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Unfolding transitions of fibronectin and its domains

Stabilization and structural alteration of the *N*-terminal domain by heparin

M. Yahiya KHAN,*‡ Marvin S. MEDOW† and Stuart A. NEWMAN*§

*Department of Cell Biology and Anatomy and †Department of Pediatrics, Division of Pediatric Gastroenterology and Nutrition, New York Medical College, Valhalla, NY 10595, U.S.A.

Changes in the conformational state of human plasma fibronectin and several of its fragments were studied by fluorescence emission, intrinsic fluorescence polarization and c.d. spectroscopy under conditions of guanidinium chloride- and temperature-induced unfolding. Fragments were chosen to represent all three types of internal structural homology in the protein. Low concentration (< 2 M) of guanidinium chloride induced a gradual transition in the intact protein that was not characteristic of any of the isolated domains, suggesting the presence of interdomain interactions within the protein. Intermediate concentrations of guanidinium chloride (2–3 M) and moderately elevated temperatures (55–60 °C) induced a highly co-operative structural transition in intact fibronectin that was attributable to the central 110 kDa cell-binding domain. High temperatures (> 60 °C) produced a gradual unfolding in the intact protein attributable to the 29 kDa *N*-terminal heparin-binding and 40 kDa collagen-binding domains. Binding of heparin to intact fibronectin and to its *N*-terminal fragment stabilized the proteins against thermal unfolding. This was reflected in increased ΔH for the unfolding transitions of the heparin-bound *N*-terminal fragment, as well as decreased accessibility to solvent perturbants of internal chromophores in this fragment when bound to heparin. These results help to account for the biological efficacy of the interaction between the fibronectin *N*-terminal domain and heparin, despite its relatively low affinity.

INTRODUCTION

The fibronectins are a group of high-molecular-mass glycoproteins having a variety of functions related to cell adhesion, extracellular-matrix organization and tissue morphogenesis (Akiyama & Yamada, 1987; Mosher, 1988; Ruoslahti, 1988). Plasma fibronectin is the soluble form of the protein, consisting of two similar but non-identical subunits of about 225 kDa. The subunits are connected close to their *C*-terminal ends by two disulphide bonds (Mosesson *et al.*, 1975; Engvall *et al.*, 1978). Each subunit contains a number of discrete functional domains, which are made up, in turn, of three different 'homology types' or motifs (Petersen *et al.*, 1983; Skorstengaard *et al.*, 1986; Akiyama & Yamada, 1987; Hynes, 1985).

Electron microscopy shows fibronectin as a long thin strand about 2 nm in diameter, having two arms each approx. 61 nm in length, joined to each other at an angle of about 70° (Koteliansky *et al.*, 1980, 1981; Engel *et al.*, 1981; Erickson *et al.*, 1981; Odermatt *et al.*, 1982; Price *et al.*, 1982; Tooney *et al.*, 1983; Erickson & Carrell, 1983). In solution, however, the shape of fibronectin can vary from an almost fully extended state to a more compact conformation (Alexander *et al.*, 1979; Koteliansky *et al.*, 1980, 1981; Engel *et al.*, 1981; Erickson *et al.*, 1981; Odermatt *et al.*, 1982; Price *et al.*, 1982; Williams *et al.*, 1982; Marković *et al.*, 1983; Tooney *et al.*, 1983; Erickson & Carrell, 1983; Rocco *et al.*, 1984). The compact form is favoured in physiological or near-physiological buffers (Williams *et al.*, 1982; Rocco *et al.*, 1987). In contrast, partial opening of the structure is observed upon increasing the ionic strength or glycerol content, and at extreme pH values (Alexander *et al.*, 1979; Lai *et al.*, 1986). These reversible transitions occur without any significant changes in the secondary structure, suggesting that electrostatic interactions are responsible for these structural re-arrangements (Williams *et al.*, 1982; Rocco *et al.*, 1987).

Very little is known about the three-dimensional structure of fibronectin, and c.d. and i.r. spectroscopy have indicated only limited elements of conventional secondary structure (Colonna *et al.*, 1978; Koteliansky *et al.*, 1980, 1981; Venyaminov *et al.*, 1983). However, proteolytic cleavage of the protein yields a number of fragments capable of independent binding to ligands such as heparin and collagen (Odermatt *et al.*, 1982; Hayashi & Yamada, 1983; Hörmann & Richter, 1986), and the relative positions of the corresponding domains in intact fibronectin are well characterized (Hynes, 1985; Akiyama & Yamada, 1987). Useful information concerning the structure of the intact protein can therefore be gained by comparing its unfolding behaviour with that of its isolated domains. With such information, structural changes in intact fibronectin upon binding a ligand such as heparin can also potentially be attributed to specific domains.

