Modification of Carboxyl Groups Induces Self-association of Fibronectin *in vitro*

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Soluble fibronectin deposits *in vitro* and *in vivo* as fibrillar protein network. Fibrillar protein polymers form in fibronectin preparations during storage, and also when fibronectin interacts with polyamines [Vuento *et al.*, *Eur. J. Biochem.*, 105 (1980) 33]. We have studied the self-association of fibronectin as a direct binding of ¹²⁵I-labelled fibronectin to fibronectin-Sepharose. This binding had an apparent dissociation constant of 10^{-11} M. It was enhanced by lowering pH to the protonation range of carboxyl groups, by chemically blocking carboxyl groups with carbodiimide, and by spermine at low ionic strength. The results support the view that deposition of fibronectin is essentially a self-assembly process, which could be initiated by modification of the molecular charge of the protein.

Fibronectin is a dimeric protein with a mol. wt. of 440 000 which is present in a concentration of 300 mg/l in blood plasma, but also as an insoluble (deposited) protein in the pericellular matrix of *in vitro* cultured cells and in connective tissue.¹⁻³ The origin of this dual existence could reside in molecular differences between the circulatory and the cell surface forms of the protein. This hypothesis is supported by some studies^{4,5} showing distinct differences in the migration rates of the two forms in polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate. However, the biochemical and physicochemical properties of circulatory and cell surface fibronectins are very similar.⁶⁻⁹ Quite recently it has become obvious that the circulatory protein is capable of forming protein deposits indistinguishable from those observed in cell cultures. This deposition of soluble fibronectin has been demonstrated by immunochemical methods both *in vitro*¹⁰ and *in vivo*.¹¹ The findings indicate

that soluble and deposited fibronectins could have a common precursor protein. The subsequent deposition of fibronectin could be controlled by interactions of fibronectin with other molecules. Several interactions potentially important in this respect are already known. Fibronectin is capable of binding to collagen, fibrin, glycosaminoglycans and actin (reviewed in Ref. 3). It has been suggested³ that the deposition of fibronectin might be affected by multiple interactions with some of these macromolecules.

We have earlier suggested that self-association of fibronectin could be an important factor in the deposition of this protein.¹² Electron microscopy studies have demonstrated the presence of protein filaments in the preparations of purified fibronectin and in fibronectin aggregates formed with polyamines.¹² These results indicate that the deposition of fibronectin in a polymeric form would not necessarily need a macromolecular matrix, but could proceed as a self-assembly process. In this paper we show that protonation or modification of the carboxyl groups of fibronectin enhances the self-association of the protein. The results support the view that post-translational modification of fibronectin is sufficient for its deposition.

MATERIALS AND METHODS

Fibronectin was purified from citrated human plasma as described previously.¹³ The purity of the material was controlled by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate.¹⁴ Concentrations of solutions of purified fibronectin were determined by photometry¹⁵ assuming A_{280} (1%; 1 cm)=12.8. The

method of Lowry *et al.*¹⁶ was used in other protein determinations. Fibronectin was labelled with ^{125}I by lactoperoxidase catalyzed iodination.¹⁷ Details of the procedures used will be given elsewhere.¹⁸ Proteins were coupled to CNBr-activated agarose gel as described.¹⁸ Approximately 0.5–1 mg of protein was bound per ml of gel. Some of the experiments described below were carried out with immobilized proteins further modified chemically. In these instances a suspension of Sepharose-linked protein was treated with the modifying reagents which were then removed by washing the gel with buffer. The experiments involving modification of carboxyl groups were carried out with soluble fibronectin or with fibronectin coupled to Sepharose. Carboxyl groups were modified with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Sigma, St. Louis, MO., U.S.A.) at pH 4.75 in the presence of glycine ethyl ester.¹⁹ Fibronectin was first dialyzed against 0.9% NaCl, or fibronectin-Sepharose was washed with this solution. Neutralized glycine ethyl ester was added, and pH was adjusted to 4.75. The mixture was slowly stirred on a magnetic stirrer. Carbodiimide was added to a final concentration of 0.1 M, and pH was kept at 4.75 by adding dilute HCl. After an appropriate incubation time (routinely 1 h was used), the reaction was stopped by dialysis against 10 mM Tris-HCl, pH 7.5/0.9% NaCl/0.02% NaN_3 . The extent of the reaction was measured by analyzing the incorporation of radioactive ^{14}C -labelled glycine ethyl ester (glycine residue labelled, specific activity 1.65 GBq/mmol, New England Nuclear, Dreieich, Germany). In these experiments the total concentration of glycine ethyl ester was 20 mM to ensure a high enough specific radioactivity in the reaction mixture. In other experiments glycine ethyl ester was used at a concentration of 1 M. The concentration of fibronectin was initially 1 mg/ml.

