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Reversible Independent Unfolding of the Domains of Urokinase Monitored by ^{1}H NMR†

Michael J. Bogusky,‡,§ Christopher M. Dobson,*,‡ and Richard A. G. Smith‖

Inorganic Chemistry Laboratory and the Centre for Molecular Sciences, University of Oxford, South Parks Road, Oxford OX1 3QR, U.K., and Biosciences Research Centre, Beecham Pharmaceuticals, Great Burgh, Yew Tree Bottom Road, Epsom, Surrey KT18 5XQ, U.K.

Received September 27, 1988; Revised Manuscript Received May 1, 1989

ABSTRACT: Human urinary-type plasminogen activator (urokinase) and proteolytic fragments corresponding to the kringle, EGF–kringle, and protease domains have been examined by ^{1}H NMR spectroscopy. The intact protein shows a very well-resolved spectrum for a molecule of this size (MW 54000), with resonance line widths not greatly increased from those of the isolated domains. On increasing the temperature, the protein at pH values close to 4 was found to undergo two distinct and reversible conformational transitions. These were identified, by comparison with spectra of the proteolytic fragments, as the unfolding of the kringle (and EGF) domains (at ~42 °C) and of a segment of the protease domain (at ~60 °C). The remaining segment of the protease domain showed persistent structure to at least 85 °C at pH 4; only at lower pH values could complete unfolding be achieved. The results indicate that the structures and stabilities of the isolated domains are closely similar to those in the intact protein and suggest that there is a degree of independent motion at least between the kringle and protease domains.

A group of about ten proteins involved in hemostasis and fibrinolysis, including prothrombin, tissue plasminogen activator (t-PA),[1] factor XII, plasminogen, and urokinase (u-PA), have been found to be composed of mosaics of distinct structural domains (Sottrup-Jensen et al., 1978). The latter are classified according to disulfide bridge arrangement and show remarkable homology between the different proteins. They include epidermal growth factor (EGF) like domains, kringle domains, calcium binding domains, and serine protease

† This work was supported in part by the U.K. Science and Engineering Research Council. M.J.B. was supported by a National Science Foundation postdoctoral fellowship (INT-8701333).
‡ University of Oxford.
§ Present address: Merck Sharp and Dohme Research Laboratories, West Point, PA 19486.
‖ Beecham Pharmaceuticals.

[1] Abbreviations: u-PA, urinary-type plasminogen activator; EGF, epidermal growth factor; NMR, nuclear magnetic resonance; t-PA, tissue plasminogen activator; GGACK, L-glutamylglycyl-L-arginine chloromethyl ketone; NpGB, *p*-nitrophenyl *p*-guanidinobenzoate hydrochloride; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

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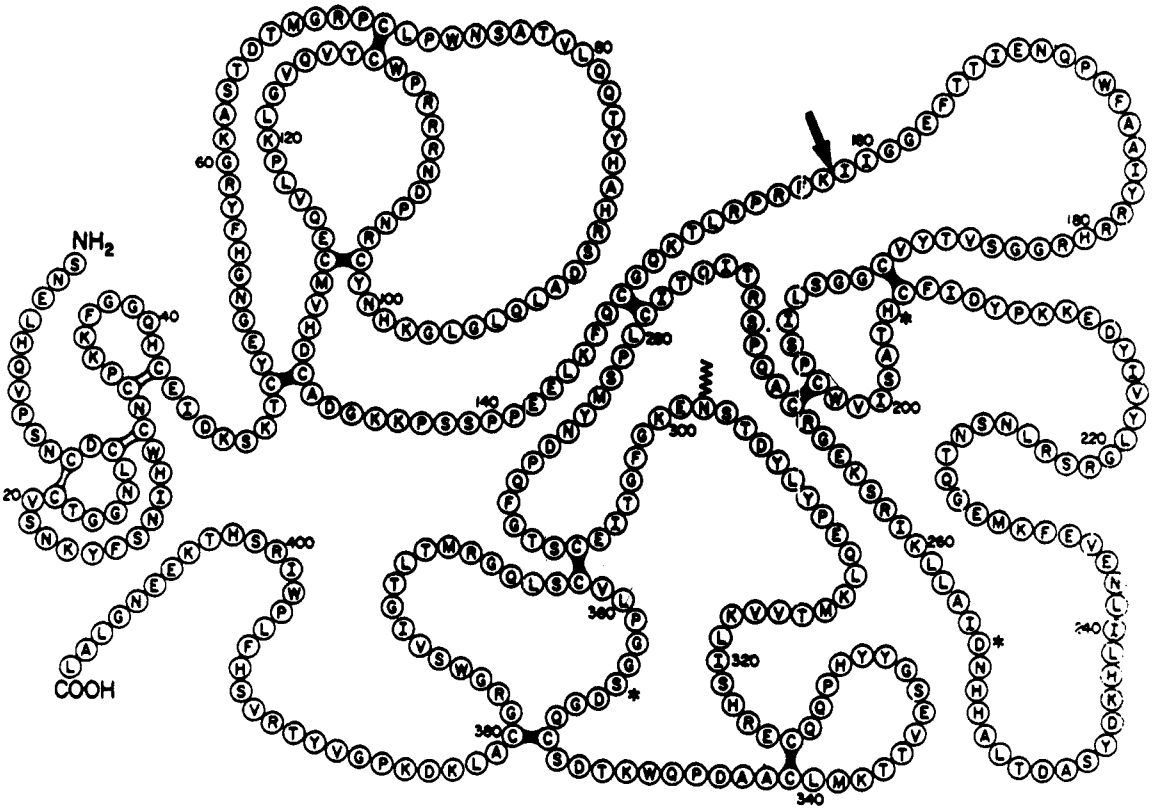