In the present investigation we have undertaken a systematic study of fibronectin and three of its important fragments, representing all the different internal homology types of the protein. Using the techniques of fluorescence emission, intrinsic fluorescence polarization and c.d. spectroscopy in the presence of guanidinium chloride, elevated temperature and solvent perturbants we have found strong evidence for interdomain interactions in fibronectin. We have also found that heparin alters and significantly stabilizes the structure of fibronectin's *N*-terminal domain, confirming and extending our earlier findings with c.d. spectroscopy (Khan *et al.*, 1988). Moreover, the stabilizing effect of heparin on the structure of intact fibronectin is confined to a portion of its denaturation profile that is dominated by the *N*-terminal domain. These results are discussed

Abbreviations used: FnNTD, fibronectin 29 kDa *N*-terminal domain; GdnHCl, guanidinium chloride.

‡ Visiting Fellow of the Fogarty International Center. Permanent address: Department of Biochemistry, North-Eastern Hill University, Shillong, India.

§ To whom correspondence should be addressed.

in relation to potential biological roles of the interaction of fibronectin with heparin-like molecules.

MATERIALS AND METHODS

Materials

Human plasma fibronectin was purchased from the New York Blood Center. Sucrose added to the commercial fibronectin preparation as a solubilizing/stabilizing agent was removed by Sephadex G-25 column chromatography, which was also employed for exchange of buffers wherever needed. The fibronectin 29 kDa *N*-terminal domain (FnNTD) was prepared by digestion with tosylphenylalanylchloromethane-treated trypsin according to the procedure of Hayashi & Yamada (1983), and its homogeneity was established by Sephadex G-100 column chromatography as well as by SDS/PAGE on 10% polyacrylamide gels. The identity of the FnNTD was confirmed by amino acid analysis (N. S. Jaikaria & S. A. Newman, unpublished work) and comparison with the published sequence (Kornblihtt *et al.*, 1985). This digestion procedure also yielded two fragments that bound tightly to gelatin–Sepharose (Hayashi & Yamada, 1983), which were eluted with 4 M-urea and further resolved by gel filtration on a Sephadex G-100 column. The smaller fragment (approx. 40 kDa), corresponding to the collagen-binding domain, was used in our studies. A 110 kDa cell-binding thermolysin fragment from the central region of fibronectin was a gift from Dr. S. K. Akiyama of the National Cancer Institute (Akiyama & Yamada, 1987). Fig. 1 shows a schematic map of the functional domains of the fibronectin molecule, and the relative positions of the fragments used in the present study. Guanidinium chloride (GdnHCl; ultrapure, enzyme grade) was obtained from Bethesda Research Laboratories. Other reagents were of analytical grade. All experiments were performed in phosphate-buffered saline (0.02 M-sodium phosphate buffer, pH 7.2, containing 0.15 M-NaCl).

Protein concentration

Protein concentrations were determined by the method of Bradford (1976), with BSA as a standard, or spectrophotometrically, by using the specific absorption coefficient ($A^{1\%}_{1\,cm}$) of 12.8 at 280 nm for fibronectin (Mosesson & Umfleet, 1970).

Reduction of disulphide bonds

Disulphide bonds of fibronectin and the FnNTD were reduced with 2-mercaptoethanol and the resulting thiol groups were modified by reaction with iodoacetamide (Hirs, 1967). Excess reagent was removed on a Sephadex G-25 column.

C.d. spectroscopy

C.d. measurements were made on a Jasco model 500C spectropolarimeter equipped with a data processor. The details were the same as described previously (Khan *et al.*, 1988).

