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Domain interactions in human plasminogen studied by proton NMR

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The NMR spectrum of miniplasminogen (V^{443}-plasminogen) under conditions of acidic pH reveals a subset of particularly well-resolved resonances whose chemical shift values are closely similar to those of isolated kringle 5. The temperature dependence of the spectrum indicates that this set of resonances disappears in a single cooperative unfolding transition appropriate for kringle 5, whilst other broader resonances from the protease domain persist to higher temperature. These results provide evidence for significant structural and motional independence of the kringle and protease domains in spite of the short linker between these domains. The NMR spectrum of Glu_1-plasminogen is closely similar to that of miniplasminogen under the same conditions. This suggests that the domain independence observed in miniplasminogen is maintained in the intact molecule.

NMR; Protein structure; Plasminogen; Kringle; Domain

1. INTRODUCTION

It has been demonstrated that it is possible to obtain well-resolved NMR spectra from urokinase, a member of the family of multi-domain fibrinolytic proteins [1,2]. Here we report that NMR techniques can be extended to another member of this family of proteins, plasminogen, where in spite of considerably greater molecular complexity, many resolved resonances can be observed. Glu_1-plasminogen is a large multi-domain glycoprotein (M_r 92000 Da) comprising a trypsin-like serine protease domain and five homology units known as 'kringles' [3]. Two forms of plasminogen exist, with and without the presence of a 77 residue pre-activation peptide, known as Glu_1- and Lys_{78}-plasminogen, respectively, as a consequence of the nature of the N-terminal residue. Activation of the zymogen involves both the removal of this pre-activation peptide and cleavage between Arg-561 and Val-562 (plasminogen numbering based on that identified in [4]) in order to unmask the functional active site. The two-domain fragment miniplasminogen, M_r 38000 Da, is formed from plasminogen by proteolytically cleaving between Val-442 and Val-443 and comprises the protease and fifth kringle domains with an N-terminal extension. This molecule is of particular interest because it is a simple model for the study of interactions between kringle and protease domains which are present in all the fibrinolytic proteins. In urokinase, it has been suggested that there exists significant segmental motion between the kringle and protease domains [1]. This was proposed to explain well resolved features in the NMR spectrum whereby resonances arising from the smaller kringle domain were narrower than would have been expected for a molecule of this size. It has been of interest, therefore, to see whether this dynamical property of urokinase is more widespread in the family of fibrinolytic proteins. In particular, studies of plasminogen and its fragments have enabled us to initiate studies of domain-domain interactions in the intact molecule and have demonstrated the usefulness of NMR in aiding the investigation and dynamical properties of a class of molecules of major therapeutic interest for which little physical and crystallographic information is at present available.

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Abbreviations: NMR, nuclear magnetic resonance; ϵ-ACA, 6-aminohexanoic acid; HPLC, high-performance liquid chromatography; DQF COSY, double quantum-filtered correlated spectroscopy; ppm, parts per million

2. MATERIALS AND METHODS

Human Glu_1- and Lys_{78}-plasminogen of pooled human plasma origin were obtained from Kabi (Stockholm, Sweden) and Immuno AG (Vienna, Austria). Porcine elastase, aprotinin and aprotinin-agarose were obtained from Sigma Chemical Co., Dorset, UK. Miniplasminogen was isolated from Lys_{77}-plasminogen by a method based on that of Sottrup-Jensen et al. [5]. Approximately 100 mg of Lys_{77}-plasminogen together with 200 μl aprotinin solution (23 TIU/ml, 440 μM) were dissolved in 1 ml of 0.2 M ammonium bicarbonate buffer, pH 7.5, containing 50 μM ϵ-ACA and incubated with 100 μl porcine elastase solution (3 mg/ml in 0.2 M ammonium bicarbonate buffer, pH 7.5 containing 50 μM ϵ-ACA) for 70 min at 25°C. 100 μl of phenylmethylsulphonyl chloride solution (50 mM in acetone) were added and the mixture was held at 25°C for 10 min to terminate the reaction. Digestion products were separated by HPLC using a TSK G3000SW derivatised silica column eluting with ANT buffer [6] (arginine HCl 0.5 M, sodium chloride 0.5 M, Trizma base 20 mM, Tween-80 0.01% v/v, pH 7.0). The resolved

