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Characterization of the interaction between human plasma fibronectin and collagen by means of affinity electrophoresis

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ABSTRACT

The interaction between human plasma fibronectin and different types and forms of collagen were analysed by affinity electrophoresis at different pH values. The fibronectin bound tightly to collagen type I, III and IV, but not to type V. The fibronectin interacted better with the denatured form of collagen type I (gelatin) than with the native form. At pH < 5.5 the fibronectin exhibited much lower affinity to gelatin than at pH > 8.0. The interaction between the fibronectin and gelatin was further analysed by affinity electrophoresis in which apparent dissociation constants (K_d) of the fibronectin for gelatin were calculated, and effects of urea, 2-mercaptoethanol and temperature on the interaction were examined. The fibronectin markedly diminished its affinity to gelatin at 3 *M* urea to give $K_d = 2.5 \cdot 10^{-6}$ *M*, which was 1000 times larger than the value without urea. The fibronectin dissociated into its monomers and the monomers diminished their affinity to gelatin in a stepwise fashion with increase in concentration of 2-mercaptoethanol. The fibronectin diminished the affinity to gelatin by elevating temperature, and van't Hoff plots of log K_d values against the reciprocal of absolute temperature (T) showed that log K_d was inversely proportional to $1/T$ in the range 15–50°C, and the thermodynamic parameters of the standard enthalpy change, the standard free energy change and the entropy change at 37°C for association of fibronectin and gelatin were all negative. At 60°C the affinity of fibronectin to gelatin was not detectable. These results suggest that a hydrophilic interaction, such as hydrogen bonding or van der Waals interaction, plays an important role in the binding of fibronectin and gelatin, and conformational changes in the fibronectin molecule may occur owing to the stepwise reduction of inter- and intra-chain disulphide bonds or to the heat of denaturation at 60°C.

INTRODUCTION

Human plasma fibronectin is a high-molecular-mass glycoprotein ($450 \cdot 10^3$) consisting of a disulphide bonded dimer which has multiple domains playing different roles [1]. One domain binds to different types of collagen, and it binds better to denatured collagen (gelatin) than native collagen [2]. One of the biological functions of fibronectin binding to gelatin is to enhance blood clotting, wound healing and phagocytosis, which are mediated by reticuloendothelial or macrophage clearance of collagen debris as well as that of fibrin microaggregates, and other bacterial and non-bacterial particles [3]. The interaction between the fibronectin and gelatin has been mainly analysed by affinity chro-

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matography [4] and enzyme-linked immunosorbent assay [5], but the nature of the interaction has been a matter of controversy [6,7].

In this paper we present the results obtained by detailed analyses of the interaction between the fibronectin and different types and forms of collagen by means of affinity electrophoresis, which was developed for analyses on biospecific interactions of enzyme with substrates, lectin with carbohydrates, antibody with antigen and fibronectin with gelatin [8–11], by calculation of dissociation constants from the decrease in the mobility of the protein as a function of the concentration of the ligand immobilized in the gel matrices. We also examined the effects of pH, urea, 2-mercaptoethanol and temperature on the dissociation constants of fibronectin for gelatin to elucidate the nature of the interaction between fibronectin and gelatin.

EXPERIMENTAL

Human plasma fibronectin

Human plasma and serum were obtained from a healthy adult male and stored at −20°C until use. The concentrations of fibronectin in plasma and serum were determined to be 361 and 273 mg/l, respectively, by an immonodiffusion method using LCP Partigen fibronectin (Behring).

Affinity electrophoresis

Affinity electrophoresis was carried out with 4.11%T, 2.6%C[a] polyacrylamide gel containing gelatin from bovine bone (EIA grade, Bio-Rad Labs.) at a desired concentration for separation and 3.12%T, 5.0%C polyacrylamide gel without gelatin for stacking in a discontinuous buffer system as described by Davis [12] and Ornstein [13]. Electrophoresis was run at 200 V and the temperature during electrophoresis was maintained with an accuracy of ±0.1°C by a thermostated apparatus [14].

Samples were prepared by dilution of human serum with 20 m*M* Tris–HCl buffer (pH 6.7) containing 10% sucrose and 2.5% carrier ampholyte (Ampholine, pH 5–7; Pharmacia) to yield a final concentration of 10 μg/ml of fibronectin. After electrophoresis, fibronectin was directly transferred to a nitrocellulose membrane (Millipore, 0.2 μm) by an electroblotting in 50 m*M* glycine–HCl buffer (pH 2.5). The fibronectin was stained by an immunostaining method using sheep anti-human plasma fibronectin antibody (The Binding Site) as first antibody and peroxidase-conjugated swine anti-sheep IgG (The Binding Site) as second antibody.

