Tissue-Type Plasminogen Activator Domain-Deletion Mutant BM 06.022: Modular Stability, Inhibitor Binding, and Activation Cleavage†

Chih-Kao Hu,‡ Ulrich Kohnert,§ Olaf Wilhelm,‖ Stephan Fischer,§ and Miguel Llinás*,‡

Department of Chemistry, Carnegie Mellon University, Pittsburgh, Pennsylvania 15213, Boehringer-Mannheim GmbH, Werk Penzberg, D-82377 Penzberg/Obb., Germany, and Frauenklinik, Technische Universität München, D-81675 München, Germany

Received May 31, 1994; Revised Manuscript Received July 18, 1994®

ABSTRACT: Recombinant BM 06.022 (M_r 39 589) is a domain-deletion mutant of the human tissue-type plasminogen activator (tPA) structured by the kringle 2 and protease modules. Unfolding under various conditions was investigated via ^{1}H-NMR spectroscopy by monitoring the well-resolved high-field methyl resonances at ~−0.97 ppm (kringle 2) and ~−0.29 and −0.54 ppm (protease). Reversible acid/base unfolding is manifest under low pH (<4.8) conditions. It is observed that, relative to the protease, the kringle exhibits higher overall stability at low pH. At pH* 4.6, BM 06.022 undergoes two distinct thermal melting transitions, at ~334 and ~352 K, assigned to an irreversible denaturation of the protease and a reversible unfolding of the kringle 2, respectively. Under the same conditions, the protease reacted with the active site inhibitor 1,5 dansyl-L-glutamylglycyl-L-arginine chloromethyl ketone (EGRck) exhibits a higher (~10 K) thermal stability than the inhibitor-free protease. Upon acidification, the EGRck-modified protease unfolds irreversibly around pH* 3.4. As exemplified by BM 06.022, a single-chain protein, as defined by continuity of the polypeptide backbone, can exhibit simultaneous folding reversibility and irreversibility for autonomous segments of the sequence. Conversion of the isolated (single-chain) protease or intact BM 06.022 to their catalytically active two-chain forms via plasminolytic cleavage of the Arg275–Ile276 peptide bond leaves the kringle 2 spectrum unaffected while perturbing the resolved high-field methyl resonances stemming from the protease. The latter also shift when the protease is reacted with EGRck, indicating that these signals are sensitive to events at the binding pocket. It is suggested that activation cleavage converts the protein from a "tight" to a "relaxed" state such that the conformation of the latter, favorable for catalysis, can be imposed upon the single-chain protease by forcefully binding a covalent inhibitor (substrate analog) to the active site.

An important step in physiological fibrinolysis is the activation of plasminogen to plasmin which, in turn, degrades the fibrin matrix of blood clots. This step is catalyzed by tissue-type plasminogen activator (tPA),[1] a mosaic protein consisting of five distinct modules: the finger domain (Ser1–His44), an epidermal growth factor-like domain (Ser50–Asp87), two kringle domains (Cys92–Cys173 and Cys180–Cys261), and the C-terminal protease (Cys264–Pro527) which affords the catalytic module proper (Pennica *et al.*, 1983; Bányai *et al.*, 1983; Ny *et al.*, 1984). tPA is a trypsin-like serine proteinase that favors peptide bond hydrolysis on the C-side of Arg and Lys residues, with Asp371, His322, and Ser478 configuring the catalytic triad (Pennica *et al.*, 1983). It consists of a single-chain (sc) polypeptide of 527 amino acid residues which upon activation via hydrolysis of the Arg275–Ile276 peptide bond assumes a two-chain (tc) form. The tc-tPA exhibits a relatively high catalytic efficiency against peptide substrates, and its inhibition by peptidyl chloromethyl ketones is much faster than with the sc form (Andreasen *et al.*, 1991). The binding of tPA to blood clots is attributed to interactions of the kringle 2 and finger domains with fibrin (van Zonneveld *et al.*, 1986; Verheijen *et al.*, 1986; Gething *et al.*, 1988).

The protease module of tPA exhibits about 35–40% homology with prototypic serine proteases such as trypsin and chymotrypsin, with the highest homology within the regions flanking the active site residues His322 and Ser478. The regions of lowest homology are around sites 298, 330, and 470, for which there are no corresponding residues in the prototypes. Synthetic competitive inhibitors for enzymes of the trypsin family have been found among Arg and Lys derivatives and among structurally related benzamidines (Stürzebecher *et al.*, 1976). 1,5 dansyl-L-glutamylglycyl-L-arginine chloromethyl ketone (EGRck) is a serine protease inhibitor which reacts to form a covalent, active site inhibited derivative (Kettner & Shaw, 1979).

Several variants of tPA have been investigated in order to develop fibrinolytic agents with improved pharmacological properties [reviewed by Haber *et al.* (1989), Higgins and Bennett (1990), and Robinson and Browne (1991)]. BM 06.022 (Figure 1) is a domain-deletion variant of human tPA comprising the kringle 2 and the protease modules only, tPA*del*(V4–E175) (Pannekoek *et al.*, 1990). It is produced as inactive inclusion bodies in *Escherichia coli* and converted into the native form by an *in vitro* refolding process (Kohnert *et al.*, 1992). Analogous constructs have been expressed by other investigators (Burck *et al.*, 1990; Obukowics *et al.*, 1990) and suggested to be suitable for structure–function studies as the protein is uncomplicated by the effects of glycosylation.

† This research was supported by Boehringer-Mannheim GmbH and by the U.S. Public Health Service, NIH Grant HL-29409.

‡ Carnegie Mellon University.

§ Boehringer-Mannheim GmbH.

‖ Frauenklinik, T.U.M.

® Abstract published in *Advance ACS Abstracts,* September 1, 1994.

