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Inter-Sulfhydryl Distances in Plasma Fibronectin Determined by Fluorescence Energy Transfer: Effect of Environmental Factors†

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ABSTRACT: Human plasma fibronectin, a dimeric glycoprotein, contains two cryptic free sulfhydryl groups per chain. Recent observations revealed that upon binding to a gelatin-coated surface the SH_1 site, located between the DNA-binding and cell-binding domains, is partially exposed, while the SH_2 site, situated within the carboxyl-terminal fibrin-binding domain, remains buried. Utilizing this newly discovered property of plasma fibronectin, we have developed a procedure to introduce maleimide derivatives of fluorescent probes such as *N*-(1-pyrenyl)maleimide, 7-(diethylamino)-3-(4′-maleimidylphenyl)-4-methylcoumarin, or fluorescein 5-maleimide selectively into either the SH_1 or SH_2 site of the fibronectin molecule and have measured the inter-sulfhydryl distances in fibronectin by fluorescence energy transfer methods. The results show that the distance between the SH_1 site of one subunit and the SH_1 site of the other subunit is between 35 and 44 Å, indicating the close proximity of the two subunits near the SH_1-containing regions. On the other hand, the distance between the SH_2 site of one subunit and the SH_2 site of the other subunit is found to be greater than 95 Å, suggesting that the two SH_2-containing regions are well separated. Additionally, the distance between the SH_1 and SH_2 sites within each subunit is estimated to be 42–53 Å, assuming no intersubunit energy transfer between the probes. Heparin or high salt, which drastically affects the hydrodynamic properties of fibronectin, had virtually no effect on the distance between the SH_1–SH_1 or the SH_1–SH_2 pair. In contrast, upon adsorption of the protein to Cytodex microcarriers, the energy transfer between the SH_1 sites was markedly reduced, implying a surface-mediated separation of the two subunits of fibronectin.

Fibronectin (Fn) is a large glycoprotein present in blood plasma and other body fluids and in tissues. It plays a part in numerous biological phenomena including cell adhesion and spreading, wound healing, phagocytosis, and differentiation [see Akiyama and Yamada (1987), McDonagh (1985), and Mosher (1984) for reviews]. The protein contains two nearly identical subunits of 240–250 kDa each, linked near their carboxyl termini by two disulfides. Within each subunit are several distinct structural domains that can be isolated from proteolytic digests with retention of specific binding affinities toward a host of macromolecules including fibrin, heparin, collagen, DNA, and cell surface molecules. Relatively little is known concerning the spatial arrangement of these structural domains of this complex protein.

Fn contains two free sulfhydryl groups per subunit: one located in a type III homology region between the DNA-binding and cell-binding domains (designated SH_1) (Skorstengaard et al., 1986) and the other also located in a type III homology region but within the carboxyl-terminal fibrin-binding domain (designated SH_2) (Garcia-Pardo et al., 1985). Neither of these sulfhydryl groups is accessible to sulfhydryl reagents in the absence of chaotropic agents (Smith et al., 1982; Lai & Tooney, 1984). Recently, we have shown that upon adsorption of Fn to a gelatin-coated surface the SH_1 site is partially exposed, while the SH_2 site remains buried (Narasimhan & Lai, 1989b). Here we have used this new observation to develop a procedure that allows the differential labeling of either SH_1 or SH_2 in Fn with maleimide derivatives of fluorescent probes (see Figure 1) and have measured inter-sulfhydryl distances of Fn by fluorescence energy transfer methods (Lakowicz, 1983; Stryer, 1978).

Materials and Methods

Fn was purified from fresh-frozen human plasma, obtained from the Blood Center of Southeastern Wisconsin, on a gelatin–Sepharose 4B affinity column (Engvall & Ruoslahti, 1977). The integrity and purity of the protein, before and after labeling with the sulfhydryl reagents, were routinely examined with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The fluorescence probes *N*-(1-pyrenyl)maleimide (P), 7-(diethylamino)-3-(4′-maleimidylphenyl)-4-methylcoumarin (C), and fluorescein 5-maleimide (F) were purchased from Molecular Probes (Eugene, OR). Polystyrene latex beads with surface carboxyl groups were purchased from Polysciences (Warrington, PA). The beads used in this study had a diameter of 0.5 μm. Tris(hydroxymethyl)aminomethane (Tris), phenylmethanesulfonyl fluoride (PMSF), 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (carbodiimide), trypsin (bovine pancreas), and heparin (porcine

