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Evidence That the Two Amino Termini of Plasma Fibronectin Are in Close Proximity: A Fluorescence Energy Transfer Study†

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ABSTRACT: A fluorescence energy transfer technique has been used to study the intramolecular distance between the two amino termini of human plasma fibronectin. The glutamine-3 residue near the amino terminus of each chain was labeled enzymatically with either monodansylcadaverine or monofluoresceinylcadaverine by use of coagulation factor XIIIa. The nonradiative fluorescence energy transfer between the dansyl (donor) and fluorescein (acceptor) pair in the same protein molecule was determined from steady-state fluorescence measurements. On the basis of the transfer efficiency of 78%, the intramolecular distance between two glutamine-3 residues of fibronectin was estimated to be approximately 23 Å, suggesting that the two amino termini of plasma fibronectin are in close proximity. High salt, which affects the hydrodynamic properties of the protein, has no effect on the measured distance. The results support the contention that both compact (in low salt) and expanded (in high salt) conformers of fibronectin are relatively spherical in shape.

Plasma fibronectin (Fn) is a glycoprotein, consisting of two subunits of about 250 kDa each which are joined at their carboxyl termini by two disulfides [for reviews, see McDonagh (1985) and Yamada (1983)]. The protein is known to participate in blood coagulation, cell adhesion, differentiation, and embryonic development.

The solution structure of this multifunctional, multidomainal

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protein, however, is not yet known. It is generally thought that Fn exists in two interchangeable forms, namely, compact and extended. Conversion of the protein from the compact form to the extended form can be readily achieved by high ionic strength or pH as demonstrated by sedimentation velocity and light scattering studies (Alexander et al., 1979; Erickson & Carrell, 1983; Lai et al., 1984; Williams et al., 1982), although the changes in solution structure induced by high salt or pH are not well characterized.

A fluorescence energy transfer technique has been shown to be a sensitive method for measurement of intramolecular distances (Lakowicz, 1983). By using this approach, we demonstrate in this paper that the two amino termini of plasma Fn are juxtaposed and separated only by 23 Å under physiological conditions.

Materials and Methods

Fn was isolated from human plasma on a gelatin–Sepharose 4B affinity column (Engvall & Ruoslahti, 1977). The purity of the protein preparations was examined routinely with 5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Fluoresceinylcadaverine (F) and dansylcadaverine (D) were from Molecular Probes (Junction City, OR). Thrombin and trypsin were purchased from Sigma (St. Louis, MO), and coagulation factor XIII was obtained from Behring Diagnostics (San Diego, CA). Guanidine hydrochloride (Gdn·HCl) was obtained from Pierce Chemical Co. (Rockford, IL).

Labeling of Plasma Fibronectin. Labeling of Fn with the fluorescent probes was carried out with thrombin-activated coagulation factor XIIIa as described by Mosher et al. (1980) with minor modifications. Fn (2 mg/mL) was incubated with D or F probe (0.5 mM) in the presence of factor XIIIa at 37 °C for 6 h. The reaction mixture was applied to a gelatin–Sepharose 4B column and washed with 0.02 M tris(hydroxymethyl)aminomethane (Tris) containing 0.15 M NaCl, pH 7.4, to remove unreacted probes and other proteins. The labeled protein retained on the column was eluted with 3 M urea and then dialyzed against a buffer containing 0.02 M Tris–0.15 M NaCl, pH 7.4. The intactness of the labeled protein was examined by gel electrophoresis; the presence of thrombin during the labeling procedure did not degrade Fn. The double-labeled Fn was prepared by mixing equimolar amounts of D and F probes (0.25 mM each) in the same reaction vessel. The double-labeled protein was isolated as described above for the single-labeled proteins.

Estimation of the Bound Probes. The amount of the probes incorporated into the protein was estimated by measuring the ratio of absorbances on a Perkin-Elmer UV–visible spectrophotometer. An extinction coefficient of 1.28 mg mL^{-1} cm^{-1} at 280 nm (Mosesson et al., 1975) was used for Fn, and molar extinction coefficients of 5.7×10^4 M^{-1} cm^{-1} at 494 nm for F probe and 3.4×10^3 M^{-1} cm^{-1} at 340 nm for D probe (Chen, 1968) were used.

