A comparative study of structural properties of fibronectin and its 180 kDa fragment

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Fibronectin from human plasma and its 180 kDa fragment which retained collagen-binding, cell-attachment and heparin-binding activities, were studied by velocity centrifugation and ^{1}H-NMR methods. The fibronectin hydrodynamic radius strongly increased at pH 11 while the hydrodynamic properties of the fragment did not change noticeably. ^{1}H-NMR spectroscopy also showed differences in the molecular properties of fibronectin and its 180 kDa fragment. Under physiological conditions the structure of fibronectin differs from that of its 180 kDa fragment. At pH 11 and in 4 M urea no differences in their structures are observed. It is suggested that interdomain and intersubunit interactions play an important role in maintaining the native conformation of intact fibronectin.

Fibronectin *180 kDa fibronectin fragment* *^{1}H-NMR*

1. INTRODUCTION

Fibronectins, high molecular mass glycoproteins, play an important role in the numerous functions of the cell [1,2]. Digestion of fibronectin by various proteases permitted the isolation of functionally active domains of the protein which interact with the collagen, heparin, cell, bacteria and DNA [1,2]. The study of fibronectin structure by different methods has shown that the fibronectin molecule, consisting of 2 subunits, contains compact globular domains which are connected by flexible polypeptide segments [3–6]. The secondary structure of fibronectin is formed exclusively by the antiparallel β-form (35%) which is more or less uniformly distributed along the protein molecule [5,7]. It has been shown recently that the isolated structural and functional domains of fibronectin retain their native conformation in solution [6,7]. This work is devoted to a study of the structural properties of fibronectin by velocity centrifugation and ^{1}H-NMR spectroscopy methods.

2. MATERIALS AND METHODS

Fibronectin was isolated from human plasma by affinity chromatography on gelatin-Sepharose with following chromatography on DEAE-cellulose [8,9]. The fibronectin fragment containing gelatin-, heparin-binding and cell-attachment domains was prepared by trypsin digestion. Trypsin in a 1:3000 ratio was added to 0.8 mg/ml of fibronectin and the mixture was incubated for 20 min at 22°C. The products of digestion were chromatographed on heparin-Sepharose and the absorbed material passed through a column (2.6 × 100 cm) with Sephacryl C-300. The obtained fragment had a purity greater than 98% by polyacrylamide gel electrophoresis in the presence of SDS and was completely absorbed on gelatin- and heparin-Sepharose. This fragment was denoted as the 180 kDa fragment.

For studies by the ^{1}H-NMR method the proteins were transferred into D_2O through a column 0.5 × 15 cm) with Sephadex G-25, equilibrated in a buffer containing 0.015 M sodium phosphate, pH 7.4,

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0.15 M NaCl in D_2O. The protein concentration was 3–4 mg/ml. ^{1}H-NMR spectra were obtained in a Bruker M500 spectrometer. Chemical shifts were measured using 2,2-dimethyl-2-silapentane-5-sodium sulfonate as an internal standard.

Sedimentation experiments were done in a Beckman model E (USA) analytical ultracentrifuge using UV optics with a scanner at 280 nm.