Detection was carried out by incubation of the membrane with a peroxidase staining solution consisting of 4-chloro-1-naphthol (60 mg) dissolved in a mixture of 20 ml cold methanol with 100 ml of Tris–HCl buffer (pH 6.7) containing 60 μl of 30% hydrogen peroxide.

The apparent dissociation constant (K_d) for the interaction between the fibronectin and gelatin was calculated from an affinity plot in which the reciprocal of the relative migration distance of fibronectin was plotted against the concentration of gelatin in the separating gel [10]. The K_d values were expressed as the molar concentration of gelatin by dividing the gelatin concentration (g/l) by the molecular mass of collagen monomer, based on the fact that fibronectin has a single binding site per collagen monomer which is located primarily within the residues 757–791 in the α 1 (I) chain [15].

Affinity titration curve

Affinity titration of human plasma fibronectin was performed as described by Ek *et al.* [16]. Electrophoresis was carried out with polyacrylamide gels (5 × 5 × 0.1 cm, 4.11%T, 2.6%C) containing 3 *M* urea, 10% glycerol, 2.4% carrier ampholyte (Sepaline, pH 3.5–10; Fuji Film) and an affinity ligand of collagen from human placenta type I, III, IV or V or gelatin. Isoelectric focusing for the first dimension was run at 6.4 W for 30 min with 1 *M* NaOH and 1 *M* H_3PO_4, as electrode solutions for the cathode and anode, respectively. After the focusing, the gel was turned through 90°, and the trench was filled with 10 μl of serum sample containing 1 μg of fibronectin, 10% glycerol and 2.4% Sepaline, pH 3.5–10. Affinity electrophoresis for the second dimension was run at 600 V for 10 min at 10°C. The fibronectin was then transferred to a nitrocellulose membrane and was detected by immunostaining method as described under *Affinity electrophoresis*.

[a] C = g N,N'-methylenebisacrylamide (Bis)/%T; T = g acrylamide + g Bis per 100 ml of solution.

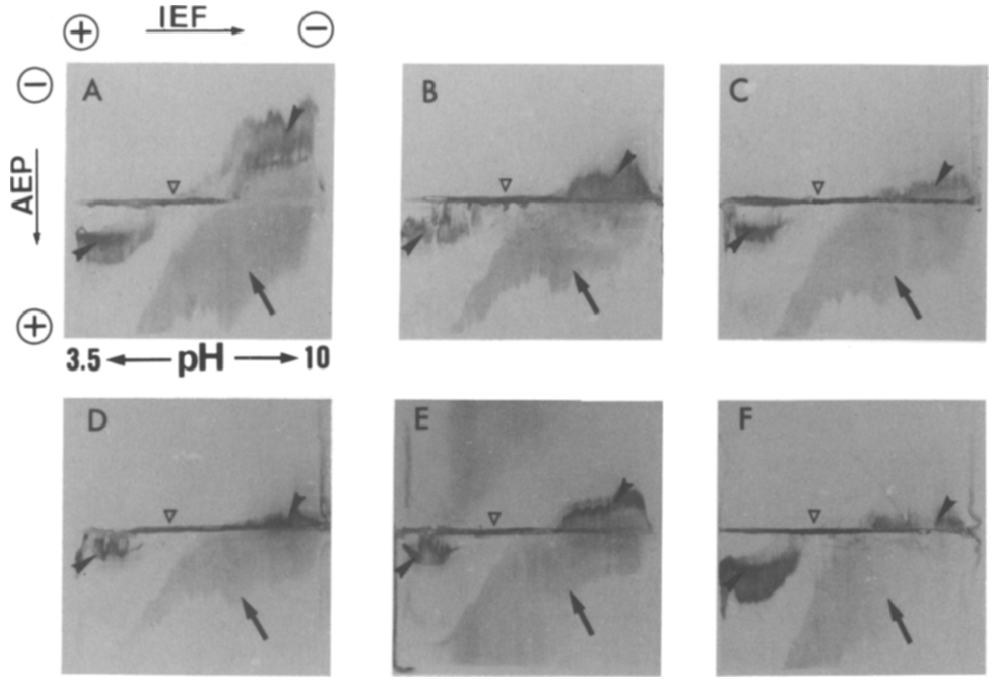

Fig. 1. Affinity titration curve of human plasma fibronectin. Electrophoresis was carried out in 4% polyacrylamide gel (A) in the absence and in the presence of 0.01% of collagen (B) type I, (C) type III, (D) type IV, (E) type V and (F) gelatin as described under *Affinity titration curve*. The arrow head indicates the pattern of fibronectin. The lightly stained broad pattern indicated with an arrow corresponds to polyclonal human immunoglobulin G which did not change its mobility in the presence of the ligands.

