Heparin Binding Is Necessary, but Not Sufficient, for Fibronectin Aggregation

A FLUORESCENCE POLARIZATION STUDY*

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Analysis of parameters governing heparin binding to fibronectin indicates that heparin binding is a necessary, but insufficient, condition for fibronectin cryoprecipitation. Heparin binding to fibronectin is a rapid, readily reversible event which can occur under several conditions which prohibit fibronectin cryoprecipitation. While cryoprecipitation of fibronectin is abolished at temperatures in excess of 10 °C, appreciable heparin binding to fibronectin does occur even at 40 °C. While increasing ionic strength and pH inhibit both heparin binding and cryoprecipitation of fibronectin, heparin binding can still occur at high ionic strengths and pH values which completely abolish cryoprecipitation. Scatchard analysis of fluorescent polarization data reveals a biphasic heparin binding curve with high and low affinity $K_d$ values of $3.5 \times 10^{-8}$ and $10^{-6}$ M, respectively. In contrast to heparin binding, fibronectin aggregation is a cooperative phenomenon. Fibronectin cryoprecipitation is greatly reduced at temperatures above 10 °C, at pH values above pH 10, and at ionic strengths above 0.3 M. Thus, heparin binding and protein aggregation are separate events which occur during fibronectin cryoprecipitation. Results obtained here via fluorescence polarization in conjunction with other physical measurements suggest that a decrease in flexibility of the fibronectin molecule is associated with the protein aggregation step of cryoprecipitation. The role of heparin in the mechanism of fibronectin cryoprecipitation is discussed.

Fibronectin is a 460,000-dalton dimeric glycoprotein involved in cell adhesion, phagocytosis, and the organization of the extracellular matrix (1, 2). Fibronectin is the major cell-surface protein of many normal cell types; however, following malignant transformation, fibronectin is either lost or greatly reduced in quantity on the cell surface (3). A slightly lower molecular weight form of fibronectin found in plasma and serum is probably a pre- (or post-) translational modification of cell-surface fibronectin (4). Both forms of fibronectin promote cell attachment (5, 6) and display affinities for several cell surface-associated molecules (1, 2, 7).

The biological properties of fibronectin depend on the interaction of fibronectin with several other macromolecules. Fibronectin possesses binding sites for collagen, heparin, hyaluronic acid, fibrin, gangliosides, actin, DNA, blood clotting factor XIIIa, and certain bacterial species (1, 2, 7). Binding of native fibronectin to the cell surface occurs following the binding of fibronectin to collagen or a suitable substrate (8, 9). Several fibronectin-mediated biological effects either depend on or are enhanced by heparin and certain other sulfated polysaccharides (10). For example, heparin enhances the rate of binding of fibronectin to collagen and stabilizes a collagen-fibronectin complex (11). Heparin also enhances fibronectin-mediated phagocytosis of gelatin-coated particles (12), and macromolecular heparin and dextran sulfates alter early events in fibronectin-mediated cell attachment (13). In this investigation, the parameters governing heparin binding to fibronectin are studied directly via fluorescence polarization analysis.

Fibronectin contributes to the formation of at least two types of cold-insoluble complexes, i.e. the heparin-induced cryoprecipitate (14), studied here, and cryofibrinogen, a cold-insoluble complex formed in the presence of fibronectin and fibrin/fibrinogen mixtures (15). Both types of cold-insoluble complexes are important from pathological and therapeutic standpoints. Plasma cryoprecipitate prepared with heparin is useful in treatment of both hemophilia A (16) and von Willebrand's disease (17) and has been used in opsonin therapy for sepsis accompanying surgery or other trauma (18). The cryofibrinogen complex is associated with the chronic intravascular coagulation syndrome (19).