Fluorescence measurements

Fluorescence measurements were made with a Shimadzu RF540 spectrofluorophotometer equipped with a thermoregulated sample chamber and automatic polarizers (C. N. Wood, Newtown, PA, U.S.A.). Excitation and emission wavelengths were 280 nm and 320 nm respectively unless otherwise indicated. For fluorescence anisotropy measurements, emission intensities at $\lambda_{max.}$ (325–335 nm, depending on the protein) parallel and perpendicular to the excitation plane were recorded sequentially, and the anisotropy, A, was determined from the equation:

$$A = (I_{vv} - I_{vh})/(I_{vv} + 2I_{vh})$$

where I is the intensity, and the first and second subscripts refer to the plane (v, vertical; h, horizontal) of polarization of the excitation and emission beams respectively (Cantor & Schimmel, 1980). Temperature-dependence of anisotropy was recorded in a continuous fashion between ambient temperature and 80 °C. All measurements were performed on at least three independent preparations. To minimize scattering effects, all solutions were filtered through Millipore filters (pore size 0.45 μm) before fluorescence measurements. The protein concentrations were chosen so that the A_{280} value never exceeded 0.06, and the fluorescence intensity was a linear function of concentration. The spectra were routinely corrected for Raman emission by subtraction of appropriate blanks.

RESULTS

Structural transitions of fibronectin and its fragments during GdnHCl-induced unfolding

The characteristic emission maximum, $\lambda_{max.}$, of an aromatic chromophore is highly sensitive to its environment (Freifelder, 1982) and can be used as an index of protein unfolding under various conditions. Fig. 2 shows the GdnHCl-induced unfolding of fibronectin, the FnNTD, the 40 kDa collagen-binding fragment and a central 110 kDa fragment that includes the cell-binding domain. All four proteins were found to undergo single-step transitions having similar midpoints at about 2.6 M-GdnHCl. However, $\lambda_{max.}$ for the intact protein exhibited a gradual decrease between 0 M- and 2 M-GdnHCl not seen with any of its fragments.

When polarization of fluorescence, a technique that is sensitive

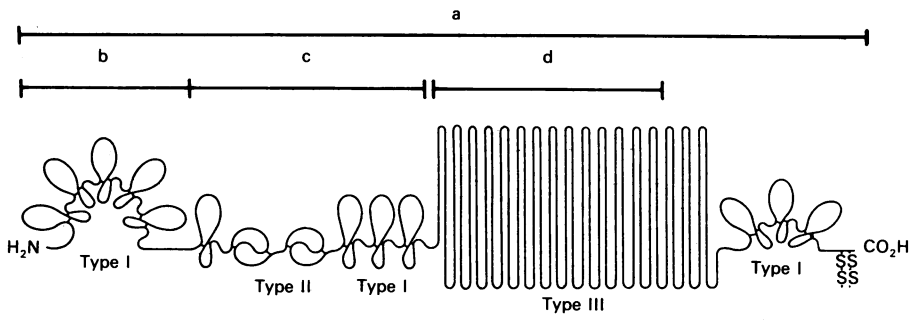

Fig. 1. Domain organization of human plasma fibronectin

Positions of type I, type II and type III internal homologies, positions of interchain disulphide bonds and relative locations of the proteins and fragments used in this study are indicated. Protein a consists of the entire dimeric molecule, fragment b is the 29 kDa FnNTD, fragment c is the 40 kDa collagen-binding domain and fragment d is the 110 kDa cell-binding domain. [Modified from Akiyama & Yamada (1987) with permission of the authors.]

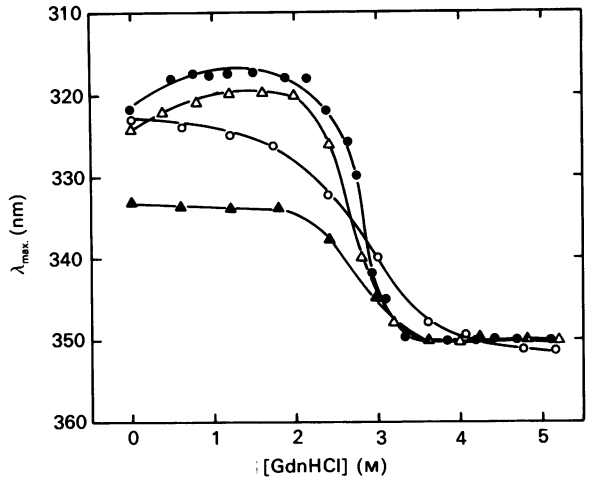

Fig. 2. Effect of GdnHCl concentration on fluorescence emission maxima of human plasma fibronectin and its fragments

○, Intact fibronectin; ●, FnNTD; ▲, 40 kDa collagen-binding fragment; △, 110 kDa cell-binding fragment.