[1] Abbreviations: BM 06.022, single-chain tPA*del*4–261; EGRck, 1,5-dansyl-L-glutamylglycyl-L-arginine chloromethyl ketone; pH*, glass electrode pH reading uncorrected for the deuterium isotope effect; sc, single chain; tc, two chain; tPA, human tissue-type plasminogen activator; uPA, human kidney-type plasminogen activator (urokinase).

0006-2960/94/0433-11760$04.50/0

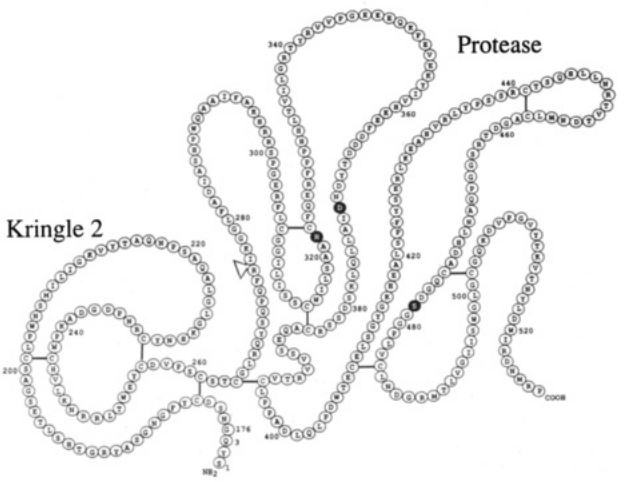

FIGURE 1: Primary structure of BM 06.022, segment 1–3 + 176–527 of tPA. Disulfide bridges are indicated by solid lines. Amino acid residues are labeled according to the single-letter code and follow the numbering of Pennica *et al.* (1983). The activation cleavage site (Arg275–Ile276 bond) (Δ) and the catalytic triad residues (reverse, white on black print) are indicated. The recombinant kringle 2 and protease modules investigated in this project are composed of the Met-Tyr-Gly176-to-Cys261 and Ser-Tyr-Gln-Ser262-to-Pro527 sequences, respectively.

Despite the deletion of the three N-terminal domains of native tPA, which affects the kinetics of plasmin formation (Kohnert *et al.*, 1993), the properties of the kringle 2 and the protease modules of BM 06.022 and tPA are rather similar (Kohnert *et al.*, 1992; Stürzebecher *et al.*, 1992).

In this paper we present a study of BM 06.022 focused on its folding stability under various conditions. As reported for urokinase (Bogusky *et al.*, 1989; Oswald *et al.*, 1989) and miniplasminogen (Teuten *et al.*, 1991, 1993), we have found that, despite the size of the protein (M_r 39 589), simple one-dimensional ^{1}H-NMR experiments can reveal unambiguous features of the conformational transitions, such as their assignment to specific domains, information that can be difficult to extract from calorimetry data. Via ^{1}H-NMR we have investigated BM 06.022 both with and without covalently bound EGRck, and we have compared the results with those obtained from intact recombinant kringle 2 and protease modules. The inactive EGRck-modified proteins afford convenient control derivatives to screen for potential self-proteolysis during the unfolding experiments. In the process, we have uncovered evidence pointing to a change in the conformational state of the protease module upon conversion from the sc to the catalytically active, tc form.

MATERIALS AND METHODS

BM 06.022 (Figure 1) and the protease module (tPA*del*4–261) were expressed as inclusion bodies in *E. coli*. The inactive molecules were transformed into the native structures by an *in vitro* refolding process and purified by affinity chromatography on ETI-Sepharose (Kohnert *et al.*, 1992). Recombinant kringle 2 domain (Gly176–Cys261 of native tPA, with an additional Met-Tyr segment at the N-terminus) was prepared according to Wilhelm *et al.* (1990). BM 06.022 and the protease module were reacted with EGRck (Calbiochem) according to the method of Higgins and Lamb (1986). The proteins were converted to their two-chain forms via hydrolytic cleavage with immobilized plasmin (Kohnert *et al.*, 1992). Benzamidine originated from Aldrich, Milwaukee, WI. All protein samples were dialyzed against distilled water, pH ~7.0, and passed through a G-50 Sephadex column (20–80 μm, Sigma, St. Louis) before use for the NMR experiments.

For the NMR experiments the protein samples were lyophilized and dissolved in 0.4 mL of ^2H_2O (99.996 atom % ^{2}H, Isotec Inc., OH) to a final concentration of 0.4–0.6 mM, and the pH was adjusted by additions of dilute ^{2}HCl or NaO^{2}H. Quoted pH* values are direct glass electrode meter readings, uncorrected for the ^{2}H isotope effect. Chemical shifts are referred to the sodium 3-(trimethylsilyl)(2,2,3,3-$^2H_4$)-propionate signal, using *p*-dioxane as an internal standard (De Marco, 1977). Probe temperature was calibrated with an ethylene glycol standard. ^{1}H-NMRspectra were recorded at 500 MHz on a Bruker AM-500 spectrometer equipped with an Aspect 3000 minicomputer. The data were processed using the program FELIX, version 2.10 (Biosym, CA). The residual solvent ^{1}H^{2}HO signal was suppressed by gated low-power irradiation during the relaxation delay of 1.5–3.0 s introduced between scans. Data were collected in the quadrature detection mode with a spectral width of 6500 Hz. A total of 16K data points were collected for each experiment. Between ~4000 and 8200 scans were combined for acceptable signal-to-noise. Prior to Fourier transformation, the data were zero-filled to 32K points and subjected to a slight digital filtering.