In this study, fibronectin cryoprecipitation has been shown to involve two events, namely a rapid heparin-fibronectin interaction and a slower protein aggregation step. We have studied the interaction of fibronectin with heparin (20, 21) by means of the fluorescence polarization technique. Fluorescence polarization permits one to observe binding events by monitoring the decrease in the rotational diffusion coefficient (22) of a relatively small labeled heparin molecule upon its binding to the much larger fibronectin molecule. Thus, fluorescence polarization provides a direct and technically simple means of measuring heparin binding in real time without prior immobilization of either constituent and without interference from protein aggregation. We have characterized several parameters involved in heparin binding to fibronectin and compared the heparin binding event to the more complex phenomenon of heparin-induced cryoprecipitation of fibronectin (14). This approach promises to make possible rigorous physicochemical investigations of heparin-fibronectin binding.
which should be useful in developing a greater understanding of the biochemical role of such interactions.

**EXPERIMENTAL PROCEDURES**

Reagents—Fibronectin was prepared from outdated human plasma by gelatin-Sepharose affinity chromatography (23). After elution with 0.1 M NaCl + 0.02 M sodium citrate, pH 5.5 (24), fibronectin was dialedyzed against 0.15 M NaCl + 0.05 M NaHPO₄, pH 7.2, aliquoted, and stored at -80°C. The Bradford protein-dye binding assay was used to determine fibronectin concentration (25); bovine serum albumin was used as a standard. The concentration of fibronectin, as determined by absorbance at 280 nm (Am) 1% protein was used to determine fibronectin concentration (25); bovine serum albumin (BSA) solution was prepared from 100% bovine serum albumin (Sigma) and stored at 4°C. All experiments were performed with a single batch of 0.3 mg/ml fibronectin solution.

Hog mucosal heparin (169 USP K-1 units/mg) was obtained from Sigma. Reagents used in fluorescein labeling of heparin were obtained from Aldrich Corp. All other chemicals were of reagent grade.

Preparation of 5-Aminofluorescein-labeled Heparin—Fibronectin heparin used in polarization experiments was prepared by a modification of the method of Ogamo et al. (26); whereby 5-aminofluorescein was coupled to the carboxyl groups of heparin. Briefly, 500 mg of hog mucosal heparin was dissolved in 20 ml (3.1, v/v, of 1 M HCl/pyridine and added to 114 mg of 5-aminofluorescein (1.6 mol eq/disaccharide unit) which was dissolved in 4 ml (1:1, v/v, of 1 M HCl/pyridine. After adjusting to pH 4.75, 390 mg (5.0 mol eq/disaccharide unit) of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide HCl dissolved in 0.32 ml of distilled water was added, and the pH was maintained at pH 4.75 by addition of 6 M HCl while stirring for 1 h at room temperature. After extensive dialysis against distilled water and two precipitations with 3 volumes of cold ethanol containing 1.25% sodium acetate, the fluorescein-labeled heparin was redissolved in distilled water, adjusted to pH 6.5, lyophilized, and stored in a foil-wrapped container at -20°C. The number of moles of bound 5-aminofluorescein/mol of heparin was calculated from the molar absorption coefficient of 5-aminofluorescein.

A fraction of fluorescein heparin with high affinity for binding to fibronectin was prepared by cryoprecipitation as described by Stathakis and Mosesson (14). It should be noted that lack of bindability of one fraction of heparin is an intrinsic property of heparin and is not an artifact of the labeling procedure. In brief, 1 mg of 5-aminofluorescein-labeled heparin was dissolved in 0.15 M NaCl + 0.05 M NaHPO₄, pH 7.2, and incubated with 3 mg of gelatin-Sepharose affinity purified human plasma fibronectin in the same buffer at 2°C for 3 h. After centrifugation at 12,000 x g for 10 min at 2°C, the pellet containing cryoprecipitated fibronectin and bound heparin was resuspended in 2 ml of 0.5 M NaCl at 37°C. Fibronectin was then removed by precipitation with 33% saturated (NH₄)₂SO₄ at 4°C followed by centrifugation for 15 min at 12,000 x g (2°C). The supernatant containing free, fluorescein-labeled heparin was dialyzed against several changes of 0.3 M NaCl + 0.05 M NaHPO₄, pH 7.2. Where necessary, fluorescein-labeled heparin solutions were concentrated by precipitation with 3 volumes of cold ethanol and resuspended in a smaller volume of buffer. The 5-aminofluorescein-labeled heparin fraction, comprising approximately 10–20% of the starting fluorescein-labeled heparin, was stored in a foil-wrapped container at 4°C. Fluorescein-labeled heparin fractions, prepared by cryoprecipitation, typically ranged in concentration from 0.15 to 0.38 mg/ml as determined by dialysis against distilled water, lyophilization, and weighing. All experiments were performed with a single batch of 0.3 mg/ml labeled heparin. When stored as a lyophilized powder at -20°C, fluorescein-labeled heparin appears to be stable; however, at 4°C, the fluorescence of aqueous solutions decays over a period of weeks. All heparin dialysis steps were performed in Spectrapor M, 2000 cut-off dialysis tubing (Spectrum Medical Industries, Los Angeles, CA). The bindable and nonbindable fractions of fluorescein-labeled heparin contained 0.2 and 0.19 mol of fluorescein/mol of heparin, respectively.