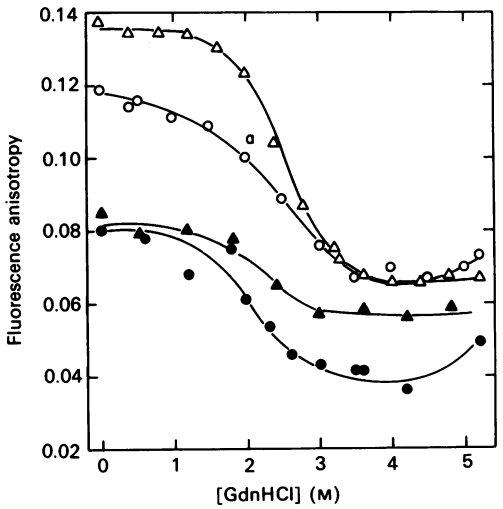

Fig. 3. Effect of GdnHCl concentration on fluorescence anisotropy of human plasma fibronectin and its fragments

○, Intact fibronectin; ●, FnNTD; ▲, 40 kDa collagen-binding fragment; △, 110 kDa cell-binding fragment.

to the rigidity of the chromophore environment, was employed, analogous results were obtained. Although there is a gradual GdnHCl-induced decrease in intrinsic fluorescence anisotropy for the intact protein between 0 and 1.5 M, the transitions for the three fragments do not begin until the concentration of GdnHCl is above 1.2 M (Fig. 3).

The utility of c.d. for determination of fibronectin structure is limited by the domination of its spectrum by a strong positive band at 228 nm that it shares with only a small number of other proteins (Woody, 1978; Welsh *et al.*, 1983). This band is due partly to spectral contributions from all three aromatic amino acid side chains, and partly to incompletely understood structural features of the protein (Khan *et al.*, 1989). We have previously used changes in the magnitude of this band as a probe of fibronectin structural changes in the presence of various polysaccharide ligands (Khan *et al.*, 1988). In the present work we have monitored the ~ 228 nm c.d. band of fibronectin and its fragments in the presence of increasing concentrations of

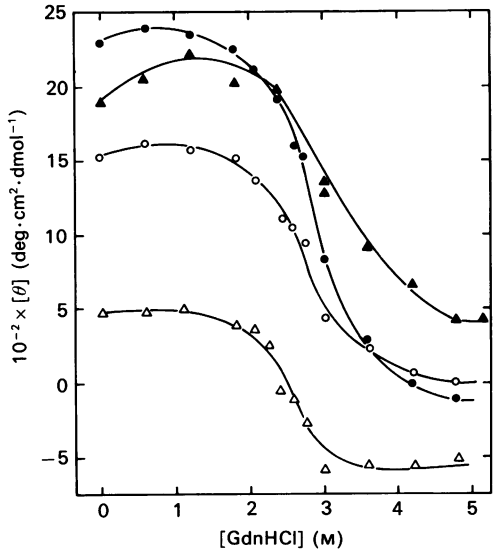

Fig. 4. Effect of GdnHCl concentration on molar ellipticity of human plasma fibronectin and its fragments

○, Intact fibronectin; ●, FnNTD; ▲, 40 kDa collagen-binding fragment; △, 110 kDa cell-binding fragment. The far-u.v. c.d. spectra of the proteins were recorded at 25 °C and their maxima (228, 229, 226 and 228 nm for intact fibronectin and the FnNTD, collagen-binding domain and cell-binding domain respectively) were utilized for computation of molar ellipticities. Protein concentrations were in the range 0.05–0.1 mg/ml.

GdnHCl (Fig. 4). Although the general characteristics of the denaturation curves shown in Fig. 4 are similar to those in Figs. 2 and 3, the c.d. transition seen with the intact molecule lacked the phase representing gradual structural perturbations at < 1.5 M denaturant concentration.

Structural transitions induced by elevated temperature

Fluorescence anisotropy measurements of fibronectin and its fragments were performed as a function of temperature under several different conditions. The anisotropy of the intact protein did not show the gradual change seen with low GdnHCl concentrations, but exhibited a highly co-operative transition between about 55 °C and 65 °C, followed by a more gradual increase in flexibility above 65 °C (Fig. 5*a*). Interestingly, the reduced and modified protein (Fig. 5*c*) behaved similarly to the non-reduced protein (Fig. 5*a*) in the co-operative melting range, but unlike the latter resisted additional change to a more flexible state above 65 °C.