FIGURE 1: Schematic representation of the structure of human urokinase. Disulfide bridges are indicated by solid lines connecting cysteine residues. The three fragments obtained by limited proteolysis are the EGF–kringle domain (residues S1–K135), the kringle domain (residues S47–K135), and the serine protease domain (residues K136–L411). The residues at which cleavage has occurred to give the two-chain form are indicated by an arrow.

domains. The individual proteins contain one or more of at least two of these domains. The structure of none of the proteins is, however, known in detail; crystallographic studies of the intact proteins have not yet proved successful. In certain cases, however, it is possible to isolate fragments of the proteins which contain one or more of the individual domains. Crystal structures of one of these, fragment I of prothrombin (Olsson et al., 1982; Park & Tulinsky, 1986; Harlos et al., 1987; Tulinsky et al., 1988), which consists of a calcium binding and a kringle domain along with an associated carbohydrate moiety, have been determined. In addition, detailed NMR studies have been performed on the kringle 1, 4, and 5 domains isolated from plasminogen (Trexler et al., 1983, 1985; De Marco et al., 1982, 1985; Llinas et al., 1983; Ramesh et al., 1986, 1987; Thewes et al., 1987, 1988; Mabbutt & Williams, 1988); these appear broadly in accord with the crystallographic structure of the kringle domain in prothrombin fragment I. Further, it seems highly probable that the overall architecture of the EGF domains resembles those of the isolated growth factors, several of which are known in detail from NMR structure determinations (Cooke et al., 1987; Montelione et al., 1987; Kohda et al., 1988), and that the structures of the serine protease domains resemble those of trypsin and related proteins (De Häen et al., 1975).

There is considerable evidence that the structures of the isolated domains are at least broadly unperturbed when incorporated into the intact proteins (Castellino et al., 1981). In plasminogen, the binding properties of the kringle domains and the activity of the protease domains are largely retained (Sottrup-Jensen et al., 1978). In addition, thermal unfolding of the different domains in plasminogen has been observed by calorimetric measurements and shown to be similar to the unfolding of the isolated fragments (Castellino et al., 1981; Novokhatny et al., 1984). There is relatively little information, however, on the identity of interdomain contact regions or on the modulation of domain function during binding of ligands (such as fibrin) at remote sites.

Human urinary-type plasminogen activator (urokinase, u-PA, EC 3.4.21.31) is a serine protease of restricted specificity whose principal physiological function appears to be the activation of plasminogen to plasmin. The latter enzyme is a major source of proteolytic activity in physiological fibrinolysis, fertilization, tissue remodeling, and tumor growth. Urokinase has been used for the treatment of acute myocardial infarction and pulmonary embolism; there is therefore substantial clinical interest in the development of multidomain plasminogen activators based upon urokinase and t-PA as thrombolytic agents. A detailed understanding of domain interactions in these proteins will aid the design of novel therapeutic agents of that type.

Structural studies on both urokinase itself (Gunzler et al., 1982; Steffens et al., 1982) and the urokinase gene (Holmes et al., 1985) have revealed that it is normally expressed as a single-chain form (scu-PA, prourokinase) containing just three distinct domains (see Figure 1), EGF, kringle, and serine protease. Single-chain urokinase possesses catalytic activity (Lijnen et al., 1986), but urokinase can also be isolated in a two-chain form that arises through cleavage at lysine-158. This form (MW 54 000) is known as high-MW urokinase. Most of the N-terminal (A-chain) region of high-MW u-PA can be removed by proteolytic cleavage at lysine-135 to yield a low-MW (MW 33 000) fully active form containing the protease domain linked to a short interdomain peptide by a disulfide bridge. The activity of low-MW urokinase and the ability to construct functional hybrid proteins containing the urokinase protease domain by use of either chain recombi-

nation (Robbins & Tanaka, 1986) or exon shuffling (De Vries et al., 1988) techniques all suggest a substantial degree of functional independence of the three domains of urokinase. The protease domain appears to contain all the features necessary for specific recognition and cleavage of plasminogen; the roles of the A-chain domains are less clear although it has also been shown (Appella et al., 1987) that the N-terminal EGF region is involved in interaction with cellular receptors. The function of the central kringle domain is currently undefined. It does not appear to confer fibrin-binding capacity on high-MW urokinase, and this distinguishes it from other kringle-containing fibrinolytic proteins, notably plasminogen and t-PA (Suenson & Thorsen, 1981; Higgins & Vehar, 1987).