miniplasminogen-containing pool was concentrated by centrifugation using Centricon-10 concentration cells (Amicon, Gloucestershire, UK), buffer exchanged into 0.1 M ammonium bicarbonate buffer, pH 8.5 and lysine binding contaminants were removed by passage through a small lysine-Sepharose column. The small amount of miniplasmin present was removed by table rotation at 4°C with aprotinin-agarose (approximately 1 ml of packed gel, 0.36 TIU/ml, per 5 mg of protein) until the amidolytic activity against the chromogenic substrate S-2251 (H-D-Val-Leu-Lys-NH-Ph-NO_2·HCl, KabiVitrum) was reduced to less than 0.5% of that of fully active material. Samples for NMR were prepared at pH 4.0 by adjusting the pH with 2% and 0.2% NaOD and DCl, and were exhaustively desalted by cycles of concentration and dilution with D_2O using Amicon Centricon-10 concentration cells. The sample for the thermal unfolding experiment shown in Fig. 3 was buffer-exchanged into 100 mM d_4-acetic acid/sodium d_3-acetate by further concentration and dilution steps.

NMR spectra were acquired on Bruker 500 and 600 MHz AM series spectrometers at the Oxford Centre for Molecular Sciences. Sample concentrations varied between 0.4 and 1.2 mM and 500–3200 scans were acquired to give adequate signal-to-noise. One-dimensional spectra were acquired with quadrature detection over 4K data points and a spectral width of 14.92 ppm. Spectra are shown resolution-enhanced using a Lorenz-Gauss transformation with parameters GB 0.2, LB −10 followed by zero filling to 8K data points. For the double quantum-filtered 2D-correlated spectrum [7,8], 512 t_1 increments of 2K data points were acquired over a spectral width of 7462 Hz using time-proportional phase incrementation with saturation of the residual water by low-power irradiation during the 1 s relaxation delay introduced between scans. The data set was processed on a Sun 4/110 workstation using software provided by Dr D.R. Hare (FTNMR, Hare Research Inc.) and was resolution-enhanced by trapezoidal multiplication prior to zero filling to 4K data points in both dimensions.

3. RESULTS

The one-dimensional NMR spectrum of miniplasminogen is shown in Fig. 1. It is well resolved and shows a high degree of chemical shift dispersion. It can readily be seen, however, that the spectrum consists of some particularly well resolved resonances and a number of broader resonances. The two-dimensional COSY spectrum shows many intense cross-peaks; part of the aromatic region is shown in Fig. 2 and one can clearly identify a number of spin systems. Several resonances can be assigned to particular residues in the kringle 5 domain by comparison with the spectrum of isolated kringle 5 under similar conditions [9,10]. Indeed, from this comparison, it is apparent that almost every narrow resonance detected in the spectrum of miniplasminogen is present also in the spectrum of the isolated kringle 5 domain at a closely similar chemical shift position. In particular, the resonances assigned to the highly downfield shifted His-33 H2 proton, all the Trp-25 and Trp-62 ring protons and the Leu-46 methyl protons (consensus kringle numbering used here) have chemical shifts invariant to within 0.05 ppm in the two molecules.

As part of this work, we have performed thermal unfolding studies on miniplasminogen to investigate the stability of domains in this molecule. The unfolding of regions of the protein is manifested by the disap-

