Domain Structure and Domain-Domain Interactions of Recombinant Tissue Plasminogen Activator*

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The melting of recombinant tissue plasminogen activator (rtPA) has been investigated by differential scanning calorimetry and fluorescence spectroscopy. At neutral pH, rtPA melts with only partial reversibility in a single sharp peak that can be deconvoluted into four transitions. By contrast, at acidic pH the melting process is spread over a broad range of temperature and is highly reversible. Under these conditions five transitions are resolved by deconvolution analysis. Additional measurements in 6 m guanidinium chloride reveal a sixth transition representing an extremely stable domain. Comparison of the melting curves of several fragments with those of the parent protein allowed all of the transitions to be assigned. The results indicate that rtPA is comprised of six independently folded domains. Two of these domains correspond to the two kringle modules whose thermodynamic properties are similar to those of the kringle in plasminogen. Additional domains are formed by the epidermal growth factor (EGF)-like and finger modules, the latter of which is extremely stable, requiring the presence of a chemical denaturant for its melting to be observed. The serine protease module contains two more domains which at neutral pH melt cooperatively in a single transition but at low pH melt independently, accounting for the greater number of transitions observed there. Measurements with a 50-kDa fragment lacking the C-terminal half of the serine protease module and with a variant lacking the finger and EGF domains indicate that the serine protease domains interact strongly with and are stabilized by the finger and/or EGF domains in the intact protein. This interaction between domains located at opposite ends of the molecule produces a more compact structure. A better understanding of such interactions may enhance efforts to engineer plasminogen activators with improved thrombolytic properties.

Tissue plasminogen activator (tPA)† has attracted much attention because of its physiological importance and its potential value as a therapeutic agent (1-3). It is the main physiological activator of plasminogen and through its binding to fibrin serves to localize the activation process to the region of the clot. It is a glycoprotein of molecular mass 68 kDa, approximately 10% of which is due to carbohydrate. The molecule is synthesized as a single chain of 527 amino acids and may be converted by plasmin or similar enzymes to a form containing two chains, A and B, the second amino terminus occurring at Ile-276. tPA differs from other serine proteases such as plasminogen and some blood clotting factors in that both the single and two chain forms are enzymatically active.

tPA is a mosaic protein consisting of five modules, homologous forms of which are also found in other proteins. A fibronectin type I finger (F), an epidermal growth factor-like module (EGF), and two kringle modules (K1 and K2) form the A chain which functions to mediate the interaction of tPA with other proteins, while the B chain comprises a serine protease module (SP) which is responsible for the enzymatic conversion of plasminogen to plasmin. Numerous attempts have been made to improve the specificity and/or catalytic efficiency of tPA by manipulation of the modular composition through recombinant techniques (4-8). Success in the insertion or substitution of a module depends on whether that module constitutes an independently folded domain. Only then could it be expected to retain its structure and function when transposed to a new environment. In some cases, the interaction between modules may also be important for the manifestation of specific functions.

The independent folding status of the K2 module in tPA has been established by Kelly and Cleary who expressed it in Escherichia coli and showed that it exhibits a reversible unfolding transition by differential scanning calorimetry (DSC) (9). The independence of kringle modules in plasminogen was also demonstrated (10, 11). Therefore it is likely that K1 in tPA is also independently folded although it has not yet been proven. Recent work (12) has demonstrated the independent folding status of several fingers in fibronectin, two of which appear to interact strongly with each other and some of which are extremely stable. The status of the finger in tPA has not been determined. There are numerous examples of serine proteases that are homologous to the SP module in tPA and are obviously independently folded. However, in plasminogen, this module was found to consist of two independently folded but interacting domains (10). The status of the SP module in tPA is undetermined. Thus one might expect each module in tPA to form an independently folded domain with possible interactions between some of them.

Radek and Castellino (13) provided evidence for independent melting of two separate regions of the recombinant tPA molecule (rtPA). However, they did not assign the observed transitions to specific modules. Furthermore, the total en-
thalpy reported by these authors is unusually low for a compact protein the size of tPA suggesting that some domains may not have been detected. The goal of the present work was to identify all of the independently folded domains in rtPA and to detect possible interactions between them. Clarification of the domain structure and domain-domain interactions of this protein is important for efforts to express modified forms having improved properties for therapeutic purposes.

