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Immunochemical characterization of human plasma fibronectin

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Human plasma fibronectin has been purified by a non-denaturing affinity chromatography procedure [Vuento & Vaheri, (1979) *Biochem. J.* **183**, 331–337], and antisera have been raised by immunizing rabbits with the native protein. The antisera reacted strongly with native fibronectin, but only weakly with reduced and alkylated fibronectin or with heat-denaturated fibronectin. Denaturation also affected the haemagglutinating and gelatin-binding activities of fibronectin and increased its susceptibility to proteolytic degradation. The antisera reacted with fragments of fibronectin obtained by proteolysis with plasmin. Large fragments (mol.wt. 180000–200000), lacking the region harbouring the interchain disulphide bridges but containing the sites responsible for gelatin-binding and haemagglutinating activity, showed as intense a reaction with the antisera as intact fibronectin. Smaller peptides showed a weaker reaction. All fragments tested showed sensitivity to denaturation in their reaction with the antisera. The results were interpreted as showing that: (1) native fibronectin has an ordered conformation that is easily perturbed by denaturation; (2) most of the antigenic determinants of the protein are dependent on conformation; (3) the region of the fibronectin molecule containing the interchain disulphide bridges has only few antigenic determinants; and (4) covalent interaction of the two subunits does not contribute to the antigenic structure recognized by rabbit antisera. The observed correlation between the antigenic activity and a structural and functional intactness of fibronectin suggests that the antibodies to native fibronectin could be used as a conformational probe in studies on this protein.

Fibronectin is a high-molecular-weight glycoprotein present in soluble form in plasma and also in insoluble form in connective tissue (Yamada & Olden, 1978; Vaheri & Mosher, 1978). Fibronectin binds to collagen (Engvall & Ruoslahti, 1977; Dessau *et al.*, 1978), to fibrin (Ruoslahti & Vaheri, 1975; Stemberger & Hörmann, 1976), to acid polysaccharides like heparin (Stathakis & Mosesson, 1977) and to bacteria (Kuusela, 1978). It also agglutinates erythrocytes (Yamada *et al.*, 1975; Vuento, 1979). These various interactions suggest a role for fibronectin as an adhesion-mediating and opsonizing protein (Yamada & Olden, 1978; Blumenstock *et al.*, 1978).

The molecular weight of plasma fibronectin (also known as 'cold-insoluble globulin') has been reported to be 450000 (Mosesson *et al.*, 1975). The protein is composed of two subunits of equal or nearly equal size. The subunits are linked by disulphide bridges, which are located near to one end of the polypeptide chains (Jilek & Hörmann, 1977; Chen *et al.*, 1977; Iwanaga *et al.*, 1978). Studies involving enzymic degradation have suggested that fibronectin contains relatively proteinase-resistant domains that are interconnected by proteinase-susceptible polypeptide segments (Ruoslahti *et al.*, 1979; Balian *et al.*, 1979; Fukuda & Hakomori, 1979; Hahn & Yamada, 1979; Wagner & Hynes, 1979). Such domains could be stabilized against proteolytic attack by specific folding of the polypeptide chains. The available spectroscopic data on fibronectin support the presence of ordered structures in fibronectin, but do not give a clear picture of how these are stabilized in terms of a secondary structure (Alexander *et al.*, 1979).

Antibodies have been successfully used as tools in studies on protein structure (Crumpton, 1974). We have used rabbit antibodies to fibronectin to analyse

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the structure and molecular interactions of this protein. The results indicate that the antigenic activity reflects closely structural and functional intactness of the protein and suggest that antibodies could be used as a conformational probe in studies on this protein.

Materials and methods

Purification of fibronectin

Fibronectin was purified from human plasma by affinity-chromatography procedures under non-denaturing conditions as described previously (Vuento & Vaheri, 1979). In brief, fibronectin was adsorbed from human plasma to gelatin–agarose and eluted, after washing, with arginine at pH 7.5. A second affinity-chromatography step on arginine–agarose completed the procedure. The purity of the material used in the present experiments was controlled by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Weber & Osborn, 1969). Fibronectin was stored at 4°C at a concentration of 1–3 mg/ml in 50 mM-Tris/HCl, pH 7.5, containing 100 mM-NaCl and 0.02% (w/v) NaN_3.

