Structural and Kinetic Comparison of Recombinant Human Single- and Two-Chain Tissue Plasminogen Activator

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Abstract

We examined the similarities and differences in conformation between recombinant human single-chain tissue plasminogen activator (sct-PA) and two-chain tissue plasminogen activator (tct-PA), and compared these structural data with measurements of enzymatic activity. The intrinsic protein fluorescence of native tct-PA was 54% that of sct-PA. Differences in steady state protein fluorescence were also noted with denaturation of these plasminogen activators, as well as in the quenching of intrinsic fluorescence of the reduced, alkylated species by iodide. Using the chromogenic substrate H-D-isoleucyl-L-prolyl-L-arginine-*p*-nitroanilide (S-2288), the catalytic efficiency of sct-PA was found to be 26% that of tct-PA, and this was primarily a reflection of the difference in K_m . On addition of soluble fibrin monomer prepared with the tetrapeptide glycyl-L-prolyl-L-arginyl-L-proline (GPRP), the catalytic efficiency of both species increased by 13-fold for sct-PA and by 3.5-fold for tct-PA to approximately the same value. Using the fluorophore eosin iodoacetamide covalently coupled to the single free cysteine in the molecule, Cys 83, the microenvironment of the fibrin-binding site located near this residue was studied. On addition of soluble fibrin monomer to eosin-labeled tct-PA, no effect on eosin fluorescence was noted. Eosin-labeled tct-PA had 16% less eosin fluorescence than did sct-PA and on addition of soluble fibrin monomer to eosin-labeled sct-PA, a decrease in eosin fluorescence, approaching that of eosin coupled to tct-PA, was observed. Together, these structural and kinetic data suggest that sct-PA undergoes a conformational change on binding to fibrin monomer that leads to dramatic differences in catalytic efficiency of the single-chain species. In so doing, sct-PA bound to fibrin assumes the kinetic profile of tct-PA bound to fibrin.

Introduction

Human tissue plasminogen activator (t-PA)¹ is an essential enzymatic component in the constellation of proteins that

promote fibrinolysis (1). This enzyme is synthesized by endothelial cells in a single-chain form (sct-PA) and, by analogy with other serine proteases (2), can be converted to a two-chain form (tct-PA) by proteolysis at Arg 275. Controversy exists in the literature about the differences in activity and fibrin specificity between sct-PA and tct-PA. Three groups have reported that tct-PA is considerably more active than sct-PA (3–5), and these investigators have suggested that any measured activity in preparations of sct-PA represent contamination by small amounts of the two-chain form. Other investigators have reported that sct-PA does have significant intrinsic enzymatic activity (6–11). Interpretation of these data is complicated by several factors, including the method used to measure t-PA activity, the source of t-PA, and the method used to measure activation by fibrin. Rijken and colleagues (8) sought to overcome the problem of conversion by plasmin of sct-PA to tct-PA using high concentrations of aprotinin. They measured the plasmin produced by t-PA using quantitative autoradiography but were unable to measure directly the K_m and V_{max} in the absence of fibrin because they could not achieve substrate saturation with the native substrate. Tate and colleagues (5) overcame this latter problem using a chromogenic substrate and synthesized a noncleavable sct-PA using site-directed mutagenesis, but failed to measure activation in the presence of fibrin.

In the experiments presented here, we compare the activity of native sct-PA with that of tct-PA in the presence and absence of fibrin. We compare the activity of sct-PA with that of tct-PA against the relatively specific chromogenic substrate H-D-isoleucyl-L-prolyl-L-arginine-*p*-nitroanilide (S-2288), thereby avoiding the complications engendered by evolving plasmin activity in the assay medium. We use soluble fibrin monomer prepared according to the method of Laudano and Doolittle (12) with modification (13) to assess directly the effects of fibrin on t-PA activity. As a central aspect of this paper, we correlate these kinetic studies with conformational studies to assess the relationship between structure and function of these related molecules. Our data indicate that native sct-PA and tct-PA have different conformations and different enzymatic properties in the absence of fibrin, but that fibrin binding induces conformational changes in sct-PA that are accompanied by changes in enzymatic activity essentially similar to that of tct-PA.