MATERIALS AND METHODS

All measurements were performed with recombinant tissue plasminogen activator (rtPA) prepared by Genentech under the trade name “Activase” (1). Upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), it displayed a single band with a relative mobility corresponding to 58 kDa (nonreduced) and 66 kDa (reduced) on overloaded gels (lanes 2 and 3, Fig. 1E). The reduced protein showed a small amount of the two-chain form (lane 5, Fig. 1E). At lower loads a doublet can be resolved, presumably due to heterogeneity in carbohydrate content (1). This feature was much more evident in some of the fragments, the differences in the two forms being 3–4 kDa (see below). The protein was homogeneous by size-exclusion chromatography indicating the absence of aggregated or grossly unfolded material. The deletion variant, ΔFAE-rtPA (14) was obtained from Dr. Benjamin Isaacs of Genetics Institute (Boston, MA). It was homogeneous by SDS-PAGE with an apparent molecular mass of 45 kDa in both the reduced and nonreduced forms.

Fig. 1A shows the analysis by SDS-PAGE of the time course of digestion of purified 16-kDa fragments with plasmin (1:100 w/w) at pH 3.5 and 67°C. The fragments are generated within the first 20 min and are resistant to further proteolysis at this pH. These four fragments were purified from a 1-h digest by size-exclusion chromatography on Biogel P-60 and/or Sephadex G-75 superfine columns. Further digestion of a mixture of 16-kDa fragments with plasmin at pH 2.6 (1:150 w/w, 50 mM glycine, 37°C) resulted in the appearance of three additional fragments with apparent molecular mass of 23, 19, and 9 kDa (Fig. 1C). A 2-h digest was resolved by size-exclusion chromatography into two fractions, one containing the 19/23 kDa and the other containing the 9-kDa fragment. In both cases digestion of pepstatin was terminated by addition of pepstatin followed by rapid elevation of the pH to approximately 5.

Analysis of the purified pepstatin fragments by SDS-PAGE is shown in Fig. 1, B and D. It is apparent that some of them contain internal cleavages. The identity of each fragment and the positions of the internal cleavages were determined by N-terminal sequence analysis (see insets, Fig. 4). The 31- and 35-kDa fragments have the same N terminus as the parent protein and according to their size should contain the finger, EGF, K1, and K2 modules. The second N terminus was found corresponding to Val-391 representing a residual piece of the B chain linked to K2 by a disulfide bond. A third sequence beginning at Ile-210 in K2 was present in essentially all of the 35-kDa fragment and approximately half of the 31-kDa fragment, consistent with the SDS-PAGE analysis (Fig. 1B). Note the appearance of two major bands near 30 kDa in the reduced sample of 31 kDa, lane 5, but only one for 35 kDa, lane 4. Thus, the 31- and 35-kDa fragments have the same modular composition, the difference in apparent molecular mass being due to presumed differences in glycosylation and possible variations in the exact C terminus. This doublet feature as well as the internal cleavage in K2 is preserved in the 19- and 23-kDa fragments, each of which has an N terminus at Arg-89, between the EGF and K1 modules. Thus, the 19/23-kDa fragment contains the two kringles and a short piece of the B chain. The 9-kDa fragment retains the same N terminus as the parent rtPA and judging from its size should contain the finger and EGF modules.

This fragment also has internal cleavages at Arg-25 in the finger and Phe-71 in the EGF module.

In Fig. 1A and 1B, 15- and 17.5-kDa fragments generated by the initial digestion of rtPA are derived from the protease module with N termini at Ala-295 and Leu-401, respectively. They also contain internal cleavages, at Ile-312 and Leu-375 in 12.5 kDa and Val-493 in 17.5 kDa. The observed cleavage at Leu-401 defining the NH2 terminus of the 17.5-kDa fragment indicates that the above-mentioned residual piece of the B chain beginning at Val-391 in the 17.5- and 19/23-kDa fragments contains more than 10 amino acids.

The time-course of digestion with subtilisin (1:500 w/w in 0.3 M HEPES buffer at pH 7.4 and 37°C) is shown in Fig. 1E. A fragment of approximately 50 kDa, a 32/35-kDa doublet, and a 9-kDa fragment were rapidly produced and resisted further proteolysis. The 50- and 32/35-kDa fragments were purified from a 45-min digest by exclusion chromatography as before. Electrophoretic analysis of the purified fragments is presented in Fig. 1F. The 32/35-kDa fragment (lanes 4 and 5) is identical to the pepstatin-derived 31/35-kDa except for the absence of the internal cleavage in the K2 region (see insets, Fig. 4). The 50-kDa fragment has the same NH2-terminal sequence as the parent protein and a second one beginning at Ala-282, corresponding to cleavage between K2 and the SP module. It contains the entire A chain linked by a disulfide bond to a modified B chain from which most of the C-terminal half is missing. This is consistent with the appearance of the A chain and a smaller internal cleavage (see inset, Fig. 4) of approximately 16 kDa representing the NH2-terminal one-third of the B chain (Fig. 1F, lane 3); an additional approximately 4-kDa piece beginning at leu-470 is not visible in this photograph.