Reduction and alkylation

Fibronectin (1–2 mg) was reduced with 2-mercaptoethanol (100-fold molar excess to cysteine) in the presence of 8 M-urea and 5 mM-EDTA. After incubation for 2 h at room temperature, iodoacetamide was added in a 1.2-fold molar excess to thiol groups and the mixture was further incubated for 1 h. The excess of reagents was removed by dialysis against 10 mM-Tris/HCl buffer, pH 7.5, containing 150 mM-NaCl.

Denaturation with heat

In the thermal-denaturation experiments samples of fibronectin were incubated on a water bath at temperatures ranging from 30 to 80°C. The bath was thermostatically controlled with a precision of 0.5°C. After incubation, the samples were cooled under running cold tap water and analysed for immunoreactivity. The heat-denatured samples were also analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis under reducing and non-reducing conditions. No change in the subunit size (220000) was observed during these experiments, nor was there any fragmentation of dimeric fibronectin into monomers.

Proteolysis with plasmin

Commercial porcine plasmin (Sigma, St. Louis, MO, U.S.A.) or human plasmin (Kabi, Stockholm, Sweden) were used for proteolytic degradation of fibronectin. Samples of fibronectin (20 mg) were incubated with 1 mg of plasmin at 37°C in 10 mM-Tris/HCl, pH 7.5, containing 150 mM-NaCl. The digestions were stopped by adding phenylmethanesulphonyl fluoride to a final concentration of 1 mM. The digests were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Weber & Osborn, 1969). The cleavage pattern of native fibronectin was closely similar to that described by Jilek & Hörmann (1977), Chen *et al.* (1977) and Iwanaga *et al.* (1978). Large fragments, no longer disulphide-bonded, were first formed, and these were slowly digested into smaller fragments. In preparative experiments, the enzyme concentration and incubation time were adjusted to give a complete disappearance of the disulphide-bonded material with a mol.wt. of 450000.

The digests were fractionated on a column (2.9 cm × 70 cm) of Sephadex G-200, equilibrated with 10 mM-Tris/HCl, pH 7.5, containing 150 mM-NaCl and 0.02% (w/v) NaN_3. Protein was measured in the chromatographic fractions by photometry at 280 nm. The fractions were concentrated by ultrafiltration with a UM-10 membrane (Amicon, Lexington, MA, U.S.A.).

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

This was carried out by the method of Weber & Osborn (1969), with 5% (w/v) acrylamide. The gels were stained with Coomassie Blue. The molecular weights of fibronectin fragments were measured by using the following marker proteins, which all were reduced with 2-mercaptoethanol: purified plasma fibronectin (mol.wt. 220000), bacterial β-galactosidase (Sigma; 130000), rabbit muscle phosphorylase *a* (Worthington, Freehold, NJ, U.S.A.; 100000), bovine serum albumin (Sigma; 68000), ovalbumin (Sigma; 43000), human immunoglobulin G [Kabi; 51000 (heavy chains) and 25000 (light chains)], and pancreatic ribonuclease *a* (Sigma; 13800).

Antisera to native fibronectin

Antisera to fibronectin were obtained by immunizing rabbits with the native, purified protein. Initial subcutaneous injections of 0.5–1.0 mg of fibronectin in 10 mM-Tris/HCl, pH 7.5, containing 150 mM-NaCl were given emulsified with Freund's complete adjuvant (Difco, Detroit, MI, U.S.A.). Subsequent booster injections were given monthly without the adjuvant. Sera were collected 7–10 days after the booster injections. The antisera used in the present experiments were from animals that had been immunized for 1 year or longer. The specificity of the antisera was controlled as described previously (Vuento, 1979). The titres of sera were estimated by double-immunodiffusion analysis against purified fibronectin. The antisera gave a detectable precipitation line against fibronectin (0.3 mg/ml) at dilutions of 1:32–1:256.

