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Structural Changes in the NH_2-terminal Domain of Fibronectin upon Interaction with Heparin

RELATIONSHIP TO MATRIX-DRIVEN TRANSLOCATION*

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The effects of heparin and various related polysaccharides on the circular dichroic spectra of fibronectin and its 31-kDa NH_2-terminal tryptic fragment were studied. These effects were evaluated with respect to (i) spectral features of the native proteins that are sensitive to pH denaturation and breaking of disulfide bonds, (ii) sensitivity of spectral changes to Ca^{2+}, and (iii) the fibronectin-dependent interfacial interaction known as "matrix-driven translocation." We found that native heparin causes an attenuation of the positive CD peak at 228 nm with both the intact protein and the fragment, and causes a small but reproducible red shift in the spectrum of the fragment. All of these changes are analogous to spectral changes seen with denaturation or reduction of the proteins. In contrast to the situation with the intact protein, the heparin-induced spectral changes in the fragment were abolished in the presence of 10 mM Ca^{2+}. Desulfation of heparin lessened or destroyed its ability to induce these changes, and carboxymethylated heparin and dextran sulfate induced different kinds of spectral alterations. Fibronectin and heparin determinants required for the induction of the characteristic spectral shift of the NH_2-terminal domain corresponded to those required for matrix-driven translocation, suggesting that the associated conformational change in fibronectin plays a role in this biophysical effect.

Fibronectin is a glycoprotein found in embryonic and adult connective tissues and in blood plasma (1-3). It has specific binding sites for many important biological molecules including, but not limited to, collagen, fibrin, DNA, actin, and heparin (reviewed in Ref. 1). The interaction of fibronectin with heparin or heparin-like molecules plays an important role in the binding of collagen (4-6), the phagocytosis of gelatin (7, 8), the cryoprecipitation of fibrinogen (9, 10), cell attachment and spreading (1, 3), and in the recently described phenomenon of matrix-driven translocation (MDT)[1] (11, 12). Very little is known about the mechanism(s) by which heparin mediates all these processes.

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[1] The abbreviation used is: MDT, matrix-driven translocation.

MDT is a biophysical process involving a rapid unidirectional movement of cells or particles from one region to another of a compositionally heterogeneous collagen-fibronectin matrix (11, 12). Previously we have reported that MDT is promoted by an interaction between the NH_2-terminal (31 kDa) domain of fibronectin and heparin-like molecules on the cell or particle surface (12). We also suggested, on the basis of studies with domain-specific monoclonal antibodies, that MDT depended on a conformational change in the NH_2-terminal domain that occurred during this interaction. In this report, we describe a series of circular dichroism (CD) studies on the interaction of heparin and other polysaccharides with human plasma fibronectin, in relation to the known molecular determinants of MDT (12). The results suggest that a conformational change in the NH_2-terminal region of the fibronectin molecule consequent to the binding of heparin is indeed associated with the promotion of MDT.

EXPERIMENTAL PROCEDURES

Materials—Human plasma fibronectin was obtained from the New York Blood Center and was used without further purification. The NH_2-terminal domain of fibronectin was prepared by digesting the protein with tosylphenylalanyl chloromethyl ketone-trypsin (Worthington) at a protein to enzyme ratio of 100:1, followed by DEAE-cellulose chromatography as described (13). Heparin was purchased from Sigma. N- and N+O-desulfated heparins were prepared as described (14, 15); carboxymethylated heparin (16) was a gift from Dr. I. Danishefsky of the Department of Biochemistry of New York Medical College. Other reagents were of analytical grade. All experiments were performed in phosphate-buffered saline (0.02 M sodium phosphate buffer, pH 7.2, 0.15 M NaCl) at room temperature (about 25 °C) unless stated otherwise.

Protein Concentration—Protein concentrations were determined either by the method of Bradford (17) or by spectrophotometry using a specific extinction coefficient, $E^{1\%}_{1\,cm}$, for fibronectin of 12.8 (18).

Translocation Assays—Matrix-driven translocation assays were performed as previously described (11, 12) by recording the relative interfacial movement of two contiguous drops of soluble type I collagen, one containing suspended polystyrene latex beads ("primary gel") and one containing fibronectin or various modified fragments thereof ("secondary gel"). Each assay was performed at least three times.