Fluorescence Measurements. Steady-state fluorescence measurements were made on an SLM 8000 spectrofluorometer, equipped with a Lauda RC3 water bath for control of the sample compartment temperature as described previously (Lai et al., 1987). The sample was excited at 345 nm, and emission was recorded from 460 to 600 nm. Unless stated otherwise, the protein (0.3 mg/mL) was dissolved in 0.02 M Tris–0.15 M NaCl, pH 7.4. For the double-labeled protein, the concentration of the bound probes was about 0.35 μM for each probe during measurement. The bandwidth was 4 nm. To obviate inner-filter effects, the absorbance at 345 nm was kept at 0.03 or below. The relative quantum yield of D probe labeled Fn was determined to be 0.06 with quinine sulfate as a standard having a known quantum yield of 0.7 (Scott et al., 1970) by use of eq 1, where ϕ is the quantum yield, A is the

$$\frac{\phi[\text{protein}]}{\phi[\text{standard}]} = \frac{A(\text{protein})}{A(\text{standard})} \frac{\text{Abs(standard)}}{\text{Abs(protein)}} \quad (1)$$

area under the emission spectra, and Abs is the absorbance of the solution at the excitation wavelength (345 nm).

Ultracentrifuge Measurements. A Beckman Model E analytical ultracentrifuge was used with single-sector cells to determine sedimentation coefficients.

Energy-Transfer Measurements. Energy-transfer efficiency of the donor in the presence of the acceptor was followed by the acceptor enhancement method. The observed transfer efficiency (E_{obsd}) was calculated from eq 2, where F_{DA} is the

$$E_{obsd} = \frac{F_{DA}}{F_D + F_A} - 1 \quad (2)$$

fluorescence of the double-labeled sample and $F_D + F_A$ is the fluorescence of the mixture of the single-labeled proteins. The apparent transfer efficiency from eq 2 was corrected for the donor labeling stoichiometry with eq 3 (Fairclough & Cantor, 1978), where E_c and f_D are the corrected transfer efficiency and the fraction of the acceptor molecules having a donor molecule on the other chain, respectively. f_D was estimated to be 0.45 in this study. Data presented in this study are averaged values of two or more experiments with an error of about ±5%.

$$E_c = E_{obsd}/f_D \quad (3)$$

Results and Discussion

Characterization of the System. When D and/or F probes were covalently introduced into the Fn molecule in the presence of factor XIIIa, a stoichiometry of 1.8 to 1 (probe to protein) was reproducibly obtained [Mosher et al. (1980) and references cited herein]. No difference in the extent of incorporation between D and F probes was found. Trypsin digestion showed that the bound probes were solely associated with the amino-terminal 29-kDa domains (data not shown). Since glutamine-3 residues at the amino termini of Fn are known to serve as transglutaminase sites (McDonagh et al., 1981), it is probable that we have labeled these sites selectively with the cadaverine derivatives of the fluorescent probes.

Binding of the probes to the protein results in changes in their emission maxima; the emission maximum for F probe bound to Fn is 514 nm compared to 511 nm for F probe alone in solution, and the emission maximum for D probe bound to Fn is 508 nm compared to 515 nm for the free D probe. The observed red shift for F probe and blue shift for D probe are indicative of the bound probes being in a more hydrophobic environment (Lakowicz, 1983).