In the present study, we have utilized a previously unexploited additional interdomain proteolytic cleavage to isolate a 10-kDa fragment corresponding to the discrete kringle domain of urokinase. This fragment, together with the EGF–kringle unit obtained by autolysis of high-MW urokinase and the remaining C-terminal region (low-MW urokinase itself), has been examined by high-resolution proton NMR. Despite the high molecular weight of the intact protein, the spectra are sufficiently well resolved to permit identification of resonances attributed to specific domains in the protein. This has allowed characterization of the unfolding transitions of the intact protein.

Materials and Methods

Human high-MW two-chain urokinase of urinary origin was a product of the Japan Chemical Research Co. Ltd. and was obtained through the courtesy of Zeria Pharmaceutical Co., Tokyo, Japan. The product was >95% high-MW urokinase by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS–PAGE). Human plasmin was prepared by activation of human plasminogen (1.0 g) (Immuno, Vienna, Austria) with urokinase (10^6 IU, 100:1 molar ratio) in 0.18 M sodium phosphate, 0.26 M NaCl, 0.13 M glucose, 0.05 M L-lysine, 1.0 mM 6-aminohexanoic acid, and 33% glycerol, pH 7.4 (40 mL), at 4 °C for 4 h. The product was found to contain 180 μM plasmin by active site titration with *p*-nitrophenyl *p*-guanidinobenzoate hydrochloride (NpGB) performed by the method of Chase and Shaw (1969) and was stored at −40 °C. Aprotinin and NpGB were obtained from Sigma Chemical Co., Poole, U.K. L-Glutamylglycyl-L-arginine chloromethyl ketone (GGACK) was obtained from Cambridge Bioscience, Cambridge, U.K.

Isolation of the Urokinase Kringle Fragment (Residues 47–135). High-MW urokinase [310 μM in 0.1 M sodium phosphate, 0.60 M NaCl, 1% w/v Tween 80, and 33% v/v glycerol, pH 7.4 (4.0 mL)] was mixed with human plasmin (120 μL of 180 μM) and incubated at 37 °C for 48 h. After this time, SDS–PAGE analysis (Pharmacia Phastsep System, 20% homogenous gels) showed that little high-MW enzyme remained, and the product was subjected to high-performance gel-permeation chromatography on a 600 × 21.5 mm column of TSK G3000SW silica (Beckman, High Wycombe, U.K.) fitted with a 21.5 mm diameter precolumn. The column was eluted with 0.5 M L-arginine, 0.5 M NaCl, 20 mM Tris-HCl, and 0.01% w/v Tween 80, pH 7.0, at 1.0 mL/min, 23 °C. Under these conditions, low-MW urokinase eluted at approximately 167 mL and was recovered from fractions eluting between 162 and 174 mL. A second, enzymatically inactive, peak (kringle) was observed at 194 mL and recovered from a pool of the 191–202-mL fractions. Both pools were exchanged into 50 mM ammonium bicarbonate, pH 7.5, with small columns of Sephadex G-25 (PD-10, Pharmacia, Milton Keynes, U.K.) and lyophilized. Further details of the characterization of this fragment will be presented elsewhere. Samples for NMR were reconstituted in this buffer and subjected to a repeat cycle of gel filtration and lyophilization.

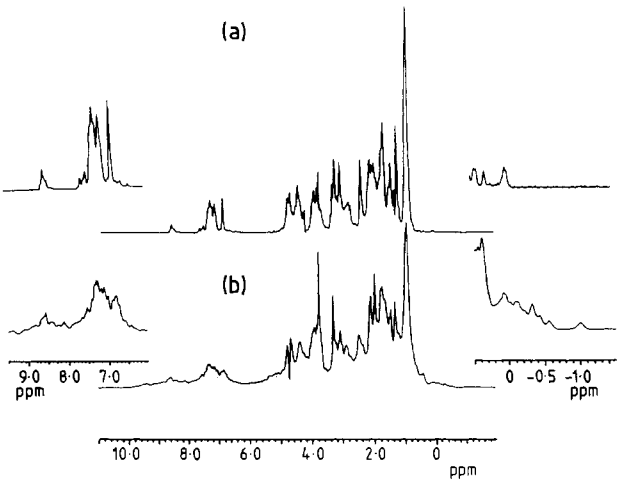

FIGURE 2: 600-MHz ^{1}H NMR spectrum of urokinase (0.45 mM protein in ^2H_2O, pH 3.8) at (a) 85 and (b) 35 °C. The inserts show expansions of the upfield and downfield regions of the spectrum.