Analysis of antigenic activity

Quantitative analysis of immunoreactivity of fibronectin was carried out by a kinetic immunoturbidometric method (Spencer & Price, 1979), with $(NH_4)_2SO_4$ (Stakenburg, 1979) as a precipitation-enhancing agent. The procedure was a modification of the method developed for determination of fibronectin from human plasma (U.-H. Stenman, unpublished work). In this method the change in turbidity caused by formation of antigen–antibody complexes was measured by a sensitive photometer at 354 nm. Samples were diluted in 10 mM-Tris/HCl buffer, pH 7.5, containing 150 mM-NaCl and 1% (w/v) bovine serum albumin. Diluted sample (0.1 ml) was mixed with 0.7 ml of 0.96 M-$(NH_4)_2SO_4$ in 10 mM-sodium phosphate buffer, pH 7.5, in a cuvette of an automated photometer (model 8600 reaction-rate analyser; LKB, Stockholm, Sweden). The apparatus was set to add 0.2 ml of antiserum, appropriately diluted with the 0.96 M-$(NH_4)_2SO_4$ solution. The antiserum was diluted so that the reaction mixture was in antibody excess; usually a dilution of 1:40 was used. The development of turbidity was recorded at 35°C. After the first 2 min the increase in turbidity was slow, and the curves reached a plateau in about 5 min. The rate of increase in turbidity was calculated by a Optilab model 432 reaction rate calculator (LKB). Standard curves were obtained by plotting the calculated rate of increase in turbidity against the concentration of the sample. For native fibronectin, standard curves were obtained for the concentration range of 1–20 μg/ml (final concentration in the assay mixture).

Double-immunodiffusion analysis was carried out with 1% agarose (L'Industrie Biologique Française, Gennevilliers, France) in 10 mM-Tris/HCl, pH 7.5, containing 150 mM-NaCl and 0.1 mg of merthiolate/ml. The plates were photographed after an incubation time of 16 h at room temperature.

Assay of haemagglutinating activity

The haemagglutinating activity of fibronectin was assayed as described previously (Vuento, 1979) by using trypsin-treated rabbit erythrocytes.

Analysis of gelatin-binding activity

The gelatin-binding activity of fibronectin was analysed as the ability of the protein to bind to gelatin–Sepharose (Vuento & Vaheri, 1978). Samples [0.3 mg in 0.1–0.3 ml of 10 mM-Tris/HCl (pH 7.5)/150 mM-NaCl] of fibronectin (or peptides derived from fibronectin by proteolysis with plasmin) were applied on columns (0.5 cm × 1.2 cm) of gelatin–Sepharose, equilibrated with the above buffer. The columns were first eluted with equilibrating buffer, then with 1 M-NaCl in the equilibrating buffer, and finally with 1 M-L-arginine hydrochloride buffered at pH 7.5. The amount of fibronectin bound to the column was measured from the difference in amount of protein applied and the amount of protein eluted with the first two buffers.

Determination of protein

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Results

Effect of denaturation by heat on the antigenic activity

The immunoturbidometric method used in the present experiments measured the increase in turbidity that resulted from formation of antigen–antibody complexes. It has been shown that, in titration of antibody with antigen, the curves of

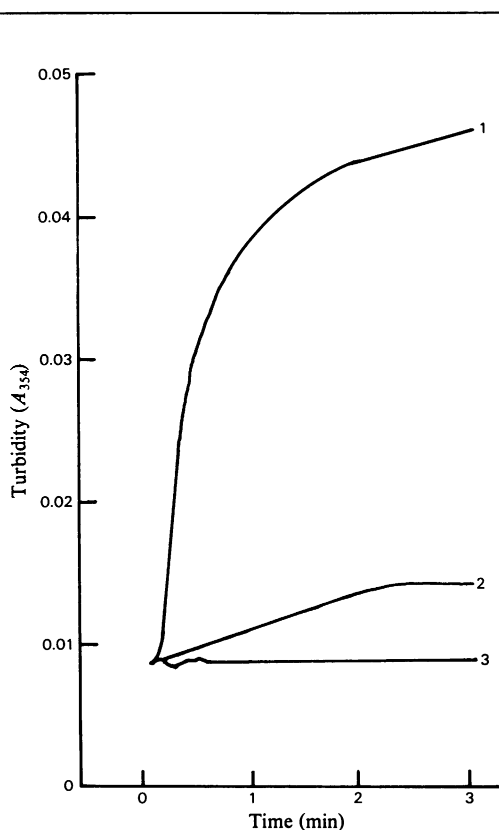

Fig. 1. *Analysis of native and denatured fibronectin by kinetic immunoturbidometry*

The Figure shows the development of turbidity from samples (7 μg) of native (curve 1) and denatured (30 min at 65°C, curve 2) fibronectin after mixing with antiserum to native fibronectin. Curve 3 was obtained with buffer containing no fibronectin.

turbidity versus antigen concentration are similar to the classical titration curves [amount of antigen precipitated versus concentration of antigen (Spencer & Price, 1979; Stakenburg, 1979)].