The FnNTD, a relatively flexible structure even at 40 °C, underwent a gradual biphasic temperature-induced loss of fluorescence anisotropy between 40 °C and 70 °C (Fig. 6*a*). Although its unfolding profile differed in detail from that of the FnNTD, the collagen-binding fragment also exhibited a gradual biphasic thermal unfolding transition in fluorescence anisotropy (Fig. 6*f*). These changes contrasted markedly with the highly co-operative transition undergone by the intact fibronectin molecule (Fig. 5*a*) and its cell-binding fragment (Fig. 6*e*).

The extensive unfolding of the FnNTD that occurred in the presence of GdnHCl (Fig. 6*d*) could not be achieved by elevated temperature alone (Fig. 6*a*). Furthermore, reduction and modification of the FnNTD substantially decreased its flexibility (Fig. 6*c*), suggesting that the FnNTD acquires additional secondary structure upon reduction (see also Fig. 3 in Khan *et al.*, 1988). Even at temperatures as high as 80 °C the flexibility of the reduced and modified FnNTD was less than that of the thermally unfolded disulphide-bonded fragment (Fig. 6*a*).

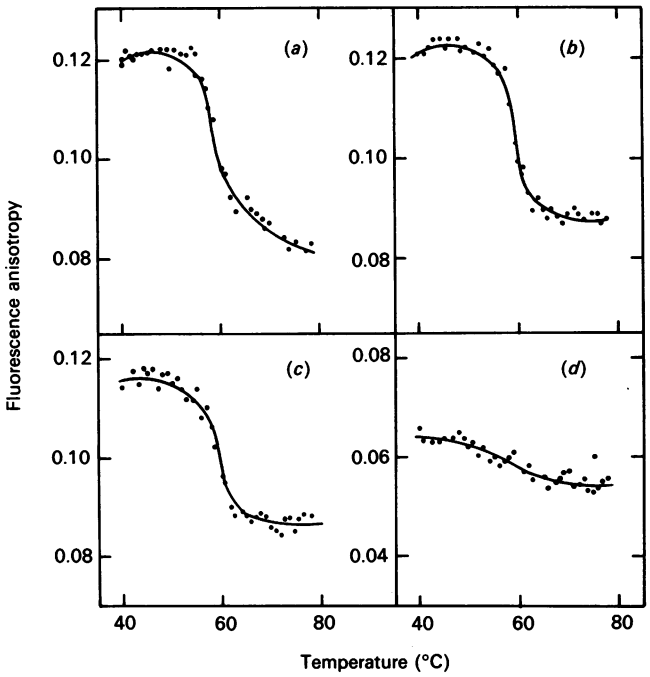

Fig. 5. Temperature-dependence of fluorescence anisotropy of human plasma fibronectin under various conditions

(*a*) Human plasma fibronectin; (*b*) fibronectin–heparin complex; (*c*) reduced and modified fibronectin; (*d*) GdnHCl (6 M)-denatured fibronectin. Fluorescence emission intensities parallel and perpendicular to the excitation plane were sequentially recorded with the automatic polarizer while the temperature in a thermoregulated sample chamber was raised from 40 °C to 80 °C at a rate of 0.5 °C/min. Points shown are representative of data collected over a continuum of temperature values.