RESULTS

The interaction of human plasma fibronectin with different types of collagen and gelatin at different pH values was analysed by means of the affinity titration curve with 0.01% of collagen and gelatin as shown in Fig. 1. The fibronectin strongly interacted with collagen type I (Fig. 1B), III (C) and IV (D) at pH >8.0, as indicated by the marked retardation of the mobility of fibronectin as compared with the pattern of fibronectin without collagen (Fig. 1A). The fibronectin interacted with collagen type V at pH >8.0, but the retardation in the migration was significantly smaller than that with collagen type I (Fig. 1E). The fibronectin interacted better with gelatin (Fig. 1F) than with native form of type I, as indicated by the stronger retardation of the mobility of fibronectin at pH >8.0. In contrast, the fibronectin exhibited much lower affinity to gelatin and the collagen at pH <5.5, as indicated by slight retardation of the mobility of fibronectin.

The presence of urea in the gel deminished the affinity of fibronectin to gelatin in a dose-dependent manner, and the k_d values at 37°C were $1.49 \cdot 10^{-7}$, $2.50 \cdot 10^{-6}$ and $3.58 \cdot 10^{-6}$ M at urea concentrations of 2, 3 and 4 M, respectively (Fig. 2).

The reduction with 2-mercaptoethanol dissociated the fibronectin into monomers having a lower affinity to gelatin, and the monomers diminished the affinity in a stepwise fashion with increase in 2-mercaptoethanol concentration, and finally the monomers lost the affinity to gelatin at 500 mM 2-mercaptoethanol (Fig. 3). The monomers showed microheterogeneity consisting of several spots which had different isoelectric points and different affinities to gelatin at 100 mM 2-mercaptoethanol (Fig. 4).

The fibronectin diminished the affinity to gelatin with increase in temperature, and the affinity was completely lost at 60°C. The Van 't Hoff plots of log K_d against the reciprocal of absolute temperature (T) indicated that log K_d was inversely proportional to $1/T$ in the temperature range 15–50°C, as shown in Fig. 5. The standard enthalpy change (ΔH^0), the

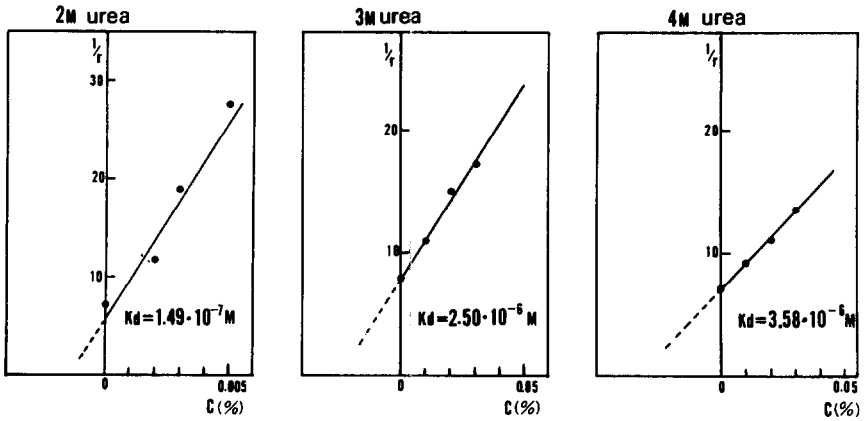

Fig. 2. Effect of urea on the dissociation constant (K_d) for fibronectin and gelatin. Ordinate: reciprocal of relative migration distance of fibronectin ($1/r$). Abscissa: concentration of gelatin (%) in the polyacrylamide gel (C).