In antibody excess the turbidity developed is proportional to the concentration of reactive antigen present in the reaction mixture. This is demonstrated in Fig. 2, which shows the relationship between turbidity and fibronectin concentration in our assay. The ability of the immunoturbidometric method to distinguish between antigen preparations with different antigenic activities is demonstrated in Fig. 1. It is seen that curves of turbidity versus time are different with native and with denatured fibronectin. Denatured fibronectin developed less turbidity and gave a curve with a shallow slope compared with the curve given by native fibronectin.

When fibronectin was incubated at elevated temperatures for periods of 30 min, it was found that the antigenic activity of the protein remained unchanged up to 55°C. A rapid loss of antigenic activity took place when this temperature was exceeded. As shown in Fig. 3, the transition curve was quite steep between 55 and 60°C. At temperatures higher than 60°C, further decrease in antigenic activity was slow. Most of the antigenic activity of native fibronectin was lost by incubating for 30 min at 60–65°C (Figs. 2–4). However, there was a small, but consistently present, residual activity left even after 30 min incubation at 80°C. This activity amounted to about 10–15% of the initial activity. When samples of human plasma and serum were similarly incubated, the immunoreactivity of fibronectin present in these samples was lost at the same temperature range as that of purified fibronectin (results not shown). Fig. 4 shows a time curve of inactivation of antigenicity at 65°C. The inactivation appeared to be a rapid process, being completed in 15–20 min at this temperature. Samples analysed immediately after denaturation or after an incubation for 24 h at 4 or at 22°C showed the same low antigenic activity, indicating that no significant renaturation of the heat-denatured fibronectin occurred.

The effect of heat denaturation was also studied by immunodiffusion analysis. Fig. 5 demonstrates a decrease in the antigenic activity of fibronectin

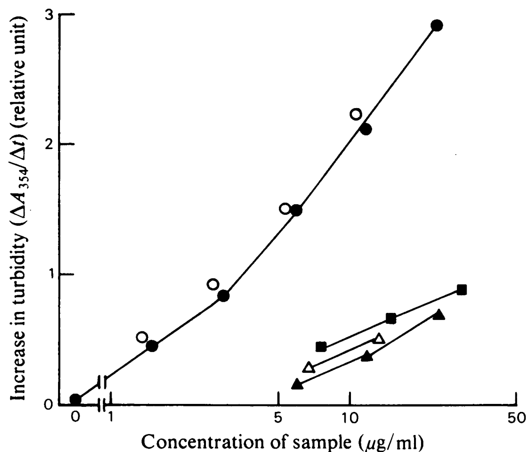

Fig. 2. *Kinetic immunoturbidometry of native and modified fibronectins*

The increase in turbidity was recorded when a constant amount of antiserum reacted with various amounts of native and modified fibronectin. The Figure demonstrates a low antigenic activity of reduced and alkylated fibronectin (■) and of heat-denatured fibronectin (30 min at 65°C, ▲) as compared with native fibronectin (●). The antigenic activity of monomeric fragments of fibronectin (mol.wt. 180000–200000, ○) is similar to that of native fibronectin. Small fragments (mol.wt. 30000, △) have a low activity.

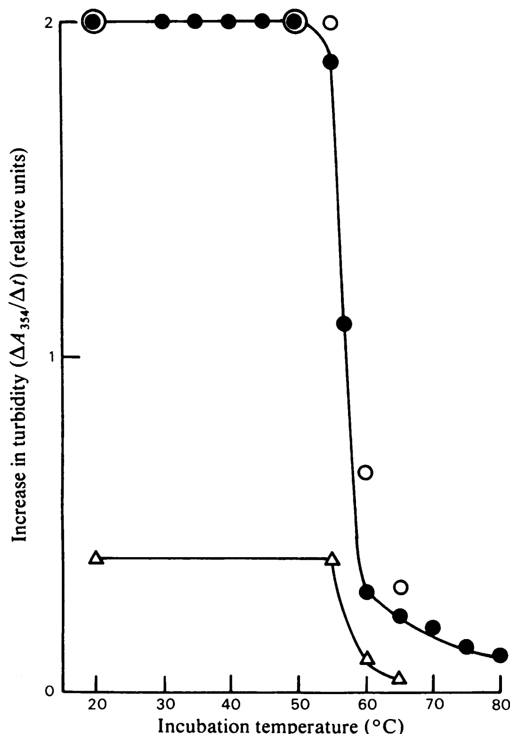

Fig. 3. *Heat-inactivation of native fibronectin*

Fibronectin (●), large monomeric (mol.wt. 180000–200000) plasmin fragments of fibronectin (○) or small fragments (mol.wt. 30000, △) were incubated for 30 min at the indicated temperatures. The samples were analysed by kinetic immunoturbidometry at a concentration of 10 μg/ml.

inactivated at 65°C. The precipitin lines formed by the denatured protein were weaker than those formed by the native protein.