Cleavage of Disulfide Bonds—Reduction of disulfide bonds was carried out by treating the protein in phosphate-buffered saline with 100 molar excess β-mercaptoethanol for 4 h under nitrogen, in the absence or presence of 6 M guanidine hydrochloride. The sulfhydryl groups were subsequently blocked by reaction with an excess of *N*-ethylmaleimide for 30 min (19). Excess reagent was removed by Sephadex G-25 column chromatography.

Circular Dichroism—CD measurements were made on a Jasco model 500C spectropolarimeter equipped with a microcell and a data processor. All spectra in the far UV region between 270 and 200 nm were obtained with a time constant of 2 s, a sensitivity of 2 millidegree cm^{-1}, and a scan rate of 25 nm min^{-1}. Protein concentrations varied from 0.05 to 0.15 mg/ml. Weight ratios of heparin or dextran sulfate

to protein ranged from 0.01 to 1.0. Quadruplicate scans were made, and the buffer blanks were subtracted from the average spectrum for each sample; each experiment was repeated at least three times. Mean residue ellipticities (deg cm^2 $dmol^{-1}$), $[\theta]$, were calculated by,

$$[\theta] = \frac{[\theta]_{obs} \times 100\ MRW}{lc}$$

where $[\theta]_{obs}$ is the observed ellipiticity in degrees; *MRW* is the mean residue molecular weight, calculated from amino acid composition to be 108 (20) and 112 (21) for intact fibronectin and its NH_2-terminal domain, respectively; l is the optical pathlength (cm); and c is the concentration of the protein in mg/ml.

RESULTS

Characteristics of the NH_2-terminal Domain—Before carrying out physical studies we made certain that the fragment corresponding to the 31-kDa NH_2-terminal region of fibronectin (13) was homogeneous, active in the MDT assay, and that it retained its characteristic secondary structure after isolation. The fragment migrated as a single band by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (not shown). We have previously shown that the isolated 31-kDa NH_2-terminal is sufficient to promote MDT of cells or particles containing heparin-like determinants on their surfaces (12). That the fragment used in the present study was active in this assay system is indicated in Fig. 1*b*, where there was extensive translocation of particles across the primary-secondary gel interface, in contrast to the fibronectin-free control (Fig. 1*a*). The interaction of the NH_2-terminal domain of human plasma fibronectin with heparin is diminished as the concentration of Ca^{2+} is increased (22). Correspondingly, we found that raising the Ca^{2+} concentration from 0.3 mM in the standard MDT assay system to 10 mM completely eliminated translocation in the presence of the fragment (Fig. 1*c*). We made use of this calcium effect in the physical studies described below.

The CD spectra of the fragment and of intact fibronectin were compared at physiological pH and under acidic conditions at which native structure would be expected to be disrupted. Both the proteins have positive and negative CD bands at about 228 and 212 nm, respectively. These spectral features are in general agreement with previous fibronectin studies (23–26). The molar ellipticities at 228 nm were calculated from the CD spectra at different pH values. The results obtained are shown in Fig. 2. Structural transitions appear complete by pH 2.0, where the value of $[\theta]$ is about 400 for both proteins. However, the pH-induced transition of the NH_2-terminal domain is more cooperative than that of the intact fibronectin molecule, with a midpoint at about pH 2.85 for the former, in contrast to pH 3.4 for the latter. Additionally, the pH at which the transition begins is much lower for the fragment (pH 4.8) than for the intact molecule (pH 5.6). These results clearly show that the NH_2-terminal fragment we have used in these studies contains a very high degree of structure which is lost during its pH denaturation. The greater steepness of the *curve* in Fig. 2, *upper*, as compared with Fig. 2, *lower*, taken together with the higher molar ellipticity of the fragment relative to the intact molecule at neutral pH, suggests that the former contains a greater degree of structure than the latter and probably constitutes one of the most highly organized regions of the fibronectin molecule.