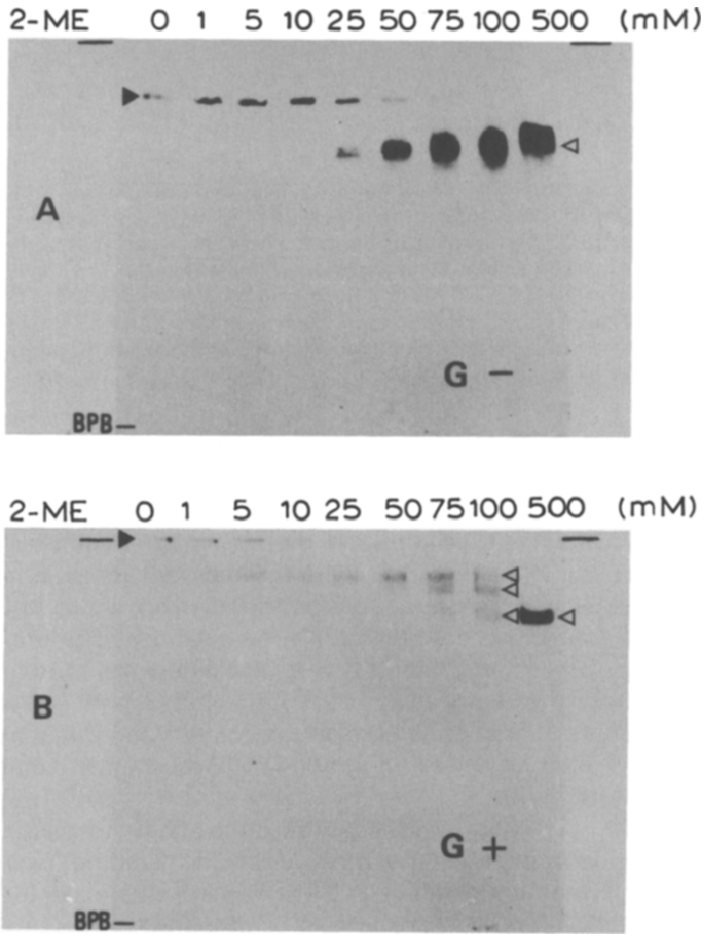

Fig. 3. Reduction of fibronectin with 2-mercaptoethanol. Dissociation of fibronectin into monomers and stepwise reduction in the affinity of fibronectin to gelatin. The fibronectin was reduced with 2-mercaptoethanol for 30 min at 37°C prior to electropphoresis in the gel containing 3 *M* urea (A) in the absence and (B) in the presence of 0.12% gelatin. The numbers above each lane indicate the concentration of 2-mercaptoethanol (m*M*). ▼ = Dimeric form of fibronectin; ▽ = monomeric form of fibronectin.