Preparation of two-chain rtPA was accomplished by incubation with plasmin, 1:100 w/w, for 1 h at room temperature in 0.3 M HEPES, pH 7.4. The mixture was acidified to pH 2, and dialyzed against 50 mM glycine, pH 3.4, for 2 h.

Protein concentrations were determined from the absorbance at 280 nm using molar extinction coefficients (ε cm⁻¹M⁻¹, 280 nm) that were calculated from the amino acid sequences by the formula 0.97
Fluorescent labeled rtPA was prepared by reacting 1 mg of the protein in 1 ml of 0.02 M Tris, pH 7.8, containing 4 M urea, with a 20-fold molar excess of fluorescein maleimide (Molecular Probes) for 1 h at room temperature. The sample was dialyzed extensively against 0.3 M HEPEs, pH 7.4, and passed over a 9-ml column of Sephadex G-25 to remove residual dye. The degree of labeling was determined spectrophotometrically to be 0.7 mol of dye per mol protein, presumably attached to the single free sulfhydryl at position 83 (16). A preparation that was first reacted with DTNB, incorporating 0.8 mol/mol, subsequently incorporated only 0.2 mol of dye when labeled with fluorescein maleimide. When chromatographed on Superose-12 (Phar-macia) the labeled protein eluted as a single sharp peak identical to that obtained with the unlabeled one.

SDS-PAGE analysis was performed with the Pharmacia Phast system. The apparent molecular mass values are based on measured relative mobilities of the fragments on 8-25% gradient gels except for the 9-kDa fragment for which a 20% homogeneous gel was used to assess molecular mass.

Differential scanning calorimetry measurements were made with a MicroCal MC-2, DASM-IA, or DASM-4 instrument at a scan rate of 1 °C/min and protein concentrations ranging from 1 to 10 mg/ml in the absence of denaturants and 11-16 mg/ml in 6 M GdmCl. The solvent was buffer in many cases, the solvent used in many cases was 0.1 M Tris containing 0.15 M NaCl at pH 7.4. However, the protein was quite soluble at pH 7.4 in 0.3 M HEPEs and in 0.05 M MOPS containing 0.25 M NaCl and also at pH 3 or 4 in 0.05 M glycine. Deconvolution analysis was performed by the recurrent procedure of Freire and Biltonen (17) using software provided by MicroCal, selecting options whereby the computer (as opposed to the user) determines the number of component two-state transitions contributing to a given endo-
term. This procedure is based on fundamental thermodynamic equations which allow the number of underlying independent transitions contributing to a complex endotherm to be determined without any a priori assumptions. Baselines for the purposes of deconvolution were determined by the straight-line method or by the method of splines using software provided by MicroCal. An example of a baseline determined by the latter approach is given in Fig. 2B. The relative error of the total enthalpy values is estimated at ±10% and that of the Tm values at ±0.5 °C. The corresponding errors in the parameters of individual transitions obtained by deconvolution of curves representing multiple domains is estimated at ±15% and ±2°C.

GdmCl titrations were performed with a motor-driven syringe (Harvard Instruments) controlled with a personal computer programmed for slow addition (20 μl/min) of 8 M GdmCl to a stirred 1-
×1-cm cuvette containing 1.6 ml of protein at 0.2 mg/ml while monitoring the anisotropy of the fluorescein fluorescence at 520 nm, excited at 493 nm, with an SLM 8000-C fluorometer controlled by the same computer. The temperature of the sample was controlled at 25 ± 0.1 °C with a circulating water bath. After addition of 1.6 ml of GdmCl stock, the program was interrupted, 1.6 ml was removed from the cuvette, and the titration was continued to complete the unfolding transition. Thermal unfolding was detected in the fluorometer by monitoring the fluorescence intensity at 524 nm while heating at 1 °C per min.

Analytical exclusion chromatography was performed at room temperature in 0.3 M HEPEs, pH 7.4, with a Pharmacia fast-protein liquid chromatography system on a column of Superose-12 at 1 ml per min monitoring elution by intrinsic fluorescence with excitation at 280 and emission at 340 nm.