RESULTS

Expansions of the low-field (A) and high-field (B) ^{1}H-NMR spectra of intact BM 06.022 (a), and of the isolated protease (b) and kringle 2 (c) modules, are shown in Figure 2. The spectral linewidths are in the order (a) > (b) > (c), reflecting the proteins' relative molecular weight (M_r 39 589, 30 160, and 9763, respectively). By comparing the various spectra, the methyl doublet shifted to ~–0.97 ppm can be assigned to the Leu226 δ-methyl group in kringle 2 (Byeon *et al.*, 1989). Similarly, the two broad resonances at ~–0.29 and –0.54 ppm in BM 06.022, which become three resolved signals at ~–0.28, –0.37, and –0.48 ppm in the EGRck-modified BM 06.022 (see below), arise from the protease. K2 and P labels identify these and other kringle 2 and protease resonances, respectively, in Figure 2 and elsewhere.

Thermal Unfolding Studies. Figure 3 B displays the high-field, methyl region of the intact protease. The two resonances at ~–0.29 and –0.54 ppm lose intensity upon raising the temperature to become undetectable at ~328 K. Beyond this point, the spectrum remains essentially unaltered and is not recovered upon lowering the temperature (upper trace, 305 K). Thus, at pH* 4.6, the protease undergoes irreversible thermal unfolding. In order to avoid cumulative effects of denaturation in the course of the thermal unfolding experiments, the protease samples were freshly dissolved in ^2H_2O before recording each ^{1}H-NMR spectrum at the different temperatures. Although at pH < 5.5 the catalytic activity of BM 06.022 is negligible (U. Kohnert, unpublished observations), a separate experiment was run in which a stoichiometric amount of benzamidine was added in order to preclude self-proteolyis as a potential factor contributing to protein degradation. Benzamidine is a mild BM 06.022 inhibitor (Stürzebecher *et al.*, 1992). In the presence of benzamidine the protease spectrum showed similar characteristics to those it exhibits in the absence of inhibitor. Furthermore, while benzamidine stabilized the protease against gradual degradation at constant temperature (311 K), the overall temperature profile of BM 06.022 remained essentially unaltered. Hence, denaturation of the protease is concomitant to its thermal unfolding. In the aromatic region (not shown), most of the

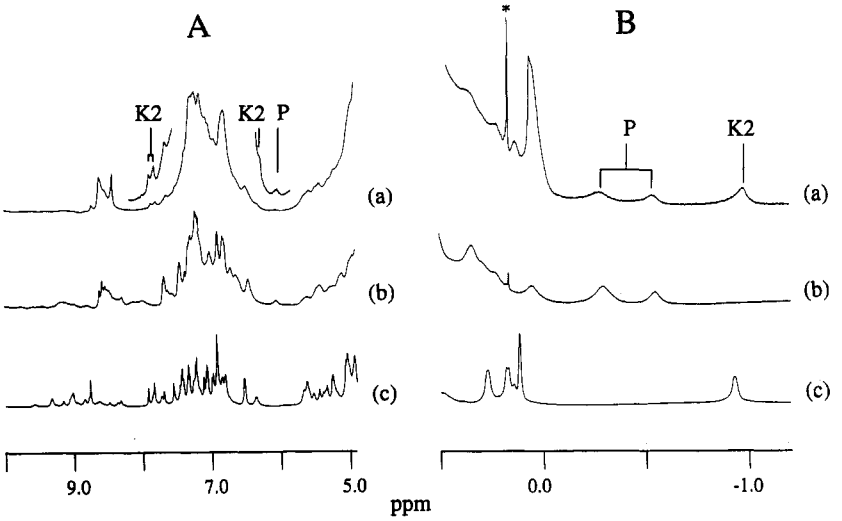

FIGURE 2: ^{1}H-NMR spectra of BM 06.022 (a) and the isolated protease (b) and kringle 2 (c) modules. (A) Low-field region; (B) high-field region. Resonances stemming from the protease and kringle 2 modules are indicated by "P" and "K2", respectively. An asterisk (*) indicates an impurity peak. Resonances stemming from partially exchanged amide NH protons are apparent at $\delta > 8$ ppm. Vertical expansion traces are shown above spectrum A (a). Protein concentrations are 0.4–0.6 mM in ^2H_2O; spectra recorded at pH* 4.6, 311 K.

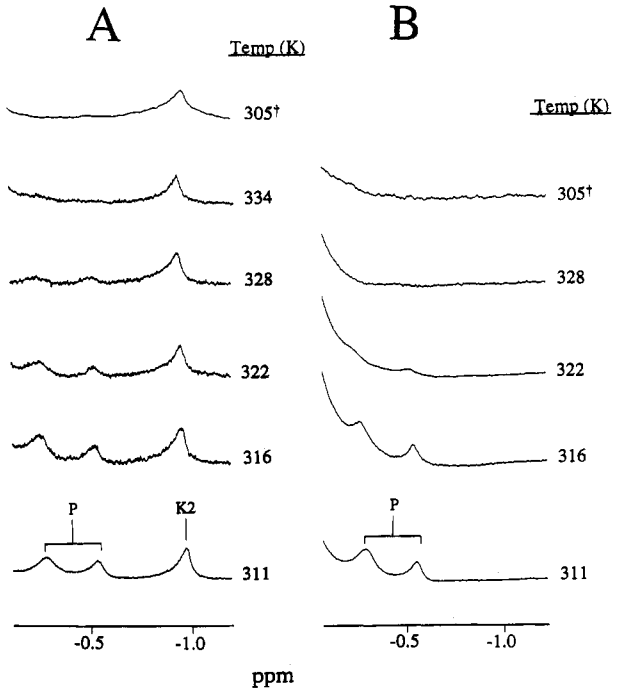

FIGURE 3: High-field region ^{1}H-NMR spectrum of intact BM 06.022 (A) and the protease module (B): temperature profiles. "K2" and "P" label methyl resonances from kringle 2 and protease, respectively. The top traces (†) show spectra recorded at 305 K after the protein had been thermally unfolded. Except for temperature (indicated), the experimental conditions are as for Figure 2.