3. RESULTS AND DISCUSSION

To elucidate the structural organization of fibronectin a study was done of the intact fibronectin and its 180 kDa fragment which retained the basic functional properties of fibronectin: binding with collagen and heparin, stimulation of cell adhesion. The sedimentation analysis of fibronectin and 180 kDa fragment has shown that fibronectin (dimer) has a molecular mass of 430 kDa and a sedimentation constant of 14 S. With an increase of the solution pH to 11, the sedimentation constant of the protein decreased to 8 S. At the same time it was noted that its secondary structure did not vary essentially [4]. The 180 kDa fragment (monomer) has a sedimentation constant of 6.8 S. In contrast to the native protein the sedimentation constant of the fragment did not decrease noticeably with an increase of pH. The increase of the hydrodynamic radius for fibronectin with the increase of the pH indicates a significant unfolding of the molecule. That this parameter does not change for the 180 kDa fragment is evidence of the absence of unfolding with a change of pH. The question is: what is the difference between the structure of fibronectin and its 180 kDa fragment? We have assumed that in the intact fibronectin interdomain and intersubunit interactions play an important role in the compactization of the protein. To test this assumption fibronectin and 180 kDa fragment were studied by NMR spectroscopy. Fig.1 presents the ^{1}H-NMR spectra of fibronectin and 180 kDa fragment. The spectra are characterized by the presence of wide overlapping lines against the background of which narrow signals are observed. Such spectra are typical for large proteins [10]. Line widths in the NMR spectrum are determined by the mobility of parts of the protein molecule. Consequently, there are regions in the fibronectin molecule with different mobilities. The narrow component of the spectrum is most likely due to the amino acid residues located on the surface of the domains and in the flexible non-structured regions of fibronectin and 180 kDa fragment. We did not carry out a detailed study of the ratio between the integral intensity of the narrow and broad component, but according to a rough estimate the narrow component in the fibronectin spectrum composes not more than 20% of the total signal. It can be assumed that the greater part of the fibronectin amino acid residues is not very mobile and in a compact structure. The ^{1}H-NMR spectrum of the 180 kDa fragment is

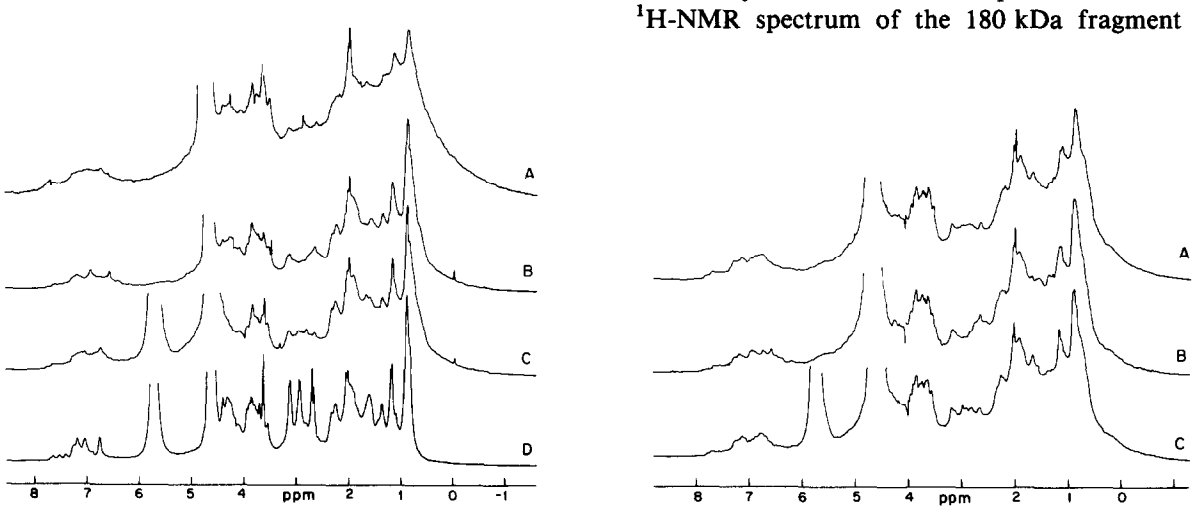

Fig.1. ^{1}H-NMR spectra of intact fibronectin (left) and 180 kDa fragment (right). A, 0.015 M sodium phosphate, pH 7.4, 0.15 M NaCl; B, (A) + 4 M urea; C, (A) but pH 11; D, (A) + 4 M urea + 0.005 M DTT.

close to that of the intact protein. However, the broad component in the fragment spectrum is less pronounced than that in the fibronectin spectrum. This is evidence that the 180 kDa fragment, just as fibronectin, contains regions with a compact structure.