Effect of reduction and alkylation

Reduction of the disulphide bonds of fibronectin in the presence of 8M-urea, followed by alkylation with iodoacetamide, decreased the antigenic activity of fibronectin to about 10% of the original value (Fig. 2, Table 1). In contrast, the antigenicity was not significantly changed in control experiments, where the protein was incubated with iodoacetamide in the presence of 8M-urea, or subjected to the reducing and alkylating conditions without the presence of urea.

Distribution of antigenic activity in proteolytic fragments of fibronectin

The distribution and conformational dependence of antigenic activity within proteolytic fragments were studied by immunodiffusion and immunoturbidometric methods. A sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of a plasmin digest of native fibronectin is shown in Fig. 6. Fractionation of the digest on Sephadex G-200 is shown in Fig. 7. The eluted fractions were pooled as

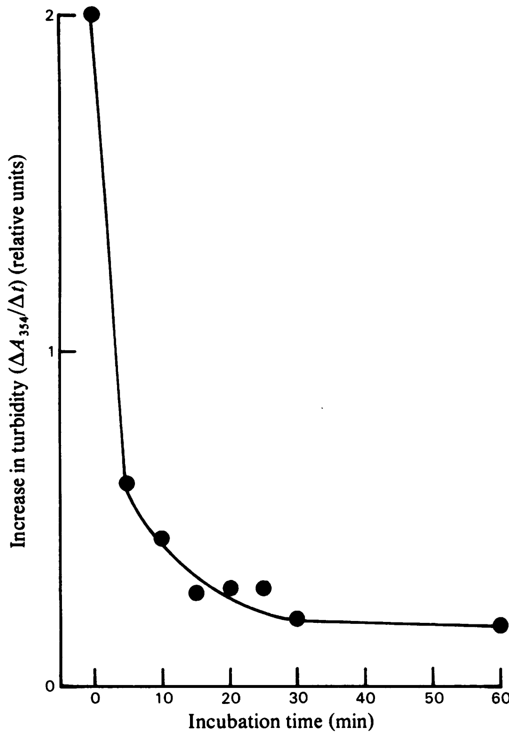

Fig. 4. *Time course of inactivation of fibronectin at 65°C*
Fibronectin (0.4mg/ml in Tris-buffered saline, pH7.5) was incubated at 65°C for the indicated periods of time and assayed for antigenic activity by immunoturbidometry method. The final concentration of fibronectin was 10μg/ml.

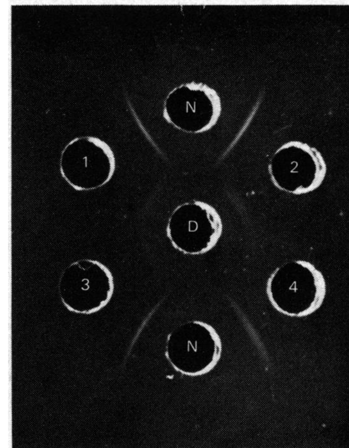

Fig. 5. *Analysis of native and denatured fibronectin by double immunodiffusion*
Native (N) and heat-denatured (D) fibronectin were tested at a concentration of 1mg/ml against four antisera (1–4) to native fibronectin, each from a different rabbit. The plate was photographed after an incubation time of 16h.

Table 1. *Effect of reduction and alkylation on the antigenic activity of fibronectin*
Fibronectin (2mg) was reduced with 2-mercaptoethanol with or without the presence of 8M-urea and alkylated with iodoacetamide. Control experiments were made with iodoacetamide only. The excess of reagents were removed by dialysis, and the preparations were tested for antigenic activity by kinetic immunoturbidometry, as described in the Materials and methods section.