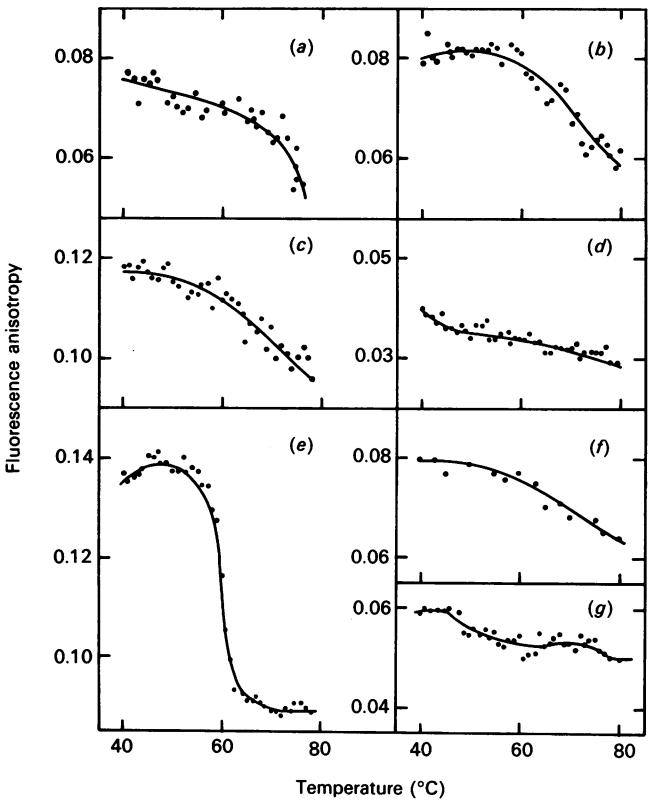

Fig. 6. Temperature-dependence of fluorescence anisotropy of fragments of human plasma fibronectin under various conditions

(*a*) FnNTD; (*b*) FnNTD–heparin complex; (*c*) chemically reduced and modified FnNTD; (*d*) FnNTD in the presence of 6 M-GdnHCl; (*e*) 110 kDa cell-binding fragment; (*f*) 40 kDa collagen-binding fragment; (*g*) cell-binding fragment in the presence of 6 M-GdnHCl. Other details are the same as described in the legend to Fig. 5.

Effect of heparin binding

Both intact fibronectin and the FnNTD were more resistant to the loss of fluorescence anisotropy with increasing temperature in the presence of heparin (Figs. 5*b* and 6*b*) than in its absence (Figs. 5*a* and 6*a*). This effect was much more pronounced for the fragment, where the maximum anisotropy was retained over the 40–60 °C temperature range in the presence of heparin (Fig. 6*b*), in contrast with the gradual loss of anisotropy with increased temperature in the absence of heparin (Fig. 6*a*).

These results suggested that heparin induces a conformational change in the FnNTD, consistent with our earlier suggestions based on c.d. (Khan *et al.*, 1988). We performed a van't Hoff analysis of the thermal unfolding equilibrium of the FnNTD, using a two-state transition model (Pace, 1986). Because the unfolding transition would not be expected to be reversible at the highest temperatures tested, we excluded data points that were obtained above 75 °C. Under this assumption, we estimated that the enthalpy of unfolding of the FnNTD is 117.2 kJ/mol (28.0 kcal/mol) (Fig. 7). In the presence of heparin, ΔH increases to 311.3 kJ/mol (74.4 kcal/mol), indicating a stabilization against thermal unfolding in the complex of 194.1 kJ/mol (46.4 kcal/mol) relative to the isolated domain.

The total ΔG for the unfolding transition of the FnNTD, and the extent to which it may be changed in the presence of heparin, depend on entropic as well as enthalpic considerations, but the former are difficult to ascertain directly. In order to gain some insight into the nature of the structural rearrangement undergone by fibronectin and the FnNTD in the presence of heparin, we performed a series of solvent perturbation studies (Herskovits & Laskowski, 1962; Williams *et al.*, 1965). The FnNTD shows a

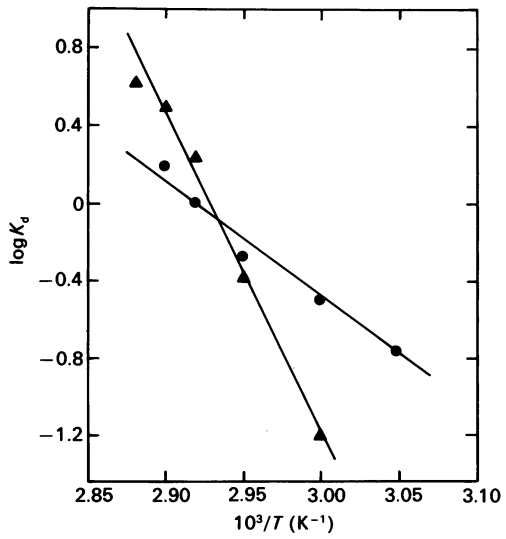

Fig. 7. van't Hoff analysis of FnNTD unfolding in the presence and in the absence of heparin

●, FnNTD; ▲, FnNTD–heparin complex. Straight lines were determined by a least-squares-fit computer program.

