Interaction of Gelatin with a Fluorescein-labeled 42-kDa Chymotryptic Fragment of Fibronectin*

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A 42-kDa gelatin binding fragment of human plasma fibronectin was labeled with fluorescein and its fluid-phase interaction with gelatin was investigated. At 25 °C in 0.1 M Tris, 0.15 M NaCl, pH 7.3, a dissociation constant, \( K_d = 0.6 \mu M \), was obtained from the dependence of fluorescence polarization on gelatin concentration. An identical value was obtained for the unlabeled fragment by competition. Binding was unaffected by higher concentrations of NaCl up to 1.0 M, but increased as much as 20-fold at low ionic strength. The dependence of \( K_d \) on temperature revealed that dissociation of the complex is accompanied by an increase in entropy. Thus, the interaction is not dominated by either hydrophobic or electrostatic forces; an important role for hydrogen binding is proposed.

Fibronectin is a large glycoprotein found in plasma and on the surface of numerous cell types (see Refs. 1–6 for reviews). It is comprised of two similar chains which are disulfide-linked near the C termini. Fibronectin interacts with a variety of macromolecules including several constituents of the extracellular matrix where it mediates the adhesion and spreading of cells. Work in many laboratories has demonstrated that treatment with selected proteolytic enzymes produces different fragments which retain affinity for specific macromolecules. In addition to reinforcing the concept of multiple independent domains held together by connecting strands, this approach has provided information about the linear arrangement of domains within the polypeptide chains.

A rigorous understanding of the structure and function of fibronectin will require quantitative knowledge of its various binding properties. Of the numerous interactions which have been reported, that with collagen has received the most attention. Of particular interest is the observation that denatured collagen (gelatin) exhibits a stronger interaction with fibronectin than do native collagens, suggesting that the active regions of the latter are partially masked in the native fibrillar forms (7-11). Studies with purified collagen chains and cyanogen bromide fragments thereof suggest multiple gelatin binding sites within the collagen molecule, although a specific region near the collagenase-sensitive site of the \( \alpha_1 \) chain appears to dominate (9, 12, 13). Most of the published studies have utilized methods in which various soluble species are examined for their ability to inhibit the binding of fibronectin to surfaces coated with gelatin or collagen. We have taken the approach of using fluorescent labels to examine the binding under conditions where both species are in the fluid phase (14). In this report, we examine the interaction between gelatin and a fluorescein-labeled 42-kDa fragment obtained by chymotryptic digestion of purified fibronectin. Fluorescence polarization was used to monitor the interaction.

**EXPERIMENTAL PROCEDURES**

**Materials**—Fibronectin was isolated from human plasma or cryoprecipitate by affinity chromatography on gelatin-Sepharose according to method B of Miekka et al. (15). Bovine pancreatic \( \alpha \)-chymotrypsin and soybean trypsin inhibitor were purchased from Calbiochem-Behring. Phenylmethylsulfonyl fluoride and Celite-bound fluorescein isothiocyanate were made from Sigma. Swine skin Type I gelatin (300 Bloom) was also obtained from Sigma. Analysis of reduced samples by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate confirmed the dominance of \( \alpha \) chains with lesser amounts of \( \beta \) and \( \gamma \) components and little evidence of low molecular weight degradation products.

**Preparation of the 42-kDa Gelatin Binding Fragment**—The 42-kDa gelatin binding fragment of fibronectin was prepared by a modification of the method of Hahn and Yamada (16). Purified fibronectin (400 mg) was bound to a column of gelatin-Sepharose (100 ml) in 0.1 M Tris, 0.15 M NaCl, 0.02% azide, pH 7.3, hereafter referred to as Tris-buffered saline. About 90 ml of a solution of \( \alpha \)-chymotrypsin (0.12 mg/ml) in TBS was passed through the column at a rate of 1.5 ml/min followed by an equal volume of soybean trypsin inhibitor containing 40 \( \mu \)M phenylmethylsulfonyl fluoride in TBS. The column was then washed with 0.1 M Tris containing 0.4 M NaCl, 40 \( \mu \)M phenylmethylsulfonyl fluoride, and 0.02% azide at pH 7.3 until the A	extsubscript{280} reached baseline values. Bound protein was eluted with 6 M urea in TBS, concentrated in an Amicon stirred cell with a PM-10 membrane, and chromatographed on an LKB Ultrogel ACA44 exclusion column (1 x 58 cm) equilibrated with TBS. The protein eluted in a single peak with \( K_w = 0.35 \). Fractions were pooled and used without further processing. Gel electrophoresis of reduced fragment in 6.5% polyacrylamide containing 0.10% sodium dodecyl sulfate revealed a single band which migrated slightly behind ovalbumin, with an apparent mass of 42 kDa in agreement with Hahn and Yamada (16).