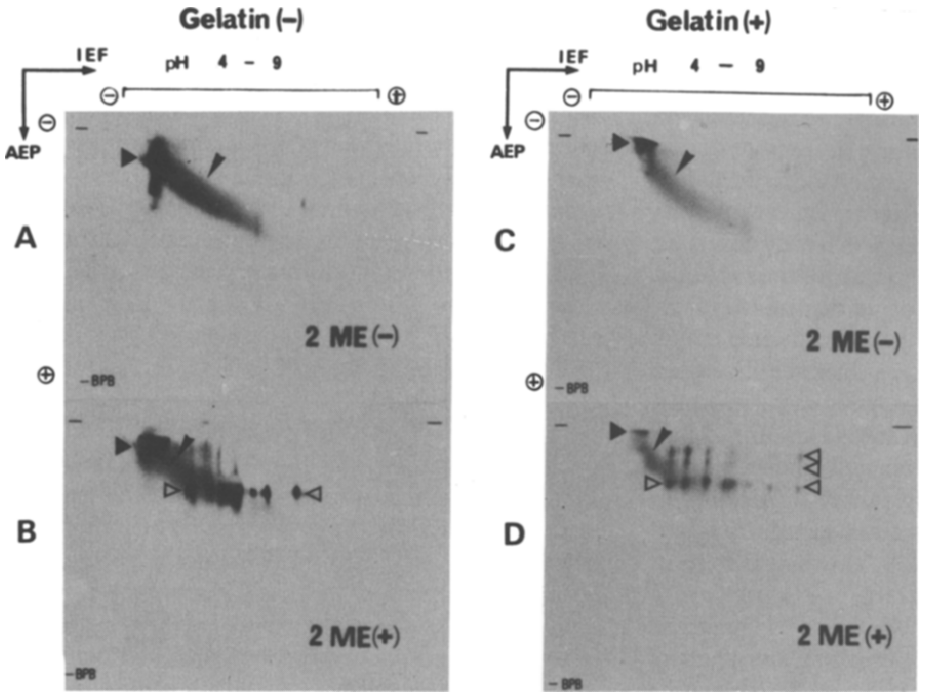

Fig. 4. Two-dimensional affinity electrophoresis of fibronectin. A 4-μl volume of human serum was incubated with an equal volume of 10 m*M* phosphate buffer (pH 7.4) containing 200 m*M* 2-mercaptoethanol (2-ME) for 30 min at 37°C for application to electrophoresis. Isoelectric focusing was carried out in the first dimension with 5% polyacrylamide gel containing 2.5% Pharmalyte, pH 4–9, 10% glycerol, 0.21% lysine and 6 *M* urea using electrode solutions of 0.1 *M* NaOH for the cathode and 0.1 *M* H_3PO_4 for the anode at 250 V for 15 min, 500 V for 15 min, 1000 V for 15 min and 2000 V for 2 h. Affinity electrophoresis was carried out in the second dimension with 4% polyacrylamide gel containing 3 *M* urea in the absence (A, without 2-ME; B, with ME) and the presence (C, without 2-ME; D, with ME) of 0.05% gelatin at 200 V until bromophenol blue marker dye reached the bottom of the separating gel. The fibronectin was stained by the immunostaining method as described under *Affinity electrophoresis*. ▼ = Dimeric form of fibronectin; ▽ = monomeric form of fibronectin; arrow head = human immunoglobulin G.

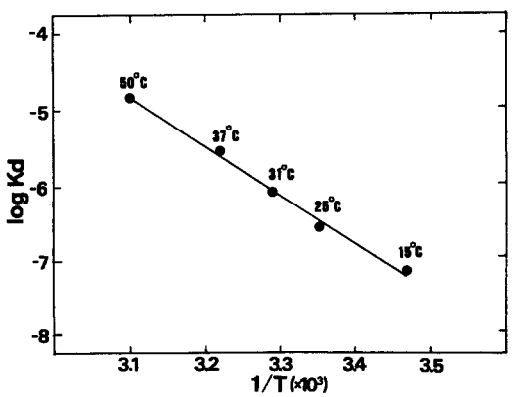

Fig. 5. Van 't Hoff plots of log K_d calculated by affinity electrophoresis at different temperatures against the reciprocal of absolute temperature ($1/T$).

standard free energy change (ΔG^0) and the entropy change (ΔS^0) for the association of the fibronectin and gelatin were calculated from the following equations:

$$K_a = 1/K_d$$
$$\Delta H^0 = [2.303RT_1T_2/(T_2 - T_1)]\log(K_{a_2}/K_{a_1})$$
$$\Delta G^0 = -2.303RT\log K_a$$
$$\Delta S^0 = (\Delta H^0 - \Delta G^0)/T$$

where K_a is the association constant and R the gas constant (8.3166 J/K · mol). ΔH^0 was calculated to be −124 kJ/mol and ΔG^0 and ΔS^0 at 37°C were −33.23 kJ/mol and −295.1 J/mol · K, respectively.

DISCUSSION

Plasma fibronectin interacts with different types and forms of collagen [5]. It has been reported that interstitial collagens, types I and III, interact better with the fibronectin than does the basement membrane collagen, type IV. However, our results indicate that the fibronectin interacts with type IV as strongly as with types I and III at pH >8.0. This type of interaction is pH dependent and much weakened at pH <5.5. On the other hand, it has been reported that the fibronectin interacs better with the denatured form of type I (gelatin) than with the native form. However, the nature of the interaction between the fibronectin and gelatin is still a matter of controversy [6,7,17]. Our results show that the fibronectin bound more tightly to gelatin than to type I at pH >8.0, but the interaction was much weakened at pH <5.5.

The interaction was also inhibited by low concentrations of urea, which did not bring about gross conformational changes of fibronectin. This suggests a small contribution of a hydrophobic interaction to the binding, and the protonation of the imidazole group in the histidine residue of fibronectin [18] may be unfavourable for the binding to the collagens.

The reduction with 2-mercaptoethanol dissociated the fibronectin into monomers. The monomers diminished the affinity to gelatin in a stepwise fashion with increase in 2-mercaptoethanol concentration, and finally the monomers lost the affinity at 500 mM of 2-mercaptoethanol. This suggests that conformational changes in the fibronectin molecule occur owing to the stepwise reduction of inter- and intra-chain disulphide bonds in the dimeric form of fibronectin.

The examination of the effect of temperature on the interaction between fibronectin and gelatin showed that the thermodynamic parameters ΔH^0, ΔG^0 and ΔS^0 calculated from the van 't Hoff plots had negative values for the association of fibronectin and gelatin. This suggests that a hydrophilic interaction, such as hydrogen bonding or Van der Waals interaction, plays an important role in the interaction [19] between fibronectin and gelatin.

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