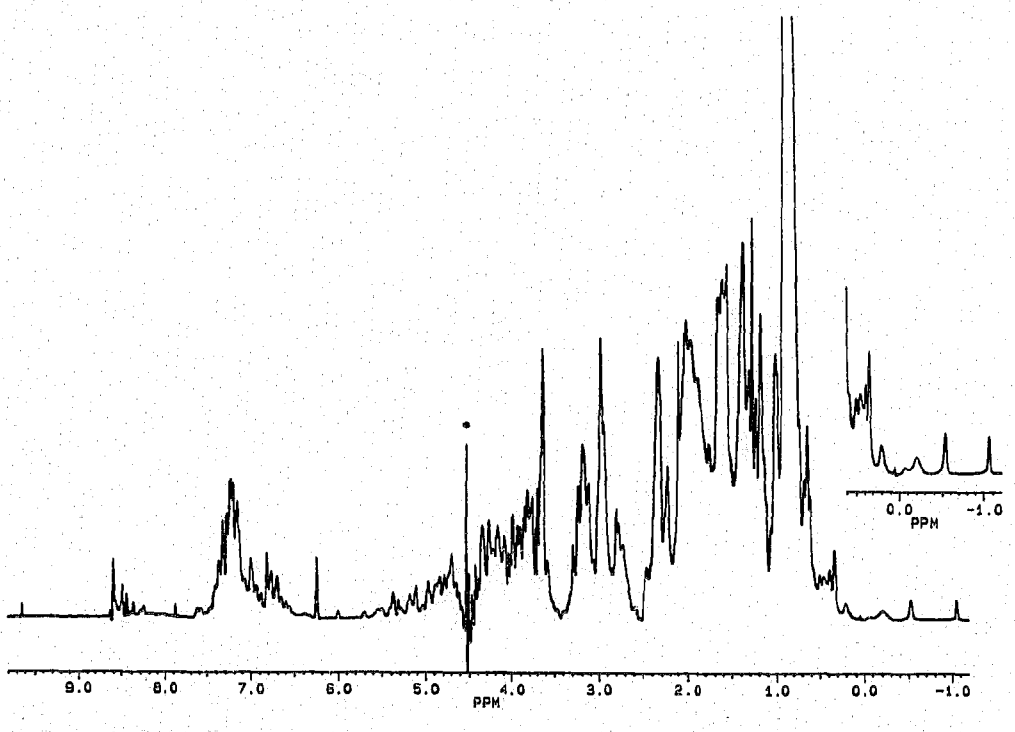

Fig. 1. 600 MHz spectrum of miniplasminogen in D_2O at 47°C, pH 4.0. All exchangeable protons have been exchanged for deuterons in this spectrum.