**Labeling with Fluorescein**—The fragment, at a concentration of 1.5 mg/ml, was incubated for various times in a suspension containing 7.5 mg/ml Celite-FITC in 0.05 M NaHCO\(_3\) at pH 8.5 and 25 °C. Excess reagent was removed by ultrafiltration followed by chromatography on a Bio-Gel P-2 column. The degree of labeling was determined optically as previously described (17). A value of \( A_{280} = 11.6 \) was used for the fragment (18). Both the initial polarization (\( P \)) and the change in \( P \) upon titration with gelatin, but not the \( K_d \), decreased with an increasing degree of labeling. All of the data presented herein were obtained with preparations having \( <1 \) mol of dye per mol of fragment. Preparations having a higher degree of labeling would still bind to gelatin-Sepharose but were not as useful.

*This work was supported by National Institutes of Health Grant HL-21791. This represents Publication 650 from the American Red Cross Biomedical Research and Development Labs, Bethesda, MD 20814. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: TBS, Tris-buffered saline; 0.1 M Tris, 0.15 M NaCl, pH 7.3; Fn, fibronectin; FITC, fluorescein isothiocyanate.
for spectral measurements of the fluid-phase interaction because of the smaller polarization response.

**Polarization Measurements**—Measurements of fluorescence polarization (P) were made as previously described (14) with vertical excitation at 495 nm with an SLM 8000 fluorometer set in the “T” format, with OGS15 cut-off filters (SLM) in place to isolate the fluorescence emission. Corrections for background were made using samples equivalent in all respects to the sample in which P was being determined except for the absence of fluorophore. All measurements were made at 25 °C in TBS unless otherwise indicated. Titrations with gelatin were done by the addition of small volumes of concentrated stock solutions, whose concentrations were based on the weight of lyophilized material. Molar concentrations are expressed in terms of 100,000-Da α chain equivalents.

**Analysis of Data**—Titration data were analyzed according to the following equation for binding of gelatin to α equivalent and independent sites per fragment:

\[ \Delta P/[G] = \Delta P_{\text{max}}/[G]_0 - \Delta P/K_d \]

(1)

where \( \Delta P \), the observed change in polarization, is assumed to be proportional to the fractional saturation, approaching \( \Delta P_{\text{max}} \) at saturation, and \([G]_0\) is the concentration of free gelatin expressed in terms of α chain equivalents. In deriving Equation 1 from the familiar Scatchard equation (20), the parameter \( \tilde{r} \) = [bound gelatin]/[total fragment] was set equal to \( \Delta P/\Delta P_{\text{max}} \), such that \( \tilde{r} \) was eliminated. If the concentration of labeled fragment is low relative to that of gelatin, then the concentration of free gelatin approaches that of total gelatin \([G]_0\) and plots of \( \Delta P/[G]_0 \) versus \( \Delta P \) will be nearly linear with a limiting slope equal to \( K_d^{-1} \). Since the fluorescence intensity did not change by more than ±10% during the course of titration, no correction was made for differences in quantum yield between free and bound fragment. Linear best fits were obtained with a linear regression computer program using the method of least squares.

Competition between labeled and unlabeled fragments was analyzed by the method of Bock and Shore (21) using the following equation:

\[ \frac{[\text{Frag}]}{\Delta [G]} = K_d' \left( 1 - \alpha \right) + \frac{1}{n} \]

(2)

where \([\text{Frag}]\) is the total concentration of unlabeled fragment, \( K_d' \) is the dissociation constant for unlabeled fragment, \( \Delta [G] \) is the fractional saturation of labeled fragment, and \( K_d \) is the dissociation constant for unlabeled fragment, \( \alpha = \Delta P/\Delta P_{\text{max}} \) is the fractional saturation of labeled fragment, and \( \Delta [G]_0 \) is the difference in total gelatin concentration required to produce a given \( \alpha \) in the absence and presence of unlabeled fragment. The ratio of \( K_d' \) to \( K_d \) can be obtained from the slope of a plot of \([\text{Frag}]_0/\Delta [G] \), versus \( (1 - \alpha)/\alpha \).