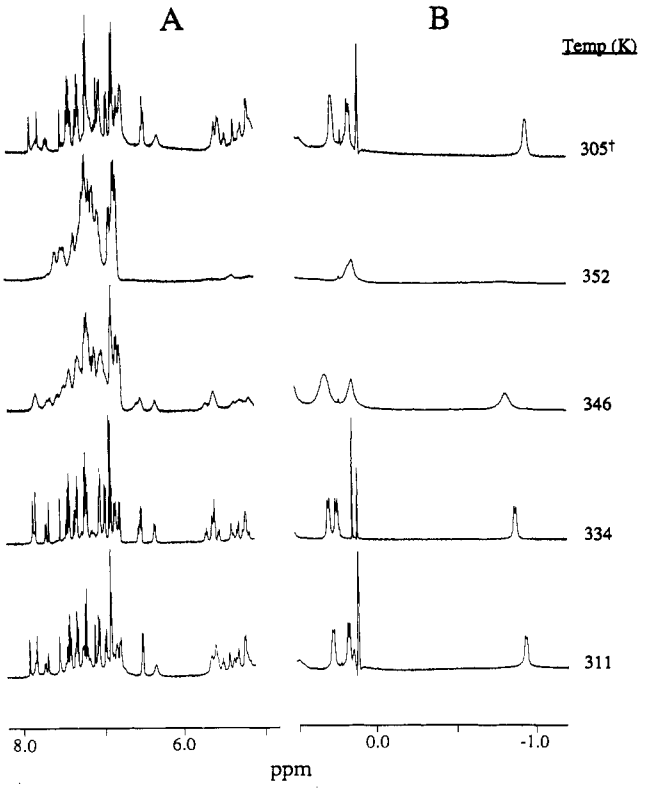

FIGURE 4: ^{1}H-NMR spectra of the kringle 2 domain: temperature profile. Low- (A) and high- (B) field spectral regions are shown expanded. The top traces (†) show spectra recorded at 305 K after the protein had been thermally unfolded. Except for temperature (indicated), the experimental conditions are as for Figure 2.

resonances between 5 and 6 ppm disappear, and at 328 K, the spectrum exhibits characteristics typical of a random coil polypeptide.

High-field spectra of BM 06.022 at various temperatures, pH* 4.6, are shown in Figure 3A. Upon raising the temperature, the protease resonances decrease their amplitudes while shifting to lower fields, toward the random coil spectral positions, thus indicating gradual unfolding. In contrast, up to 334 K, the kringle 2 resonances are essentially unaffected. Thus, two distinct transitions for BM 06.022 become apparent: one for the protease domain, which is mostly unfolded at ~334 K, and another for the kringle, which melts at ~352 K, implying that the two modules unfold independently. Subsequent gradual cooling after melting the protein (top trace, 305 K) anneals the kringle domain to its native conformation, as indicated by the reappearance of the Leu226 δ-methyl group at its original chemical shift (~–0.97 ppm). In contrast, resonances from the protease fail to recover. The invariance of the kringle 2 linewidths with temperature suggests that aggregation effects are not significant.

Figure 4 shows the temperature dependence of the kringle 2 spectrum. In the high-field region (Figure 4B) resonances shift to the low fields and, above 334 K, gradually broaden and decrease their intensities. It is apparent by monitoring both the high-field methyl resonances, as well as the low-field

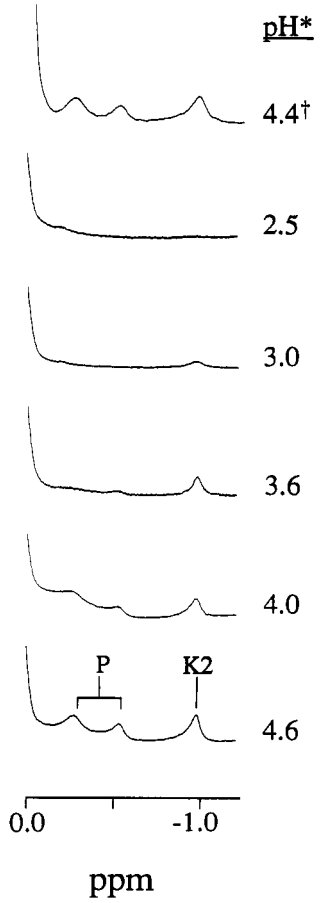

FIGURE 5: High-field region ^{1}H-NMR spectra of BM 06.022: acidic pH profiles. **"K2"** and **"P"** label methyl resonances from kringle 2 and protease, respectively. The top traces (†) show spectra recorded at 311 K after the protein had been acid-unfolded. Except for pH* (indicated), the experimental conditions are as for Figure 2.

aromatic region (Figure 4A), that the kringle is completely unfolded at ~352 K. Subsequent cooling to 305 K (upper trace) recovers the native form spectrum. These results are in agreement with the melting profile of the isolated kringle 2 domain as determined by differential scanning calorimetry (Kelley & Cleary, 1989). Hence, under the experimental conditions, the thermal unfolding of the kringle is reversible whether or not it is part of the intact BM 06.022 (Figures 3A and 4). In contrast, a distinct behavior is exhibited by the protease module. Comparison of BM 06.022 and intact protease spectra (Figure 3, panels A and B) reveals that while the isolated protease is completely denatured at 328 K, a measurable fraction remains folded in the intact BM 06.022 at the same temperature. This difference suggests a stabilization of the protease structure in BM 06.022 and hints at domain–domain interactions in the intact protein.