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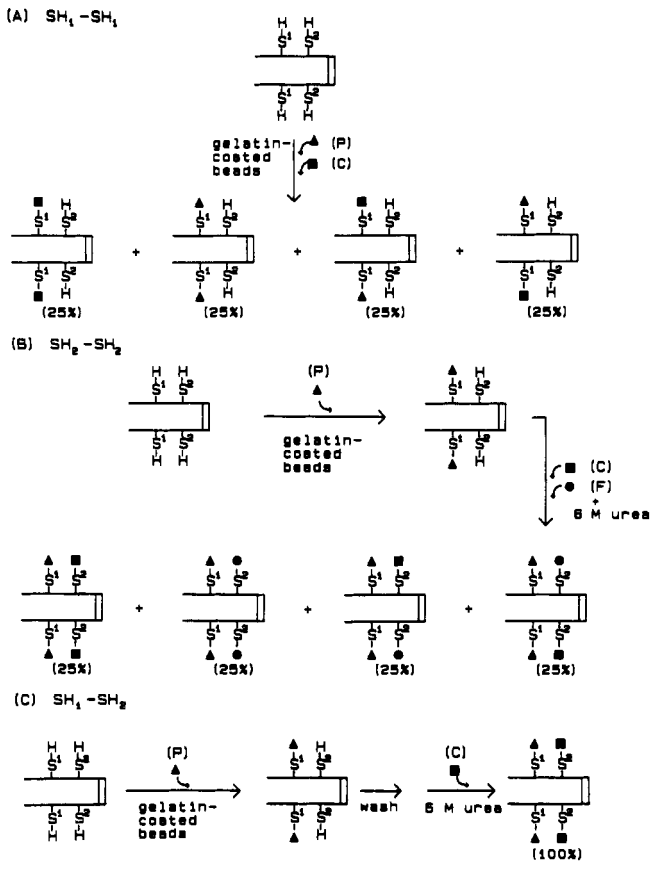

FIGURE 1: Schematic diagrams depicting the experimental strategies to label specifically the free sulfhydryl groups of Fn with maleimide derivatives of fluorescent probes. (▲) P; (■) C; (●) F. The percentage indicates the fraction of the labeled protein with that particular labeling pattern. Detailed procedures are described under Materials and Methods.

intestinal mucosa, grade I) were from Sigma (St. Louis, MO). Guanidine hydrochloride was obtained from Pierce (Rockford, IL). Cytodex 1 and Cytodex 3 microcarriers were purchased from Pharmacia (Piscataway, NJ). All other chemicals were of high-quality reagent grade.

The buffers used in this study were 0.02 M Tris–0.15 M NaCl, pH 7.4 (TBS), and 0.02 M NaH_2PO_4/Na_2HPO_4–0.15 M NaCl, pH 7.4 (PBS).

Labeling of Fn with Fluorescent Probes. The schematic diagram in Figure 1 outlines the procedures used for the preparations of the (a) SH_1–SH_1 pair, (b) SH_2–SH_2 pair, and (c) SH_1–SH_2 pair, each with a donor–acceptor pair of fluorescent probes present in the same protein molecule. Single-labeled control samples containing only the donor probe or the acceptor probe in the protein molecule were prepared as described for the preparations of the double-labeled samples.

(*a*) *SH_1–SH_1 Pair.* Labeling of SH_1 was performed by following the procedure developed by Narasimhan and Lai (1989b). Briefly, gelatin was coupled to polystyrene beads after activation of the beads with carbodiimide (Molnar et al., 1987). Fn (3 mg) was incubated with 15 mL of the gelatin-coupled beads for 15 min at room temperature with constant mixing. After incubation the unbound Fn was removed by centrifugation and quantitated by the absorption at 280 nm; the amount of Fn bound was calculated on the basis of the amount recovered in this step. To the beads, resuspended in TBS, were added the fluorescent probes, either the C or P probe alone or equal amounts of each at a 2:1 molar ratio of probe to Fn, and the reaction mixture was incubated for 30 min at room temperature. Following reaction, a 20-fold excess of cysteine was added to block any unreacted probes, and the mixture was incubated for an additional 30 min at room temperature. After the beads had been washed twice by centrifugation and resuspension with TBS, the recovery of Fn differentially labeled at SH_1 was carried out by resuspending the beads in 8 mL of TBS containing 8 M urea for 15 min at 37 °C. The reaction mixture was centrifuged to produce a clear solution containing the labeled Fn, which was collected and filtered with a 0.2-μm filter to remove any residual beads. The sample was then dialyzed extensively against TBS to remove urea. Recovery of SH_1-labeled Fn was typically 60–65% of the amount bound.

(*b*) *SH_2–SH_2 Pair.* Differential labeling of SH_2 was achieved by first blocking the SH_1 sites with the P probe according to the procedure outlined above and labeling the SH_2 sites with either the C or F probe or equal amounts of each according to the procedure described previously by Wolff and Lai (1989).

(*c*) *SH_1–SH_2 Pair.* This pair was labeled in two separate steps: First, the SH_1 labeling with the P probe was performed as described above for the preparation of the SH_1–SH_1 pair. After recovery of the labeled sample, the labeling of the SH_2 sites with the C probe was done essentially as described above for the preparation of the SH_2–SH_2 pair.

Estimation of Labeling Stoichiometry. The stoichiometry of the probe to protein for each probe was determined by measurement of absorbances on a Perkin-Elmer 320 or a Hewlett-Packard HP-8451A spectrophotometer. An absorption coefficient of 1.28 mL mg^{-1} cm^{-1} at 280 nm was used for Fn (Mosesson et al., 1975). A molar absorption coefficient of 2.8×10^4 M^{-1} cm^{-1} at 396 nm was used for the C probe conjugated to Fn (Wolff & Lai, 1989). Molar absorption coefficients of 2.2×10^4 M^{-1} cm^{-1} at 344 nm and 5.2×10^4 M^{-1} cm^{-1} at 494 nm were determined for the P and F probes, respectively. These values were obtained by comparison of the absorbances of the probe conjugated to Fn in 4 M guanidine hydrochloride, with the absorbance of the known concentrations of the cysteine conjugates of the corresponding probe in TBS containing 4 M guanidine hydrochloride. We have shown previously that under these conditions the free sulfhydryl groups of Fn are exposed to solvent (Narasimhan et al., 1988). In the calculations of stoichiometry, corrections were made for probe absorbance at 280 nm and for absorption due to other probes.