Energy-Transfer Measurements. The emission spectra of the double-labeled Fn and a mixture of two single-labled Fn (F-Fn and D-Fn) are shown in Figure 1A. It is noted that the emission intensity of the double-labeled Fn (Figure 1A, solid line) was 35% greater than that of the single-labeled Fns (Figure 1A, dotted line). Since the concentrations of the protein and of the probes in both samples were identical, the increase in emission intensity may be attributed to the energy transfer from D (donor) to F (acceptor) in the double-labeled samples. Addition of trypsin, which is known to fragment plasma Fn (McDonagh, 1985), reduces the emission intensity of the double-labeled Fn (Figure 1B, solid line) to the same level as that of the single-labeled Fn (Figure 1B, dotted line), suggesting that trypsin digestion results in releasing peptides

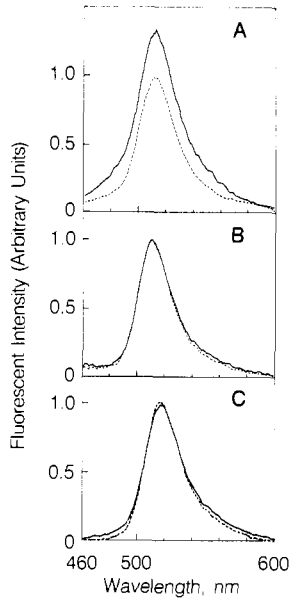

FIGURE 1: Fluorescence emission spectra of double-labeled Fn (solid line) and a mixture of F-Fn and D-Fn (dotted line): (A) in the absence of either trypsin or Gdn·HCl; (B) in the presence of 0.5% trypsin; (C) in the presence of 4 M Gdn·HCl. In these experiments the fluorescent-labeled protein solutions were diluted with equal volumes of either the buffer solution alone (A), 1% trypsin (B), or 8 M Gdn·HCl (C). The final concentrations of the proteins as well as of the probes in all the samples were identical. Fluorescence measurements were carried out at 25 °C in a buffer containing 0.02 M Tris–0.15 M NaCl, pH 7.4. Excitation wavelength was 345 nm.

containing bound probes into the solution, thereby abolishing the observed energy transfer. This argues that the observed increase in emission intensity as shown in Figure 1A is a result of energy transfer between the bound D and F probes on the intact molecule and that this process is an intramolecular event. It is worth noting that the emission maxima of both samples blue shift to 511 nm (Figure 1B), which is consistent with the notion that upon trypsin digestion the F probes released into the solution are exposed to a more hydrophilic environment. The time course of trypsin effects on the observed energy transfer is shown in Figure 2. The energy transfer from D to F on the protein molecule is completely diminished upon addition of trypsin for 30 min. The rate at which the observed energy transfer is decreased by trypsin is correlated with the rate of Fn degradation by trypsin as demonstrated by gel electrophoresis (data not shown).

To further ascertain that the observed energy transfer is the result of intramolecular juxtaposition of the two amino termini, we have determined the effect of Gdn·HCl, a widely used protein-denaturing agent, on the measured energy transfer. Our rationale is that if the observed energy transfer is due to an intramolecular event, the unfolding of the protein by the chaotropic agent should extend the amino termini to greater than 70 Å apart, thereby abolishing the energy transfer. Indeed, addition of 4 M Gdn·HCl reduces the emission intensity of the double-labeled Fn to the same extent as that of the single-labeled Fn, Figure 1C, similar to the trypsin effect shown in Figure 1B. The red shift in the emission spectra obtained for denatured proteins, Figure 1C, probably results from the guanidine effect as described previously in other protein systems (Jullien & Garel, 1983).

Estimation of the Fraction of DF- and FD-Labeled Fibronectin. In double-labeled Fn preparations, about 50% of the bound probes were found to be F probes, and the other 50% were D probes. There are at least nine possible combinations for bound probes to appear on the protein molecule,

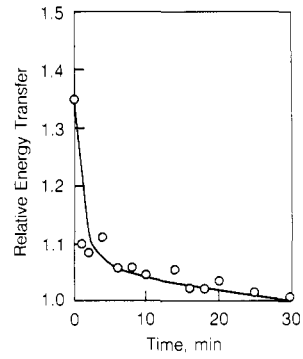

FIGURE 2: Time dependence of trypsin effects on the relative energy transfer of double-labeled Fn at 25 °C. Trypsin was added to double-labeled Fn and to a mixture of F-Fn and D-Fn, respectively. The changes in the relative energy transfer, which is defined as the ratio of the relative fluorescence intensities of doubled-labeled Fn and of a mixture of F-Fn and D-Fn at 514 nm, as shown in Figure 1A, were monitored as a function of time. The concentration of trypsin was 0.5%. Other experimental conditions were as described in Figure 1.