The experimental mean harmonic rotational relaxation time, \( \rho_H \), was estimated from measurements of polarization as a function of temperature. The Perrin equation (22) for vertically polarized excitation is given by:

\[ \rho_H = \frac{3 \tau (1/P_0 - 1/3)}{(1/P - 1/P_0)} \]

(3)

where \( \tau \) is the fluorescence lifetime and \( P_0 \) is the limiting polarization in the absence of rotation. The observed polarization is dependent on viscosity, \( \eta \). \( P_0 \) can be obtained by extrapolation of a plot of \( 1/P \) versus \( T/\eta \) according to the modified Perrin equation (23). A value of 4.0 ns was taken for \( \tau \) (24–26). Theoretical values for hydrated spheres were estimated from the equation:

\[ \rho_0 = 3 M (\bar{c} + h) \]

(4)

\[ R \]

where \( M \) is the molecular weight, \( \bar{c} \) is the partial specific volume of the protein, \( h \) is the degree of hydration, and \( R \) is the gas constant (23). Since \( \bar{c} \) for proteins is usually around 0.72 ml/g and since proteins tend to be hydrated to the extent of 0.3–0.6 g of H_{2}O/g of protein, a lower limit of 1.0 ml/g was assumed for the quantity \( (\bar{c} + h) \) (27, 28).

**RESULTS**

Rechromatography on Gelatin-Sepharose: Inhibition by Fluid-phase Gelatin—Fig. 1A illustrates the binding of the labeled 42-kDa fragment to gelatin-Sepharose. All of the fluorescence bound to the column and could be eluted with 6 M urea (Fig. 1A) or by lowering the pH to 4 (not shown).

Thus, the labeling procedure did not diminish the ability of the fragment to interact with immobilized gelatin. Control experiments with free dye showed that it has no affinity for the column. When the labeled fragment was preincubated with excess gelatin, none of the fluorescent material bound to the column, indicating that fluid-phase gelatin competes with solid phase gelatin for binding to the fragment.

Exclusion Chromatography with and without Gelatin: Demonstration of a Soluble Complex—Further evidence for a fluid-phase interaction between gelatin and the labeled fragment comes from the exclusion chromatography results shown in Fig. 1B. In the absence of gelatin, the fragment eluted as a single peak with \( K_a = 0.35 \). The position of elution was slightly behind that of ovalbumin (\( M_0 = 45,000 \)) on the same column. When preincubated with excess gelatin, the labeled fragment eluted close to the void, providing unequivocal evidence for complex formation.

**Polarization Measurements**—Titration with Gelatin—Titration of labeled fragment with increasing amounts of gelatin caused a hyperbolic increase in P (Fig. 2A, circles) which, when plotted according to Equation 1, produced a straight line whose slope corresponds to \( K_a = 5.8 \times 10^{-7} \) M and whose x-intercept predicts \( \Delta P_{\text{max}} = 0.032 \) (Fig. 2B). The increase in P caused by gelatin could be reversed by dilution and also by addition of 4 M urea (not shown).
Addition of unlabeled fragment up to a concentration of 49.5 \mu M had no effect on \( P \) in the absence of gelatin, suggesting no tendency for the unlabeled fragment to associate with the labeled one under these conditions. It did, however, strongly inhibit the increase in \( P \) caused by the addition of gelatin. Titration data obtained in the presence of several concentrations of unlabeled fragment, when plotted according to Equation 2, gave a set of lines having essentially equivalent slopes and intercepts (Fig. 2C). From these, an average ratio of 4.95 was obtained, conclusively demonstrating that the labeling process does not significantly alter the binding ability of the fragment. From the intercept, a fractional value of \( n = 0.72 \pm 0.04 \) was obtained for the number of sites per fragment.

**Effects of Salt**—The interaction between the labeled fragment and gelatin was sensitive to the ionic composition of the medium. Dissociation constants obtained in phosphate and Tris buffers at various levels of NaCl are summarized in Table I. The strongest interaction was seen at low ionic strength in 0.01 M phosphate buffer where \( K_d = 3 \times 10^{-8} \) M. A 2-fold higher value was seen in 0.02 M Tris. Increasing NaCl concentration to about 0.15 M in either buffer produced about a 3-fold increase in \( K_d \), which remained nearly unchanged by higher salt concentrations up to 1 M. However, increasing the Tris concentration from 0.02 to 0.1 M in the presence of 0.15 M NaCl caused a further increase, suggesting that the interaction may also be influenced by specific ions.