The specificity of labeling was verified by SDS–PAGE followed by visualization of the fluorescent bands with a UV source (Narasimhan & Lai, 1989b). Both C and P probes used to label the SH_1 sites are virtually nonfluorescent as the maleimide derivative but become highly fluorescent upon reaction of their olefinic bond with the cysteine residue (Sippel, 1981). We have shown previously that the hydrodynamic properties of Fn, including sedimentation constants, and circular dichroism behaviors are not affected by labeling with sulfhydryl reagents (Lai & Tooney, 1984; Lai et al., 1984b).

Fluorescence Measurements. Steady-state fluorescence measurements were made with an SLM 8000 spectrofluorometer, equipped with a Lauda RC3 circulating waterbath for control of sample compartment temperature. All spectra were acquired at 25 °C and corrected for wavelength-dependent instrument response. Excitation wavelengths of 345 and 395 nm were used for the P and C probe labeled samples, respectively. An excitation wavelength of 495 nm was used for the F probe labeled samples. TBS buffer was used for all solution studies, and PBS was used for all experiments involving Cytodex microcarriers. In solution studies initial

protein concentrations were between 0.05 and 0.1 mg mL^{-1}. To reduce inner-filter effects, the absorbance at the excitation wavelength was kept below 0.03. Relative quantum yields were determined for the P and C probes by comparison to that of quinine sulfate (Parker & Reese, 1966), which has a quantum yield of 0.7 in 0.1 N H_2SO_4 (Scott et al., 1970). Polarization measurements were made with the same instrument, arranged in the "T-format", with an excitation wavelength at the absorption maximum, the emission was monitored at the emission maximum, and appropriate filters were used in the reference channel.

Coating of Cytodex Microcarriers with Labeled Fn. Fluorescently labeled Fn was coated onto the Cytodex bead surface as described previously (Wolff & Lai, 1989). The ratio of the protein to bead was 0.2–0.5 $\mu g/10^3$ beads.

Energy-Transfer Measurements. The efficiency of energy transfer, from an energy donor to an energy acceptor, can be followed from both the quenching of donor fluorescence and the enhancement of acceptor fluorescence. The observed efficiency (E_{obs}) for the donor quenching method can be calculated from the ratio of the donor fluorescence, in the presence and absence of the acceptor, eq 1, where F_{DA} is the

$$E_{obs} = 1 - F_{DA}/(F_D + F_A); (\lambda_d, \lambda_d) \quad (1)$$

fluorescence of the double-labeled sample and $F_D + F_A$ is the fluorescence of the mixture of single-labeled proteins and (λ_d, λ_d) denotes that the excitation wavelength used is at the donor's excitation maximum and the emission is monitored in the region of the donor's emission maximum. The efficiency of energy transfer can also be determined by the acceptor enhancement method. Here, the efficiency is expressed as the ratio of the acceptor fluorescence, in the presence and absence of the donor, eq 2, where F_{DA} and $F_D + F_A$ are as defined

$$E_{obs} = [F_{DA}/(F_D + F_A) - 1](\epsilon_A/\epsilon_D); (\lambda_d, \lambda_a) \quad (2)$$

above and (λ_d, λ_a) denotes that excitation is at the donor's maximum and that the emission is monitored in the region of the acceptor's emission maximum. The term ϵ_A/ϵ_D is the ratio of the molar absorption coefficients of the acceptor and the donor, respectively, at the wavelength of excitation. The inclusion of this term allows for correction due to the direct excitation of the acceptor which occurs when the acceptor's absorbance is appreciable at the excitation wavelength. In the case where donor–acceptor stoichiometry is not exactly 1:1, the corrected efficiency (E_c) can be calculated from the observed efficiency as shown in eq 3, where f_a and f_d are the

$$E_c = E_{obs}/f_a \text{ (or } f_d) \quad (3)$$

fractions of available sites occupied by the acceptor and donor, respectively.

Calculation of R_0. To calculate the distance between two sites by energy transfer, the Förster critical distance R_0, at which the transfer efficiency is 50%, has to be determined, eq 4, where κ^2 is the orientation factor between the donor and

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

the acceptor, 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. To evaluate the overlap integral, Simpson's rule for integral approximation was used for summation of the integral at 1-nm intervals. Initially, a value of 2/3, the dynamic average, was assumed for κ^2 in the calculation of R_0. Subsequently, a probable range of values and the expected extreme values for the distance were determined on the basis of a likely range of κ^2 estimated from measured polarization values (Haas et al., 1978). The distance, r, is calculated according to

$$r = (1/E_c - 1)^{1/6} R_0 \text{ (Å)} \quad (5)$$

Results

Characterization of Systems. The efficiencies of labeling of plasma Fn with maleimide derivatives of fluorescent probes, as shown schematically in Figure 1, were 0.95 ± 0.05 for SH_1 per subunit and 0.90 ± 0.10 for SH_2 site per subunit, respectively (Narasimhan & Lai, 1989b). The differences in labeling efficiency among the three probes were small. However, it is of interest to note that under similar labeling conditions the fluorescein maleimide reacted poorly with the newly exposed SH_1 sites (data not shown). Since the maleimide derivative of either the C or P probe is more hydrophobic than the corresponding fluorescein probe (inasmuch as the latter contains a carboxyl group), the difference in the labeling efficiency could be due simply to the hydrophobicity of the cleft-like structures in which the free sulfhydryls are situated (Lai et al., 1984a; Wolff & Lai,1989), which are presumably more accessible to hydrophobic probes. The differential accessibility of the maleimide derivatives of fluorescent probes to the newly exposed SH_1 site, upon binding of Fn to a gelatin-coated surface, argues that the SH_1 site is only partially exposed under these experimental conditions.