Instrumentation—Fluorescence measurements were made using an L-format spectrofluorometer assembled in the laboratory from commercial components. The sample compartment (SLM Instruments, Inc., Urbana, IL) was equipped with Jarrell Ash (Jarrell Ash, Waltham, MA) quarter-meter monochromators at the excitation and emission ports. Excitation and emission wavelengths were set at 495 and 540 nm, respectively; all slit widths were maintained at 8 nm. Fluorescence was excited and viewed through rotatable Glan-Thompson polarizers. The detection system consisted of a Spex digital photometer (Spex Industries, Inc., Metuchen, NJ) which interfaced with a Franklin Ace 1200 personal computer (Franklin Computers, Framingham, NJ) via an Adalab data acquisition system (IMI, State College, PA). The cuvette chamber was equipped with a magnetic stirrer and had both temperature control and dehumidifying capabilities.

Fluorescence Polarization Measurements—The basic phenomenon which allows fluorescence polarization measurements to be used to assay ligand binding is the increase in fluorescence polarization of a low molecular weight fluorescent ligand as it becomes relatively immobilized upon binding to a high molecular weight molecule (22). In the present system, the binding of approximately M, 12,900 fluorescein-labeled heparin to M, 430,000 fibronectin has been studied and, hence, nearly the theoretical maximum increase in fluorescence polarization could be expected.

The polarization (P) was computed and corrected for grating induced artifacts in the standard way using the expression:

\[ P = \frac{I_{VV} - I_{HV}}{I_{VV} + I_{HV}} \]

where I is the fluorescence intensity observed through a given orientation of the polarizer placed in the excitation and emission beam. The first subscript of each intensity is the orientation of the excitation polarizer, and the second subscript denotes the orientation of the emission polarizer. These orientations were either vertical (V) in the laboratory frame or horizontal (H).

In general, measurements of heparin binding to fibronectin were performed by taking an initial recording of polarization values of unbound, fluorescently labeled heparin. Measurements were performed in 3 ml 1-cm path length plastic cuvettes (Evergreen Scientific, Los Angeles, CA), containing 1.5 ml of 0.10 M NaCl + 0.05 M NaHPO₄, pH 7.2, and a variable amount of fluorescein-labeled heparin as specified in the text. Increases in fluorescence polarization were recorded upon addition of fibronectin.

The increase in fluorescence polarization of fluorescein-labeled heparin upon binding to fibronectin permits several types of measurements to be performed. First, the kinetics of heparin binding to fibronectin can be monitored by simply determining the time course of the increase of fluorescence polarization at constant fluorescein-labeled heparin and fibronectin concentration. The kinetics of dissociation of a heparin-fibronectin complex can also be studied by observing the time course of decrease in fluorescence polarization upon addition of agents which cause dissociation of heparin from fibronectin. Conditions affecting the binding of heparin to fibronectin can also be studied by permitting fluorescently labeled heparin to bind to fibronectin at various pH values, ionic strengths, or temperatures.