Role of Disulfide Bonds—Fibronectin contains a number of intrachain disulfide bonds in addition to the two that connect the two chains of the dimer (1). There is the potential presence of two such bonds in each of the 12 "type 1" structural homologies present in the fibronectin molecule, of which five constitute the 31-kDa NH_2-terminal domain (1, 2). We were

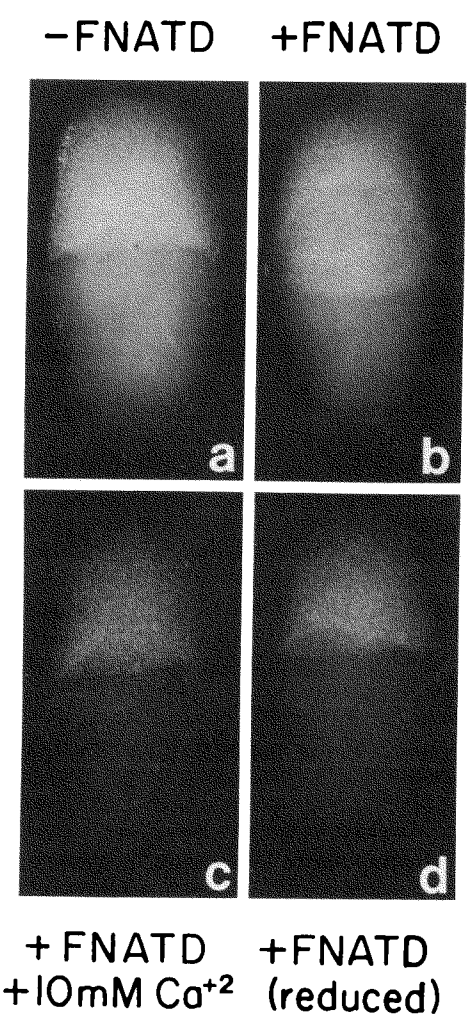

FIG. 1. **Effect of excess of Ca^{2+} or reduction and modification of disulfide bonds on MDT-promoting activity of fibronectin NH_2-terminal domain (*FNATD*).** All primary gels contained 2.5×10^6 polystyrene latex beads/ml. Secondary gels contained no fibronectin (*a*), or 12.5 μg/ml of the 31-kDa NH_2-terminal fibronectin fragment under standard assay conditions (*b*), in the presence of 10 mM Ca^{2+} (*c*), and after reduction and modification of disulfide bonds in the presence of 6 M guanidine hydrochloride (*d*). Long dimension of all primary-secondary gel preparations was approximately 12 mm.

therefore interested in whether such bonds serve to stabilize the secondary structure of the protein and whether their reductive cleavage changes its CD spectrum and other conformation-sensitive properties. We found that the magnitude of the major positive CD peak in the reduced and modified protein was not only substantially attenuated but that the position of the band was also red shifted by several nanometers. A similar effect was observed when the protein was subjected to acid denaturation (Fig. 3). When the fibronectin that was reduced and modified in the presence of guanidine hydrochloride was assayed for its ability to promote MDT, a functional test of its NH_2-terminal domain, it was found to be totally inactive (Fig. 1*d*). Fibronectin that was reduced and modified in the absence of the denaturant exhibited some residual MDT-promoting activity (not shown).

Effect of Heparin and Dextran Sulfate on the Structure of Fibronectin and Its NH_2-terminal Fragment—Circular dichroic spectra of fibronectin and its NH_2-terminal fragment were compared in the presence and absence of unmodified heparin, desulfated or carboxymethylated heparin, and dextran sulfate. Consistent with previous reports (24, 27) we

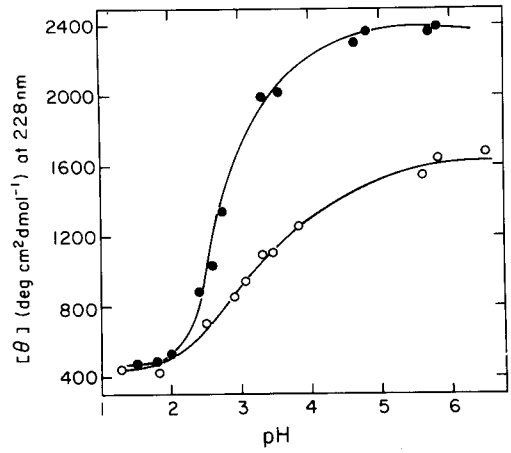

FIG. 2. **Dependence on pH of mean residue ellipticity of intact fibronectin (*open circles*) and its 31-kDa NH_2-terminal fragment (*filled circles*).** The pH of the protein solutions in phosphate-buffered saline (adjusted to different pH) was determined before and after the CD measurements were made. The pH value shown is the mean of the two determinations, which did not differ by more than 0.03 pH unit.