EGF–Kringle Fragment (Residues 1–135) and Protease Fragment (Residues 136–411). Urokinase (250 μM in 0.15 M sodium phosphate, 0.14 M NaCl, and 0.01% w/v Tween 80, pH 7.4, 2.5 mL) was incubated at 25 °C in the presence of aprotinin (4 μM). After 5 days the product was fractionated as described above. Low-MW urokinase was recovered from fractions eluting between 163 and 171 mL, and an enzymatically inactive peak at 184 mL was recovered from fractions 181–189 mL. Both pools were processed as described above.

Inactivated Intact Urokinase. This was prepared by treating stock high-MW urokinase (2.5 mL of 208 μM in the above glycerol-free phosphate buffer) with GGACK (85 μL of 12 μM in water) at 25 °C for 1 h. The enzyme was 98% inactivated and was then dialyzed against 50 mM ammonium bicarbonate, pH 7.5 (3 × 500 mL), over 16 h at 4 °C and lyophilized.

NMR Studies. Solutions for NMR studies were 0.4–1.6 mM in protein as indicated. Lyophilized protein samples were dissolved in 0.5 mL of ^2H_2O (99 atom % ^2H, Sigma, St. Louis), and the pH was adjusted to the required value within the range 3.8–4.7 by the addition of dilute ^2HCl. All pH measurements are direct meter readings uncorrected for isotope effects.

^{1}H NMR spectra were recorded at 600.13 MHz on a Bruker AM-600 spectrometer at the temperatures indicated with weak coherent irradiation to suppress the residual H^2HO resonance. Between 128 and 2048 scans were acquired for acceptable signal-to-noise ratios.

Results

The 600-MHz ^{1}H spectrum of urokinase in ^2H_2O at 35 °C is shown in Figure 2b. Intact high-MW urokinase was studied in a form irreversibly inactivated with a tripeptide chloromethyl ketone in order to eliminate possible autolysis during data acquisition. The spectrum is indicative of a highly structured globular protein with a substantial number of resonances having chemical shifts significantly perturbed from the values characteristic of unstructured peptides. The resulting dispersion of resonances is clearly observed in the region of the spectrum where resonances of aromatic protons are found, between 6.0 and 9.0 ppm, and in the region upfield (between 0.4 and −1.0 ppm) and downfield (between 5.0 and 6.0 ppm) of the main aliphatic region of the spectrum. Most notable

is the group of resonances between 5.0 and 5.5 ppm, which is highly indicative of C_α protons of residues in β-sheet structure, and the resonances between 0 and −1.0 ppm, which are characteristic of aliphatic protons, particularly methyl groups, adjacent to aromatic residues in the folded structure. Several well-resolved resonances are observed in both these regions, most notably the resonance at −1.0 ppm.

A number of the resonances in Figure 2 visible between 7.5 and 10.0 ppm downfield of the major envelope of aromatic protons arise from amide protons. The ^1H NMR spectrum of urokinase freshly dissolved in ^2H_2O shows the presence of a substantial subset of amide protons that are slow to exchange with solvent. This subset is highly resistant to exchange with solvent deuterons for periods of days at 35 °C and pH 4. Many of the amide protons remain unexchanged at temperatures in excess of 50 °C for periods of hours. Such slowly exchanging hydrogens are often involved in interpeptide hydrogen bonds and are usually indicative of stable secondary structure (Englander & Kallenbach, 1984).

The spectrum of urokinase at 85 °C shown in Figure 2a differs substantially from the spectrum of the protein at 35 °C. The overall dispersion of resonances is greatly reduced, and the majority of resonances are found in distinct bands, characteristic of the random coil chemical shift values found for unstructured peptides (Wüthrich, 1987). This is particularly evident in Figure 2 for the resonance at ca. 1.0 ppm attributable to the methyl groups of valine, leucine, and isoleucine residues and the resonance at ca. 6.8 ppm attributable to the aromatic 3,5-protons of tyrosine residues. There are, however, several resonances well shifted from the chemical shift positions anticipated for a random coil structure. Most notable are up to six strongly upfield-shifted resonances between 0.0 and 0.5 ppm. Additionally, several resonances are visible between 6.3 and 6.8 ppm, just upfield of the tyrosine 3,5-proton resonances, and between 4.5 and 5.5 ppm, in the region characteristic of C_α protons in β-sheet structure. The spectrum remains essentially unchanged even at temperatures above 85 °C, except for a slight loss in intensity observed for the shifted resonances relative to the remaining resonances. The spectral changes occurring between 35 and 85 °C are fully reversible under these conditions; upon reduction of the temperature to 35 °C, the resulting spectrum is identical with that in Figure 2b. The spectrum at 85 °C suggests that while the majority of the protein appears unstructured, there is a residual folded region or regions associated with a subset of residues whose resonances are strongly perturbed from the random coil values. No amide resonances can be observed at this temperature, indicating that exchange with solvent is now rapid, taking place within minutes of exposure to ^2H_2O.