To determine the effect of ionic strength on binding of heparin to fibronectin, fluorescein-labeled heparin was dialyzed against several changes of 0.30 M NaCl + 0.05 M NaHPO₄, pH 7.2. Where necessary, fluorescein-labeled heparin solutions were concentrated by precipitation with 3 volumes of cold ethanol and resuspended in a smaller volume of buffer. The 5-aminofluorescein-labeled heparin fraction, comprising approximately 10–20% of the starting fluorescein-labeled heparin, was stored in a foil-wrapped container at 4°C. Fluorescein-labeled heparin fractions, prepared by cryoprecipitation, typically ranged in concentration from 0.15 to 0.38 mg/ml as determined by dialysis against distilled water, lyophilization, and weighing. All experiments were performed with a single batch of 0.3 mg/ml labeled heparin. When stored as a lyophilized powder at -20°C, fluorescein-labeled heparin appears to be stable; however, at 4°C, the fluorescence of aqueous solutions decays over a period of weeks. All heparin dialysis steps were performed in Spectrapor M, 2000 cut-off dialysis tubing (Spectrum Medical Industries, Los Angeles, CA). The bindable and nonbindable fractions of fluorescein-labeled heparin contained 0.2 and 0.19 mol of fluorescein/mol of heparin, respectively.

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Data Analysis—Several assessments of 5-aminofluorescein-labeled heparin binding could conveniently be made directly in terms of the measured polarization. For quantitative analysis of fluorescein-labeled heparin binding, an equation was derived based on the law of addition of polarization values for the fraction of added heparin that
PM
ployed.

polarization of free, fluorescein-labeled heparin susceptible upon addition of fibronectin, indicating that the 85% of the maximum polarization value that would be reached upon addition of fibronectin to the bindable fluorescein-labeled heparin fraction, certain proteins (29-31), may also be composed of subpopulations of molecules that vary in their ability to bind fibronectin. In the studies reported here, the product of the total fluorescein-labeled heparin added and the fraction of heparin bound was assessed from the amount of heparin bound was assessed from the product of the total fluorescein-labeled heparin added and the fraction of heparin bound.

RESULTS

Measurements of Heparin Binding to Fibronectin and Cryoprecipitation—Since an increase in polarization over the polarization of free, fluorescein-labeled heparin (P_F) indicates an increase in bound heparin, heparin binding is reported here in terms of measured polarization values. The aggregation of fibronectin molecules which occurs during cryoprecipitation was monitored by light scattering and is reported here in A_580 units.

Initially, polarization experiments were conducted using 5-aminofluorescein-labeled heparin that had not been prepared by cryoprecipitation with fibronectin (see "Experimental Procedures"). Only a small increase in the polarization of the fluorescein-labeled heparin/fibronectin mixture (P_M) was perceptible upon addition of fibronectin, indicating that the majority of heparin was not bound. This observation suggested that heparin, a heterogeneous substance with regard to molecular weight, charge density, and binding affinity for certain proteins (29-31), may also be composed of subpopulations of molecules that vary in their ability to bind fibronectin. To test this hypothesis, fluorescein-labeled heparin was used to cryoprecipitate fibronectin, and the bound fluoro-

rescein-labeled heparin recovered from the cold-insoluble pellet was used in the polarization assay (see "Experimental Procedures"). This fraction of heparin is referred to here as "bindable fluorescein-labeled heparin." Upon addition of fibronectin to the bindable fluorescein-labeled heparin fraction, P_M approached 0.35, indicating that nearly all of the bindable fluorescein-labeled heparin had bound to fibronectin. Thus, cryoprecipitation of fibronectin with fluorescein-labeled heparin probably produces a fraction enriched with heparin possessing a higher binding affinity for fibronectin. In the studies reported here, bindable fluorescein-labeled heparin was employed.