Energy-Transfer Measurements. (a) SH_1–SH_1 Pair. The P (donor) and C (acceptor) probes were introduced into the SH_1 sites of Fn as outlined in Figure 1A. The emission spectrum of this double-labeled Fn is shown as the solid line in Figure 2A, in which the sharp peaks at 375 and 395 nm and a shoulder at 415 nm are characteristic of the emission maxima for the P probe conjugated with macromolecules (Weltman et al., 1973), and the peak at 476 nm arises from the emission maximum of the C probe (Wolff & Lai, 1989). The dashed line in Figure 2A is the composite emission spectrum of the single-labeled control mixture. Care was taken during the preparation of the samples to ensure that the concentrations of the P and C probes in both samples were identical. This was routinely verified by monitoring the relative emission intensities of the C probe in both double-labeled and single-labeled samples with the excitation wavelength of 395 nm at which only the C probe is excited so that there is no contribution from the P probe. Thus, the observed decrease in the emission intensity of the P probe, concomitant with the increase in the emission intensity of the C probe in the double-labeled Fn (solid line), compared to those in the single-labeled mixture (dashed line) in Figure 2A, may be attributed to energy transfer from the P to C probe in the double-labeled Fn sample.

Two approaches, namely, treatment with guanidine hydrochloride and digestion with trypsin, were used to ascertain whether the observed energy transfer is an intramolecular event (Wolff & Lai, 1988). It was reasoned that, if the energy transfer is an intramolecular event, addition of guanidine hydrochloride, a potent denaturing agent, would unfold the protein molecule and separate the donor from the acceptor by more than 70 Å, thereby destroying the energy transfer. Indeed, addition of 4 M guanidine hydrochloride resulted in the abolition of the observed energy transfer, as shown in Figure 2B, in which the spectra of the double-labeled and single-labeled samples became superimposable. Digestion of the samples with trypsin, a proteolytic enzyme that cleaves Fn, produced similar results (data not shown; Wolff & Lai, 1988). Thus, it is conceivable that the observed energy transfer in Figure 2A is an intramolecular event, resulting from the energy transfer from the P to the C probe in the same protein molecule. It is noted that the emission maximum of the C probe conjugated with Fn was red-shifted from 476 to 483 nm in

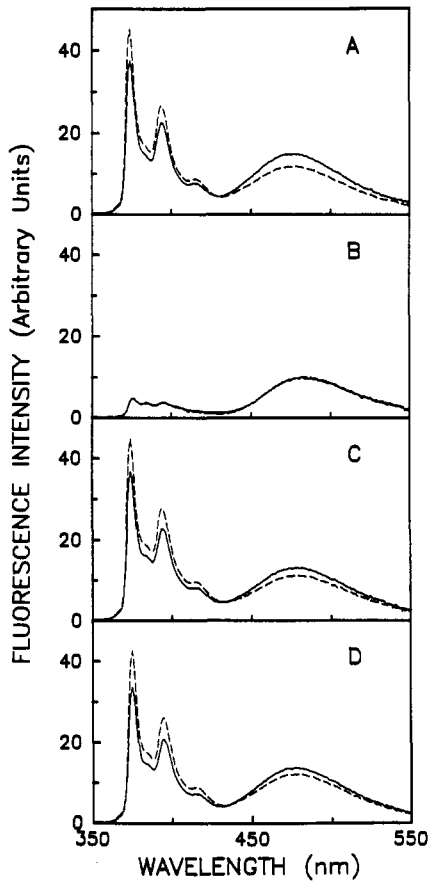

FIGURE 2: Corrected fluorescence emission spectra of the double-labeled Fn with the P and C probes labeled at the SH_1 sites (solid line) and a mixture of the single-labeled Fn (dashed line) in buffer containing 0.02 M Tris–0.15 M NaCl, pH 7.4 (TBS) (A), in the presence of 4 M guanidine hydrochloride (B), 1 M NaCl (C), or heparin (200:1 molar ratio of heparin to Fn) (D). The concentrations of Fn (0.1 mg/mL) were identical in all samples. All spectra were acquired at 25 °C, with an excitation wavelength of 345 nm and an acquisition time of 2 s/nm.