In order to characterize the various changes in the protein as a function of temperature, the detailed temperature dependence of the spectrum was examined. Expansions of the upfield region of the urokinase spectrum as a function of temperature are shown in Figure 3. Several marked changes are clearly evident. In particular, between 35 and 39 °C the resonance at −1.0 ppm begins to broaden. As the temperature is increased, the resonance continues to broaden and diminish in intensity, completely disappearing from the spectrum by 55 °C. A second resonance at 0.13 ppm can also be observed to diminish in intensity over the same temperature range. The remainder of the resonance intensity in the region of the spectrum upfield of 0.5 ppm appears largely unchanged apart from a gradual narrowing of the peaks with increasing temperature. Between 45 and 55 °C only minor changes are evident in the spectrum; these are likely to be due to small

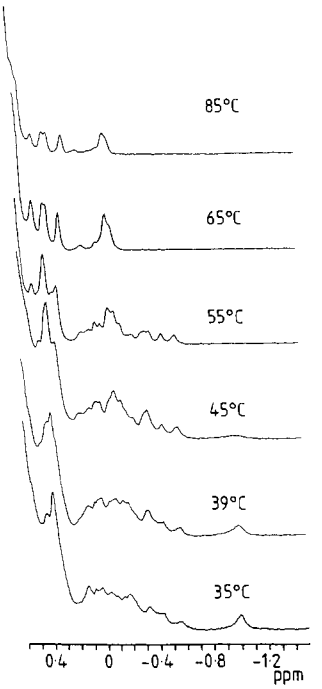

FIGURE 3: Upfield region of the 600-MHz ^1H NMR spectrum of urokinase at the temperatures indicated. The sample conditions were as in Figure 2.

shifts of resonances with temperature. A second major change in the spectrum is, however, evident between 55 and 65 °C as indicated by the disappearance of numerous resonances between 0.5 and −0.6 ppm to leave approximately six resonances. The spectrum then remains largely unchanged up to 85 °C with these well-resolved resonances remaining in this region of the spectrum as previously noted. At lower pH values, however, the disappearance of this subset of resonances can be observed at lower temperature; at pH 2.5 above 70 °C the spectrum resembles closely that anticipated from random coil values (R. E. Oswald, M. J. Bogusky, M. Bamberger, C. M. Dobson, and R. A. G. Smith, unpublished experiments).

The corresponding expansions of the low-field region of the spectrum, which includes both aromatic and C_α resonances, are shown in Figure 4. Although somewhat less clearly observed, changes in the spectrum as a function of temperature are again evident. The most notable change between 35 and 45 °C is the increase in resonance intensity at ca. 6.8 ppm, the random coil chemical shift of the tyrosine 3,5-protons. Between 45 and 55 °C, no significant changes are observed in this region other than small shifts of resonances. Above 55 °C the spectrum is, however, characterized by the disappearance of numerous resonances with increased resonance intensity at positions associated with random coil chemical shift values. At 85 °C the spectrum is largely characteristic of an unfolded protein, except for the previously noted resonances which remain between 6.5 and 6.9 ppm and between 5.0 and 5.7 ppm; at least six distinct resonances are present in both these regions of the spectrum. As with the persistent resonances in the upfield region, however, these are absent from the spectrum at elevated temperatures at lower pH values. The differences observed in the protein spectrum between 35 and 85 °C are clearly evident from the detailed temperature dependence and can now be seen to occur not gradually but in several discrete steps. The results indicate that the protein undergoes at least three independent structural transitions. The first transition occurs between 39 and 45 °C and is followed by a second at approximately 60 °C. Only at lower pH can the third transition, resulting in apparently complete un-

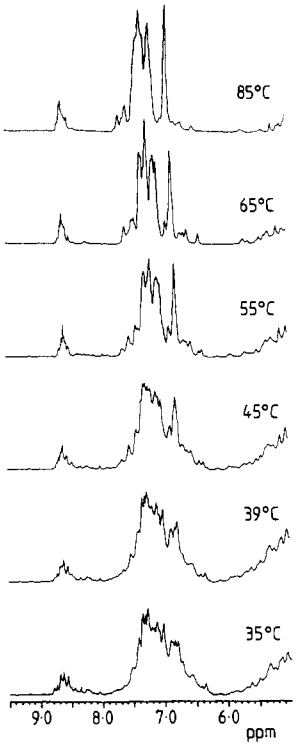

FIGURE 4: Downfield region of the 600-MHz ^1H NMR spectrum of urokinase at the temperatures indicated in the figure. The sample conditions were as in Figure 2.

folding, be clearly observed. The protein remains with some residual structure even above 85 °C at pH values above about 4.0. The structural transitions are all fully reversible under the experimental conditions described.

In order to interpret the observed spectral changes in more detail and to identify specific regions within the intact protein associated with each unfolding transition, we have compared the ^1H NMR spectra of urokinase with those of the individual domain fragments, which have been obtained by limited proteolysis of the intact protein. Shown in Figures 5 and 6 are expanded regions of the ^1H NMR spectra recorded at 35 °C of the kringle, EGF–kringle, and protease domains of urokinase, as well as of the intact protein. Each fragment gives high-quality spectra indicative of a structured, globular protein. In addition, each domain fragment when freshly dissolved in ^2H_2O contains a subset of slowly exchanging amide protons.