namely, DD, FF, DF, and FD (double-labeled), D–, –D, F–, and –F (single-labeled), and –– (nonlabeled). Since the stoichiometry of labeling was 0.9 probe per chain, we estimate that about 40% of the total proteins were FD and DF labeled. Another 40% were DD and FF labeled, and 10% each were for D–/–D and F–/–F labeled proteins. The estimation is based upon the assumption that the nonlabeled fraction (––) is negligible. Judging from the total possible unoccupied sites on all single-labeled fractions, the above assumption may be valid. It is therefore conceivable that about 40% of the proteins being labeled with F probe on one chain and D probe on the other chain or vice versa may be responsible for the observed energy transfer reported in this work. This has been taken account for in the calculation of transfer efficiency (see a later section).

Calculation of R_0. For calculating the distances between two sites by energy transfer, the Förster energy transfer distance R_0, at which the efficiency of energy transfer between a donor-acceptor pair is 50%, has to be estimated. R_0 can be calculated from eq 4 (Stryer & Haugland, 1967), where κ^2

$$R_0 = (9.79 \times 10^3)(\kappa^2 \eta^{-4} \phi_D J_{DA})^{1/6} \text{ (Å)} \qquad (4)$$

is an orientation factor, η is the refractive index of the medium, ϕ_D is the quantum yield of the donor in the absence of the acceptor, and J_{DA} is the spectral overlap integral, which is defined as in eq 5 (Fairclough & Cantor, 1978), where $F_D(\lambda)$

$$J_{DA} = \frac{\int_0^\infty F_D(\lambda)\epsilon_A(\lambda)\lambda^4 \, d\lambda}{\int_0^\infty F_D(\lambda) \, d\lambda} \text{ (cm}^3 \text{ M}^{-1}) \qquad (5)$$

and $\epsilon_A(\lambda)$ are the donor fluorescence and acceptor extinction coefficient, respectively. Simpson's rule for integral approximation was used for summation of the integral at 1-nm intervals, and the value of J was calculated to be 5.93×10^{-14} cm^3 M^{-1}. The quantum yield of the donor was 0.07 (see Materials and Methods). A value of 1.4 for the refractive index of the medium was used for the calculation. κ^2, the square of the geometric part of the dipole–dipole interaction, is dependent upon the relative orientation of donor and acceptor. This factor can range from 0 to 4. κ^2 is generally assumed to be equal to $^2/_3$, the value for donor and acceptors which randomize by rotational diffusion prior to energy transfer. To evaluate the rotational diffusion of the bound probes, we have determined the effective rotational correlation time of Tempo–cadaverine, a nitroxide spin-label primary

amine, bound to Fn using ESR spectroscopy. The effective correlation time of Tempo–cadaverine, similar in structure to D or F probe, bound to the amino termini of Fn was found to be 5×10^{-10} s (Narasimhan and Lai, unpublished observation) which is much shorter than the lifetime of either F or D probe. Thus, the assumption that the value of the orientation factor is $^2/_3$ for randomly orienting dipoles seems to be valid. From the values of J_{DA}, ϕ_D, κ^2, and η, the R_0 value of 28.6 Å was calculated, which is within the ranges of the R_0 value for the D–F pair reported in the literature (Lakowicz, 1983).