Differential absorption measurements were made at room temperature in an Aviv Cary 118 spectrophotometer. Small volumes of 1 M HCl were added incrementally to the sample cell containing protein at a total absorbance of 0.5 at 280 nm and equivalent volumes of buffer (50 mM HEPEs, pH 7.4) were added to the reference, measuring the pH and scanning the spectrum after each addition. The optical measurements were complicated by the appearance of turbidity, especially in the pH range from 5 to 6.5. This is due to the low concentration of HEPEs used in this experiment to allow changes in pH to be effected without excessive dilution. It was not possible to estimate the net ΔOD at 285 nm by arbitrarily connecting the baseline before and after the main differential absorption band. The use of uncorrected measurements near its isoelectric point, accounting for the gap in the titration curve between pH 5.3 and 6.5.
The domain structure of tissue plasminogen activator (tPA) is examined in detail. FIG. 3 shows deconvolution analysis of excess heat capacity functions obtained at pH 7.4 (A), pH 4.0 (B), and pH 3.4 (C) with single chain rtPA. $T_m$ and $\Delta H$ values for the component transitions, designated by numerals next to each curve, are summarized in Table I.

The foregoing analysis indicates the presence in tPA of at least five domains which unfold in the temperature range examined. They behave more or less independently at low pH but two of them appear to interact to form a single cooperative unit at neutral pH. The sharpness of the peak at pH 7.4 demands that one of the underlying transitions be of high enthalpy. Attempts to forcibly replace transition 4 with two superimposed transitions of lower enthalpy dramatically reduced the quality of the fit because of the increased width of the lower enthalpy transitions.

To determine which parts of the molecule are responsible for the various transitions, several fragments were prepared from single chain rtPA as described under “Materials and Methods.” The melting of all fragments was examined at pH 3.4 in 50 mM glycine buffer, as these are the conditions where the component transitions were best resolved. The three smallest pepsin-derived fragments, namely 9, 12.5, and 17.5 kDa, failed to show any detectable melting transition below 90°C by either DSC or intrinsic fluorescence measurements. This suggests that the one or more internal cleavages present in each of these fragments results in the abolition of their compact structure. However, the larger fragments all showed complex endotherms in spite of the presence of internal cleavages in the K2 region of two of them. The results of deconvolution of melting curves obtained at pH 3.4 are presented in B through F of Fig. 4 and in Table II. The results for intact rtPA are reproduced in A for comparison.

The melting curve of the 50-kDa subtilisin fragment, which is missing most of the C-terminal half of the SP module, deconvolutes into four transitions (Fig. 4B). The 32/35-kDa subtilisin fragment, which is missing the entire SP module, exhibits only three transitions (Fig. 4C). This indicates that the SP module comprises two domains, whose melting would appear to be responsible for transitions 1 and 4 in the parent protein (Fig. 4A). Transition 4 corresponds to the C-terminal region (SP-C), most of which is missing from the 50-kDa fragment, and transition 1 to the N-terminal portion (SP-N), which is absent in 32/35-kDa and smaller fragments. Similar deconvolution patterns were obtained with the 50- and 32/35-kDa fragments at pH 3.0 where the endotherm was shifted to slightly lower temperature (not shown).

Fig. 4D refers to the 31-kDa pepsin-derived fragment which differs from the subtilisin form by the presence of an internal

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**Fig. 3.** Deconvolution analysis of excess heat capacity functions obtained at pH 7.4 (A), pH 4.0 (B), and pH 3.4 (C) with single chain rtPA. $T_m$ and $\Delta H$ values for the component transitions, designated by numerals next to each curve, are summarized in Table I.

**Fig. 4.** Deconvolution of excess heat capacity functions obtained with rtPA and several fragments at pH 3.4. The identity and modular composition of the melted species is shown schematically in the upper right corner of each panel. The numerals designate the two state transitions obtained by the deconvolution analysis; $T_m$ and $\Delta H$ values are summarized in Table II. The arrows designate the position of internal cleavage in the K2 module.
cleavage in a portion of its K2 module. The DSC profiles are nearly identical except in the region of the lowest transition, i.e., transition 2, which has a lower $T_m$ and enthalpy in the partially cleaved fragment (see also Table II). This decrease is even more pronounced with the purified 35-kDa fragment whose K2 module is completely cleaved (Fig. 4E). This strongly suggests that transition 2 represents the melting of the K2 module. Fig. 4E presents results obtained with the 19/23-kDa fragment which is devoid of the EGF and finger modules. Since transition 5 has disappeared, it can be assigned to the melting of one or both of these modules. The 19/23-kDa fragment is also completely cleaved in the K2 region. The lower $T_m$ and enthalpy of the first transition in this fragment is therefore consistent with its assignment to the K2 domain. Transition 3 must then be due to the melting of K1, the only other module present in the 19/23-kDa fragment. The parameters of this transition are not significantly influenced by the loss of the finger and EGF modules or by the loss of the protease module, suggesting the absence of strong interactions between K1 and these modules. Similarly, the parameters of transition 2 are the same in the parent protein and in the uncleaved subtilisin-generated 32/35-kDa fragment (Fig. 4, A and C) indicating the absence of strong interaction between the K2 and serine protease modules under these conditions.