Methods

Materials. sct-PA was obtained from Genentech, Inc., South San Francisco, CA. S-2288, H-D-valyl-L-leucine-L-lysine-*p*-nitroanilide

lysine; PN-Sepharose, plasmin immobilized on Sepharose 4B; PPACK, D-phenylalanyl-L-prolyl-L-arginine chloromethylketone. 2HCl; sct-PA, single-chain tissue plasminogen activator; SFM, soluble fibrin monomer; S-2288, H-D-isoleucyl-L-prolyl-L-arginine-*p*-nitroanilide; S-2251, H-D-valyl-L-leucyl-L-lysine-*p*-nitroanilide; tct-PA, two-chain tissue plasminogen activator.

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1. *Abbreviations used in this paper:* eos-t-PA, 5-(iodoacetamido)eosin-labeled tissue plasminogen activator; FM-Matrex, Matrex bead-immobilized fibrin monomer; GPRP, glycyl-L-prolyl-L-arginyl-L-proline; PBST, phosphate-buffered saline with 0.006% Tween 80; PLL, poly-L-

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(S-2251), human plasminogen, human plasmin, and human fibrinogen were purchased from KABI Vitrum, Stockholm, Sweden. Matrex Pel 102 beads were obtained from Amicon Corp., Danvers, MA. Plasminogen-free bovine thrombin was purchased from Miles Pharmaceuticals, Naperville, IL. Aprotinin and glycyl-L-prolyl-L-arginyl-L-proline (GPRP) were obtained from Sigma Chemical Co., St. Louis, MO. D-Phenylalanyl-L-prolyl-L-arginine chloromethylketone.2HCl (PPACK) was purchased from Calbiochem-Behring Corp., La Jolla, CA. Iodo-beads were obtained from Pierce Chemical Corp., Rockford, IL. 5-(Iodoacetamido)eosin was purchased from Molecular Probes, Inc., Junction City, OR. Na¹²⁵I was obtained from Amersham Corp., Arlington Heights, IL. [¹⁴C]iodoacetamide was obtained from New England Nuclear, Boston, Ma. Cyanogen bromide-activated Sepharose 4B was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. All other chemicals were reagent grade or better. Deionized water was used throughout.

Preparation of plasmin-Sepharose. Purified plasmin was coupled to cyanogen bromide-activated Sepharose 4B in 0.1 M NaHCO₃, pH 8.3, 0.5 M NaCl. 900 µg plasmin was added to 5 ml of activated beads. The reaction was allowed to proceed at room temperature for 2 h, after which the beads were washed twice with 50 mM Tris, pH 8.0, 0.15 M NaCl, then alternately with 0.1 M sodium acetate, pH 4.0, 0.5 M NaCl, and 0.1 M NaHCO₃, pH 8.3, 0.5 M NaCl. The washed plasmin-coupled beads were finally suspended in 10 mM sodium phosphate, pH 7.4, 0.15 M NaCl, 0.006% Tween 80 (PBST) and stored at 4°. When prepared in this fashion, 25 µl of a 30% slurry of plasmin immobilized on Sepharose 4B (PN-Sepharose) routinely hydrolyzed 1.0 mM S-2251 at 37°C at a rate of 0.077 ODU/min.

Conversion of sc-tPA to tct-PA. Predominantly sct-PA (76% by NH₂-terminal amino acid analysis using a gas phase sequenator [Applied Biosystems, Foster City, CA] at the Peptide Facility of Brigham and Women's Hospital, as well as by electrophoretic analysis and densitometry) was converted to tct-PA by incubating 50 µl of the PN-Sepharose slurry with 1 ml of 0.9 mg/ml sct-PA at room temperature for 2 h with rocking. At the end of the incubation, the PN-Sepharose was removed by centrifugation at 8,700 g for 1 min in an Eppendorf microfuge.

Completeness of conversion was monitored by SDS-PAGE performed in the presence of 2-mercaptoethanol (2-ME). Under the conditions of our assay, conversion was complete at 2 h.