Comparison of the spectra of the kringle and EGF–kringle fragments shows that the majority of the well-resolved resonances in the latter arise from the kringle domain. These are essentially identical in the two spectra, indicating a close similarity of the kringle structures in the two fragments, and provide no evidence of the perturbation of the chemical shifts of the resolved kringle resonances as a consequence of contacts with the EGF domain. Only two well-resolved resonances in the EGF–kringle fragment are not seen in the kringle spectra; these are the resonance of a methyl group at 0.4 ppm and a resonance from a single proton at 5.7 ppm. It is likely, therefore, that these arise from the EGF domain, but the possibility that these arise from kringle residues whose resonances are shifted by the presence of the EGF domain cannot be completely eliminated at this time. Firm assignment of these resonances must await either a detailed analysis of the component spectra or isolation of the EGF fragment.

To a first approximation, the spectrum of intact urokinase appears to be a superposition of the spectra of the EGF–kringle and protease fragments, except that some increase in line width is evident in the intact protein. A number of resonances can

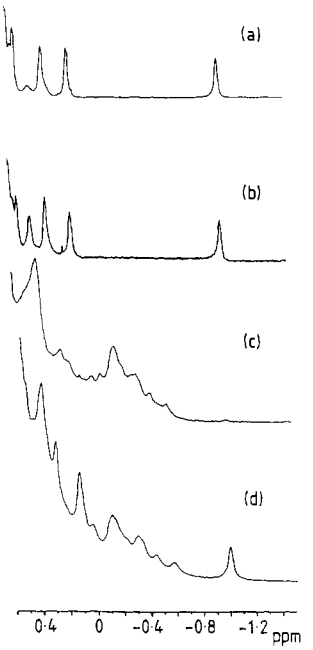

FIGURE 5: Upfield region of the 600-MHz ^1H NMR spectra of intact urokinase and its proteolytic fragments: (a) kringle domain, residues S47–135; (b) EGF–kringle domain, residues S1–L135; (c) protease domain, residues K136–L411; (d) intact urokinase. All spectra were of samples between 0.5 and 1.6 mM in concentration and were recorded at 35 °C, pH 4.5 ± 0.4 in ^2H_2O.

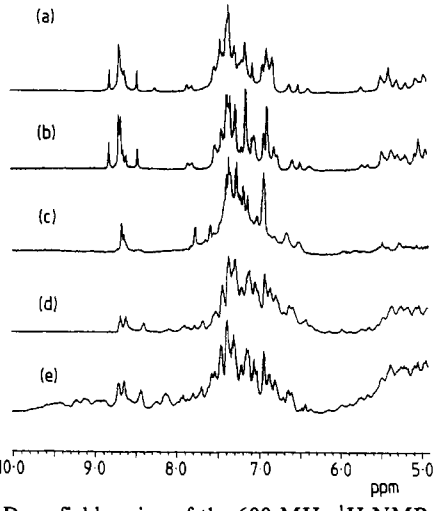

FIGURE 6: Downfield region of the 600-MHz ^1H NMR spectra of intact urokinase and its proteolytic fragments: (a) kringle domain; (b) EGF–kringle domain; (c) protease domain; (d) intact urokinase in which the labile hydrogens were replaced by deuterons by warming the sample to 80 °C in ^2H_2O for 15 min; (e) intact urokinase in ^2H_2O, for which the spectrum was recorded immediately following dissolution of the lyophilized protein and shows the presence of a subset of slowly exchanging amide protons.

be identified with individual domains within the intact protein. The resonance at −1.0 ppm in the spectrum of the intact protein, for example, is seen to have an exact counterpart in the spectrum of the EGF–kringle and kringle fragments; it can therefore be assigned as belonging to the kringle domain. A similar resonance is also present in spectra obtained on both the kringle 1 (De Marco et al., 1982), kringle 4 (Trexler et al., 1983), and kringle 5 (Thewes et al., 1987) domains isolated from human plasminogen as well as bovine prothrombin fragments 1 and 2 (Esnouf et al., 1980a,b) and has been assigned to a leucine residue corresponding to Leu-94 in intact urokinase. This residue is conserved in all known kringle sequences to date (Trexler & Patthy, 1983; Park & Tulinsky,

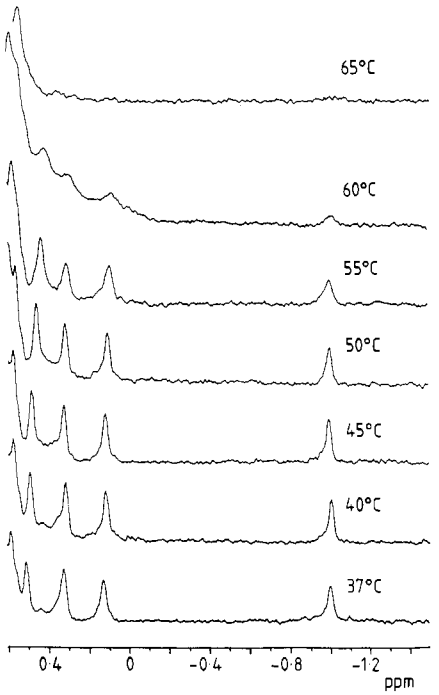

FIGURE 7: Upfield region of the 600-MHz ^1H NMR spectrum of the kringle fragment at the temperatures indicated in the figure. The protein was 1.6 mM in ^2H_2O, pH 4.5.