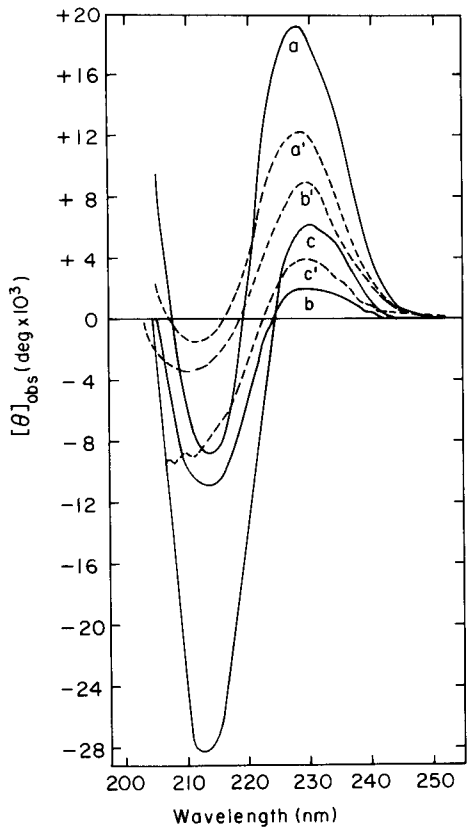

FIG. 3. **Far UV-CD spectra of intact fibronectin (*solid lines*) and its 31-kDa NH_2-terminal fragment (*broken lines*) under different conditions.** *a* and *a'*, proteins in phosphate-buffered saline under native conditions; *b* and *b'*, proteins reduced and modified in the presence of 6 M guanidine hydrochloride; *c* and *c'*, proteins under acid-denaturing conditions (pH 2.0). The concentrations of intact fibronectin and its fragment were 0.125 and 0.06 mg/ml, respectively.

found that heparin induces a conformational change in intact fibronectin such that the magnitude of the positive peak at 228 nm is decreased (Fig. 4*A*). However, in contrast to the spectrum of the intact protein, that of the fragment undergoes a heparin-induced change in which the signal normally cen-