The assignment of transition 2 to the K2 domain is further supported by the fact that the melting temperature and enthalpy of the uncleaved form, as deduced from the deconvolution results of A—C in Fig. 4 (see also Table II, lines 1–3), coincide with the values published by Kelly and Cleary (9) for isolated recombinant K2 under similar conditions. This coincidence also provides further support for the conclusion that the K2 domain does not interact strongly with any of the other domains under these conditions since its melting parameters when completely isolated are very close to those in the whole protein.

In summary, two of the five transitions can be assigned to the melting of the SP module, two to the kringle modules, and one to the EGF and/or finger modules. It is possible that the latter two modules cooperate to form a single domain responsible for transition 5. However, EGF itself is extremely stable, requiring the presence of a denaturant for its melting to be observed (18). The same is true for several finger modules in fibronectin (12). Thus, it is necessary to test for the possibility that either the finger or the EGF-like domain in rtPA is also extremely stable and therefore not observed in the above measurements.

**Further Examination of the Finger/EGF Region**—In 6 M GdmCl, pH 3.4, all modules associated with the previously measured transitions would be expected to be unfolded at room temperature. When rtPA and the 31/35-kDa fragments were heated under these conditions, a new endotherm, designated transition 6, with a midpoint near 60 °C and an enthalpy of 26–28 kcal/mol was obtained (Fig. 5, Table II). This endotherm is well described by a two-state transition indicating the melting of an extremely stable cooperative unit. It can thus be concluded that transitions 5 and 6 reflect the separate melting of the finger and EGF domains, but their assignment remains to be determined.
Domain Structure of Tissue Plasminogen Activator

TABLE II

<table>
<thead>
<tr>
<th>Protein or fragment no.</th>
<th>Transition number</th>
<th>Total curve</th>
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</thead>
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<tr>
<td></td>
<td>$T_m$</td>
<td>$\Delta H$</td>
</tr>
<tr>
<td>-------------------------</td>
<td>-------</td>
<td>-------------</td>
</tr>
<tr>
<td>1. rtPA (single chain)</td>
<td>45.9</td>
<td>48</td>
</tr>
<tr>
<td>2. 50-kDa subtilisin fragment</td>
<td>46.8</td>
<td>48</td>
</tr>
<tr>
<td>3. 32/35-kDa subtilisin fragment</td>
<td>51.3</td>
<td>62</td>
</tr>
<tr>
<td>4. 31-kDa pepsin fragment</td>
<td>48.0</td>
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</tr>
<tr>
<td>5. 35-kDa pepsin fragment</td>
<td>38.6</td>
<td>31</td>
</tr>
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<td>6. 19/23-kDa pepsin fragment</td>
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</tr>
<tr>
<td>7. AFAE-rtPA</td>
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<td>8. rtPA (single chain) in 6 M GdmCl</td>
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<td>9. 32/35-kDa pepsin fragment in 6 M GdmCl</td>
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</table>

**Assignment**

- **SP-N**
- **K2**
- **K1**
- **SP-C**
- **EGF**
- **FINGER**

| Transition enthalpy ($\Delta H$) is given in kcal/mol. Transition temperature ($T_m$) is given in °C.
| GdmCl, guanidinium chloride.
| The enthalpy is not corrected for GdmCl binding.

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Fig. 6. **Unfolding of fluorescein-maleimide-labeled rtPA.**

**A.** Titration with GdmCl while monitoring the fluorescence anisotropy; B, heating at 1 °C per min in the presence of 0, 1, 2, or 6 M GdmCl while monitoring the fluorescence intensity.