Radiiodination of t-PA. sct-PA was radioiodinated using Iodo-beads. One Iodo-bead was incubated with 0.5 mCi of Na¹²⁵I at 25°C for 15 min. To this was added 1.0 ml of 0.1 mg/ml sct-PA in PBST and the incubation continued for 20 min with gentle rocking. The protein solution was then applied to a 10 × 0.7 cm Sephadex G-25 column that had been washed with a solution of 5 mg/ml BSA. 12 0.3-ml fractions were collected and assayed for total and 25% TCA-precipitable radioactivity. Routinely, column fractions four through six contained maximal protein-bound counts with a specific activity of ~ 0.1–0.2 µCi/µg. These fractions were either used immediately or stored at 4°C for up to 1 wk without appreciable loss of protein-bound counts. In our hands, radioiodination did not affect either the enzymatic activity of t-PA or the enhancement of that activity by fibrin. When radiolabeled tct-PA was required for an experiment, ¹²⁵I-sct-PA was treated with PN-Sepharose as described above.

Labeling of t-PA with 5-(iodoacetamido)-eosin. Eos-t-PA was produced by incubating sct-PA with a 25-fold molar excess of fluorophore (solubilized in acetone) in PBST at room temperature in the dark for 2 h. Excess unreacted fluorophore was removed by extensive dialysis at 4°C in the dark. t-PA has only a single cysteine (Cys 83) that is not involved in a disulfide bond (14), and under the conditions of labeling (pH > 7.0), only this free thiol will react with the iodoacetamide moiety. The extent of incorporation of label was assessed by the method of Franzen and colleagues (15). In addition, the labeled molecule was treated with [¹⁴C]iodoacetamide in an effort to measure directly unreacted cysteine groups. Compared with the native sct-PA molecule, in which 0.97 mol of [¹⁴C]iodoacetamide were bound per mol of protein, 0.04 mol of [¹⁴C]iodoacetamide were bound per mol of

eos-sct-PA, confirming the essential completeness of the labeling reaction. Labeling t-PA with eosin iodoacetamide had no effect on intrinsic enzymatic activity against S-2288 (see below) or on binding to fibrin (see below).

Preparation of Matrex bead-immobilized fibrin monomer (FM-Matrex). FM-Matrex was prepared essentially according to the method of Loscalzo and colleagues (13). Fibrinogen was covalently coupled to solid beads of polymerized acrylonitrile with free carboxyl and *N*-hydroxysuccinimide carboxylate ester groups on the surface (Matrex 102) essentially according to the method of Collier (16). Fibrinogen covalently coupled to the beads was then converted to fibrin monomer by incubation with 4 U/ml of bovine thrombin for 1 h at 25°C. At the end of the incubation, PPACK was added to a concentration of 48 µM to inhibit further thrombin activity. The beads were then washed three times with PBST and stored for up to 2 wk at 4°C without a detectable loss of bound protein. Using ¹²⁵I-fibrinogen, ~ 4.0 × 10⁵ molecules of fibrinogen bound per 3-µm-diam bead with a 95% coupling efficiency. Because of steric constraints induced by the coupling process, fibrin monomer was unable to assemble into polymer on the bead surface, and this immobilized fibrin monomer bound to Matrex was used in the binding experiments described below.

Preparation of soluble fibrin monomer (SFM). SFM was prepared as described previously (12, 13), starting with human fibrinogen purified free of plasminogen by standard methods (17, 18). The SFM was maintained in the monomeric state for up to 1.5 h at room temperature in the presence of 2 mM GPRP. In the absence of GPRP, greater than 95% of the fibrinogen was clottable.

Measurement of t-PA activity. The enzymatic activity of t-PA was assessed using the relatively specific chromogenic substrate S-2288. Substrate hydrolysis was measured spectrophotometrically with a Gilford Response UV/Vis Spectrophotometer (CIBA-Corning, Oberlin, OH). Activity was measured at 37°C in PBST using substrate concentrations ranging from 0.075 to 1.5 mM and a t-PA concentration of 9.20 × 10⁻⁸ M. Kinetic parameters were determined from the initial rates by double-reciprocal plot analysis. Since the sct-PA preparation contained 24% tct-PA by amino-terminal analysis, constants for sct-PA were determined by correcting the reaction velocity at each substrate concentration for the percentage contamination with tct-PA.