Table I: Transfer Efficiencies (E_{obs}) between SH Groups in Fn[a]

condition	FnP_1C_1[b]	FnC_2F_2	FnP_1C_2
TBS (7.4)	0.21 ± 0.03	0.01 ± 0.01	0.19 ± 0.04
1 M NaCl	0.18 ± 0.01	nd	0.20 ± 0.05
heparin[c]	0.19 ± 0.02	nd	0.24 ± 0.01
Cytodex 1	0.08 ± 0.03	nd	0.33 ± 0.15

[a] The data for E_{obs} were averages of the values calculated separately from eq 1 and 2, which were different within 5–10%. Each data point was an average of at least two independent measurements. [b] Abbreviations: FnP_1C_1, Fn labeled at SH_1 with both the P and C probes; FnC_2F_2, Fn labeled at SH_2 with both the C and F probes; FnP_1C_2, Fn labeled at SH_1 with the P probe and at SH_2 with the C probe. [c] Heparin was added at a 200:1 molar ratio of heparin to Fn.

the presence of guanidine hydrochloride; the latter value is the same as that of the cysteine conjugate of the C probe in solution (Rychlik et al., 1983), suggesting that the C probe attached to Fn is located in a hydrophobic environment (Wolff & Lai, 1989).

The observed energy-transfer efficiency (E_{obs}) and corrected energy-transfer efficiency (E_c) were estimated to be 0.21 and 0.41, respectively (Tables I and II). The latter value was calculated with eq 3, with the assumption that only 50% of the double-labeled sample contains both the P and C probes in the same protein molecule (see Figure 1A). The values of the spectral overlap integral (J) and the quantum yield of the P probe (ϕ) are summarized in Table II. By use of eq 4, an R_0 of 37 Å was estimated for the P and C pair, which is in

Table II: Energy-Transfer Parameters for Inter-Sulfhydryl Distances

parameter	FnP_1C_1[a]	FnC_2F_2	FnP_1C_2
donor (site)	P (SH_1)	C (SH_2)	P (SH_1)
acceptor (site)	C (SH_1)	F (SH_2)	C (SH_2)
ϕ_{donor}	0.40	0.68	0.40
polarization (donor)	0.28	0.42	0.28
polarization (acceptor)	0.43	nd	0.42
$J \times 10^{14}$ (M^{-1} cm^3)	4.19	15.54	4.19
R_0 (2/3) (Å)	37.0	50.3	37.0
E_{cor}	0.41 ± 0.03	0.02 ± 0.01	0.19 ± 0.04
r (Å)	39	>95	47
probable range (Å)	35–44	>85–109	42–53
extreme range (Å)	24–49	>60–122	29–59

[a] The definitions of the abbreviations were described in Table I.

accord with values reported in the literature (Richter et al., 1985). From the values of E_c and R_0, the distance between the donor and acceptor calculated with eq 5 was found to be between 35 and 44 Å. Considering a molecular dimension of 300 × 500 Å for the compact form of Fn as revealed by electron microscopy (Price et al., 1982; Erickson & Carrell, 1983), the result suggests the close proximity of the two SH_1-containing regions of the Fn molecule.

It is known that the sedimentation coefficient of Fn decreases with an increase in ionic strength (Alexander et al., 1979). This phenomenon has been interpreted to mean that Fn exists in either a compact form (low salt) or an extended form (high salt). We conducted experiments to see whether high ionic strength affects the intramolecular distance between the two SH_1 groups. Addition of 1 M NaCl slightly reduced the difference in signal intensity between the emission spectra of the double-labeled sample (solid line) and the single-labeled mixture (dashed line of Figure 2C), which corresponds to a decrease in E_{obs} from 0.21 to 0.18 (Table I). It appears that high ionic strength exerts only a minor effect on the intersubunit distance between the two SH_1 sites in Fn.

Fn has at least two distinct heparin-binding sites, one located at the amino-terminal 29-kDa region and the other located near the carboxyl terminus (Hayashi & Yamada, 1982). Heparin binding is thought to induce a conformational change of Fn as reported previously (Ankel et al., 1986). However, similar to the results obtained with high salt, inclusion of heparin at a molar ratio of 200:1 (heparin to Fn) produced only a small change in the emission spectra between the double-labeled (solid line) and the single-labeled mixture samples (dashed line of Figure 2D): E_{obs} decreased only from 0.21 to 0.19 (Table I). The presence of heparin does not seem to have a major effect on the measured distance between the two SH_1 regions. Thus, it is probable that neither high ionic strength nor the binding to heparin causes any major structural change in the regions containing the SH_1 sites.

(*b*) *SH_2–SH_2 Pair.* The SH_2 sites of Fn were labeled selectively with the C (donor) and F (acceptor) probes as outlined in Figure 1B. Figure 3A shows the corrected emission spectrum of the double-labeled sample (solid line) and that of the single-labeled mixture (dashed line). The superimposability of the two spectra in Figure 3A revealed virtually no evidence of energy transfer between the C and F probes in the double-labeled sample. If energy transfer occurs between the C and F probes, one would expect to see a decrease in the emission intensity of the C probe of 473 nm, concomitant with an increase in the emission intensity of the F probe at about 520 nm, as observed previously with similar fluorophores attached at different sites of the Fn molecule (Wolff & Lai, 1989). Figure 3B demonstrates that upon digestion with trypsin there is essentially no change in the relative fluorescence intensities of the two samples. The red-shift in the