The self-association of fibronectin was studied using a solid-phase radioassay. A detailed description of the assay will be published elsewhere.¹⁸ Aliquots of Sepharose-linked proteins (20 μl) were incubated in plastic tubes (7 $\times$ 50 mm) with labelled fibronectin. The volume of the incubation mixture was 1.0 ml. Bovine serum albumin (Sigma) was added to buffers to a concentration of 5 mg/ml. This was necessary in order to prevent binding of labelled protein to tube walls. The tubes were mixed by end-over-end rotation. After incubation for 16–20 h the gel was sedimented by centrifugation (1000g, 1 min) and

supernatant was carefully removed by suction. The gel was rapidly washed once with 1 ml of buffer. The tubes with their contents were counted in a scintillation counter. Initial experiments showed that the binding was reversible. We therefore used the method of Scatchard^{20,21} for estimation of dissociation constants. Fibronectin used in these experiments was freshly prepared. The binding experiments were done within 1–2 days after radio-labelling.

Affinity chromatography was performed at room temperature using columns (3 ml) of fibronectin-Sepharose or albumin-Sepharose equilibrated with 10 mM Tris-HCl, pH 7.5/0.9% NaCl/0.02% NaN_3 . Fifty ml of freshly prepared pooled human serum was passed through the columns, which were then extensively washed with the above buffer. Elution was achieved with 8 M urea in the above buffer. Eluted proteins were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate,¹⁴ and transferred electrophoretically from polyacrylamide gel to nitrocellulose membrane²² (Bio-Rad, Richmond, CA., U.S.A.). The

Fig. 1. Scatchard plot of binding of ^{125}I -labelled fibronectin to fibronectin-Sepharose. The ratio of the concentrations of bound and free labelled fibronectin (B/F) has been plotted against the concentration of bound labelled fibronectin (B). The linear portions of the curve fitted to the experimental points have been used to estimate dissociation constants K . The slope of such a line is equal to $-K^{-1}$. Abscissa intercept of the line is equal to the concentration of binding sites.

Fig. 2. Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate. Protein material bound from serum to and eluted from fibronectin-Sepharose (A) or albumin-Sepharose (B) was analyzed after reduction with mercaptoethanol. Migration is from top to bottom. Migration positions of molecular weight markers are shown on the left. The gel was stained with Coomassie Blue.

protein bands were then visualized using pre-determined dilution of anti-fibronectin anti-serum²³ and peroxidase-conjugated antibodies against rabbit immunoglobulins²² (Dako, Denmark).

RESULTS AND DISCUSSION

Previous studies using electron microscopy have demonstrated formation of filamentous protein

polymers from purified fibronectin.¹² In accord with these findings, we demonstrate here a direct binding of labelled fibronectin to fibronectin-Sepharose (Fig. 1). In Fig. 1, the ratio of concentrations of bound and free ¹²⁵I-labelled fibronectin (B/F) has been plotted against concentration of bound ¹²⁵I-labelled fibronectin.²¹ The curve shows deviation from linearity, indicating binding site heterogeneity.²¹ The linear portions of the curve were used to estimate dissociation constants. The slope of the line is equal to $-K^{-1}$, K being the dissociation constant. Abscissa intercepts of the lines (not shown) are equal to the concentrations of binding sites. Such calculations indicated that the "high" affinity binding sites have an apparent dissociation constant of 3×10^{-11} M. The "low" affinity sites have an apparent dissociation constant of 1×10^{-10} M. This is actually a fairly high affinity. As the immobilization of fibronectin to solid phase may affect the binding and make the reaction mechanism complicated, the values obtained for dissociation constants should be taken as approximative.

Self-association of fibronectin was also observed in affinity chromatography experiments. Approximately 1 and 0.5 mg of protein were recovered from columns of fibronectin-Sepharose and albumin-Sepharose, respectively. Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate revealed two closely spaced polypeptide bands having mobility similar to that of fibronectin in the protein material eluted from fibronectin-Sepharose (Fig. 2). Immunoblotting using antibodies against fibronectin confirmed the presence of fibronectin in the material eluted from fibronectin-Sepharose column, while the material bound to and eluted from albumin-Sepharose did

Table 1. Effect of spermine and heparin on the binding of ¹²⁵I-labelled fibronectin to fibronectin-Sepharose. Experimental details are given in the Materials and Methods section. The diluent buffer used in these experiments was 5 mM Tris-HCl, pH 7.5, containing 5 mg/ml of bovine serum albumin. Concentration of fibronectin was 5 ng/ml. Results for albumin-Sepharose and plain Sepharose serve as controls. Abbreviation: n.d., not determined.