Ultraviolet (UV) spectrophotometry. Absorption measurements were performed in a Gilford Response UV/Vis spectrophotometer. Spectra were recorded at 25°C in PBST. Difference spectra were obtained by subtracting spectra taken at pH 7.4 from those recorded at pH 11.5.

Fluorescence spectrophotometry. Fluorescence measurements were performed on a spectrofluorometer (Fluorolog 2 model F111X; Spex Industries, Inc., Edison, NJ) equipped with a thermostatted cell holder. The absorbance of solutions was < 0.1 at exciting wavelengths. A reference cell containing rhodamine B was used to correct for variation and nonlinearity in the excitation source.

Quantum yields were derived from fluorescence intensity measurements at 280 or 295 nm, using the method of Parker and Rees (19):

$$A_p/A_r = Q_p D_p / (Q_r D_r) \quad (1)$$

where *A* is the area under the fluorescence spectrum and *D* is the optical density at the exciting wavelength. The subscripts *p* and *r* refer to protein and reference solution, respectively. A value of 0.20 for *Q_{trp}* was used (20, 21).

Since tyrosine quantum yields cannot be measured directly from recorded spectra, they were calculated from the protein and tryptophan quantum yields, according to the method of Kronman and Holmes (21):

$$Q_{tyr} = Q'_{tyr} / [1 + (m/n)(\epsilon_{trp}/\epsilon_{tyr})] \quad (2)$$

where *m* and *n* are the number of tryptophan and tyrosine residues per protein molecule, respectively, $\epsilon_{trp}/\epsilon_{tyr}$ is the ratio of the extinction coefficients of solutions of the free amino acids, *Q'_{tyr}* is estimated from

$$Q'_{tyr} = Q_{pro} - Q_{trp} \quad (3)$$

and Q'_{trp} is derived from the amplitude (F) and area (A) of the emission spectra as

$$Q_{\text{trp}} = Q'_{\text{trp}}[(F/A)_{\lambda=280}/(F/A)_{\lambda=295}] \quad (4)$$

Binding of t-PA to FM-Matrix. The binding of sct-PA and tct-PA to FM-Matrix was measured at 25°C in PBST. Increasing concentrations of radioiodinated t-PA were incubated with 1×10^4 FM-Matrix beads/ μl in a 300- μl assay volume of PBST at room temperature with rocking for 20 min. At the end of the incubation period, the beads were collected by centrifugation at 8,700 g for 2 min, washed twice with 1.5 ml of PBST, after which bound radioactivity was measured in a RIA-Gamma Counter Model 1274 (LKB Instruments, Gaithersburg, MD). Nonspecifically bound t-PA was distinguished from specifically bound t-PA by addition of a 20-fold excess of unlabeled t-PA.

Reduction and alkylation of t-PA. Both sct-PA and tct-PA were reduced and alkylated by carboxyamidation in the following manner. The proteins were incubated at room temperature for 1 h with 50 mM 2-ME in PBST without any denaturing agents, after which 60 mM iodoacetamide was added in the same buffer. Extensive dialysis against PBST was then performed to remove unbound reactants. Under these conditions, essentially all of the 35 sulfhydryl groups per molecule of t-PA were exposed (only one of which is not disulfide linked in the native molecule) as assayed by the 5,5'-dithiobis-(2-nitrobenzoic acid) method (22, 23). A molar extinction coefficient of $14,200 \text{ M}^{-1}/\text{cm}^{-1}$ for the nitrothiophenolate ion was used to calculate the concentration of free sulfhydryl groups (24). Precipitating t-PA and showing that 5,5'-dithiobis-(2-nitrobenzoic acid) failed to react with the modified protein demonstrated that alkylation was complete under these conditions.

PAGE. SDS-PAGE was performed as described by Weber and Osborne (25) and modified by Laemmli (26) using 7.5% gels. Gels were stained with Coomassie brilliant blue in 50% methanol and 5% acetic acid, and destained by diffusion. Molecular weight standards were processed similarly and apparent molecular weights (M_r) were estimated by interpolation. Quantitative reflectance densitometry was performed using a videodensitometry system (model 620; Bio-Rad Laboratories, Richmond, CA).