When fibronectin was added to 5-aminofluorescein-labeled heparin, an immediate increase in the measured polarization (P_M) was observed over the polarization of free, fluorescein-

labeled heparin (P_F). At the earliest time (15 s after addition of fibronectin), the polarization had increased to 85% of the maximum polarization value that would be reached under these experimental conditions (Fig. 1). Heparin binding to fibronectin, as measured by the increase in polarization values, reached a plateau within 2 min and remained constant for at least 1 h. In contrast, heparin-induced cryoprecipitation of fibronectin occurred more slowly (Fig. 1). Cryoprecipitate formation typically required 60-90 min to reach maximum as assessed by light scattering measurements. In the absence of heparin, cryoprecipitate formation did not exceed 0.05 absorbance units, even at time points as long as 6 h (data not shown). Thus, heparin binding to fibronectin clearly occurred more rapidly than protein aggregation.

Addition of excess, unlabeled heparin to solutions containing the fibronectin-fluorescein-labeled heparin complex eluted fluorescein-labeled heparin rapidly, as indicated by an immediate decrease in P_M (Fig. 1). Thus, both the association and dissociation of heparin and fibronectin occur rapidly and reversibly. The polarization of bound fluorescein-labeled heparin (P_M) was not reduced to the polarization of free fluorescein-labeled heparin (P_F), even in the presence of a 33-fold excess of unlabeled heparin equivalent to an 8-fold increase over the K_d. That excess unlabeled heparin eluted some, but not all, of the bound labeled probe supports the hypothesis that not all heparin molecules possess identical binding affinities for fibronectin.

Effect of Fibronectin Concentration—In order to observe the effect of increasing fibronectin concentration on heparin binding (Fig. 2), 5-aminofluorescein-labeled heparin was titrated with 50-μg aliquots of fibronectin. P_M increased significantly and immediately upon addition of each aliquot of fibronectin. However, each increase in P_M was progressively smaller, indicating a decrease in the amount of available bindable heparin (Fig. 2).

Increasing the fibronectin concentration in the presence of 50 μg/ml of unlabeled heparin gave a different response for cryoprecipitation measured at 350 nm. A sigmoidal curve was generated showing little cryoprecipitate formation until a fibronectin concentration of 150 μg/ml had been attained (Fig. 2). At the 50 μg/ml heparin concentration employed (a suboptimal concentration, see Fig. 3), no further increase in turbidity was observed at fibronectin concentrations greater than 300 μg/ml, in agreement with the observations of Statkakis and Mosesson (14). The results presented in Fig. 2 indicate that while heparin and fibronectin will bind at low concentrations, cryoprecipitation requires high fibronectin concentrations for protein aggregation to occur within the 1-h time frame studied. Thus, heparin binding to fibronectin and the protein aggregation event involved in cryoprecipitation are kinetically distinguishable.

Effect of Heparin Concentration—When fibronectin was titrated with aliquots of fluorescein-labeled heparin (3 μg),
Heparin Binding to Fibronectin

### FIG. 2. Effect of fibronectin concentration on heparin binding and heparin-induced cryoprecipitation.

Using procedures described under "Experimental Procedures," the effect of fibronectin concentration on heparin binding to fibronectin (closed circles) and heparin-induced cryoprecipitation (open circles) was studied. 3 μg (0.01 ml) of fluorescein-labeled heparin was titrated with 50-μg aliquots of fibronectin. For cryoprecipitation, the concentrations of fibronectin indicated were incubated with 50 pg/ml heparin for 1 h at 2°C.

### FIG. 3. A, Effect of heparin concentration on heparin binding to fibronectin. Fibronectin (300 μg/ml) was titrated with aliquots of fluorescein-labeled heparin at 2°C. O—O, indicates polarization data; A—A, indicates the amount of fluorescein-labeled heparin in μg bound to fibronectin; O—O, indicates P<sub>M</sub>, P<sub>F</sub>. B, effect of heparin concentration on cryoprecipitation. Fibronectin (300 μg/ml) was incubated with increasing concentrations of unfractionated heparin at 2°C for 60 min. Polarization values were initially high but decreased with added fluorescein-labeled heparin, a consequence of decreasing the ratio of bound to free fluorescein-labeled heparin (Fig. 3A). The polarization data was converted to the amount (in micrograms) of bound heparin as described under "Experimental Procedures." With increasing fluorescein-labeled heparin, the amount of heparin bound to fibronectin approached plateau values, indicating near-saturation of heparin-binding sites (Fig. 3A). In contrast, cryoprecipitation of fibronectin did not increase appreciably above background until a heparin concentration of 10 μg/ml had been reached (Fig. 3B). Cryoprecipitate formation then increased steadily to an optimum at 300 μg/ml heparin. Increasing the heparin concentration above peak concentrations resulted in a progressive decrease in the observed turbidity.