Reagent added		Binding of ¹²⁵ I-labelled fibronectin to derivatized Sepharose expressed as % of total radioactivity		
		Fibronectin-Sepharose	Albumin-Sepharose	Sepharose
None		18.7	3.0	0.8
Spermine	0.1 mM	50.1	9.9	2.2
	1.0 mM	51.6	14.2	6.1
	10 mM	27.2	4.9	2.0
Heparin	1 µg/ml	18.6	n.d.	0.8
	20 µg/ml	2.6	n.d.	0.6

Fig. 3. Immunoblotting of the material eluted from fibronectin-Sepharose and albumin-Sepharose. Material eluted from fibronectin-Sepharose (A) or from albumin-Sepharose (B) were fractionated by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate, and then transferred electrophoretically from polyacrylamide gel to nitrocellulose membrane. Immunoreactive fibronectin was visualized on nitrocellulose membrane after reaction with anti-fibronectin antibodies (anti-FN) by using peroxidase-conjugated antibodies against rabbit immunoglobulins. Control experiment was performed using nonimmune rabbit serum (NRS) with subsequent staining with peroxidase-conjugated anti-immunoglobulin antibodies. Migration positions of molecular weight markers are shown on the left (arrows).

not seem to contain fibronectin (Fig. 3). Other investigators also have demonstrated fibronectin-fibronectin-interactions using affinity chromatography.²⁴

Also in accordance with previous results¹² we observed an enhancement of the self-association of fibronectin by spermine at low ionic strength (Table 1). Interestingly, no enhancement but a clear inhibition was observed when heparin was included in the incubation buffer (Table 1). Because heparin has been shown to interact with fibronectin,²⁵ this result may indicate the involvement of the same fibronectin domain in self-assembly and in the interaction with heparin.

The binding of labelled fibronectin to fibronectin-Sepharose was strongly dependent on pH, as shown in Fig. 4. The optimum pH appeared to be between 4–6. The results obtained with different buffers varied to some extent. Thus more extensive binding was observed in phosphate buffer than in citrate buffer. The reason for this behaviour is not known. Binding of labelled fibronectin to nonsubstituted Sepharose gel was not significant at any pH value tested (Fig. 4). Similar low binding (approximately 1% of total radioactivity) was observed with albumin-Sepharose (not shown). As the pK values

Fig. 4. Effect of pH on the binding of ¹²⁵I-labelled fibronectin to fibronectin-Sepharose. Aliquots of fibronectin-Sepharose (20 μ l, 20 μ g of fibronectin) were equilibrated with ¹²⁵I-labelled fibronectin (5 ng) for 16 h at room temperature in various buffers (1.15 ml). The concentration of the buffers was 50 mM. All buffers contained 5 mg/ml of bovine serum albumin. The buffer systems used were glycine-HCl, pH 2.0–3.0 ($\square$), sodium citrate, pH 3.0–5.5 ($\blacktriangle$), potassium phosphate, pH 5.5–8.0 ($\triangle$), Tris-HCl, pH 7.0–9.5 ($\circ$), and glycine-NaOH, pH 8.5–10.0 (∇). Sepharose 4B gel was used as a control ($\bullet$).

for dissociation of free carboxyl groups of glutamic acid and aspartic acid are in the pH range of 4–5 in proteins, the results suggest that protonation of some critical carboxyl groups enhances the self-association of fibronectin. Our previous studies have shown that fibronectin binds to carboxyl-modified proteins¹⁸ and that such modified

Fig. 5. Scatchard plot of binding of ¹²⁵I-labelled fibronectin to carboxyl-modified fibronectin-Sepharose. Experimental details are given in the Materials and Methods section and in the legend to Fig. 1.

Fig. 6. Modification of carboxyl residues of fibronectin with carbodiimide. Modification of fibronectin (1 mg/ml) was carried out at pH 4.75 with 0.1 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide in the presence of 20 mM glycine ethyl ester. The reaction mixture also contained 3.7 kBq/ml of ^{14}C -glycine ethyl ester. The reaction was stopped after indicated incubation times by dialysis against 10 mM Tris-HCl, pH 7.5 – 0.9% NaCl – 0.02% NaN_3 . After an extensive dialysis, the protein concentration and radioactivity of the samples were determined. The extent of modification (residues modified per fibronectin subunit) was calculated using a mol. wt. of 220 000 for the fibronectin subunit.

proteins bind selectively fibronectin from serum.¹⁸ In accordance with these findings, the binding of labelled fibronectin to carbodiimide-treated fibronectin-Sepharose was greatly enhanced as compared with binding to native fibronectin-Sepharose (Fig. 5). Carbodiimide-treatment of fibronectin gave rise to modification of up to 50 carboxyl residues per fibronectin subunit, as shown in Fig. 6. Scatchard analysis of binding data (Fig. 5) again suggested the presence of more than one type of binding sites on modified fibronectin-Sepharose, with apparent dissociation constants of 3×10^{-11} M ("high" affinity sites) and 3×10^{-10} M ("low" affinity sites). These values are similar to those obtained with native fibronectin-Sepharose (Fig. 1). Because the same parent fibronectin-Sepharose gel was used in both experiments, and because the volumes of the gels used in the experiments were known, we could calculate the apparent increase in the amount of high-affinity binding sites occurring during modification to be about 100-fold.

The present results are in agreement with the suggestion¹² that the deposition of fibronectin could be essentially a self-association process which would not necessarily need a pre-existing macromolecular matrix. Our results raise the possibility that such a self-association could be initiated by modification of the molecular charge of fibronectin.

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