linear increase of fluorescence emission in the presence of increasing concentrations of both the small perturbant ethylene glycol and the larger perturbant, poly(ethylene glycol) (300 Da) (Fig. 8), indicating that both molecules have access to

chromophores in the FnNTD. The slope of this increase with ethylene glycol is significantly smaller for the fragment–heparin complex than for the fragment alone (Fig. 8*a*). This suggests that the small perturbant has less access to the chromophores in the complex than in the free FnNTD. This could have been the result of steric hindrance by heparin or of a structural reorganization in the FnNTD. However, the slope of the perturbant effect on fluorescence emission of the FnNTD–heparin complex was unchanged when poly(ethylene glycol) was used (Fig. 8*b*). This indicates that access to aromatic groups closer to the surface of the protein is not hindered by the presence of heparin, and argues against a steric effect. Corresponding results were obtained for the solvent-perturbed emission spectra of intact fibronectin in the presence and in the absence of heparin (results not shown). These observations suggest that heparin binding makes the interior of the fibronectin molecule, especially the FnNTD, more compact, without substantially altering its outer surface.

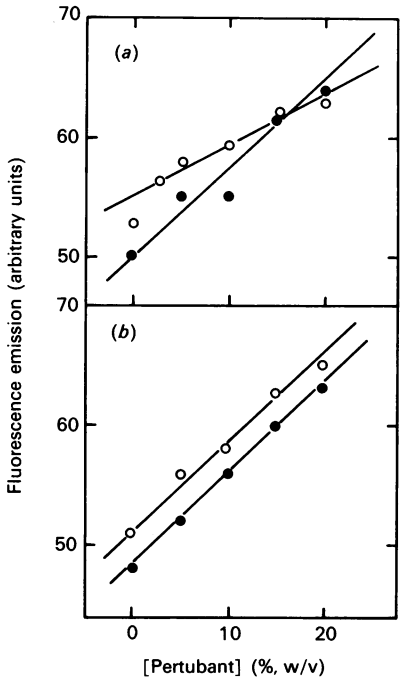

Fig. 8. Solvent-perturbed fluorescence emission spectra of the FnNTD

In (*a*) the perturbant was ethylene glycol, and in (*b*) the perturbant was poly(ethylene glycol) (300 Da). Solvent-perturbed spectra were recorded in the presence (○) and in the absence (●) of heparin.

DISCUSSION

Previous studies of fibronectin conformation established that high or low pH caused fibronectin to become more extended (as determined by sedimentation) while causing only small or moderate change in the secondary structure (as determined by c.d.) of the protein and some of its multifunctional fragments (Marković *et al.*, 1983). By measuring changes in several spectral properties of fibronectin, and fragments representing independent functional domains and all homology types, over a wide range of ionic and thermal unfolding conditions, the present study has rigorously established that intact dimeric fibronectin undergoes a gradual structural transition under conditions too mild to unfold any of its functional domains. The gradual transition is detected by techniques sensitive to the average chromophore environment (fluorescence emission) and overall protein flexibility (fluorescence polarization), but not by a technique sensitive to protein secondary structure (c.d.), providing further support for the idea that the compact form of the intact protein is due to interdomain interactions. Moreover, the induction of the gradual transition in fluorescence anisotropy by GdnHCl, but not by elevated temperature, supports the idea that the domains interact on the basis of charge (Marković *et al.*, 1983; Rocco *et al.*, 1987).

A schematic model for the progressive unfolding of fibronectin, based on previous suggestions (Marković *et al.*, 1983; Hörmann & Richter, 1986; Rocco *et al.*, 1987) and the results of the present study, is shown in Fig. 9.

Our studies also provide information concerning the conformational properties of the individual functional domains. For example, the FnNTD and the collagen-binding domain have equal fluorescence anisotropy values at ambient temperature in the absence of denaturant (Figs. 3 and 6*a*). However, the change in this parameter for the collagen-binding domain is considerably less than that of the FnNTD under conditions of both GdnHCl-