Treatment of fibronectin	Relative antigenic activity (%)
None	100
Reduction in the presence of 8M-urea, alkylation	10
Reduction without urea, alkylation	90
Alkylation in presence of 8M-urea	95

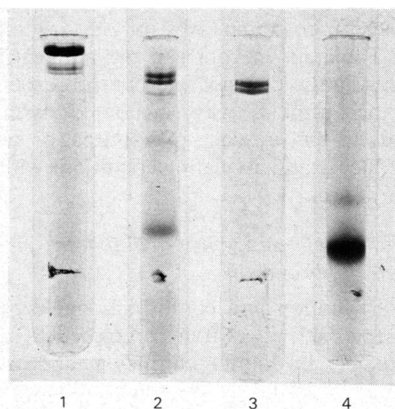

Fig. 6. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis under non-reducing conditions of plasmin fragments derived from native fibronectin*

Gel 1 shows dimeric fibronectin as a strong band near the top. In addition, several faint bands are visible below the major band; these probably represent contaminating monomeric fibronectin. Gel 2 shows a digest obtained from native fibronectin with porcine plasmin. Gels 3 and 4 show fractions separated from the digest by gel chromatography on Sephadex G-200. A comparison of the mobilities of the polypeptide bands with those of standards (see the Materials and methods section) suggested mol.wts. of 180000–200000 for the monomeric fragments eluted close to the void volume of the column (gel 3) and a mol.wt. of 30000 for the material eluted at $K_{av.} = 0.55$ (gel 4). The same molecular weights were obtained under non-reducing and reducing (results not shown) conditions. The gels were loaded with 15μg (1–3) or 30μg (4) of protein. The markings at the lower ends of the gels are at the migration position of the marker dye Bromophenol Blue.

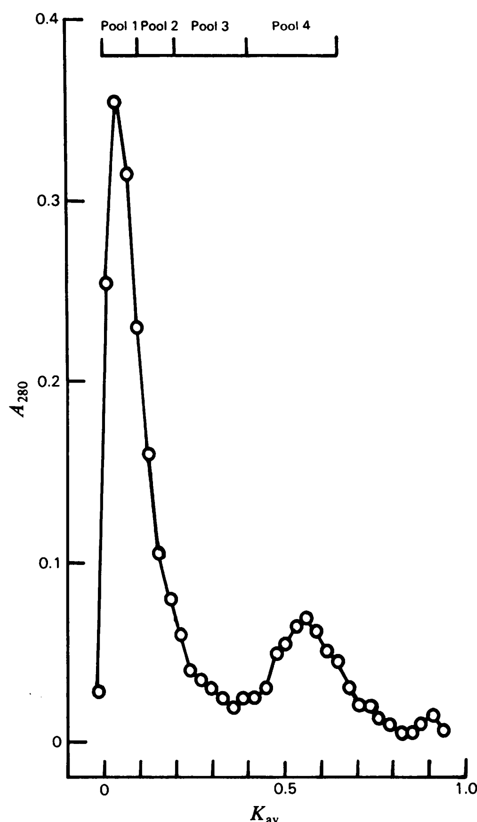

Fig. 7. *Fractionation of a plasmin digest of fibronectin on Sephadex G 200*

Fibronectin (20mg) was digested with pig plasmin as described in the Materials and methods section, and subsequently fractionated on a column (2.9cm × 70cm) of Sephadex G-200 equilibrated with 10mM-Tris/HCl, pH7.5, containing 150mM-NaCl and 0.02% NaN_3. The eluted fractions were pooled as indicated.

shown in Fig. 7. The pools 1 and 4 represented material well-defined in terms of molecular weight. The peptides in pool 1 had an apparent mol.wt. of 180000–200000 as judged from sodium dodecyl sulphate/polyacrylamide-gel electrophoresis under non-reducing (Fig. 6) and reducing conditions (results not shown). These peptides bound effectively to gelatin–Sepharose. Pool 4 contained peptides with an apparent mol.wt. of 30000. These peptides did not bind to gelatin–Sepharose. Pools 2 and 3 contained a heterogeneous group of peptides having mol.wts. between 30000 and 180000.

All fragment pools showed antigenic activity as determined by immunodiffusion (Fig. 8). Quantitative measurements by immunoturbidometry showed that large monomeric fragments (mol.wts. 180000–200000) displayed antigenic activity similar to that of intact fibronectin. Some preparations, like that shown in Fig. 2, had an activity slightly higher than intact fibronectin. The antigenicity of the shorter fragments was weaker, and the peptides of pool 4 had an activity (on a weight basis) of only 10–20% of that of intact fibronectin. Incubation of the fragments at 65°C for 30min decreased their antigenic activity to about 10–20% of the original value. All fragments studied were sensitive to denaturation by heat. As seen in Fig. 3, the temperature range for the antigenic inactivation of the fragments (pools 1 and 4) was identical with the temperature range of inactivation for intact fibronectin.