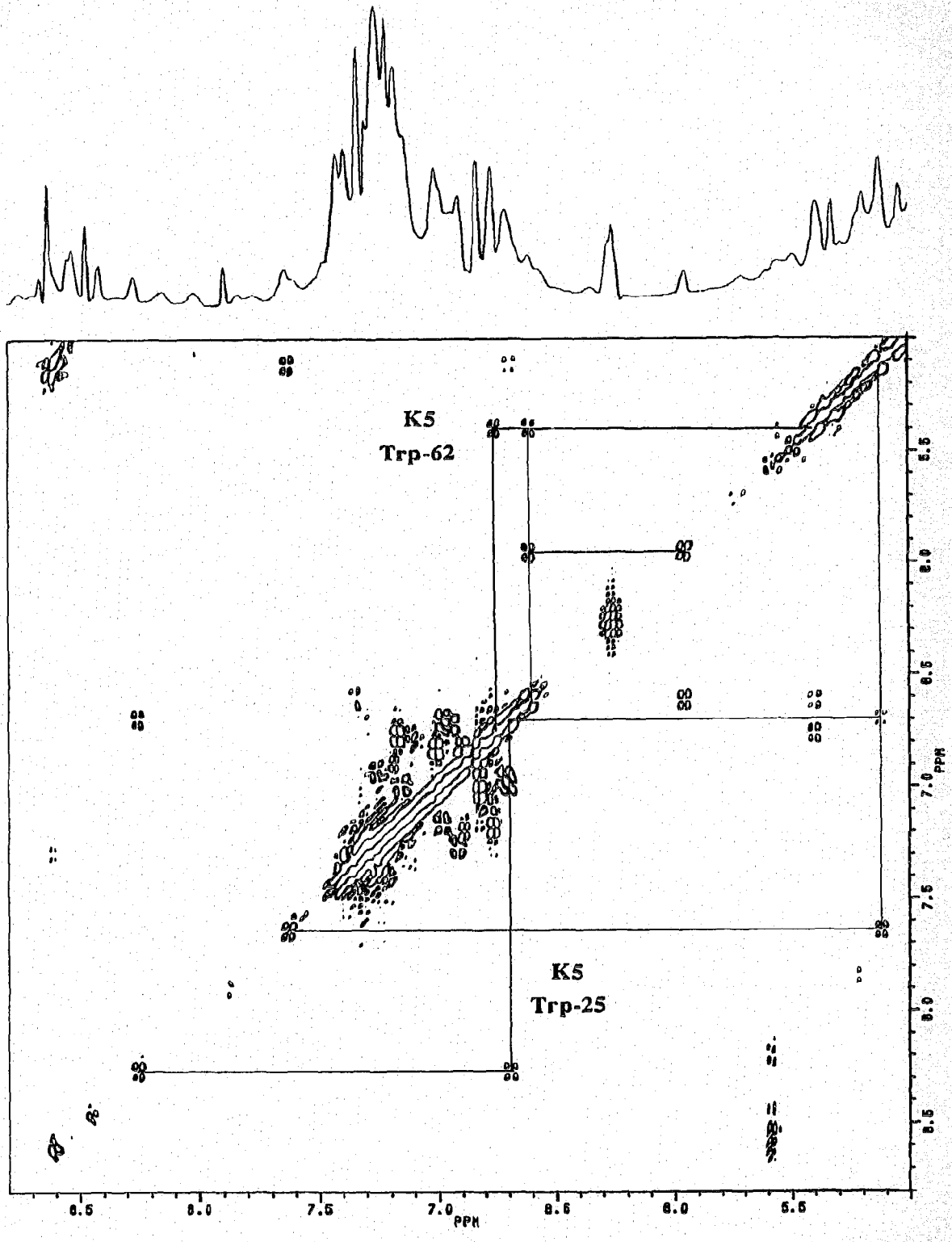

Fig. 2. 500 MHz NMR DQF COSY spectrum of miniplasminogen in D_2O at 37°C, pH 4.0. The two kringle 5 tryptophan spin systems are highlighted.

pearance of resolved resonances in both upfield and downfield regions of the NMR spectrum where these shifts in resonance position from those characteristic of unstructured peptides arose from perturbations of the local environment around the proton due to neighbouring residues and especially aromatic residues. The ther-

mal unfolding of miniplasminogen is shown in the upfield region of the NMR spectrum in Fig. 3. It can be seen that two sets of resonances disappear from the spectrum quite independently, corresponding to two unfolding transitions. One set has an unfolding transition midpoint of approximately 62°C and comprises all the identified resonances also present in the spectrum of kringle 5; in Fig. 3 this can be seen most clearly for the resonance at −1.06 ppm. The other set has an unfolding transition midpoint at higher temperature, at approximately 70°C; resonances disappearing at this temperature arise, by exclusion, from the protease domain. The lack of total cooperativity between the unfolding of the kringle and protease domains is in accord with the results of calorimetric studies [11]. Our conclusion differs from that of the calorimetry, however, in that we identify that the kringle is less rather than more stable than the protease domain. At this stage, we are unable to establish whether the protease domain shows totally cooperative unfolding, or whether it exhibits some non-cooperative behaviour as observed in urokinase [2] and suggested by calorimetry [11]. Further experimentation will be required to resolve both of these issues.

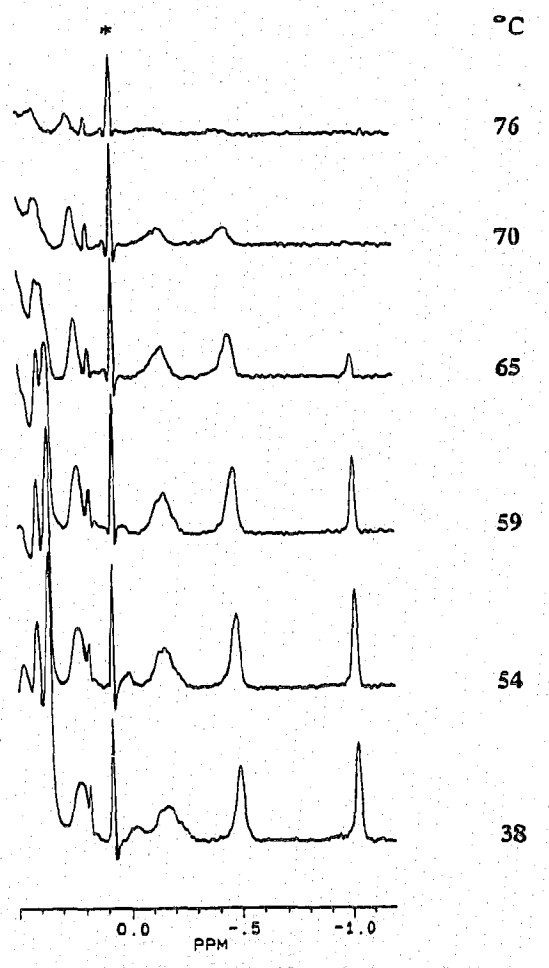

Fig. 3. Thermal unfolding of miniplasminogen at 500 MHz, pH 4.0 in 100 mM d_4-acetic acid/sodium d_3-acetate in D_2O.

In Fig. 4 are shown regions of the spectrum of Glu_1-plasminogen and a comparison with miniplasminogen under identical conditions. Although most of the lines in the spectrum of plasminogen are broad, a number of resonances are unusually well resolved for a molecule of molecular weight nearly 100000 Da. All the well-resolved resonances in the spectrum of miniplasminogen are also present in the spectrum of plasminogen at closely similar chemical shift positions. The narrowest of these resonances in miniplasminogen are the ones which have been assigned in the above to the kringle 5 domain; it seems likely, therefore, that these resonances in plasminogen also arise from the kringle 5 domain. This proposition is supported by the results of unfolding experiments performed on plasminogen using NMR. These have shown that these resonances persist to approximately 62°C, the same temperature as that associated with the unfolding of the kringle 5 in miniplasminogen, at which temperature they disappear in a single cooperative transition.