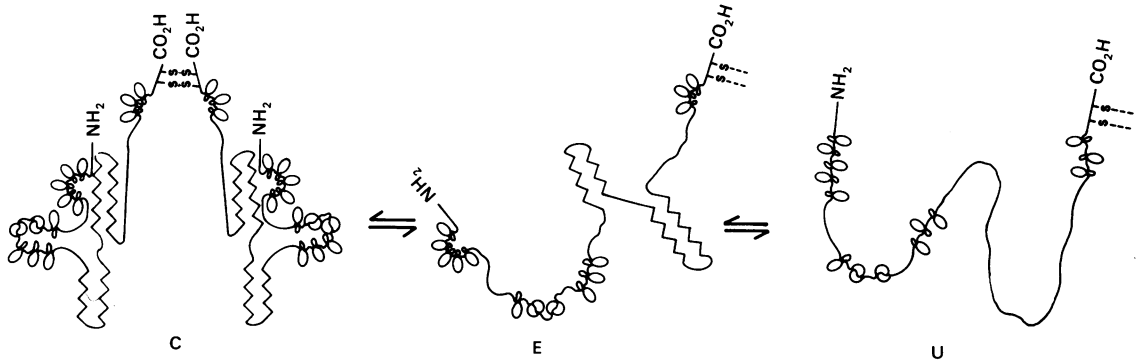

Fig. 9. Schematic representation of the proposed structure of fibronectin under different conditions

The compact form, C, is proposed to represent the soluble form of the protein found in plasma. This form is maintained by interdomain interactions and is presumed to be capable of binding heparin at the FnNTD. The extended form, E, corresponds to the configuration of the protein achieved by relatively mild changes in solvent composition, and is probably the form that binds collagen (Price *et al.*, 1982) and forms fibrils in tissue matrices (McDonald *et al.*, 1987; Woods *et al.*, 1988). In this state most of the secondary structure of the protein would remain intact. The completely unfolded state, U, is the unfolded form of the protein obtained under very strong denaturing conditions. This form results from the elimination of virtually all β-structure in fibronectin, which we suggest is largely confined to the non-disulphide-bonded central region of the protein. Some disulphide-stabilized secondary structure may still be retained in the U state. Only one of the subunits has been shown in states E and U. The disproportionate changes in different spectroscopic parameters in earlier denaturation studies (Alexander *et al.*, 1979; Odermatt *et al.*, 1982; Ingham *et al.*, 1984), as well as the non-superimposability of different unfolding transition curves in the present study, suggest that the E⇌U transition is a complex process that may involve several intermediate states.

induced (Fig. 3) and thermal (Fig. 6*a*) unfolding. This confirms, by different means, the finding by Isaacs *et al.* (1989) that the disulphide-bonded collagen-binding domain resists extensive change in its conformation even under denaturing conditions. Because the outstanding structural difference between the FnNTD and the collagen-binding domain is the presence in the latter of two type II homologies interrupting the type I homologies that otherwise exclusively comprise both domains (Skorstengaard *et al.*, 1986), the differences in unfolding behaviour of the two fragments can probably be attributed to the type II motifs.

We also note the apparent reorganization to a less flexible state that occurs when the cysteine residues of the FnNTD are reduced and modified (Fig. 6*b*). This supports our earlier suggestion, on the basis of c.d. studies (Khan *et al.*, 1988, 1989) that the FnNTD acquires additional secondary structure upon reduction of disulphide bonds. The reduced and modified intact protein exhibits similar resistance to unfolding in a region of the denaturation profile dominated by the behaviour of the disulphide-bonded domains (Figs. 5*a*, 5*c*, 6*a* and 6*f*).

The present studies also confirm our previous finding that heparin induces a conformational change in the FnNTD (Khan *et al.*, 1988). First, the heparin-bonded FnNTD is markedly stabilized against thermal unfolding (Fig. 6*b*) relative to the free fragment (Fig. 6*a*), with an estimated enthalpy difference between the two transitions of 194.1 kJ/mol (46.4 kcal/mol). Secondly, the solvent perturbation studies indicate that the stabilization of the FnNTD by heparin is accompanied by a compaction of the protein that hinders access of perturbant to interior chromophores (Fig. 8).

Conflicting reports on the strength of interaction between the FnNTD and heparin have appeared in the literature (Thompson *et al.*, 1986; Benecky *et al.*, 1988). The interaction between this domain of fibronectin with heparin-like molecules on the surfaces of cells or latex particles demonstrably promotes morphogenetic effects both in living tissues (Frenz *et al.*, 1989*a*,*b*) and in model extracellular matrices (Newman *et al.*, 1985, 1987; Khan *et al.*, 1988). Moreover, the overall strength of this interaction need not be large for it to function in morphogenesis (Forgacs *et al.*, 1989). In any case, the structural re-arrangements in the FnNTD required for its biological role, such as the compaction that occurs upon binding heparin, may have a cost in terms of decreased entropy, reducing the magnitude of the overall negative free-energy change during this binding interaction.

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