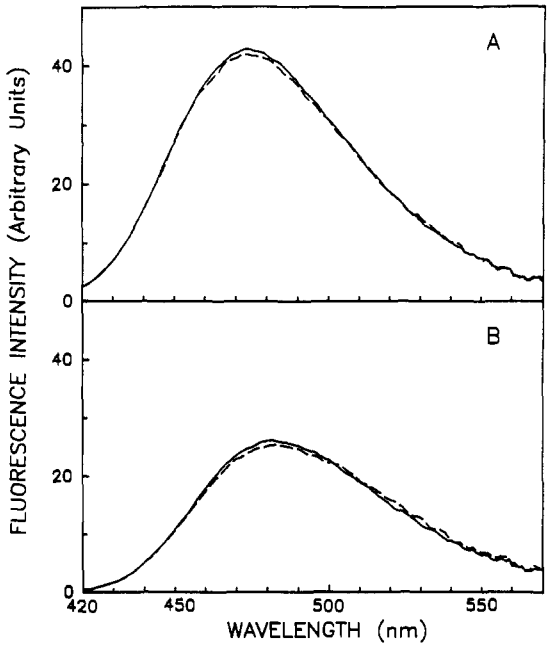

FIGURE 3: Corrected emission spectra of the double-labeled Fn with the C and F probes labeled at the SH_2 sites (solid line) and a mixture of single-labeled Fn (dashed line) in TBS (A) and following digestion with 0.05% trypsin (B). Trypsin was added from a 5% stock solution in TBS. The spectra in panel B were acquired after 1 h, after which no further changes were observed. The temperature was maintained at 25 °C throughout the measurement. The excitation wavelength was 395 nm.

emission maximum from 473 to 483 nm after trypsin digestion is consistent with an increased exposure of the C probe to solvent, which in turn reduces the overall intensity as observed previously in other protein systems (Rychlik et al., 1983).

The quantum yield and the overlap integral for the C probe were 0.68 and 15.54×10^{-14} M^{-1} cm^3, respectively (Table II). Using these values an R_0 of 50.3 Å was calculated for the donor (C) and acceptor (F) pair (Snyder & Hammes, 1985; Richter et al., 1985). The averaged energy-transfer efficiency of this donor–acceptor pair was estimated to be 0.01 (Table I). Indeed, in some experiments an apparent negative transfer efficiency was obtained. Following correction for only 50% double-labeled sample containing both C and F probes in the same protein molecule (see Figure 1B), a corrected transfer efficiency of less than 0.02 is realized (Table II), which yielded a distance of greater than 95 Å between the SH_2 sites, suggesting that the SH_2-containing regions are well separated from each other. Skorstengaard et al. (1986) suggested previously that the intersubunit disulfide bridges of plasma Fn are arranged in an antiparallel pattern. The large separation between the SH_2 regions is consistent with such an arrangement.

(*c*) *SH_1–SH_2 Pair.* The double-labeled Fn sample with the SH_1 sites labeled with the P probe (donor) and the SH_2 sites labeled with the C probe (acceptor) was prepared as shown schematically in Figure 1C. The decrease in the intensity of the P probe emission, concomitant with the increase in the intensity of the C probe emission for the double-labeled sample (solid line of Figure 4A), compared to those of the single-labeled mixture (dashed line), is indicative of an energy transfer between the P and C probes in the double-labeled sample. The observed energy transfer was abolished completely either by addition of 4 M guanidine hydrochloride (Figure 4B) or by digestion with trypsin (data not shown), suggesting that it is an intramolecular event. An energy-transfer efficiency of 0.19 was estimated (Table I). Note added in passing: The observed

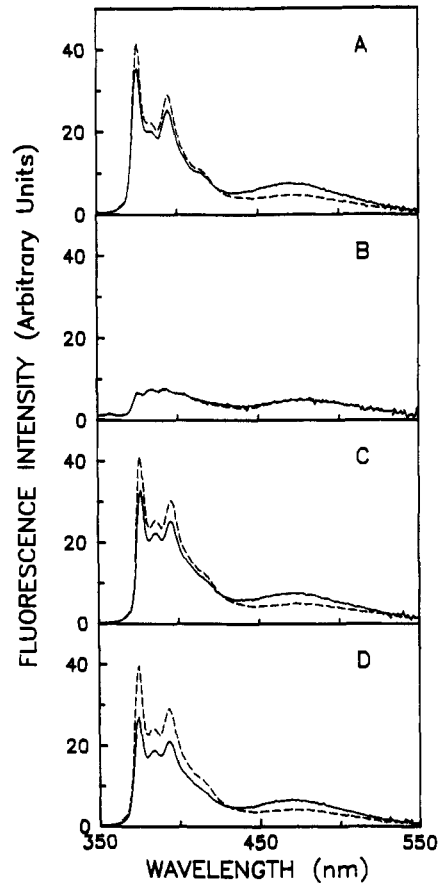

FIGURE 4: Corrected fluorescence emission spectra of the double-labeled Fn with the P probe at the SH_1 sites and the C probe at the SH_2 sites (solid line) and a mixture of the single-labeled Fn (dashed line) in TBS (A), in the presence of 4 M guanidine hydrochloride (B), 1 M NaCl (C), or heparin (200:1 molar ratio of heparin to Fn) (D). The protein concentration was 0.1 mg/mL in all samples.

and corrected energy-transfer efficiencies are the same in this case because the fraction of either probe is unity during the labeling (Figure 1C). We estimated that the distance between the SH_1 and SH_2 sites within each subunit is between 42 and 53 Å (Table II). This value is valid, only if there is no contribution of an intersubunit energy transfer between the probes. However, the possibility of such an event cannot be ruled out. The estimated distance, therefore, is subject to the uncertainty of the contribution from the intersubunit energy transfer (see Discussion).