Protein determinations. Protein concentrations were determined according to the method of Lowry and colleagues (27).

Results

Secondary and tertiary structure

UV absorption. The absorbance difference spectra in the far-UV region for sct-PA and tct-PA are shown in Fig. 1. These spectra are similar, showing positive bands at 244 and 296 nm. The positive band at the longer wavelength is probably due to vibrational structure (28).

Figure 1. Difference absorption spectra of t-PA in the near-UV region. Difference spectra were obtained for sct-PA (—) and tct-PA (---), both at 0.09 mg/ml, by subtracting spectra at pH 7.8 from those at pH 11.5 at 25°C in PBST in 1-cm pathlength cells.

Fluorescence. The endogenous protein fluorescence spectra of sct-PA and tct-PA in PBST at 25°C are shown in Fig. 2. The spectrum for sct-PA is corrected for contamination with tct-PA; all subsequent fluorescence parameters are similarly corrected. Clearly, the intrinsic protein fluorescence of native tct-PA is less than that of native sct-PA. The quantum yields and emission maxima for sct-PA and tct-PA and their reduced, denatured forms are shown in Table I. The quantum yields measured by exciting at 280 nm are protein quantum yields and are produced by tryptophan and tyrosine fluorescence, whereas the quantum yields measured by exciting at 295 nm are produced primarily by tryptophan fluorescence. The tyrosine quantum yields were obtained by subtracting tryptophan from protein quantum yields and are also listed in Table I. The tryptophan quantum yields were derived using Eq. 4, in which the ratio of tryptophan to tyrosine was calculated from the known amino acid composition (29) and in which a value of 4 was used for the ratio $\epsilon_{\text{trp}}/\epsilon_{\text{tyr}}$ (30).

The protein quantum yield of native tct-PA is 54% of that of sct-PA, while the tryptophan quantum yield is 44% of that of the single chain form. With denaturation, protein quantum yields decrease for both sct-PA and tct-PA by equivalent amounts (27 and 21%, respectively), whereas the tryptophan quantum yield decreases by 11% for sct-PA but does not change significantly for tct-PA. In the native form, the quantum yield of tyrosine is 40% greater in tct-PA than in sct-PA, whereas it is 52% greater in the corresponding denatured species.

The maximal emission wavelengths are comparable in all cases, approximating 360 nm for the native species. On denaturation, these emission maxima increase in each case, to the 364–369-nm range.

Fluorescence quenching by sodium iodide. The effect of increasing concentrations of the ionic fluorescence quencher iodide on the intrinsic fluorescence of t-PA is shown in Fig. 3, A and B. The Stern-Volmer plots suggest that principally dynamic (collisional) processes contribute to quenching of intrinsic protein fluorescence. No upward curvature is noted, implying that the static quenching parameter is small. No significant downward curvature is noted, implying an absence of significantly different multiple emission components (31). The slope of the Stern-Volmer plots for native sct-PA and native tct-PA are similar, suggesting similar quencher access to fluorophores in these molecules.

Quenching is enhanced somewhat with reduction and alkylation, more with tct-PA than with sct-PA. Denaturation greatly enhances the quenching by iodide, particularly for the

Figure 2. Protein fluorescence emission spectra of t-PA. Spectra were recorded for 0.009 mg/ml sct-PA (—) and tct-PA (---) in PBST at 25°C. Samples were excited at 280 nm and emission was recorded at 360 nm with an excitation slit width of 1.25 nm and an emission slit width of 5.00 nm.

Table I. Protein, Tryptophan, and Tyrosine Quantum Yields of Recombinant Human t-PA*

	$Q_p (\lambda_{max})^\dagger$	$Q_{trp} (\lambda_{max})^\ddagger$	Q_{tyr}
Native			
sct-PA	0.093 (360)	0.075 (361)	0.040
tct-PA	0.050 (360)	0.033 (360)	0.056
Denatured [†]			
sct-PA	0.068 (364)	0.067 (365)	0.032
tct-PA	0.039 (367)	0.035 (369)	0.021

* The values of the quantum yields and emission maxima are averages obtained from two experiments performed in duplicate with deviations of $\pm 5\%$ and ± 1 nm, respectively. Values for sct-PA have been corrected for contaminant tct-PA as described in Methods.