Stability against Acidic Conditions. The stabilities of BM 06.022 and of its component modules to acidic pH were investigated at constant temperature (311 K). The pH profile of the high-field spectrum of BM 06.022 is shown in Figure 5. As the pH* is lowered from 4.6 to 3.4, the signals stemming from the protease domain proper broaden (suggesting conformational exchange between native and unfolded forms), decrease their intensities, and eventually disappear from this section of the spectrum. In contrast, the kringle 2 Leu226 methyl resonance at ~–0.97 ppm remains distinct down to pH* 3.4 to fade out at pH* ~2.5. Thus, relative to the kringle 2 domain, folding of the protease is less resistant to acidic conditions. In a separate experiment, where BM 06.022 was

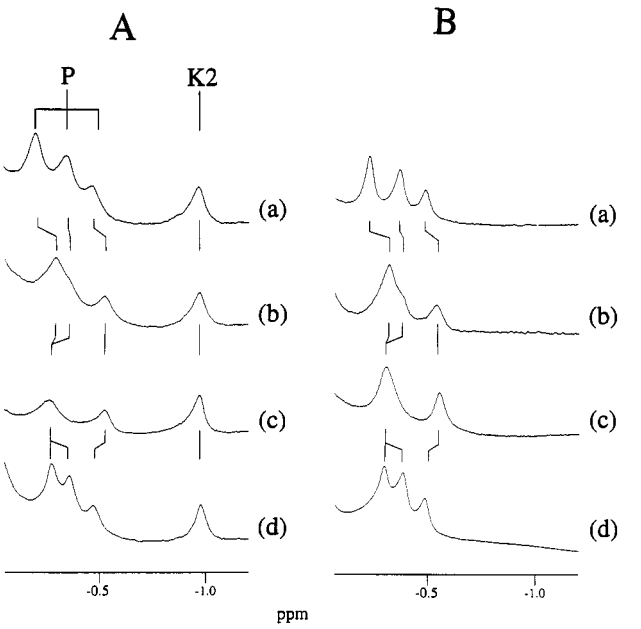

FIGURE 6: High-field region ^{1}H-NMR spectra of BM 06.022 (A) and protease (B), in their single-chain (c, d) and two-chain (a, b) forms. (d) and (a) are spectra for the proteins reacted with EGRck. **"K2"** and **"P"** label methyl resonances from kringle 2 and protease, respectively. Resonance shifts induced by reaction with EGRck or conversion from single-chain to two-chain forms are indicated by kinked vertical lines. Experimental conditions are as for Figure 2.

dissolved at pH* 2.8 and the pH* subsequently raised to 4.6, the spectra of both the protease and the kringle were recovered (Figure 5, upper trace). Therefore, the acidic unfolding of BM 06.022 is fully reversible.

Control experiments on the intact kringle 2 and protease modules demonstrated that the acidic unfoldings of the isolated units are also reversible and identical to the acidic unfolding of the corresponding modules in BM 06.022. This contrasts the pattern of cooperativity indicated by the thermal unfolding discussed above, which suggests that the kringle 2–protease interaction is mediated by interdomain hydrophobic forces rather than by electrostatic effects.

Effects of Inhibitor Binding. Figure 6B illustrates the spectrum of the protease module reacted (trace d) and unreacted (trace c) with the active site inhibitor EGRck. A similar experiment on the intact BM 06.022 is shown in Figure 6A (traces d and c). While the kringle 2 methyl resonance at ~–0.97 ppm is left unperturbed, it is observed that binding of EGRck affects the methyl groups resonating at ~–0.5 ppm, verifying, as advanced above, the assignment of these signals to methyl groups within the protease module.

Upon raising the temperature, most of the native structure resonances from the EGRck-protease remain detectable at 334 K (not shown), which is at least 10 deg higher than for the native, unreacted protease module (Figure 3B). Hence, the protease whose catalytic site is covalently occupied with the substrate analog is thermally more stable than the unmodified protein. Similarly, the thermal unfolding of the EGRck-modified protease within the intact BM 06.022 occurs between 334 and 340 K, while for the unmodified BM 06.022 the protease transition is observed below 334 K (Figure 3A). In contrast, the kringle 2 unfolding within BM 06.022 (Figure 3A) is essentially unaffected by reacting BM 06.022 with EGRck. It follows that the two domains of BM 06.022 unfold independently and that stabilization of one, *e.g.*, the protease with EGRck, leaves the other unaffected.

Table 1: Results from Acid and Thermal Unfolding Experiments on BM 06.022 and the Isolated Recombinant Modules

	unfolding[a]	
	acid	thermal
kringle 2	reversible (pH* <2.5)	reversible (T > 345 K)
sc-protease	reversible (pH* ~ 3.4)	irreversible (T < 328 K)
BM 06.022	protease: reversible (pH* ~3.4) kringle 2: reversible (pH* <2.5)	irreversible (T ~ 334 K) reversible (T > 345 K)
tc-protease	irreversible (pH* ~3.2)	irreversible (T ~ 330 K)
tc-BM 06.022	protease: irreversible (pH* ~3.2) kringle 2: reversible (pH* <2.5)	irreversible (T ~ 330 K) reversible (T ~ 342 K)
EGRck sc-protease	irreversible (pH* ~3.4)	irreversible ($T \leq$ 340 K)
EGRck BM 06.022	protease: irreversible (pH* ~3.4) kringle 2: reversible (pH* <2.5)	irreversible ($T \leq$ 340 K) reversible (T > 345 K)
EGRck tc-protease	irreversible (pH* ~3.2)	irreversible (T ~ 340 K)
EGRck tc-BM 06.022	protease: irreversible (pH* ~3.2) kringle 2: reversible (pH* <2.5)	irreversible (T ~ 340 K) reversible ($T \geq$ 342 K)

[a] Temperature (at pH* 4.6) or pH* (at 311 K) mid-transition points are indicated in parentheses.