Interestingly, similar to that of the SH_1–SH_1 pair, the distance of the SH_1–SH_2 pair is not affected by either high ionic strength (Figure 4C) or heparin (Figure 4D). The averaged transfer efficiency shows little change in the presence of 1 M NaCl when compared to that observed in physiological salt (Table I). Likewise, the addition of a 200:1 molar ratio of heparin to Fn, Figure 4D, results in a fairly small change in transfer efficiency (Table I). This finding suggests that, although high salt or heparin induces hydrodynamic changes in Fn structure, it does not affect the distance between the SH_1- and SH_2-containing regions of the Fn molecule.

Surface Effects. We examined the effects of the surface binding on the distance between the two SH_1 sites. To do so, the double-labeled Fn prepared as described in Figure 1A was adsorbed onto Cytodex microcarriers (Wolff & Lai, 1989). The emission spectra of the double-labeled Fn (solid line) and the single-labeled mixture (dashed line) coated onto the surface of Cytodex beads were found to be nearly superimposible (Figure 5A), a result suggesting that the energy transfer be-

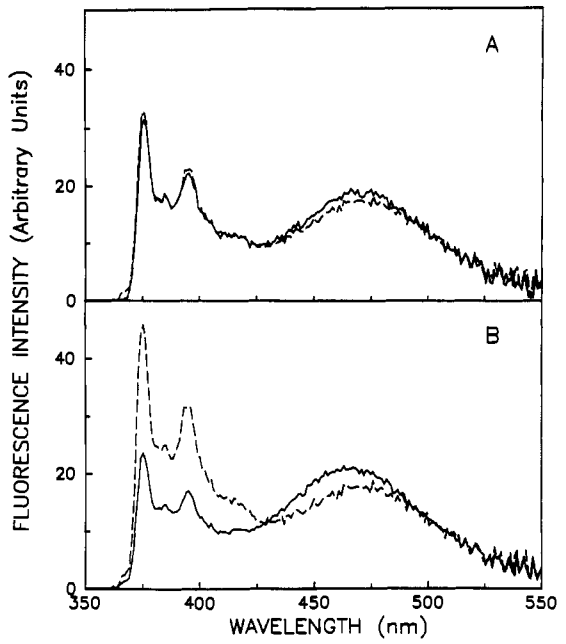

FIGURE 5: (A) Corrected emission spectra of double-labeled Fn with the P and C probes labeled at the SH_1 sites (solid line) and a mixture of single-labeled Fn (dashed line) adsorbed on Cytodex 1 microcarrier beads. (B) Corrected emission spectra of the double-labeled Fn with the P probe at the SH_1 site and the C probe at the SH_2 site (solid line) and a mixture of the single-labeled Fn (dashed line) adsorbed on Cytodex 1 microcarrier beads. Spectra were recorded at 25 °C, with an excitation wavelength of 345 nm and an acquisition time of 5 s/nm.

tween the SH_1–SH_1 pair observed in solution (Figure 2A) was greatly reduced upon adsorption of the labeled protein to Cytodex beads. As usual, the equal concentrations of the probes in both samples were verified by measuring the emission intensity of the C probe with an excitation wavelength of 395 nm at which no contribution of the P probe occurred (data not shown). However, because of the poor signal to noise associated with the light scattering properties of Cytodex beads, the estimation of actual changes in the distance of the SH_1–SH_1 pair on the surface is rather problematic.

Similar experiments were performed to determine the effect of the surface binding on the distance between the SH_1–SH_2 pair. Figure 5B shows the emission spectra of the double-labeled Fn with the P probe on the SH_1 and the C probe on the SH_2 (the solid line) and the single-labeled control mixture (the dashed line) absorbed onto Cytodex beads. In comparison to the emission spectra of the same labeled protein in solution shown in Figure 4A, a considerable amount of energy transfer is still observable between the SH_1 and SH_2 sites even when adsorbed on the bead surface. Again, due to the poor signal to noise ratio and the uncertainty of the quantum yields for the probes, while the labeled protein is coated onto the Cytodex bead surface, the calculation of actual distances on the surface is difficult. Thus, in a qualitative way it is safe to say that the distance between the SH_1 and SH_2 sites is largely unaffected by adsorption of Fn to the bead surface.

Evaluation of κ^2. In evaluation of intramolecular distances by the Förster mechanism one must estimate independently the value of the geometrical orientation, κ^2, between the donor and the acceptor pair. κ^2 is frequently assumed to be equal to 2/3; this is only valid, rigorously speaking, for donors and acceptors that randomize by rotational diffusion within the lifetime of the donor. For fluorophores that are rather rigidly attached to a macromolecule the assumption of a value of 2/3 is not valid. By measurment of the dynamic depolarization of the fluorophores in the system under investigation the range of κ^2 can be estimated (Dale & Eisinger, 1974, 1975). Nonetheless, as shown by Haas et al. (1978), even for polarizations as high as 0.4, the differences between r (the actual distance) and r' (the distance erroneously calculated by assuming κ^2 to be 2/3) are rather small. In the present system the sulfhydryl groups are known to be located in cleft-like structures and are inaccessible to solvent in the absence of chaotropic agents (Smith et al., 1982; Lai et al., 1984a). The motions of the maleimide nitroxide spin-label attached to these sulfhydryl sites are about 17 ns; in contrast to 70 ps for the same spin probe free in solution (Narasimhan & Lai, 1989a). It is conceivable that the fluorophores used in this study are strongly immobilized. Indeed, the polarization of the P probe at the SH_1 site was found to be 0.28, and those for the C probe at the SH_1 and SH_2 sites were 0.43 and 0.42, respectively (Table II). Approximating these values to 0.3 for the P probe and 0.4 for the C probe, the probable range of r and the extreme limits of r were estimated by dividing our own calculated r by the ratios of r'/r presented in Haas et al. (1978). These refined values presented in Table II do not affect any major conclusions presented in this work.