[†] Excitation at 280 nm.

[‡] Excitation at 295 nm.

^{||} In PBST.

[†] In 6 M guanidine hydrochloride-PBST.

reduced, alkylated species. There appears to be no significant difference between sct-PA and tct-PA as to quenching by iodide in the denatured states.

Comparison of enzymatic activity of sct-PA and tct-PA. The hydrolytic activity of sct-PA and tct-PA against the chromogenic substrate S-2288 was assessed in the presence and absence of SFM. As seen in Table II, sct-PA has a K_m for substrate that is 3.3-fold greater than tct-PA and a similar k_{cat} , yielding a catalytic efficiency (k_{cat}/K_m) that is only 26% of that of tctPA in the absence of SFM. In the presence of 33 $\mu\text{g/ml}$ SFM, however, the K_m of both sct-PA and tct-PA decrease significantly, ~ 16 - and 4-fold, respectively. The k_{cat} of both sct-PA and tct-PA decreased very slightly (16 and 19%, respectively). These changes lead to a catalytic efficiency that is dramatically enhanced for sct-PA (13-fold) and significantly increased for tct-PA (3.5-fold). Most interestingly, with these changes in catalytic constants induced by SFM, sct-PA manifests a catalytic efficiency that is not significantly different from that of tct-PA. Importantly, predominantly sct-PA did not undergo any significant (auto)conversion to tct-PA during the course of these enzymatic measurements; this was demonstrated by inhibiting further enzymatic activity at the end of the reaction with 250 kallikrein inactivator units/ml aprotinin, subjecting the reaction solution to gel exclusion chromatography on Sephadex G-100 in order to isolate t-PA from SFM and from chromogenic substrate, lyophilizing the t-PA peak, and

conducting NH_2 -terminal amino acid analysis using a gas phase sequenator as described in Methods. By this kind of analysis, predominantly sct-PA (76% single-chain at the outset of the reaction) remained as such throughout the reaction, both in the presence (75%, mean of two measurements) and absence (76%, mean of two measurements) of SFM. In addition, we used radioiodinated sct-PA in the reaction mixture and prepared the mixture with electrophoretic sample buffers with and without 2-ME immediately after adding aprotinin. Electrophoresis was performed on 10% gels and autoradiography was subsequently performed. Again, by densitometric analysis, there was no evidence for autoconversion of sct-PA to the two-chain form during the course of these very brief enzymatic reactions (75% sct-PA, mean of two measurements in the absence of SFM; 75% sct-PA, mean of two measurements in the presence of SFM).

Effect of poly-L-lysine and SFM on fluorescence intensity of eos-t-PA. To detect subtle changes in conformation that may accompany the binding of sct-PA to fibrin, we measured the effect of the binding of poly-L-lysine (PLL) and SFM on the steady state fluorescence intensity of eos-sct-PA and eos-tct-PA. Because these protein species are labeled solely at a single sulfhydryl group in the region adjacent to the fibrin-binding domain (32), changes in the fluorescence of the reporter group probably reflect local changes in the microenvironment of the molecule important for fibrin binding (the finger domain and the kringle-2 segment).

As shown in Table III, eos-tct-PA has only 84% of the fluorescence intensity of eos-sct-PA at 540 nm when excited at 519 nm. With addition of 250 $\mu\text{g/ml}$ PLL or 75 $\mu\text{g/ml}$ SFM the fluorescence intensity of eos-sct-PA decreases by $\sim 13\%$, approaching that of eos-tct-PA, whereas that of eos-tct-PA does not change significantly. Titrations of these fluorescence changes using PLL and SFM are shown in Fig. 4. Note that the data are expressed as the ratio of the change in fluorescence intensity at a given concentration of ligand to that at saturating concentrations of ligand to facilitate estimations of apparent K_d . From these data, the apparent estimated K_d for PLL ($\langle M_r \rangle = 300,000$) is 10 $\mu\text{g/ml}$, whereas that for SFM is 11 $\mu\text{g/ml}$.