Upon acidification, BM 06.022 reacted with EGRck shows two unfolding transitions at 311 K (not shown). The first one, at pH* ~3.4, represents the transition for the modified protease domain; the second transition occurs at pH* < 2.5 and stems from the kringle 2 structure. Expectedly, upon increasing the pH*, the kringle 2 refolds. However, in contrast to what is observed for the unmodified protein, the signals characteristic of the native EGRck-protease module do not reappear. Hence, EGRck binding, which at pH* 4.6 stabilizes the protease against thermal unfolding, interferes with the protease refolding at acidic pH while leaving the kringle 2 refolding essentially unperturbed. A control experiment on the intact, isolated EGRck-modified protease module shows a consistent behavior in that, upon raising the pH*, the high-field protease signals fail to reappear at their original chemical shifts. This demonstrates that the protease reacted with the covalent inhibitor unfolds irreversibly at low pH*, a result that verifies the interpretation given above for EGRck-BM 06.022.

Because the kringle folding process is largely unaffected by the state of the protease in BM 06.022, namely, whether the latter was reacted or unreacted with the inhibitor, one is led to conclude that under acidic conditions the two modules follow rather independent folding pathways. It also reinforces the hypothesis of physical autonomy for the kringle 2 and protease modules in intact BM 06.022.

Repercussions of Single-Chain to Two-Chain Conversion. An exhaustive study of the inhibition of tPA and BM 06.022 with benzamidine and a variety of benzamidine derivatives has shown that, relative to the single-chain proteins, the inhibition is facilitated (lower K_i values) when in two-chain forms (Stürzebecher *et al.*, 1992). This implies that cleavage of the Arg275–Ile276 bond causes a change in the conformational state[2] of the proteins which facilitates access of the inhibitor to the catalytic site. A similar effect has been reported for the kinetics of reaction of tPA with peptidyl chloromethyl ketones (Andreasen *et al.*, 1991).

As discussed above, we observe that upon reaction with the substrate analog EGRck the protease methyl signals at ~–0.29 ppm (area approximately two methyl resonances) and at ~–0.54 ppm (area approximately one methyl resonance) shift to generate three well-resolved methyl resonances at ~–0.28, –0.37, and –0.48 ppm in both the modified protease (Figure 6B, traces c and d) and BM 06.022 (Figure 6A, traces c and d) spectra. By echoing events at the protease active site, these effects suggest that the three methyl groups belong to the constellation of side chains surrounding the catalytic site. In view of the effect of protease activation on the accessibility of benzamidyl compounds to the active site, one would predict that conversion of the protease (and of BM 06.022) from sc to tc is likely to perturb at least some of the protease methyl groups whose signals we are monitoring. Comparison of Figure 6B, spectrum b, corresponding to the tc-protease, against spectrum c, corresponding to the sc-protease, or of the corresponding spectra of BM 06.022, Figure 6A, (b) and (c), shows that indeed the methyl resonance at ~–0.29 ppm shifts and splits into two partially overlapping resonances upon activation. This suggests a conformational drift of the protein upon conversion from sc form to tc form that enables the protease catalytic site to adjust itself to a conformation closer to the one it is driven to assume upon substrate binding. Indeed, subsequent reaction of tc-BM 06.022 or tc-protease (Figure 6A,B, spectra b) with EGRck (spectra a) affects the chemical shifts of these same methyl groups, as expected from the observations on the sc forms (spectra c and d). However, by comparison to what is observed for the latter, the spectral effect of ligand occupancy of the catalytic site is qualitatively more pronounced for the tc forms. Thus, it is suggested that upon irreversible EGRck binding the tc-protease module is more prone to adjust itself to the transition state conformation than is the sc form, as if the "tight" conformation of the sc-protease became "relaxed" upon conversion to the tc form so that the structural accommodation required for ligand binding is facilitated in the latter. This affords the first NMR spectroscopic evidence for a change in the conformational state of the protein upon conversion from thr sc to the tc form. Furthermore, as is the case for the sc forms, the protease module in the EGRck-reacted tc proteins unfolds irreversibly under both acidic and high-temperature conditions (not shown). Table 1 summarizes all the information regarding stability of the various forms of the recombinant proteins studied in this project.

DISCUSSION

Recent studies by Dobson and co-workers have shown that urokinase, the two-chain kidney-type plasminogen activator

[2] *Conformational state* refers to the conformation, stability of a given conformation, or both (Llinás, 1973). Thus, a change in the conformational state of a protein does not necessarily imply a significant or measurable change in the structure.

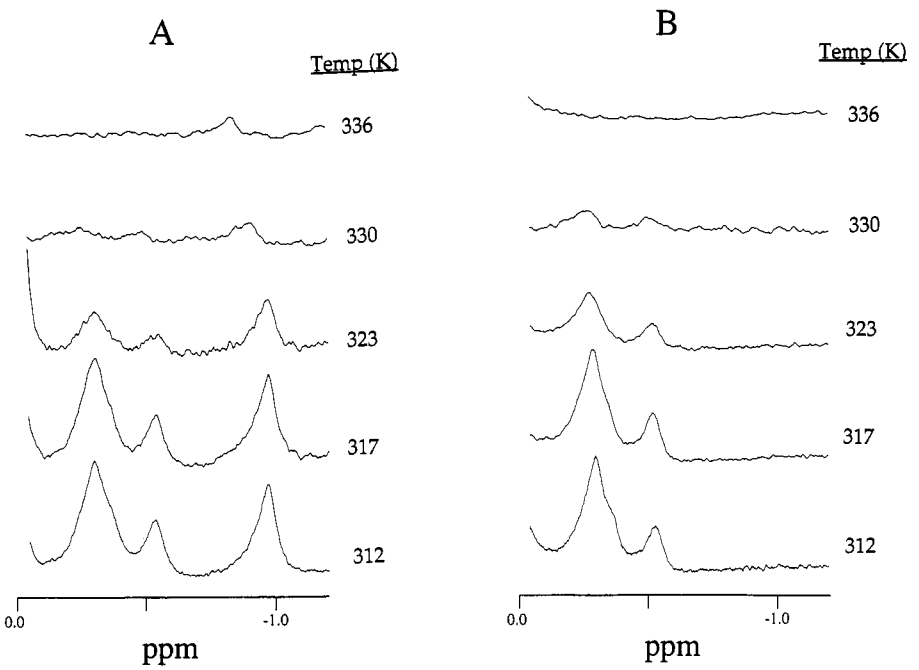

FIGURE 7: High-field region ^{1}H-NMR spectrum of tc-BM 06.022 (A) and the tc-protease module (B): temperature profiles. Resonances are identified above spectra b in Figure 6. Except for temperature (indicated), the experimental conditions are as for Figure 2.

