**, 5566 (1976); J. D. Lonsdale-Eccles, H. Neurath, K. A. Walsh, *ibid.* **17**, 2805 (1978).
19. M. Fujinaga *et al.*, *J. Mol. Biol.* **195**, 397 (1987).
20. L. Brady *et al.*, *Nature*.**343**, 767 (1990).
21. D.-L. Liao and S. J. Remington, *J. Biol. Chem.* **265**, 6528 (1990).
22. L. C. Petersen, L. R. Lund, L. S. Nielsen, K. Dano, L. Skriver, *ibid.* **263**, 11189 (1988).
23. M. Silverberg and A. P. Kaplan, *Blood* **60**, 64 (1982).
24. L. C. Petersen, E. Boel, M. Johannessen, D. Foster, *Biochemistry* **29**, 3451 (1990).
25. Oligonucleotide-mediated, site-directed mutagenesis was performed according to standard protocols (*9*). The synthetic oligonucleotides used to introduce the mutations were 5′-GGAG-AGCGGCACCTGTGCGG-3′ (F305H) and 5′-TG-GCAGGCTTCCATCTTTG-3′ (A292S). After mutagenesis, both the presence of the desired mutation and the absence of second site mutations were verified by DNA sequencing. Complementary DNAs encoding variants of tPA were ligated into the transient expression vector pSVT7, and the resulting constructs were used to transfect COS cells by the DEAE-dextran method. Conditioned media were collected, and the enzyme concentration was measured as described (*12*). Ninety-five percent of the tPA produced by this protocol is in the single-chain form (*1, 10*). This observation was verified for the tPA, tPA(F305H), and tPA(A292S,F305H) samples by protein immunoblotting. Conversion of the single-chain enzymes into their two-chain forms was accomplished by incubation with plasmin-Sepharose beads as described (*19*) and confirmed by protein immunoblotting.
26. K. Beatty *et al.*, *J. Biol. Chem.* **255**, 3931 (1980); W. E. Holmes *et al.*, *Biochemistry* **26**, 5133 (1987); E. L. Madison, E. J. Goldsmith, M. J. Gething, J. F. Sambrook, R. D. Gerard, *J. Biol. Chem.* **265**, 21423 (1990).

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