**Effect of Temperature on Affinity**—The polarization of separate samples, each containing the same amount of labeled fragment but varying amounts of gelatin, was measured at five different temperatures in TBS. Plots of the data according to Equation 1 are given in Fig. 3A. The dissocation constant increased from 3.2 to \( 10^{-7} \) M at 16°C to \( 2.0 \times 10^{-6} \) M at 37°C. There was a tendency for \( \Delta P_{max} \) to increase slightly with decreasing temperature indicating that the fragment responds differently to temperature when free than when

**Table I**

<table>
<thead>
<tr>
<th>Buffer (pH 7.3)</th>
<th>NaCl (M)</th>
<th>Ionic strength</th>
<th>( K_d \times 10^8 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.02 M Tris</td>
<td>0.00</td>
<td>0.018</td>
<td>6.3</td>
</tr>
<tr>
<td>0.02 M Tris</td>
<td>0.05</td>
<td>0.068</td>
<td>12.0</td>
</tr>
<tr>
<td>0.02 M Tris</td>
<td>0.10</td>
<td>0.118</td>
<td>16.0</td>
</tr>
<tr>
<td>0.02 M Tris</td>
<td>0.15</td>
<td>0.168</td>
<td>16.0</td>
</tr>
<tr>
<td>0.02 M Tris</td>
<td>0.30</td>
<td>0.318</td>
<td>17.0</td>
</tr>
<tr>
<td>0.02 M Tris</td>
<td>1.00</td>
<td>1.018</td>
<td>17.0</td>
</tr>
<tr>
<td>0.05 M Tris</td>
<td>0.15</td>
<td>0.156</td>
<td>24.0</td>
</tr>
<tr>
<td>0.10 M Tris</td>
<td>0.15 (TBS)</td>
<td>0.240</td>
<td>61.0 (± 3.4)</td>
</tr>
<tr>
<td>0.01 M PO4</td>
<td>0.00</td>
<td>0.021</td>
<td>3.0</td>
</tr>
<tr>
<td>0.075 M PO4</td>
<td>0.00</td>
<td>0.148</td>
<td>9.0</td>
</tr>
<tr>
<td>0.010 M PO4</td>
<td>0.15</td>
<td>0.171</td>
<td>9.0</td>
</tr>
</tbody>
</table>

* Average of 3 titrations.
interacted with gelatin. Dissociation constants are plotted in Fig. 3B according to the van't Hoff equation. The slope of the best fit straight line corresponds to $\Delta H = 16 \pm 1 \text{ kcal/mol}$. Rotational Relaxation Analysis—The effect of temperature on $P$ for labeled fragment and its complex with gelatin is plotted in Fig. 4 according to the Perrin equation. The data obtained in the absence of gelatin are linear over the temperature range investigated, with no evidence of a structural transition. Combining the value of $P_0 = 0.24$, obtained by extrapolation of the open circles to the ordinate axis, with a fluorescence lifetime of 4 ns (24-26), yields a value of $\mu H = 29$ ns for the fragment (Equation 3). This is significantly lower than the value of 43 ns estimated for an equivalent hydrated sphere using Equation 4 with $\psi = 1.0 \text{ mol/liter}$; the discrepancy would be greater if a greater degree of hydration were assumed. The data in the presence of gelatin exhibit curvature which most likely reflects progressive dissociation of the complex with increasing temperature, in accord with the previous section. Assuming that the limiting slope at lower temperatures extrapolates to the same $P$, as for the fragment alone, a value of $\mu H = 45$ ns for the complex at 25 °C was estimated. While this is larger than the value for free fragment, it is substantially lower than the value of 148 ns calculated for an equivalent hydrated sphere having a minimum molecular weight of 142,000 (fragment plus $\alpha$ chain). Thus, the fragment retains a fair amount of rotational freedom upon complexing with gelatin chains, consistent with the flexible nature of the latter.

**Discussion**

We have previously utilized the fluorescence polarization of fluorescein-labeled gelatin to detect and quantitate Fn in solution (14). The results presented here characterize the fluid-phase interaction between gelatin and the isolated 42-kDa gelatin-binding fragment of Fn using a similar method with the fluorophore attached to the fragment. The binding is reversible and saturable, most likely with one site per fragment. Although the competition data of Fig. 2C gave a stoichiometry of 0.72 $\alpha$ chains per mol of fragment, the deviation from unity may reflect uncertainty in the extinction coefficient of the fragment. The corresponding fragment in the bovine protein contains 9 Trp, 20 Tyr, and 12 cystines (29) from which an $A_{280}$ of 18 can be calculated (30). A similar value for human Fn would raise our estimate of the stoichiometry to 0.96. In any case, the binding appears to be homogeneous with no indication of multiple sites of different affinity which would cause curvature in the Scatchard-type plots (Fig. 2B).