### FIG. 4. A, the effect of temperature on heparin binding to fibronectin (O—O) and heparin-induced cryoprecipitation (A—A). 300 μg/ml fibronectin was incubated with 50 μg/ml heparin for 1 h at various temperatures. Cryoprecipitate formation was monitored at 350 nm (A—A). Polarization data representing the binding of 3 μg of fluorescein-labeled heparin to 300 μg of fibronectin in 1 ml were collected over a range from 2 to 40°C (O—O). Polarization of fluorescein-labeled heparin in the absence of fibronectin (P<sub>F</sub>) declined slightly with increasing temperature (O—O). The polarization data from Fig. 5 was plotted as P<sub>M</sub>/P<sub>F</sub> versus temperature in the inset. B, amount of bound, fluorescein-labeled heparin in micrograms at 2°C (O—O), 22°C (A—A), and 37°C (O—O). At each temperature, 300 μg/ml fibronectin was titrated with increasing amounts of fluorescein-labeled heparin. Polarization data was converted to the amount of fluorescein-labeled heparin bound to fibronectin.
provides evidence for heparin binding to fibronectin even at elevated temperatures. The decrease in $P_M$ with increasing temperature was not merely due to temperature-dependent changes in the rotational mobility or flexibility of the fluorescein-labeled heparin probe since the probe itself showed only a small decrease in $P_T$ over the temperature range studied. The ratio of $P_M/P_T$ decreased with increasing temperature, indicating a decline in the polarization of bound heparin not entirely attributable to the decrease in $P_T$ (Fig. 4A, inset). The change in $P_M$ may be attributed to a combination of increasing flexibility of the fibronectin molecule with increased temperature and decreased binding of heparin to fibronectin. Electron spin resonance investigations using spin-labeled fibronectin have provided evidence that at low temperatures fibronectin assumes a rigid, more extended shape, while a more flexible conformation is assumed as the temperature is raised (33). Circular dichroism studies also indicate that fibronectin may be capable of undergoing temperature-dependent changes in shape (34).

By titrating fibronectin with fluorescein-labeled heparin at 2, 22, and 37°C, the amount of heparin bound at the above temperatures could be calculated (Fig. 4B). The amount of heparin bound to fibronectin decreased 23% from 2 to 37°C (Fig. 4B). These results indicate that the binding constant of fibronectin for changes with temperature. Although decreased binding of heparin to fibronectin was observed, bound heparin was still evident well beyond the effective temperature range for cryoprecipitation.

**The Effect of Ionic Strength on Heparin Binding to Fibronectin and Cryoprecipitation**—In agreement with previous studies (14), maximum cryoprecipitation occurred at an ionic strength of 0.05–0.1 M and decreased rapidly to zero above an ionic strength of 0.3 M. Again, as observed previously (14), below an ionic strength of 0.05 M, cryoprecipitation of fibronectin took place even in the absence of heparin (Fig. 5).

As revealed by fluorescence polarization analysis, binding of fluorescein-labeled heparin to fibronectin decreased progressively as the ionic strength was increased from 0.05 to 0.35 M (Fig. 5). Bound fluorescein-labeled heparin was still evident at 0.5 M, but was progressively eluted at higher ionic strengths. At an ionic strength of 1 M, $P_M$ and $P_T$ were essentially identical, indicating the complete dissociation of bound heparin. Increasing ionic strength had little effect on the polarization of the unbound fluorescein-labeled probe ($P_T$). It is important to note that while heparin binding to fibronectin was still evident at ionic strengths from 0.35 to 0.5 M, no cryoprecipitation was observed in this range.