To gain additional insight into which of the two domains, finger or EGF, is responsible for transition 6, the free SH group at position 83 in the EGF domain of rtPA was selectively labeled with fluorescein maleimide. Fluorescence measurements were made at neutral pH because of the quenching effect of acid on the probe. Titration with GdmCl while monitoring the fluorescence anisotropy produced a single transition which has a midpoint near 3.5 M and is complete by 5 M GdmCl (Fig. 6A). When heated in HEPES buffer at pH 7.4 while monitoring fluorescence intensity (Fig. 6B), a single transition was observed with a midpoint near 76 °C in the absence of GdmCl, very close to the position of transition 5 observed by DSC under similar conditions, and shifting to lower temperature with increasing denaturant. In 6 M GdmCl, where transition 6 was seen by calorimetry, no transition was detected by the fluorescence label. This suggests that the EGF domain is responsible for transition 5, leaving the finger domain as the only candidate for transition 6.

**Domain-Domain Interactions in rtPA**—Having established the independent folding of individual modules in rtPA, what can be said about the interaction between them? From the results presented above, it follows that the K1 and K2 domains do not interact with each other or with other domains. However, the repertoire of fragments obtained by proteolysis did not yield information about other possible interactions, for example between the SP domains and the EGF or finger domains. Additional measurements were therefore made with a recombinant deletion variant, AFAE-rtPA, lacking the finger and EGF domains. Fig. 7 compares results obtained with the variant to those obtained with whole rtPA under the same conditions. At pH 7.4 the variant yields the same number of transitions as the whole protein, even though the EGF domain responsible for transition 5 in rtPA is missing from the variant. Transitions 2 and 3, corresponding to the kringle domains, appear to be unaffected by the deletions (see also Table I, lines 1 and 6). The other two transitions in the variant must therefore be due to the SP domains. Thus, removal of the finger and EGF domains at neutral pH has an effect that is analogous to that obtained with the intact protein by merely lowering the pH, i.e. uncoupling of the melting of the SP domains.

The **lower two panels** of Fig. 7 show that at pH 3.4, the variant again yields four transitions compared to five for the whole protein. The midpoints and enthalpies of the variant transitions are included in Table II. It is obvious that transition 5 in rtPA, the highest-temperature transition that was assigned to the melting of the EGF domain, is absent in the variant, as would be expected. The lowest transition, transition 1, corresponding to the SP-N domain, is present in both species and is slightly destabilized in the variant. Transition 4, which was assigned to the SP-C domain in rtPA, appears
Domain Structure of Tissue Plasminogen Activator

![Graph A](image1)

**FIG. 7.** Deconvolution analysis of the excess heat capacity function obtained with the ΔFAE-rtPA variant at pH 7.4 and 3.4 in comparison to rtPA under the same conditions. Transitions are assigned numbers correlating with those obtained with whole rtPA and other fragments as described in the text.

![Graph B](image2)

**FIG. 8.** Differential absorption measurements of rtPA (a) and ΔFAE-rtPA (b) as a function of pH. The gap in the curve for rtPA is due to excess turbidity in that pH range.

to be strongly destabilized in the variant where no transition of comparable stability is present. It is not immediately clear, however, to which transition it corresponds in the variant. Transition 3 with a \( T_m \) near 60 °C, corresponding to the melting of K1, was shown above to be extremely insensitive to the presence or absence of neighboring domains (see Table II) and is therefore identified with the highest transition in

ΔFAE-rtPA. The same is true for transition 2, corresponding to K2, whose melting temperature, as already mentioned, is not changed even when the module is expressed as an individual domain (9). Transition 2 is thus correlated with one of the two central transitions in the variant. This identifies the other central transition, number 4, with the melting of the strongly destabilized SP-C domain. Thus, removal of the finger and EGF domains results in decoupling and destabilization of the protease domains demonstrating a significant interaction between the latter and one or both of the former.

Additional evidence for an interaction between opposite ends of the rtPA molecule was first noticed during preparation of the 50-kDa fragment which tended to elute ahead of residual rtPA on Biogel P-60 columns at pH 4.5. Therefore, analytical exclusion chromatography measurements were made comparing the behavior of rtPA with that of ΔFAE-rtPA at neutral pH. It was found that the variant also eluted earlier than rtPA in spite of its lower mass. The absence of an intact SP-C domain in the fragment and of the finger and EGF domains in the variant eliminates the domain-domain interactions and allows them to adopt a more open conformation that is more effectively excluded from the gel matrix.