Increased susceptibility to proteolysis after denaturation

Digestion of native fibronectin by plasmin was a relatively slow process. Under our incubation condi-

Table 2. *Effect of denaturation on the haemagglutinating and gelatin-binding activities of fibronectin*
Samples of fibronectin were incubated for 30 min at the indicated temperatures or reduced and alkylated in the presence of 8 M-urea. The haemagglutinating activity was determined with trypsin-treated rabbit erythrocytes. The values for the relative-haemagglutinating titre were calculated from the maximal dilution of the sample giving a recognizable agglutination. The gelatin-binding activity was determined as binding of the sample to gelatin–Sepharose as described in the Materials and methods section. The Table shows that both activities were destroyed by reduction and alkylation. The gelatin-binding activity was stable against heat.

Treatment of fibronectin	Relative haemagglutinating titre (%)	Percentage of sample retained on gelatin–Sepharose
None	100	100
55°C	100	100
60°C	25	100
65°C	12	100
75°C	0	100
Reduction in presence of 8 M-urea and alkylation	0	0

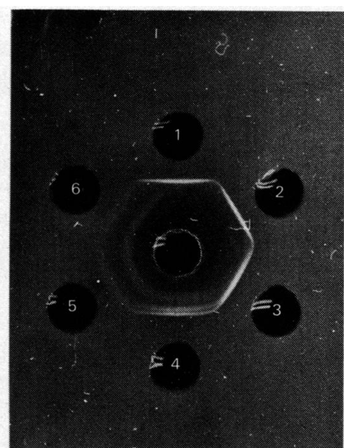

Fig. 8. *Double-immunodiffusion analysis of plasmin fragments derived from native fibronectin*
Fractions of a plasmin digest of native fibronectin prepared by gel filtration on Sephadex G-200 were tested by immunodiffusion with 20 μl of an antiserum to native fibronectin (centre well). Portions (20 μl) of the following samples (the pooling of chromatography fractions is shown in Fig. 7) were applied at a concentration of 0.3 mg/ml: 1, intact fibronectin; 2, pool 1; 3, pool 2; 4, pool 3; 5 and 6, pool 4. The plate was photographed after an incubation time of 16 h.

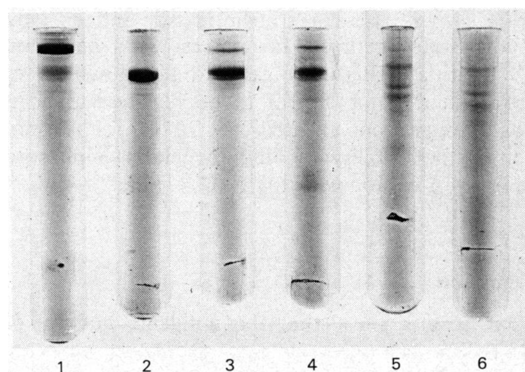

Fig. 9. *Demonstration of increased susceptibility to plasminolysis of heat-denatured fibronectin*
Native or heat-denatured fibronectin was digested with human plasmin (10 μg/mg of fibronectin) for 1 h at 37°C. Identical portions of the digests were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. All samples except that in gel 1 were reduced with 2-mercaptoethanol before electrophoresis. 1, Control fibronectin not treated with plasmin; 2, reduced control fibronectin; 3, native fibronectin treated with plasmin; 4–6, fibronectin heated at 55°C (4), 60°C (5) or 65°C (6) and subsequently treated with plasmin. The markings at the lower ends of the gels are at the migration position of the marker dye Bromophenol Blue.

tions, a complete conversion of the dimeric protein (mol.wt. 450000) to monomeric fragments (mol.wt. 200000) took about 8 h when plasmin was used in a concentration of 30–50 μg per mg of fibronectin. The conversion of the monomeric (mol.wt. 200000) fragments into smaller peptides was even slower. However, when fibronectin was incubated at 60–65°C before incubation with plasmin, an extensive degradation of the 450000- and 200000-mol.wt. species took place in a few minutes. This effect is demonstrated in Fig. 9, which shows results of sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of plasmin digests of native and denatured fibronectins. Under the conditions of the experiment, most of the protein material of the plasmin digest of native fibronectin had a mol.wt. of 200000. Fibronectin incubated at 55°C before addition of plasmin showed the same polypeptide pattern as

native fibronectin. In contrast, fibronectin heated at 60 or at 65°C before the digestion by plasmin showed only a small amount of the 200000-mol.wt. polypeptide band, but an increased proportion of smaller fragments.