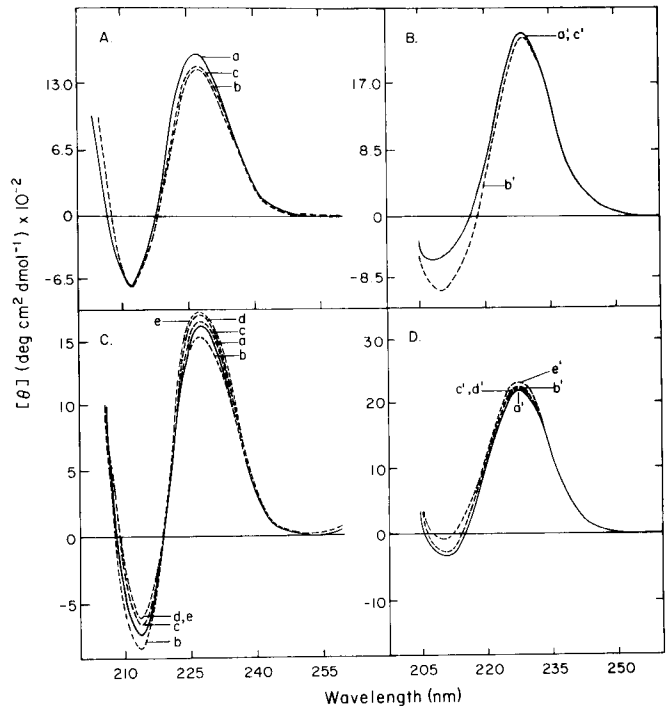

FIG. 4. **Far UV-CD spectra of intact fibronectin (*A*, *C*) and its 31-kDa NH_2-terminal fragment (*B*, *D*) under various conditions.** For clarity, spectra of untreated proteins are shown as *solid lines* in each *panel* and are designated (*a*) in the case of the intact protein and (*a'*) in the case of the fragment. Spectra were taken in the presence of intact heparin (*A*(*b*), *B*(*b'*)); intact heparin + 10 mM $CaCl_2$ (*A*(*c*), *B*(*c'*)); N-desulfated heparin (*C*(*b*), *D*(*b'*)); N+O-desulfated heparin (*C*(*c*), *D*(*c'*)); carboxymethylated heparin (*C*(*d*), *D*(d')); and dextran sulfate (*C*(*e*), *D*(*e'*)). All experiments involving $CaCl_2$ were performed in 0.05 M Tris-HCl buffer, pH 7.5.

tered at 228 nm is red-shifted to a small, but reproducible, extent in addition to being slightly attenuated. This red shift is accompanied by a marked accentuation of the minimum at 212 nm (Fig. 4*B*) which is not seen in the spectrum of the intact protein-heparin complex (Fig. 4*A*).

Modification of heparin by *N*-desulfation, N+O desulfation, or carboxymethylation altered the effect it had on the CD spectrum of both intact fibronectin and the fragment. But whereas *N*-desulfated heparin remained capable of attenuating the 228-nm peak in the intact protein (Fig. 4*C*), this polysaccharide was incapable of inducing the characteristic heparin-dependent changes in the fragment (Fig. 4*D*). Completely desulfated heparin had no effect on the spectrum of either protein (Fig. 4, *C* and *D*). Carboxymethylated heparin not only failed to cause a decrease in the signals centered at 228 nm for these proteins, it actually caused a substantial increase in this signal with the intact protein (Fig. 4*C*) and a minor increase (with no red shift) with the fragment (Fig. 4*D*). This indicates that sulfate groups and unblocked uronic acid carboxyl groups, known to be important in other heparin-protein interactions (28, 29), are critical in the capacity of native heparin to induce the characteristic conformational changes in both intact fibronectin and its NH_2-terminal domain.

Because dextran sulfate binds to fibronectin (30) and has been shown to mediate nearly all those processes which are associated with heparin binding (5, 30, 31), with the notable exception of MDT (12), we sought to determine the effect of this sulfated polysaccharide on the structure of fibronectin and its NH_2-terminal fragment. The results show that dextran sulfate, like carboxymethylated heparin, increases the 228-

TABLE I

Transitions in CD spectra of fibronectin and its NH_2-terminal fragment induced by various ligands

+, o, and − indicate increase, no change, and decrease in signal centered at 228 nm, respectively. RS indicates red shift in signal centered at 228 nm. * indicates accentuation of trough centered at 212 nm.

Ligand	Intact fibronectin	NH_2-terminal fragment
Native heparin	− − −	−; RS; *
Native heparin + Ca^{2+}	− − −	o
N-desulfated heparin	−	o
N+O-desulfated heparin	o	o
Carboxymethylated heparin	++	+/o
Dextran sulfate	++	+

nm CD signal of both fibronectin and its fragment, without inducing a red shift in the spectrum of the latter (Fig. 4, *C* and *D*). It is clear that any structural change that may be induced in these proteins by the binding of dextran sulfate is different from that induced by native heparin.

Effect of Calcium Ion—The report that heparin binding of the NH_2-terminal domain of fibronectin is inhibited by Ca^{2+} (22) led us to perform our CD studies in the presence of this ion. We found that the presence of 10 mM Ca^{2+} totally inhibits the conformational change resulting from heparin binding to the fragment. Indeed, the spectra of the fragment in the presence or absence of heparin are completely superimposable when taken in the presence of Ca^{2+} (Fig. 4*B*).

In contrast, the spectrum of the intact fibronectin molecule exhibits the same attenuation of the 228-nm peak, without a red shift, reported above (Fig. 4*A*) even when 10 mM Ca^{2+} is present. This indicates that the predominant conformational changes exhibited by the intact fibronectin molecule upon binding heparin are different from the Ca^{2+}-sensitive change(s) induced by interaction of heparin with the NH_2-terminal domain of the protein. As noted previously, the MDT-promoting function of the NH_2-terminal domain of fibronectin (12) is also completely inhibited in the presence of the same Ca^{2+} concentration (Fig. 1*c*).