**The Effect of pH on Heparin Binding to Fibronectin and Cryoprecipitation**—Cryoprecipitation of fibronectin in the presence of heparin was investigated over a broad pH range (Fig. 6). The cryoprecipitation of fibronectin was optimal at pH 7 and declined as pH increased; above pH 10, cryoprecipitation was abolished. Cryoprecipitation studies in the acidic range are complicated by the fact that fibronectin precipitates in the absence of heparin at pH 3.5–6.5 range (Fig. 6). The insolubility of fibronectin from pH 3.5 to 6.5 at low ionic strength is well-documented (35) and is probably due to isoelectric precipitation since the isoelectric point of fibronectin is between pH 5.5 and 6.0 (5). The decline in heparin-induced cryoprecipitate formation observed from pH 6.5 to 3.5 may reflect a decrease in the concentration of soluble fibronectin rather than a true decrease in cryoprecipitation due to the influence of pH. It should be noted that between pH 2 and 3, cryoprecipitation in the presence of heparin increased to values greater than observed at pH 7.0. This observation may be related, in part, to the pH-dependent transition from compact, globular fibronectin molecules at neutral pH to more extended, fibrillar structures observed at pH 3 in sedimentation velocity studies (35).

Polarization measurements representing heparin binding to fibronectin were recorded over the pH range from 7.0 to 12.0 (Fig. 6). Heparin binding to fibronectin decreased with increasing pH from a maximum at pH 7. The decline in heparin binding was gradual compared to the pH effect on cryoprecipitation as $P_M$ values at pH 11.0 were still 42% of those at pH 7. However, above pH 11.0, binding of heparin to fibronectin was virtually abolished, i.e., $P_M = P_T$. Increasing pH from 7 to 12 had no effect on the polarization of unbound, fluorescein-labeled heparin in the absence of fibronectin ($P_T$). Below pH 7.0, fibronectin precipitated, and nonspecific protein aggregation interfered with measurements of the fluorescence polarization induced by heparin binding to fibronectin. In addition, although nonspecific precipitation was not a problem at pH 2–3, we were unable to investigate the binding of heparin to fibronectin in this range due to quenching of the fluorescent probe below pH 4.

**DISCUSSION**

The present study indicates that cryoprecipitation of fibronectin by heparin solutions is a process consisting of two
independent steps, namely an initial event involving the binding of heparin to fibronectin and a subsequent event involving aggregation of fibronectin molecules to produce an insoluble complex at 2 °C. Using the technique of fluorescence polarization, we have characterized several parameters involved in heparin binding to fibronectin and have compared the effects of these parameters on heparin-induced cryoprecipitation, we have characterized several parameters independent of cryoprecipitation of fibronectin.

The heparin binding and protein aggregation events involved in cryoprecipitation can readily be distinguished from one another. The present study provides additional insight into the mechanism governing heparin-induced cryoprecipitation. Since it is clear that heparin can bind to fibronectin at low temperatures, pH values, and temperatures which do not support cryoprecipitation (Figs. 4–6), heparin binding may be regarded as a necessary but insufficient condition for cryoprecipitate formation.

We have shown that heparin binding is rapid and precedes the protein aggregation event which occurs at low temperatures (Fig. 1). The temporal requirements for heparin binding and protein aggregation differ markedly; while heparin binding occurs within seconds, maximum protein aggregation requires over an hour (Fig. 1). Temporal differences between heparin binding and cryoprecipitation may be ascribable to the time required for the diffusion of activated fibronectin molecules and their association into an insoluble latticework.