4. DISCUSSION

From the close identity of resonance positions in the various fragments and in the intact molecule we can deduce that close structural similarities must exist between the isolated, miniplasminogen and plasminogen kringle 5 domains. In miniplasminogen, the kringle resonances are significantly narrower than would have been expected for a globular molecule of this size. Although such line-narrowing could in principle arise from substantial internal motion, the resonances involved are located in the core of the kringle domain and have large chemical shift dispersions indicating the existence of highly ordered structure. Further, there appears to be no evidence from the crystal structure of prothrombin fragment 1 for disorder in this region [12]. We propose, therefore, that under these experimental conditions of acidic pH, the narrowness of the kringle lines results from significant freedom of motion of the entire domain about the kringle 5–serine protease linker. Such segmental motion causes the kringle protons to have an effectively reduced correlation time and thereby reduced line-widths. Our analysis of resonance linewidths has shown that the correlation time for kringle protons is almost half that of protease protons giving rise to peaks in the upfield region (A.J. Teuten, X. Li and C.M. Dobson, unpublished results). This effect is shown particularly clearly in the two-dimensional COSY spectrum (Fig. 2) which is highly selective for narrow resonances because of phase cancellation of cross-peaks arising from broad resonances. This is analogous to the mobility of the kringle domain reported in urokinase [1,2]. It is signifi-