(tc-uPA), reacted with EGRck exhibits independent thermal unfolding under acidic conditions for its various modules: growth factor, kringle, and protease (Bogusky *et al.*, 1989; Oswald *et al.*, 1989; Nowak *et al.*, 1993). This parallels our observations on (single-chain) BM 06.022 to the extent that the EGRck-protease module remains stable up to ~333 K in both EGRck-uPA and EGRck-BM 06.022 homologs. A difference, however, is that, in the case of BM 06.022, but not tc-uPA, the protease undergoes irreversible thermal denaturation. Also, while at pH* ~4.5 the uPA kringle melts at ~333 K, the tPA kringle 2 is somewhat more stable, requiring a temperature of ~350 K to become fully unfolded; yet, in both cases, the unfolding of the kringle is reversible.

Our results on BM 06.022 are also consistent with differential scanning calorimetry studies of tPA and related domain-deletion mutants indicating an irreversible denaturation component in thermograms recorded under various experimental conditions (Radek & Castellino, 1988; Novokhatny *et al.*, 1991). Although BM 06.022 and wild-type tPA differ in the deletion of three domains, our study affords unambiguous evidence for assigning the reported ~322 K thermal transition (at pH 3.4) to the protease module. In contrast, the acidic unfolding of the protease and intact BM 06.022 is reversible, although such is not the case for the EGRck-modified derivatives as spectra corresponding to their native forms were not recovered upon returning the media to pH* ~4.5. However, the solutions do not opalesce, indicating no significant coagulation/precipitation as is observed in the course of thermal unfolding at pH* ~4.5. This suggests that the covalent attachment of EGRck to His322 at the catalytic center prevents the protein from regenerating at low pH by interfering with the folding pathway.

From the temperature and pH perturbation experiments on BM 06.022 it is concluded that the kringle 2 and protease modules behave as autonomous units. This does not imply, however, a total relative dynamics freedom between the two, in contrast to what is indicated for the various domains in tc-uPA (Nowak *et al.*, 1993). Indeed, our experiments reveal a measurable degree of domain–domain interactions in BM 06.022. Thus, the thermal unfolding experiments show that while the isolated protease denatures at <328 K, in BM 06.022 the protease module denatures at >328 K (Figure 3, panels A and B). A similar trend is observed for the EGRck-reacted protease both as an isolated module and as a part of BM 06.022 (not shown). This indicates an extent of stabilization of the protease structure in the intact BM 06.022.

The serine proteases are likely to undergo distortions upon complexation of the substrate (Steitz & Shulman, 1982). The spectrum of the protease domain linked to EGRck shows a number of differences and, overall, is somewhat narrower and better resolved than that of the unmodified protease (compare Figure 6, traces c and d). This may reflect interference of the ligand with intermolecular aggregation mediated by the protease substrate binding site. On the other hand, the shift of resonances, such as the high-field methyl signals at ~–0.28, –0.37, and –0.48 ppm, suggests that the structure of the EGRck-protease is perturbed relative to its native state, echoing events at the catalytic locus. We note that these effects mimic what is observed upon conversion of the protein from the sc to tc form by cleavage of the Arg275–Ile276 peptide bond and suggest a more relaxed structure in the latter relative to the former.

The pH* 4.6 temperature profile of the protease domain reacted with the active site covalent inhibitor (not shown) reveals that its folding is markedly stabilized relative to its native state, qualitatively similar to what has been observed for tc-uPA (Novokhatny *et al.*, 1992). This thermodynamic effect should be contrasted with the destabilization of the acidic refolding process discussed above in the context of low-pH acid/base response. Thus, the latter is likely to reflect a kinetic (activation barrier) effect since, relative to the unmodified module, at pH* 4.6 the EGRck-protease is thermally more stable.

To conclude, while the two modules in BM 06.022 behave as thermodynamically highly independent units, to the extent that their relative thermal and pH profiles are modulated by complexing a specific ligand at the protease catalytic locus, the protein is endowed with a degree of domain–domain interaction. The latter is likely to relax in the process of conversion to the tc, catalytically active form, which suggests

an important change in the conformational state of the protein upon activation. Indeed, thermal denaturation experiments on the isolated tc-protease and tc-BM 06.022 (Figure 7) reveal no significant difference between these two molecules in what concerns the protease module thermal unfolding profile, which supports a low degree of cooperativity between the kringle 2 and protease domains in the tc form of BM 06.022.

As previously reported (Kohnert *et al.*, 1993), kinetic data suggest that (a) those regions of the kringle 2 and protease modules that are important for the interaction with plasminogen are the same in BM 06.022 and tPA and (b) fibrin monomer and fragments thereof stimulate the activator efficiency of BM 06.022, mimicking the response of tPA to these effectors. It is thus tempting to speculate that the similar catalytic efficiency of tPA and tc-tPA in the presence of fibrin cofactor (Tate *et al.*, 1987; Higgins *et al.*, 1990) may be a consequence of a weakened interdomain protease–kringle 2 interaction in the fibrin-bound sc form, as binding of the kringle to the fibrin polymer may structurally alter the coupling between the two modules in a manner that resembles what we observe in BM 06.022 on going from the sc to the tc form.