Distance between Donor and Acceptor. From the corrected energy transfer efficiency (E_c) and R_0, the distance between the donor and acceptor can be determined from eq 6 (Wu & Stryer, 1972). E_{obsd} and E_c were estimated to be 0.35 ± 0.02 and 0.78 as calculated from eq 2 and 3, respectively. The intramolecular distance between two glutamine-3 residues at the amino termini of plasma Fn was estimated to be 23 Å. Thus, the two amino termini of plasma Fn in solution appear to be in close proximity. The distance estimated here, however, is only qualitative rather than quantitative due to several unknown factors including the orientation and flexibility of the spacer (the five methylene groups) between the fluorophore and the primary amine group and the exact percentage of the dual-labeled proteins in the samples. In addition, since eq 4 and 6 contain a $1/r^6$ term, the amplitude of the "wobble" motions of the probes may also affect the measured distance. In other words, the possibility that in this study the closest distance rather than the averaged distance between the two amino termini of the protein is measured cannot be ruled out.

$$R = (1/E_c - 1)^{1/6} R_0 (2/3) \qquad (6)$$

Effects of Ionic Strength on the Distance between the Two Amino Termini. In 1979, Alexander et al. showed an effect of ionic strength on the sedimentation coefficient of Fn; it changed from 13.6 *s* in 0.04 M KCl to 10.8 *s* in 0.4 M KCl. Their observation has been confirmed by other investigators (Erickson & Carrell, 1983; Lai et al., 1984). On the basis of these data, it is generally assumed that Fn exists in two extreme conformations, namely, compact (in low salt) and extended (in high salt) conformers. However, because the solution structure of the protein is still not known, the nature of changes in conformation could not be deduced. We carried out several experiments to determine whether the change in conformation from the compact form to the extended form involves the alteration of the relative distance between the two amino termini of the protein. Surprisingly, the relative energy transfer remains fairly constant between 0.02 and 1.0 M salt (data not shown), indicating that the distance between the two amino termini is not affected by ionic strength. Our sedimentation results in Table I show that at physiological salt condition the *s* values for native and the labeled Fn reported here are in good agreement with the literature values (Alexander et al., 1979) and that the labeled protein undergoes the same transitions as a function of salt as does native Fn. The results of the sedimentation experiments also indicate two important points: (1) the modification of Fn with the fluorescent probes employed in this study does not change the hydrodynamic properties of the protein, and (2) the protein samples are in monomeric form and there is no indication of protein aggregation. This further confirms that the observed energy transfer reported in this work is due to an intramolecular process. The possibility that protein aggregation contributes to the observed energy transfer can be ruled out.

Thus, it is suggested here that while high ionic strength disrupts the electrostatic interactions between various regions in the protein molecule, thereby inducing the change in conformation as reflected by the change in sedimentation coefficient, the relative distance between the two amino termini of the protein may not be affected.

Table I: Sedimentation Coefficients of Native and Fluorescent-Labeled Fibronectins at pH 7.4[a]

samples	conditions	$s \pm \sigma$[b]	$s_{20,w} \pm \sigma$[c]
native Fn	0.02 M Tris, 0.15 M NaCl	12.80 ± 0.06	12.86 ± 0.06
FD-Fn[d]	0.02 M Tris, 0.15 M NaCl	12.89 ± 0.10	12.86 ± 0.04
native Fn	0.02 M Tris, 1.0 M NaCl	7.79 ± 0.31	9.13 ± 0.39
FD-Fn	0.02 M Tris, 1.0 M NaCl	8.00 ± 0.06	9.21 ± 0.20

[a] The measurements were carried out at ambient temperature with protein concentrations of 0.8–1.0 mg/mL. [b] Observed sedimentation coefficients prior to correction for solvent viscosity or temperature. The values reported here are means ± standard deviation (two independent experiments). [c] Corrected sedimentation coefficients with the equation:

$$s_{20,w} = s_{t,sol}\left(\frac{\eta_t}{\eta_{20}}\frac{\eta_{sol}}{\eta_w}\right)\left(\frac{1 - \bar{v}\rho_{20,w}}{1 - \bar{v}\rho_{t,w}}\right)$$

The partial specific volume, $\bar{v}$, was assumed to be 0.72 mL/g for plasma Fn (Alexander et al., 1979). [d] FD-Fn is the double-labeled Fn which contains all combinations as described in the text.