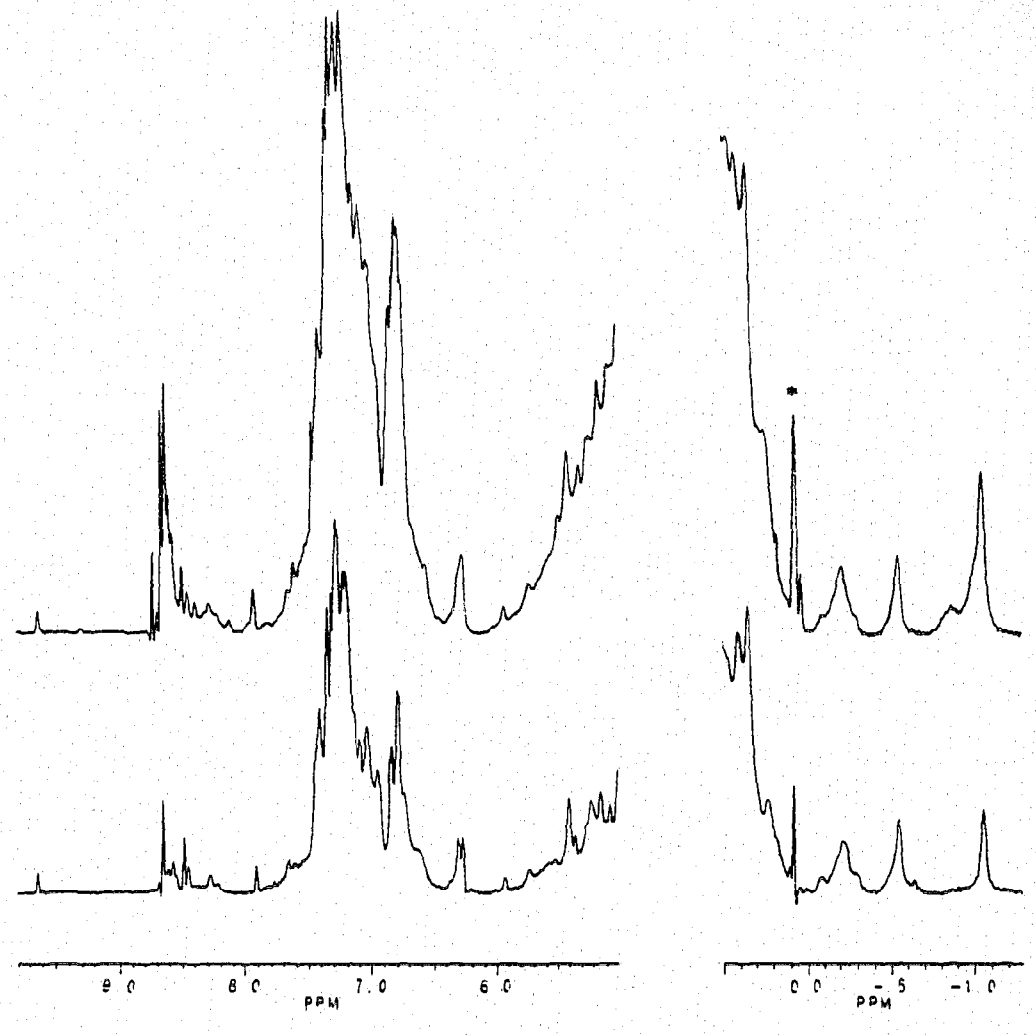

Fig. 4. Comparison of 500 MHz NMR spectra of Glu_1-plasminogen (above) and miniplasminogen (below) at 27°C, pH 4.0.

cant, however, that in plasminogen this particular linker is short, comprising just 6 residues including one proline, whereas in urokinase it is long, comprising 16 residues. Further, using NMR we have been able to observe non-cooperative thermal unfolding of the kringle and protease domains (Fig. 3) which provides additional evidence for significant independence of these domains.

In Fig. 4 it can be seen that the kringle and protease resonances in Glu_1-plasminogen are only broadened to a small degree relative to miniplasminogen in spite of the considerable increase in molecular weight. This can be explained in terms of segmental motion of the kringle and protease domains in plasminogen in the same way as it has been demonstrated here for miniplasminogen. In this case, however, the kringle 4–kringle 5 linker is present which must also permit motion of the kringle 5 domain. Nevertheless we have been unable to identify any resonances arising from the kringle 4 domain, with the possible exception of two histidine resonances (at 7.45 and 8.72 ppm) believed to arise from the surface residue His-3 [13]. Indeed, with this exception, no evidence has been obtained for any well resolved resonances arising from regions of the protein other than from the kringle 5 and serine protease domains. Each kringle, for instance, would be expected to contribute a resonance of intensity 3 protons to the peak at −1 ppm arising from the Leu-46 methyl protons. The chemical environment of these protons is highly conserved, and thus this chemical shift is very characteristic [9,14]. Although the spectrum of intact plasminogen does show intensity from more than one kringle in this region (Fig. 4), that from the kringles 1–4 appears very broad by comparison with that from kringle 5. Presumably the resonances of this portion of the molecule in the rest of the spectrum are broadened so as to be almost unobservable.

One explanation for this is that the kringles 1–4 and possibly the pre-activation peptide are strongly associated and hence undergo slow tumbling consistent with a combined molecular mass of close to 50000 Da. Close association between kringles 1, 2 and 3 would be

reasonable in view of the presence of a very short three residue linker between kringles 1 and 2 and an inter-domain disulphide bridge between kringles 2 and 3. The kringle 4 domain, however, has linkers of 24 and 26 residues on either side and if these were unstructured would give opportunity for considerable mobility. Although the possibility that intermolecular interactions make a contribution to the line-widths cannot be fully excluded at this stage, our results suggest that linker length is not of overriding importance in determining domain independence. That long linkers do not necessarily give rise to high mobility may be because of structure in such linkers, as a result of the structural requirements for an O-linked glycosylation site (here at Thr-346), or as a consequence of strong domain-domain contacts.

Our results have demonstrated that NMR can be a valuable technique for investigating thermodynamic and dynamical aspects of large multi-domain molecules. Our evidence that independent domain motion can occur even when inter-domain linker peptides are relatively short widens the possible range of proteins to which these techniques can be applied. Dynamical properties of proteins are also increasingly recognised as being of major functional importance, and the independent domain motion observed in the proteins investigated so far may have particular significance in their biological roles. In particular, the possible processive or migratory behaviour of such proteins within the fibrin matrix [15,16] must depend to some extent on independent domain function. The latter has already been suggested as an explanation for the apparent difficulty in crystallisation of multi-domain proteins [1,17]. Analysis of the NMR spectra of individual domains can lead to complete structure determination [18,19] and in conjunction with low angle X-ray scattering and modelling studies [20], this technique can be used to obtain a structural perspective even in the absence of X-ray data. In preliminary experiments we have observed changes in the NMR spectra of plasminogen and miniplasminogen on activation to plasmin and miniplasmin. As a result it may be possible to investigate structural changes associated with proteolytic cleavages and zymogen activation. It is hoped that these studies will lead to a greater understanding of the structure and properties of a number of fibrinolytic proteins of clinical and therapeutic importance.

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