Effect of denaturation on the haemagglutinating and gelatin-binding activities

The haemagglutinating and gelatin-binding activities of fibronectin were investigated under conditions that reduced the antigenic activity. It was found that reduction and carboxymethylation in the presence of 8 M-urea completely abolished both the haemagglutinating and the gelatin-binding activity of fibronectin. The effect of heat denaturation on the haemagglutinating activity is shown in Table 2. It was evident that the inactivation of the haemagglutinating activity took place in the same temperature range where the transition to an antigenically inactive state occurred. In contrast with the haemagglutinating activity, the gelatin-binding activity of fibronectin did not appear to be sensitive to heat. Thus preparations heated at 75°C for 30 min retained their ability to bind to gelatin–Sepharose and to resist elution with 1 M-NaCl (Table 2).

Discussion

Our results show that the antigenic activity of native fibronectin was strongly decreased when the protein was subjected to denaturing conditions such as temperatures of 60–65°C or reduction and carboxymethylation. This indicates that native fibronectin has a specific organized structure that is recognized by antibodies. Perturbation of this structure appears to give rise to altered molecules with which the antibodies react only weakly. There was no degradation of the polypeptide chains of fibronectin during the denaturing experiments, as indicated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Therefore, the denaturation-induced changes must involve the secondary and/or the tertiary structure. Thus the antigenic determinants of the native protein seem to be dependent on the conformation of the protein molecule, a situation which is usually associated with globular proteins (Crumpton, 1974).

Our results are in line with the spectral data reported by other workers. Transitions in the spectroscopic parameters of fibronectin have been observed at elevated temperatures or in the presence of denaturing agents (Alexander *et al.*, 1979; Colonna *et al.*, 1978). It is interesting to compare the transition curve of antigenic activity (Fig. 3) with the results obtained by Alexander *et al.* (1979) for tryptophan fluorescence polarization and for mean residue ellipticity at 227 nm. The two latter parameters have been shown to remain unchanged at pH 7.0 up to 55°C, but to undergo a strong transition in the temperature range 55–60°C, i.e. the temperature causing loss of antigenic and haemagglutinating activity.

The digestion of native fibronectin with plasmin gave rise to fragments that all showed antigenic activity (Fig. 8). The reaction with antisera given by the large monomeric fragments (mol.wt. 180000–200000) and by smaller peptides (mol.wt. 30000) were assessed quantitatively. The intense reaction given by the large monomeric peptides suggests that the region of the intact molecule containing the interchain disulphide bridges did not give rise to antibodies in rabbits. Sometimes the fragment preparations were slightly more active than intact fibronectin. This may not be significant, because the comparison was based on protein determination by the method of Lowry *et al.* (1951). The colour values of intact and modified proteins may be slightly different. These results suggest that a covalent interaction in the intact protein between the subunits plays no role in the antigenic structure.

Together with antigenic activity, the large fragments retained their gelatin-binding activity. It was shown previously (Vuento, 1979) that the haemagglutinating activity of fibronectin is also retained in similar monomeric fragments obtained with plasmin.

The reaction of the smaller fragments (mol.wt. 30000–180000) with the antisera was weaker than that given by the large fragments. This indicates that native fibronectin has different antigenic determinants distributed over the polypeptide chains. The isolated small peptides (mol.wt. 30000) did not bind to gelatin and were thus different from the gelatin-binding fragments of similar size studied by other workers (Hahn & Yamada, 1979; Ruoslahti *et al.*, 1979). In preliminary experiments we have found gelatin-binding peptides with mol.wts larger than 30000 in plasmin digests of fibronectin, but these have not been obtained in amounts sufficient for characterization.

Denaturation caused loss of antigenicity and also affected the haemagglutinin and gelatin-binding activities of fibronectin (Table 2). However, although the gelatin-binding activity was destroyed by reduction and alkylation, it remained when the protein was denatured by heat. This suggests that the gelatin-binding sites on fibronectin are associated with exceptionally stable domains. The denaturation also increased the susceptibility of the protein to proteolysis. This effect was probably caused by increased unfolding of the polypeptide chains, which exposed plasmin-susceptible sites on the protein molecule. It is noteworthy that the temperature range where unfolding occurred was the same as that where the transition to a non-antigenic

state was observed. Considering these effects, it is clear that the antigenic activity very well reflected a general native state of fibronectin. This suggests that antibodies against native fibronectin are useful as a conformational probe in studies on the structure and molecular interactions of this protein.

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