We speculate that high salt may cause a "balloon" effect on Fn structure; the two ends (the amino and carboxyl termini) of the protein remain relatively unchanged while the central regions are expanded. The model stipulates that both compact form and expanded form of plasma Fn are relatively spherical in shape; recent calculations made by Hermans showed that this is in fact the case (Hermans, 1985). This model is also consistent with the observations made by Robinson and Hermans (1984) that the two subunits of plasma Fn are folded independently and are closely associated with each other, even after partial reduction of interchain disulfide bridges. On the other hand, recent work by Skorstengaard et al. (1986) indicated that the interchain disulfide bridge pattern of plasma Fn is antiparallel, which certainly would affect the way in which the two subunits are arranged with respect to each other. It is possible that the two subunits of plasma Fn may also be arranged in an antiparallel fashion. At present, detailed analysis of the solution structure of plasma Fn, however, is hampered by the lack of information concerning the relative arrangement of various domains in each subunit.

The results obtained from electron microscopy (EM) studies suggested that in the presence of high salt Fn appears as a rather extended, V-shaped molecule joined at the carboxyl termini by disulfide bridges, implying that the two amino termini are well separated (Erickson & Carrell, 1983; Odermatt et al., 1982). The donor–acceptor pair used in this study is only sensitive to the intramolecular distance within a range of 70 Å (Lakowicz, 1983). If Fn were in an extended V-shape, the two amino termini would have been greater than 70 Å apart; therefore, no energy transfer between the amino termini could be detected. Thus, Fn in solution cannot exist in a V-shape conformation. This discrepancy between EM and this study could be due to the difference in experimental conditions: while the present study focuses on the structure of Fn in solution, EM studies deal with the structure of Fn bound to a surface. Experiments are under way in our laboratory to determine the effects of surface binding on the relative distance between the two amino termini of Fn molecule.

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Anisotropy and Anharmonicity of Atomic Fluctuations in Proteins: Implications for X-ray Analysis†

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ABSTRACT: The effects of anisotropy and anharmonicity of the atomic fluctuations on the results of crystallographic refinement of proteins are examined. Atomic distribution functions from a molecular dynamics simulation for lysozyme are introduced into a real-space (electron density) refinement procedure for individual atoms. Several models for the atomic probability distributions are examined. When isotropic, harmonic motion is assumed, the largest discrepancies between the true first moments (means) and second moments (*B* factors) of the positions calculated from the dynamics and the fitted values occur for probability densities with multiple peaks. The refined mean is at the center of the largest peak, and the refined *B* factor is slightly larger than that of the largest peak, unless the distance between the peaks is small compared to the peak width. The resulting values are often significantly different from the true first and second moments of the distribution. To improve the results, alternate conformations, rather than anharmonic corrections, should be included.

A knowledge of the functional form of the probability density functions (pdfs) of the fluctuations of the atomic position is essential for determining the structures of protein crystals by X-ray diffraction (Willis & Pryor, 1975). Moreover, the nature of the fluctuations is of considerable interest because of their possible role in protein function (Karplus & McCammon, 1981, 1983). Also, it has been suggested recently that the antigenicity and atomic mobility of proteins may be related (Westhof et al., 1984; Tainer et al., 1984), although alternative explanations of the observed correlations have been given (Novotný et al., 1986).

For the refinement of protein crystal diffraction data (Hendrickson, 1985), it is assumed in most cases that the atomic displacements are harmonic and isotropic, i.e., that the atomic fluctuations obey a three-dimensional isotropic Gaussian distribution. Anisotropic and anharmonic effects have been introduced in the refinement of small molecules (Willis & Pryor, 1975), such as Li_3N, which usually have temperature factors corresponding to root-mean-square (rms) displacements, $\langle u_r^2 \rangle^{1/2}$, of less than 0.3 Å at 294 K (Zucker & Schultz, 1982). The motion of an atom in these molecules is generally restricted to a single minimum in the local potential, and anisotropic effects can be included by the use of an anisotropic three-dimensional Gaussian pdf. There are several possible models for anharmonic distributions, which have been compared by Zucker and Schulz (1982). They considered the performance and implementation of an expansion of the potential in a power series; Edgeworth and Gram–Charlier expansions, which are expansions of a harmonic distribution in terms of Hermite polynomials; and the

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