Fig.1 shows the effect of different agents denaturing fibronectin: elevated pH, urea and reduction of disulfide bonds. Increase of the pH leads to a greater share of mobile regions, i.e. to some unfolding of the structure of fibronectin and its fragments. This is indicated by the sharpening and increase of the narrow component intensity both in the aromatic and aliphatic regions of the spectrum and by the relative decrease of the broad component intensity. Notwithstanding the unfolding of the protein, fibronectin and its fragment at pH 11 retains compact regions with a globular structure. Evidence of this is the presence of signals in the spectra from the methyl groups of aliphatic amino acid residues in a much stronger field beginning from 0.9 ppm. A corroboration of the presence of domains is a significant overlapping and wide range of chemical shifts of aromatic residue ring proton resonances in the 5.0–7.5 ppm interval. The addition of 4 M urea to fibronectin and to its fragment partially denatures the protein – the contribution of the spectrum broad component decreases and practically all the resonances sharpen. The presence of 4 M urea does not lead to complete denaturation of the protein: there are the same signs of folded structures as at pH 11. Complete denaturation of fibronectin and its fragment does not take place either with the addition of 8 M urea (not shown). After addition of dithiothreitol in the presence of urea the fibronectin spectrum dramatically changes and resembles a usual spectrum of denatured protein. In this spectrum all the lines are narrow, the signals from protons of monotypic residues having close chemical shifts. It should be noted that the spectral structure and the ratio between the narrow and broad component in the fibronectin spectra at pH 11 and in 4 M urea are similar to the spectrum of the 180 kDa fragment in the same non-physiological conditions. This indicates that fibronectin and the 180 kDa fragment at pH 11 and 4 M urea have similar structural properties. A study of the effect on fibronectin structure of such factors as the increase of pH, addition of urea and reduction of disulfide bonds by the ^{1}H-NMR method has shown that fibronectin is a highly structured protein retaining significantly compact domains under conditions usual for denaturation of globular proteins. Disulfide bonds play an important role in maintaining the structures of fibronectin domains. The basic differences between fibronectin and its 180 kDa fragment are revealed in non-denaturing conditions. This is in good agreement with sedimentation data analysis. To distinguish the differences between fibronectin and the 180 kDa fragment in physiological conditions we made their

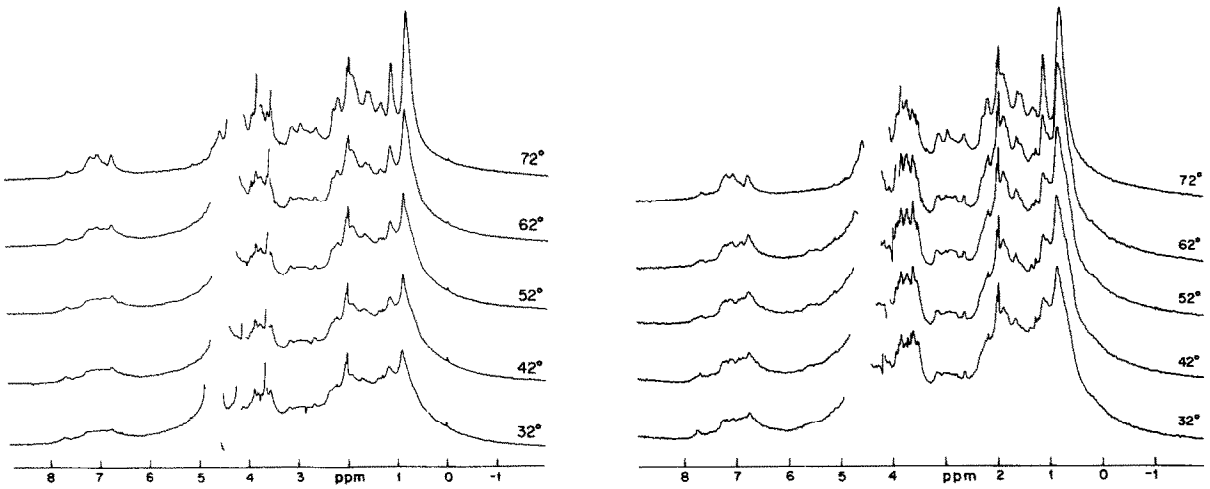

Fig.2. ^{1}H-NMR spectra of intact fibronectin (left) and 180 kDa fragment (right) at different temperatures.