Binding of t-PA to FM-Matrex. We used FM-Matrex as a ligand for t-PA binding experiments to facilitate estimations of affinity and stoichiometry. Because fibrin monomer on Matrex beads cannot polymerize, the nature of the ligand is known and stoichiometry can be determined. In addition, with fibrin monomer covalently attached to beads, bound t-PA can be readily separated from free t-PA by centrifugation. The binding data obtained with this system are shown in Fig. 5. Clearly, both sct-PA and tct-PA bind saturably and specifi-

Figure 3. Quenching of protein fluorescence of t-PA by iodide. Stern-Volmer plots were constructed for sct-PA (open symbols) and tct-PA (closed symbols) in native (circles) and reduced, alkylated (squares) species in the absence (A) and presence (B) of 6 M guanidine hydrochloride. Quenching by iodide (A and B) was measured in PBST at 25°C on excitation with 280 nm and emission at 360 nm.

Table II. Kinetic Constants for Hydrolysis of S-2288 by sct-PA and tct-PA*

	K_m	k_{cat}	k_{cat}/K_m
	<i>mM</i>	<i>s</i> ⁻¹	<i>mM</i> ⁻¹ / <i>s</i> ⁻¹
Sct-PA [‡]			
Without SFM	0.82	6.75	8.23
With SFM	0.051	5.53	108.43
tct-PA			
Without SFM	0.25	7.82	31.28
With SFM	0.061	6.57	107.71

* Enzymatic assays and analyses of data were performed as described in Methods.

[‡] Kinetic constants for sct-PA have been corrected for contaminant tct-PA as described in Methods.

cally, and both bind similarly with an approximate apparent K_d of 3.9 μ M. With 4×10^5 molecules of fibrinogen bound per bead (see Methods), at saturation, the stoichiometry of t-PA to fibrin monomer is $\sim 1.1:1$.

Discussion

In most published studies, t-PA activity is measured indirectly as plasmin activity (9, 33), and because plasmin itself can convert sct-PA to tct-PA, any quantitative assessment of t-PA activity is confounded by ongoing conversion of enzyme by substrate. Furthermore, absolutely pure preparations of sct-PA are not available and there is, as yet, no inhibitor that selectively inhibits contaminant tct-PA. We have attempted to overcome these difficulties in the experiments described here by several significant experimental alterations. We used the chromogenic substrate S-2288 to measure the kinetic constants of t-PA. In addition, however, we have derived the actual constants of sct-PA using preparations containing tct-PA by quantitative assessment of the degree of contamination. We also used a different preparation of fibrin for assessing activation. Instead of Desafib (American Diagnostica), which may manifest altered conformations and thereby have altered acti-

Table III. Fluorescence Emission Intensity of Eos-t-PA*

	Normalized emission intensity
	%
Eos-sct-PA [‡]	100
+ Poly-L-lysine [§]	87
+ SFM	85
Eos-tct-PA [‡]	84
+ Poly-L-lysine	83
+ SFM	83

* Measurements were made in PBST at 25°C with excitation at 519 nm and emission at 540 nm. Each value represents the average of two experiments.

[‡] t-PA concentrations were 69 nM.

[§] Poly-L-lysine was added to a concentration of 250 μ g/ml.

^{||} SFM was added to a concentration of 75 μ g/ml.

vating ability, we used SFM maintained in the monomeric state with the tetrapeptide inhibitor GPRP. When we attempted the kinetic experiments with Desafib, we observed only marginal activation compared with SFM.

We complemented these kinetic studies with structural studies of both sct-PA and tct-PA. Using both UV absorption and fluorescence spectroscopy of these molecules, we showed that there were both similarities and dramatic differences between single- and two-chain species in both the native and denatured states. What specific features of the protein conformation are affected by conversion of sct-PA to tct-PA cannot be determined from these data because the interpretations are limited by the fact that the measured signals represent averages from fluorophores located in multiple regions of a given protein molecule.

Despite these shortcomings, we were able to derive some useful general information about the similarities and differences between the conformations of sct-PA and tct-PA. The UV difference absorption spectra were very nearly identical. Since the absorption differences induced by alkaline denaturation are similar, these data argue that the microenvironments of the subclass of intrinsic chromophores perturbed by alkalinity must be similar.