**Acid-induced Conformational Transition in the SP-C Domain**—The previous section revealed a significant difference between rtPA and the ΔFAE variant with respect to the stability of the SP-C domain at low pH. In rtPA, lowering the pH uncoupled the melting of the two SP domains and strongly destabilized the SP-N domain while having little effect on the SP-C domain. In ΔFAE-rtPA, where the SP domains are already uncoupled at neutral pH, lowering the pH caused a large 20 °C decrease in the \( T_m \) of the SP-C domain. Thus, the presence of the finger and EGF domains seems to protect the SP-C domain from the strong destabilizing influence of acid. One interpretation of this effect is that the SP-C domain, in the absence of the finger and EGF domains, undergoes an acid-induced transition to a less stable form. The two proteins, rtPA and ΔFAE-rtPA were therefore titrated with acid while monitoring the differential UV absorption spectrum for evidence of a conformational change. The results in Fig. 8 indicate the presence in both proteins of an acid transition with a midpoint near pH 6.5. The direction of the absorbance change is consistent with a net increase in the exposure of aromatic side chains to a more polar environment. However, the amplitude is much greater for the ΔFAE variant consistent with the idea that domain-domain interactions present in the whole protein prevent the changes responsible for most of the differential absorbance. The much smaller change observed with rtPA could be related to the uncoupling of the two SP domains, without the conformational change.

**DISCUSSION**

The melting of rtPA occurs in two temperature regions. Most of the structures melt between 50 and 90 °C at neutral pH. Some of these structures are destabilized upon lowering the pH to 3.4 where the melting occurs over a broader range, between 30 and 90 °C, with better reversibility and improved separation of the individual components. The curve at pH 3.4 deconvoluted into five two-state transitions representing five independently folded domains. A sixth domain is much more stable and requires the presence of a denaturant for its melting to be observed. The total enthalpy of rtPA is much higher than that reported by Radek and Castellino (13). Our value, confirmed on three different calorimeters, when added to the contribution from the high temperature transition, is reasonable for a compact globular protein of this size. Thus, all domains are accounted for.
The correlation between domains and modules is supported in a general sense by the observation that cleavage within a module, i.e. within a disulfide loop, tends to diminish or abolish the corresponding melting transition, whereas cleavage between modules preserves their compact structure. The same is true for the fingers in fibronectin (12) and the kringle domains of plasminogen (10). Studies with various fragments and with fluorescein-labeled rtPA allowed each of the two-state transitions derived from deconvolution analysis to be assigned to a specific module, as summarized in the last row of each table. The K1 and K2 and the smaller finger and EGF modules all constitute independently folded domains. The much larger serine protease module exhibits two transitions at low pH but only one at neutral pH. Furthermore, it was possible to remove most of the C-terminal half of the SP module without destroying the compact structure of the remaining half, as shown by the retention of transition 1 in the 50-kDa fragment (Fig. 4, A and B). Thus, the SP module comprises two domains which are relatively independent at pH 3.4 and 4.0 but which at neutral pH are merged like those in trypsin and chymotrypsin to form a "highly cooperative structure that melts in an all-or-none manner" (19). The serine protease module of plasminogen is similar to that of tPA (20) and, when examined in the calorimeter at low pH, also reveals two independently folded domains, one of which is destabilized upon activation to plasmin (10). In contrast, conversion of rtPA from the single-chain to the two-chain form had very little effect on the relative stability of the two SP domains at pH 3.4 (this work) and pH 7.4 (13). NMR measurements at pH 4 and different temperatures provided evidence for a two-domain structure of the SP module of urokinase (21), which is also highly homologous to those in tPA and plasminogen (20). Calorimetric results to be published elsewhere confirm the two-domain structure for the SP module of urokinase at pH 4.5. Whether the two halves of the SP modules in plasmin(ogen) and urokinase are merged into a single cooperative unit at neutral pH has not been determined. All of these SP modules are homologous to the pancreatic serine proteases whose three-dimensional structures suggest the presence of two merged structural domains even though they melt as single cooperative units (19, 22–23). Higaki and Light (24) studied the kinetics of active site regeneration in neotrypsinogens and proposed that the two halves of the molecule fold independently prior to recombining. However, they did not isolate the separate domains and prove that they were independently folded. The present work comes closer to that goal in that the independent folding of the N-terminal half of the SP domain of rtPA has been observed in the absence of an intact C-terminal half, i.e. in the 50-kDa fragment. It seems that the interaction between the constituent domains of serine proteases is quite variable, being easier to observe in some examples than in others.