comparative study at different temperatures (figs 2,3). At an elevated temperature from 17 to 60°C the intensity of the narrow component in the fibronectin spectra increases while that of the broad one decreases without any noticeable change in the general form of the spectrum. The relative change of the intensity of the line with the center at 0.9 ppm (the intensity of this signal at 27°C is taken as unity) depending on the temperature is shown in fig.3. The signal in the range of 0.9 ppm in protein spectra is mainly due to the methyl group protons of valine, leucine and isoleucine amino acid residues. In physiological conditions the intensity in the 180 kDa fragment spectrum at a temperature increase from 17 to 60°C changes insignificantly. In denaturing conditions (pH 11, 4 M urea) changes in the spectra of fibronectin and the 180 kDa fragment proceed similarly. Up to 40°C the intensity of lines in their spectra remains unchanged and increases at higher temperatures. Thus, under physiological conditions, with an increase of temperature from 17 to 50°C, a noticeable increase of the narrow component intensity takes place in the fibronectin spectrum, while the effect is much less pronounced for the 180 kDa fragment. It has been shown previously that fibronectin structural domains under physiological conditions are stable at heating up to 55°C and that denaturation is observed at 65°C [4,5]. The 180 kDa fragment also has an analogous stability. Thus, the observed difference between fibronectin and its 180 kDa fragment under physiological conditions is not determined by the difference in the temperature of domain denaturation but by other factors.

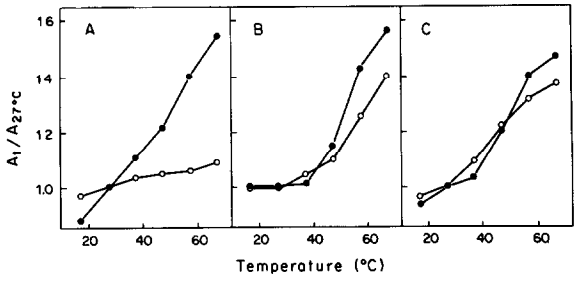

Fig.1. ^{1}H-NMR spectra of intact fibronectin (left) and 180 kDa fragment (right). A, 0.015 M sodium phosphate, pH 7.4, 0.15 M NaCl; B, (A) + 4 M urea; C, (A) but pH 11; D, (A) + 4 M urea + 0.005 M DTT.

This difference is difficult to explain using the model of fibronectin with thin elongated subunits in which domains are not interacting with each other [6,11]. Our experimental data are easy to explain if it is assumed that interdomain and intersubunit interactions in the fibronectin molecule exist. At an increase of the solution temperature up to 50°C the bonds between the domains weaken. The mobility of the domains in this case must increase with the resulting increase of the narrow component and decrease of the broad one in the fibronectin spectrum. As the changes in the fibronectin spectrum proceed under low (physiological) temperatures it follows that the connection between the domains is weak. This points to the high flexibility of the fibronectin molecule. The fact that in physiological conditions the fibronectin spectrum has a greater intensity of the broad component and a somewhat lower resolution in the aromatic region of the spectrum than in that of the 180 kDa fragment spectrum can also be explained by the presence of interactions between the domains in the intact fibronectin and the absence of such interactions in the 180 kDa fragment. It is the similarity of fibronectin and 180 kDa fragment in denaturing conditions (pH 11, 4 M urea) that explains the same temperature dependence of their spectra changes and reflects only the domain denaturation of fibronectin and 180 kDa fragment. Thus, the main difference in the structural organization of fibronectin and its 180 kDa fragment is that interdomain and intersubunit interactions play a dominant role in maintaining the native conformation of the intact protein. The presence of such interactions leads to compactization of the fibronectin molecule.

Proceeding from all the available facts in fibronectin conformation studies it is evident that the protein molecule possesses a high intramolecule (interdomain and intersubunit) flexibility and can dramatically change its shape depending on external factors and at interaction with other molecules.

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