1986). Similarly, the group of resonances between 0.0 and −0.6 ppm in the intact protein can be seen to arise from the protease domain by comparison with the spectra of the isolated protease domain; no resonances from the kringle or EGF domains are seen in this region in the spectrum of the fragments. In the low-field region of urokinase at this temperature, identification of individual resonances of the different domains is limited by extensive overlap of the comparatively broad lines. Unambiguous assignment of the two possible EGF resonances identifiable in the EGF–kringle fragment is therefore not possible in the one-dimensional spectra presented here. Interpretation of the structural changes is therefore restricted in this work to the kringle and protease domains.

This comparison of the spectra of the proteolytic fragments with that of the intact protein, in conjunction with the temperature dependence of the urokinase spectra discussed above, makes it possible to associate the conformational transition observed at a midpoint of about 42 °C with a structural change localized within the EGF–kringle domain rather than the protease domain. In a similar manner, the transition centered at 60 °C may be associated with the protease domain. In order to explore this further, and to identify the origin of the residual structure that persists above 60 °C, the temperature dependence of the spectra of the individual fragments was examined. Figure 7 illustrates the changes in the NMR spectrum of the isolated kringle fragment at pH 4.5 induced by increases in temperature. Between 45 and 55 °C the resonances upfield of 0.6 ppm are seen uniformly to broaden and diminish in intensity. Similar behavior has been observed for all the resolved resonances in the region of the spectrum below 5.0 ppm. The resonances are extensively broadened by 60 °C with marked reductions in intensity, in accord with similar studies performed on the intact protein. This broadening is attributed to chemical exchange effects; this conclusion is supported by the results of one- and two-dimensional magnetization-transfer experiments (M. J. Bogusky, R. E. Oswald, M. Bamberger, R. A. G. Smith, and C. M. Dobson, unpublished experiments). Above 65 °C the spectrum of the kringle domain is characteristic of a fully unfolded protein with no evidence of regions with residual globular structure. These changes are reversible and indicative of a highly cooperative unfolding transition. The unfolding transition of the kringle fragment was observed to be strongly pH dependent, occurring at lower temperatures at lower pH values. At pH 3.8 unfolding was found to take place with a midpoint of ~45 °C, very close to the temperature of the first transition observed in the intact protein.

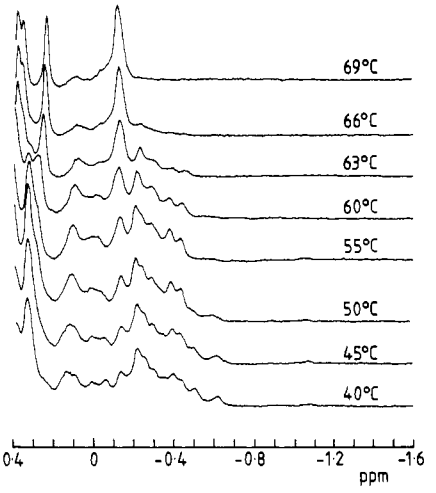

FIGURE 8: Upfield region of the 600-MHz ^1H NMR spectra of the serine protease domain at the temperatures indicated in the figure. The protein concentration was 0.5 mM in ^2H_2O, pH 4.5.

The temperature-dependent changes in the spectrum of the isolated protease domain are shown in Figure 8. The similarities between the upfield regions (0.4 to −0.6 ppm) of urokinase and of the individual protease domain at each temperature are clearly evident; all but three resonances in this region in the intact protein can be attributed to the protease domain. Between 35 and 55 °C little change is seen in the spectra other than several resonances shifting with temperature, with the exception of the broadening and disappearance of two resonances between −0.6 and −0.4 ppm. Between 55 and 66 °C, however, a dramatic loss in signal intensity is evident, which leaves a subset of resonances very similar to those observed in the intact protein at the same temperature. In the low-field region, a major transition is also seen at ~60 °C, and again, the remaining subset of resonances in this region resembles closely those observed in the intact protein at higher temperatures. No further changes in the spectrum at temperatures up to 80 °C are observed, and again the spectral changes are fully reversible. This result suggests, therefore, not only that the protease domain in urokinase is similar in structure and stability to the excised domain but also that in both cases only partial unfolding of the domain structure occurs at ~60 °C. The residual structured region in intact urokinase can therefore be identified as part of the protease domain. It is also clear that the individual kringle and protease domains show no significant deviation in folding behavior or stability as compared to the domains in the intact protein under similar conditions.