The fluorescein label, which attaches preferentially to primary amino groups (25), did not perturb the binding properties of the fragment. There are conflicting reports in the literature regarding the effect of lysine modifications on the gelatin-binding function of whole Fn (31-33). These studies probably involved more extensive modification than that produced by us. The preparations utilized here had been labeled with $\leq 1$ mol of fluorescein per mol of fragment in order to enhance the polarization response to added gelatin and could have been preferentially labeled at the N-terminal amino group (25). However, preparations having as many as 2.5 mol/mol of the bulky negatively charged label still bound quantitatively to gelatin-agarose suggesting that, if lysines are required for the interaction, the relevant ones are not particularly reactive toward FITC.

There are reports that binding of Fn to solid phase gelatin is sensitive to NaCl (31-34). Most of the effects are observed at subphysiological ionic strength. Since these two proteins have opposite net charge at neutral pH, one might expect a strengthening of their interaction at low ionic strength. This is illustrated quantitatively by the present study which reveals about a 3-fold increase in the dissociation constant of the 42-kDa fragment when NaCl is raised to physiological levels in the presence of 0.01 M phosphate or 0.02 M Tris. However, since Fn-loaded columns of gelatin-agarose can be washed with 1 M NaCl with little or no loss of Fn (15, 35-38) and since little change in $K_d$ of the fragment is caused by raising the ionic strength from physiological up to 1.0 M (Table I), the primary interaction between gelatin and the relevant domain of Fn cannot be electrostatic in nature. The further increase in $K_d$ caused by additional Tris in the presence of 0.15 M NaCl is consistent with the unique sensitivity of the Fn-gelatin interaction to amino compounds including Tris (36). The value of $K_d$ observed in TBS is in good agreement with the results of McDonald and Kelly (39) showing 50% inhibition of binding of labeled Fn to solid phase gelatin under similar conditions by similar fragments at concentrations near 1 $\mu$M. It is also identical with the value obtained when the labeled fragment interacts with a high molecular weight fraction of fluorescein-labeled gelatin but 1000-fold weaker than the interaction of that same gelatin with intact Fn (14). The stronger binding of Fn as compared to the fragment may be related to the bivalent nature of the intact protein whose two gelatin binding domains could bind simultaneously to separate sites on a gelatin molecule. Alternatively, the affinity of the fragment may be attenuated by the process of removing it from the parent protein. However, the value obtained in phosphate-buffered saline (bottom line, Table I) is very close to the value of $1.6 \times 10^{-7}$ M obtained under nearly identical conditions for a larger chymotryptic fragment, FITC-60 kDa (18).

Evidence for a hydrophobic contribution to the interaction between Fn and gelatin is provided by the fact that it can be disrupted by a variety of chaotropic agents including urea, ethylene glycol, thiocyanate, heavy halides, and amionic detergents (3, 7, 31, 33, 37, 38, and this work). Nonionic detergents appear to be less effective, an observation which led Vuento et al. (31) to suggest that "hydrophobic interactions contribute very little to the binding." These authors seem to prefer an electrostatic mechanism in spite of their own observation that differential binding persists in 1 M NaCl. Our equilibrium measurements reveal that interaction of the 42-kDa fragment with gelatin is characterized by a free energy of dissociation, $\Delta G^\circ = -RT \ln K_d = +8.1 \text{ kcal/mol in TBS at 37 } ^\circ \text{C}$. Combining this with the observed enthalpy of $+16$ kcal/mol leads to a value of $+8$ kcal/mol for $T\Delta S$ at the same temperature. Thus, dissociation is accompanied by an increase in entropy contrary to what is expected for a hydrophobic interaction (40). Much of this increase can be attributed to the greater translational and rotational freedom of the separated components (41). The fact that Fn (15) as well as the 42-kDa fragment (this work) can be eluted by simply lowering the pH in the absence of chaotropes raises further doubts about the predominance of hydrophobic forces and, given the limited electrostatic effects, suggests an important role for hydrogen bonding.

Measurements of polarization as a function of temperature produced an estimate of the rotational relaxation time for the labeled fragment which was substantially lower than that expected for an equivalent hydrated sphere of the same molecular weight. The opposite result is more commonly obtained with globular proteins because of deviations from spherical shape (42). Indeed, such behavior might be expected for Fn fragments based on sedimentation analysis and their
these subdomains are not strongly associated.

REFERENCES