Cryoprecipitation also differs from the initial binding interaction in that threshold concentrations of fibronectin and heparin are required to produce cryoprecipitation (Figs. 2 and 3). While the binding of heparin to fibronectin increases progressively as either the concentration of heparin or fibronectin is increased (Figs. 2 and 3), cryoprecipitation appears to require a threshold concentration of both fibronectin and heparin (Figs. 2 and 3), and hence, fibronectin aggregation appears to be a cooperative phenomenon. Cryoprecipitation may require that more than one of the three heparin-binding sites of fibronectin be occupied (36, 37). In contrast, no threshold concentration was noted for the initial binding of heparin to fibronectin. The concentration dependence plots of many heparin-related phenomena commonly show a maximum like that seen in Fig. 3B for cryoprecipitation. With respect to the role of heparin in the thrombin-antithrombin reaction, such a maximum has been attributed to a decrease in reaction rate at high heparin concentration which is due to the binding of thrombin and antithrombin to different heparin molecules rather than to the same molecule (38). An analogous phenomenon may be operating in cryoprecipitation. Heparin likely acts by binding more than one fibronectin molecule and thereby facilitating slow fibronectin–fibronectin interactions. Interestingly, a molecular mass of 12,000 daltons is assumed for heparin, the maximum in Fig. 3B occurs at 25 mol heterogeneous heparin/mol of fibronectin which, if corrected for the approximate 10–20% content of fibronectin-bindable heparin, is close to the stoichiometric equivalence of five heparin-binding sites/fibronectin dimer. At higher heparin concentrations, it becomes increasingly probable that individual heparin molecules will be associated with no more than one fibronectin molecule, and hence, the build-up of a network of interacting fibronectin molecules cross-linked by heparin will be lessened at high heparin concentrations.

Cryoprecipitation of fibronectin by heparin occurred only over the narrow temperature range from 0 to 6 °C (Fig. 4A); in contrast, heparin was able to bind to fibronectin over a much wider range, i.e. 2–40 °C (Fig. 4, A and B). Circular dichroism (34), electron spin resonance (33), and electron microscopic (39) studies suggest that fibronectin molecules are more rigid at low temperatures. The fluorescence polarization studies presented here have demonstrated an increase in polarization values with decreasing temperature, indicative of increased heparin binding (Fig. 4, A and B); however, this observation may also be attributable in part to a decrease in flexibility of the fibronectin molecule at low temperature (Fig. 4A). An increase in rigidity at low temperatures may stabilize a heparin–fibronectin complex and thereby enhance inter-molecular association during cryoprecipitation. Indeed, CD studies indicate preferential binding of heparin to low temperature forms of fibronectin (34).

The decrease in fibronectin cryoprecipitation observed at high pH and ionic strength is readily explained by the finding that the initial event in cryoprecipitation, heparin binding to fibronectin, is also inhibited at high pH and ionic strength (Figs. 5 and 6). The results of the current study permit some speculation concerning the nature of the heparin–fibronectin interaction. In general, the interactions between heparins and proteins can be considered to involve "functional domains" on the polysaccharide which interact preferentially with the protein or proteins (30, 40). In the case of antithrombin binding, this domain is a specific pentasaccharide (41), although the binding strength can be modified by factors outside this pentasaccharide (29, 31). In the case of thrombin binding, the interaction appears to be electrostatic (42, 43) and selective only for the more highly sulfated regions of heparin (30). While the interference of high ionic strength and pH (Figs. 5 and 6) indicates an electrostatic component in interaction of heparin with fibronectin, the mechanism by which heparin and fibronectin interact appears to require some specificity on the part of the heparin in that all heparins do not apparently have the ability to bind to fibronectin. A previous study has indicated that fibronectin displays more than one class of binding sites for heparin (20). Scatchard analysis (44) of the data in Fig. 3A reveals a biphasic curve with a Kd of 1 × 10⁻⁴ to 3.5 × 10⁻⁵ M (data not shown) similar to values in the literature (20).

In the present study, fluorescence polarization analysis has been employed to examine binding interactions between fluorescein-labeled heparin and fibronectin. Polarization measurements have recently also been used to study fibronectin binding to gelatin (45) and C1q (46). In the future, fluorescence polarization analysis should prove useful in determining the precise mechanism of fibronectin interaction with heparin, collagen, C1q, and many other biomolecules which form complexes with fibronectin.

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