ACKNOWLEDGMENT

The authors thank K. Constantine and M. Ravikumar for their assistance with preliminary NMR experiments.

REFERENCES

Andreasen, P. A., Petersen, L. C., & Danø, K. (1991) *Fibrinolysis 5*, 207–215.

Bányai, L., Váradi, A., & Patthy, L. (1983) *FEBS Lett. 163*, 37–41.

Bogusky, M. J., Dobson, C. M., & Smith, R. A. G. (1989) *Biochemistry 28*, 6728–6735.

Burk, P. J., Berg, D. H., Warrick, M. W., Berg, D. T., Walls, J. D., Jaskunas, S. R., Crisel, R. M., Weigel, B., Vlahos, Ch. J., McClure, D. B., & Grinnell, B. W. (1990) *J. Biol. Chem. 265*, 5170–5177.

Byeon, I.-J. L., Kelley, R. F., & Llinás, M. (1989) *Biochemistry 28*, 9350–9360.

De Marco, A. (1977) *J. Magn. Reson. 26*, 527–528.

Gething, M.-J., Adler, B., Boose, J.-A., Gerard, R. D., Madison, E. L., McGookey, D., Meidell, R. S., Roman, L. M., & Sambrook, J. (1988) *EMBO J. 7*, 2731–2740.

Haber, E., Quertermous, T., Matsueda, G. R., & Runger, M. S. (1989) *Science 243*, 51–56.

Higgins, D. L., & Lamb, M. C. (1986) *Arch. Biochem. Biophys. 249*, 418–426.

Higgins, D. L., & Bennett, W. F. (1990) *Annu. Rev. Pharmacol. Toxicol. 30*, 91–121.

Higgins, D. L., Lamb, M. C., Young, S. L., Powers, D. B., & Anderson, S. (1990) *Thromb. Res. 57*, 527–539.

Kelley, R. F., & Cleary, S. (1989) *Biochemistry 28*, 4047–4054.

Kettner, C., & Shaw, E. (1979) *Biochim. Biophys. Acta 569*, 31–40.

Kohnert, U., Rudolph, R., Verheijen, J. H., Weening-Verhoeff, E. J. D., Stern, A., Opitz, U., Martin, U., Lill, H., Prinz, H., Lechner, M., Kresse, G.-B., Buckel, P., & Fischer, S. (1992) *Protein Eng. 5*, 93–100.

Kohnert, U., Horsch, B., & Fischer, S. (1993) *Fibrinolysis 7*, 365–372.

Llinás, M. (1973) *Struct. Bonding 17*, 135–220.

Novokhatny, V. V., Ingham, K. C., & Medved, L. V. (1991) *J. Biol. Chem. 266*, 12994–13002.

Novokhatny, V. V., Medved, L., Mazar, A., Marcotte, P., Henkin, J., & Ingham, K. C. (1992) *J. Biol. Chem. 267*, 3878–3885.

Nowak, U. K., Li, X., Teuten, A. J., Smith, R. A. G., & Dobson, M. (1993) *Biochemistry 32*, 298–309.

Ny, T., Elgh, F., & Lund, B. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 5355–5359.

Obukowics, M. G., Gustafson, M. E., Junger, K. D., Leimgruber, R. M., Wittwer, A. J., Wun, T.-Ch., Warren, T. G., Bishop, B. F., Mathis, K. J., McPherson, D. T., Siegel, N. R., Jennings, M. G., Brightwell, B. B., Diaz-Collier, J. A., Bell, L. D., Craik, S. Ch., & Tacon, W. C. (1990) *Biochemistry 29*, 9737–9745.

Oswald, R. E., Bogusky, M. J., Bamberger, M., Smith, R. A. G., & Dobson, C. M. (1989) *Nature 337*, 579–582.

Pannekoek, H., Lijnen, H. R., & Loskutoff, D. J. (1990) *Thromb. Haemostasis 64*, 600–603.

Pennica, D., Holmes, W. E., Kohr, W. J., Harkins, R. N., Vehar, G. A., Ward, C. A., Bennett, W. F., Yelverton, E., Seeburg, P. H., Heynecker, H. L., & Goeddel, D. V. (1983) *Nature 301*, 214–221.

Radek, J. T., & Castellino, F. J. (1988) *Arch. Biochem. Biophys. 267*, 776–786.

Robinson, J. H., & Browne, M. J. (1991) *TIBTech 9*, 86–90.

Steitz, T. A., & Shulman, R. G. (1982) *Annu. Rev. Biophys. Bioeng. 11*, 419–444.

Stürzebecher, J., Markwardt, F., Wagner, G., & Walsmann, P. (1976) *Acta Biol. Med. Ger. 35*, 1665–1676.

Stürzebecher, J., Neumann, U., Kohnert, U., Kresse, G.-B., & Fischer, S. (1992) *Protein Sci. 1*, 1007–1013.

Tate, K. M., Higgins, D. L., Holmes, W. E., Winkler, M. E., Heyneker, H. L., & Vehar, G. A. (1987) *Biochemistry 26*, 338–343.

Teuten, A. J., Smith, R. A. G., & Dobson, C. M. (1991) *FEBS Lett. 278*, 17–22.

Teuten, A. J., Cooper, A., Smith, R. A. G., & Dobson, C. M. (1993) *Biochem. J. 293*, 567–572.

van Zonneveld, A. J., Veerman, H., & Pannekoek, H. (1986) *J. Biol. Chem. 261*, 14214–14218.

Verheijen, J. H., Caspers, M. P. M., Chang, G. T. G., de Munk, G. A. W., Pouwels, P. H., & Enger-Valk, B. E. (1986) *EMBO J. 5*, 3525–3530.

Wilhelm, O. G., Jasnukas, S. R., Vlahos, Ch. J., & Bang, N. U. (1990) *J. Biol. Chem. 265*, 14606–14611.