The transitions in the CD spectrum of fibronectin and its NH_2-terminal fragment induced by unmodified and modified heparins, and by dextran sulfate, are qualitatively summarized in Table I.

DISCUSSION

Analysis of the structure of fibronectin by different methods has led to contrasting conclusions, ranging from approximately 35% β-structure at one extreme (23) to nearly 100% β-structure at the other (24). A major difficulty in applying CD, the most commonly used method for determining protein secondary structure, to fibronectin arises from the presence of the positive band at 228 nm, a feature not seen in proteins which lack aromatic amino acid residues (32), and the relatively low ellipticity values in the far UV region in general. While the origin of the CD band at 228 nm remains speculative, our inference that it arises from nonrandom structure within the fibronectin molecule is based on its intensity and position (33) and its attenuation upon pH denaturation or reduction of native disulfide bonds. The origin of the far UV CD spectra of fibronectin has also been described in another recent study (34).

The circular dichroic spectra described in this study have established the following: (i) the interaction of heparin with the NH_2-terminal domain of fibronectin induces a conformational change that involves the alteration of certain structural features of the protein; (ii) the conformational change induced by heparin in the NH_2-terminal fibronectin domain is different in kind from that induced by heparin in other fibronectin domains; (iii) the heparin-induced change in the NH_2-terminal domain of fibronectin is different from changes induced by dextran sulfate.

Whereas the CD spectrum of the intact fibronectin molecule exhibits a decrease in optical activity at 228 nm upon interaction with heparin, a small red shift in the band centered at this position and an accentuation of the trough at 212 nm indicative of additional rearrangements are only seen in the spectrum of the fragment-heparin complex. Intact fibronectin has a strong heparin binding site in its COOH-terminal region in addition to the site present in the NH_2-terminal fragment (13), and it is probable that the spectral shift is overshadowed in CD measurements of the intact protein. In a similar fashion, the spectral changes induced in the fragment by heparin are sensitive to Ca^{2+}, but this is not seen with the intact protein (Fig. 4*D*).

Heparin is believed to alter protein secondary structure (35) by interactions of its sulfate and carboxyl groups with basic amino acid residues in proteins (28, 29, 36). Our results indicate that sulfate groups and unblocked uronic acid carboxyl groups of heparin are both important in its ability to induce a characteristic structural change in the NH_2-terminal domain of fibronectin. When the carboxylate groups are blocked, but the sulfate groups remain, the resulting polysaccharide induces a change in the opposite sense that is similar to that induced by dextran sulfate (Fig. 4*D*). These data are consistent with a two-step process in which sulfate groups of heparin are required for the initial interaction with the NH_2-terminal domain, while the carboxyl group(s) is/are essential for the structure-altering transition.

Our previous studies on matrix-driven translocation indicated that this interfacial effect is mediated by properties of the NH_2-terminal heparin binding domain of fibronectin that are not shared by the protein's COOH-terminal heparin binding domain (12). Because a monoclonal antibody directed against the NH_2-terminal domain inhibited MDT without interfering with heparin binding, we suggested that a conformational change in this fibronectin domain consequent to its binding of heparin-like molecules on cell or particle surfaces was required for MDT (12). Subsequently, we found that carboxymethylation of heparin did not destroy its ability to inhibit MDT, but N- or N+O desulfation did (37). On the basis of these previous studies and those reported here, we suggest that the Ca^{2+}-sensitive heparin-induced conformational change in the NH_2-terminal domain of fibronectin is central to the promotion of MDT. This effect probably requires the conformational change to occur at the particle-matrix interface in concert with translocation, since fibronectin in the presence of heparin does not promote movement (12). A conformational change of this sort may act, for example, to reduce the interfacial tension (38, 39) at the boundary between populated and vacant regions of an extracellular matrix (40). More detailed studies of the nature of this fibronectin-heparin interaction should help determine whether this interpretation is valid.

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