Discussion

Calorimetric studies of plasminogen (Castellino et al., 1981; Novokhatny et al., 1984) and t-PA (Redek & Castellino, 1988) have demonstrated that thermal unfolding of these multidomain proteins can occur in a stepwise manner. The NMR studies presented here show that similar stepwise unfolding behavior can be observed for urokinase, and in addition they enable direct identification of the structural changes associated

with each unfolding transition within the intact protein.

The similarities observed between the NMR spectra of urokinase and those of the proteolytic fragments provide clear evidence for the structural integrity of the kringle and protease domains in the fragments. Further, comparison of the transition temperatures of the kringle domain in the fragments and in the intact protein indicates that any interaction between the folded kringle and protease domain has no significant influence on the thermal stability of the kringle domain. Although the unfolding transitions in the protease domain are similarly unaffected by the presence of the kringle and EGF portions of the molecule, a similar conclusion cannot be drawn from this observation as both the kringle and EGF domains are themselves unfolded at the temperatures at which unfolding of the protease domain takes place. For a similar reason, as the EGF domain appears to unfold prior to the kringle domain under the conditions of the experiments described here, no firm conclusions may be drawn about the EGF–kringle interaction from the fact that the kringle unfolding temperature is not significantly different in the EGF–kringle fragment from that in the isolated kringle fragment. Future NMR studies should, however, enable such interactions to be explored in greater detail.

The lack of sensitivity of the unfolding temperature of the kringle domain to the presence of the folded serine protease domain suggests that specific interactions between these two domains are relatively weak. This conclusion is supported by the observation of resonance line widths in the various spectra. The NMR spectrum of urokinase is very well resolved for a protein with a molecular weight of over 50 000; numerous relatively narrow lines are observed superimposed on the overall envelope of the protein resonances. The resonances of the kringle and protease domains in the intact protein do not appear to be substantially broader than in the spectra of the isolated fragments. As NMR line widths in proteins are determined by the rate of molecular reorientation, the implication of these observations is that there is internal motion within the molecule. We propose that this motion could originate as a consequence of a lack of structural rigidity of the linker region (residues 132–147) between the kringle and proteases domains, permitting each domain to move with a degree of independence of its neighbor (Oswald et al., 1989). This conclusion is similar to that drawn from studies of the intact lac-repressor protein (Wade-Jardetsky et al., 1979) where it has been shown that the narrow resonances in the spectrum are a consequence of extensive segmental motion of the headpiece domain. The existence of the proposed segmental motion in urokinase appears to have aided significantly the present study; indeed, the line widths of the resonances of protons in the intact protein are sufficiently narrow for excellent 2-D NMR spectra to be obtained (Oswald et al., 1989).

A particularly interesting feature of the results presented in this paper concerns the observation that stepwise unfolding occurs not only for the different domains of urokinase but also within the protease domain. A similar observation of a two-step unfolding of the protease domain of plasminogen has been reported from calorimetric studies (Novokhatny et al., 1984) although the reported difference in stability was much smaller than that observed for urokinase. The protease domain of urokinase shows substantial sequence homologies with digestive serine proteases; for example, when compared with human trypsinogen, there is 38% sequence identity with a further 27% conservative substitutions. It is therefore likely that close structural relationships exist between the different proteins (De Häen et al., 1975; Holland, 1989). X-ray crystallographic studies of several serine proteases including trypsin and chymotrypsin show that the molecules are each composed of two similar structural units, themselves often referred to as domains (Richardson, 1981). We suggest that the two-step unfolding observed within the protease domain of urokinase could reflect markedly different thermal stabilities of these two segments of the domain. It is of particular interest in this regard that in neither urokinase nor plasminogen do any of the six disulfide bonds present in the protease domain link the two segments of the domain. Further experiments are in progress to test this hypothesis.

The ability to use NMR spectroscopy to probe unfolding transitions (McDonald et al., 1971; Dobson & Evans, 1984; Evans et al., 1989) and to provide structural data about unfolded and partially folded proteins (Dobson et al., 1984; States et al., 1987; Baum et al., 1989) makes it a technique of considerable potential in studies of the factors stabilizing protein structural elements and of the process of protein folding. The multidomain proteins involved in hemostasis, such as urokinase described here, could be remarkably amenable to such NMR studies despite their complexity and relatively large size. This can be seen to arise as a consequence both of their intrinsic dynamical nature, which may have major functional significance (Blasi, 1988; Oswald et al., 1989), and of the nature of their reversible stepwise unfolding behavior.

ACKNOWLEDGMENTS

We thank M. Bamberger, R. E. Oswald, C. C. F. Blake, S. K. Holland, A. J. Teuten, and R. J. P. Williams for many valuable discussions and J. Boyd for assistance with various aspects of the NMR experiments.

Registry No. u-PA, 9039-53-6.

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