The tPA finger is among the most stable of modules so far investigated. Recent studies of fibronectin showed that several of the fingers in this protein are also extremely stable with melting temperatures in the 40–70 °C range in 6 M GdmCl (12). Other fingers are more "normal," melting between 60 and 80 °C in the absence of denaturants. The EGF module is the next most stable module in rtPA and the present work provides the first calorimetric demonstration of the independently folded status and reversible unfolding of an EGF module in any protein. One or both of these modules interacts with one or both of the SP modules to have a dramatic effect on their stability and on the extent to which they cooperate in their unfolding. Removal of the finger/EGF region selectively destabilizes the SP-N domain while having little effect on the stability of the SP-C domain, an effect similar to that caused in the parent protein by simply lowering the pH (see Fig. 9). Destabilization of the SP-C domain requires the combined effects of low pH and removal of the finger and EGF domains, whereas destabilization of the SP-N domain is caused by either manipulation. In other words, the SP-C domain is intrinsically sensitive to acid, but this sensitivity is manifested only in the absence of the stabilizing influence of the finger/EGF region with which it interacts. This is consistent with spectral measurements revealing an acid transition, analogous to one observed by others in trypsin (25), that was much more prominent in the variant lacking the finger/EGF region. One possibility is that titratable groups that are important for the stability of the SP-C domain are masked by interaction with the finger/EGF domain(s).

Our results don't distinguish whether the finger, EGF, or both domains are involved in this interaction. Radek and Castellino (13) presented DSC data for a variant lacking only the EGF domain that are qualitatively similar to ours for the ΔFAE-rtPA variant. They suggested that the EGF domain may play a role in the overall stability. On the other hand, the much more stable finger domain is also a good candidate, especially in view of the speculation offered by Baron et al. (26) based on their elucidation of the three-dimensional structure of a homologous finger domain from fibronectin. These authors noted that the rtPA finger, if modeled after their expressed fibronectin finger, would have a large hydrophobic surface that might interact with one or more other modules. We suspect that such interaction would involve the SP-N and SP-C domains since both are affected by removal of the finger/EGF region, a manipulation that also increased the hydrodynamic volume providing independent evidence for an interaction.

The fact that all modules in rtPA form independently folded domains is consistent with the results of numerous experiments demonstrating the functional autonomy of these modules. However, attempts to engineer an improved fibrinolytic agent by deletion, insertion, or swapping of domains have not been as successful as hoped. One of the more promising approaches was to combine the catalytically superior SP module of urokinase with the fibrin-binding properties of the tPA heavy chain to obtain chimeric hybrids having the best of both molecules (5, 6). Although some of the products were

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indeed more active than either parent alone, their affinity and specificity for fibrin was consistently much lower than that of rtPA. In at least one case, this was attributed to improper folding of some of the rtPA heavy chain domains that are involved in fibrin binding, as evidenced by measurements with a monoclonal antibody that recognized the A chain in rtPA but not in the hybrids (6). This antibody also inhibited the ability of rtPA to activate plasminogen in the presence of a fibrin substitute while having no effect on the activity of the hybrids. The fibrin binding function of rtPA appears to involve the second kringle as well as the finger domain (4, 7, 27–31). Is it possible that the interaction of the SP module of tPA with the EGF/finger region, as documented here, affects the interaction with fibrin in a significant way that can not be duplicated by the SP domain of uPA? Evidence for such communication between domains comes from a recent study (32) with another monoclonal antibody, raised against amino acid residues 4–8 of tPA, that reacted with tPA only after its SP domain had formed a complex with α1-antitrypsin. On the other hand, removal of the finger and EGF domains has only marginal effects on the catalytic activity measured with peptide substrates or with plasminogen substrate in the absence of fibrin-like stimulators (4, 30, 31, 33). Further, Markland et al. (34) reported that the SP domain could be relocated to the amino terminus of the molecule with no effect on fibrinolytic activity. These latter observations suggest that the functional significance of domain-domain interactions in rtPA is either extremely subtle or manifested in a way not yet discerned.

The main conclusions of this work are summarized by the schematic illustration in Fig. 9. Recombinant tPA has been shown to comprise six independently folded domains. Two of these domains correspond to the kringle modules whose thermodynamic properties are similar to those of the kringles in these domains correspond to the kringle modules whose thermodynamic properties are similar to those of the kringles in fibrin-like stimulators tide substrates or with plasminogen substrate in the absence of another monoclonal antibody, raised against amino acid residues 4-8 of tPA, that reacted with tPA only after its SP domain had formed a complex with al-antitrypsin. On the other hand, removal of the finger and EGF domains has only marginal effects on the catalytic activity measured with peptide substrates or with plasminogen substrate in the absence of fibrin-like stimulators (4, 30, 31, 33). Further, Markland et al. (34) reported that the SP domain could be relocated to the amino terminus of the molecule with no effect on fibrinolytic activity. These latter observations suggest that the functional significance of domain-domain interactions in rtPA is either extremely subtle or manifested in a way not yet discerned.

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REFERENCES