Figure 4. Effect of addition of poly-L-lysine and SFM on eos-t-PA fluorescence. Fluorescence was measured for solutions of sct-PA at 0.005 mg/ml in PBST at 25°C. Samples were excited at 519 nm and emission was recorded at 540 nm with an excitation slit width of 1.25 nm and an emission slit width of 5.00 nm. The ordinate is defined by the ratio of the change in fluorescence intensity at any given concentration of poly-L-lysine or SFM to the maximal change in fluorescence intensity. At saturating concentrations of ligand, the absolute fluorescence intensity is reduced relative to that in the absence of ligand. The data are plotted in this fashion to facilitate approximate binding analyses.

Figure 5. Binding of t-PA to fibrin monomer on FM-Matrex. Binding of sct-PA (○) and tct-PA (●) to fibrin monomer immobilized on FM-Matrex was measured in PBST at 25°C. Increasing concentrations of radioiodinated t-PA

were added to 1×10^4 FM-Matrex beads/ μ l in 300- μ l assay volumes. Incubations were performed for 20 min with rocking. Beads were collected by centrifugation at 8,700 g for 2 min at the end of the incubation period, washed twice with PBST, and bound radioactivity was determined. Specific binding (plotted here) was distinguished from nonspecific binding by addition of a 20-fold excess of unlabeled t-PA to the incubation medium. Nonspecific binding amounted to no more than 35% of total bound counts.

In contrast, analysis of intrinsic protein fluorescence reveals differences between sct-PA and tct-PA. The fluorescence of t-PA predominantly originates from tryptophan moieties. Despite the presence of 24 tyrosine residues per 72,000-D molecule and only 13 tryptophan residues, the tryptophan fluorescence dominates protein fluorescence. Dramatic reductions in the quantum yield of intrinsic protein fluorescence accompany the conversion of sct-PA to tct-PA. In addition, the polar quencher iodide had greater access to fluorophores in reduced, alkylated, nondenatured two-chain than in single-chain forms.

The wavelengths of the emission maxima of sct-PA and tct-PA are also rather similar and suggest that most of the tryptophan residues are relatively exposed to solvent rather than buried in the hydrophobic matrix of the protein. Denaturation tends to shift the maxima to slightly longer wavelengths and to decrease the quantum yield. Although neither the emission maximum nor the quantum yield can be a simple function of the degree of exposure of tryptophan residues (21, 34), the combination of a modest red shift and a decrease in quantum yield argues that some tryptophan residues buried in the native molecule are exposed after denaturation.

Although both sct-PA and tct-PA bound to fibrin monomer covalently linked to Matrex beads identically, significant differences in the vicinity of the fibrin-binding domain can be inferred from the differences in fluorescence emission of eosin covalently linked to Cys 83. The fluorescence emission of this fluorophore was reduced by 16% in tct-PA compared with sct-PA. With addition of PLL or SFM to a solution of sct-PA, the fluorescence of the eosin label decreased, approaching that in tct-PA. Addition of PLL or SFM had no effect on eosin fluorescence in tct-PA.

Together, these structural and kinetic data suggest that sct-PA undergoes a conformational change on binding in a one-to-one complex to fibrin monomer. This conformational change in turn leads to dramatic differences in catalytic efficiency of the single-chain species, primarily by a significant reduction in K_m . In so doing, fibrin-bound sctPA assumes the kinetic profile of tct-PA bound to fibrin and, at least in the fibrin-binding domain as reported by eosin bound to Cys 83, adopts a conformation approaching that of tct-PA. These data argue that sct-PA is more fibrin-specific than tct-PA primarily because of its markedly reduced catalytic efficiency in the ab-

sence of fibrin. Despite similarities in fibrin affinity, fibrin binding induces a marked enhancement in the catalytic efficiency of sct-PA. Fibrin binding also increases the catalytic efficiency of tct-PA, but the extent of enhancement is much greater for the single chain form (3.5-fold vs. 13-fold). These conformation-dependent enzymatic differences may account for the observed differences between predominantly sct-PA and predominantly tct-PA preparations observed in vivo (35).

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