Discussion

A thorough understanding of the numerous functions of Fn requires knowledge on the spatial arrangement of its structural domains. The present study is our recent attempt in mapping the intramolecular distances between various regions of the Fn molecule by fluorescence energy transfer methods.

The data present in this study are summarized as follows: (i) The two SH_1 sites of soluble Fn are separated by about 35–44 Å, implying that the SH_1-containing regions of the two subunits of Fn are in close proximity. Neither high salt nor heparin affects this intramolecular distance. In contrast, upon adsorption of Fn onto Cytodex microcarriers, the two SH_1 sites become well separated, indicating that the surface binding induces the separation of the two subunits. (ii) The two SH_2-containing regions of Fn, on the other hand, are separated by at least 95 Å. Since the SH_2 site is located only about 30 kDa from the carboxyl terminus, if the two chains of Fn are arranged in a parallel fashion, one may expect the detection of the energy transfer between the probes at these two sites. The lack of observed energy transfer seems to be consistent with the notion that the two chains of Fn are arranged, instead, in an antiparallel fashion (Skorstengaard et al., 1986). (iii) The distance between the SH_1–SH_2 pair is 42–53 Å, which is not affected by either high salt or heparin or the surface binding.

Sedimentation velocity studies showed that Fn can undergo a marked and reversible conformational change from a compact to an extended form as affected by high ionic strength or extreme pH (Alexander et al., 1979). The molecular basis of this expansion is not known. The lack of the effect of high salt on the intersubunit distance between the two SH_1 sites indicates that the salt-induced molecular expansion does not involve these regions of the protein molecule and that the driving force for the interactions between the two subunits in these regions could be hydrophobic in nature, as predicted on the basis of analyses of the primary sequence (Rocco et al., 1987). Thus, the hydrophobic interactions between the SH_1 regions may constitute at least one of the specific noncovalent contacts between the two subunits that stabilize the compact conformation of soluble plasma Fn (Robinson & Hermans, 1984). Additionally, the absence of a high-salt effect on the intrasubunit distance between the SH_1 and SH_2 sites argues that the hydrophobic interactions between these regions may

contribute to the proper folding of each subunit. Previously, using a fluorescence energy transfer method, we showed the lack of a high-salt effect on the distance between the two amino-terminal regions that are juxtaposed under physiological conditions (Wolff & Lai, 1988). On the basis of all these results, we speculate that the collagen-binding region and/or the region covering the cell-binding and Hep-II domains are involved in the high salt induced expansion of the Fn molecule.

We demonstrated previously using electron spin resonance techniques that heparin induces a subtle conformational change of Fn to a more relaxed state (Ankel et al., 1986). The data presented here suggest that this heparin-induced conformational change does not involve either the SH_1–SH_1-containing regions or the SH_1–SH_2-containing regions of the protein. This is rather surprising, considering the close proximity of the SH_2 site to the strong heparin-binding domain near the carboxyl terminus (Hayashi & Yamada, 1982). Perhaps, the binding of heparin, a highly negatively charged molecule, does not affect the hydrophobic interactions between the SH_1–SH_2 regions.

Most of the functions of Fn are expressed when the protein interacts with a surface. This phenomenon has been termed the surface activation of plasma Fn, although the exact molecular mechanism is not yet known (McAbee & Grinnell, 1983; Schwarz & Juliano, 1984). We showed previously using a fluorescence energy transfer method that the two amino-terminal regions of Fn are separated upon binding of the protein to Cytodex 1 microcarriers (Wolff & Lai, 1989). In this study, we show that the surface binding also induces the separation of the SH_1-containing regions, but not the SH_1–SH_2 regions. In this study, Cytodex 1 microcarriers were used for all the surface-binding experiments. Studies using Cytodex 3 microcarriers (with collagen) were hampered due to the presence of nonreproducible fluorescent signals associated with the beads which overlapped substantially with the emission spectra of the probes used in this work.

The observations that the two SH_1 sites are well separated and the distance of the SH_1–SH_2 pair remains unchanged upon adsorption of Fn to Cytodex beads suggest that while the surface binding induces the separation of the two subunits of Fn, it exerts no measurable effect on the intrasubunit distance at least between the SH_1 and SH_2 sites. The similarity in the extent of the observed energy transfer of the SH_1–SH_2 pair between the labeled proteins in solution and on the surface, where the two subunits at least near the SH_1 sites are separated, seems to suggest that the measured distance between the SH_1–SH_2 pair for the protein in solution (Figure 4A) is due mainly to an intrasubunit event and has little contribution from the energy transfer between the SH_1 of one subunit and the SH_2 of the other subunit. However, the direct evidence for this claim is still lacking. This problem may be resolved by two approaches: One is to use time-resolved fluorescent measurement to see whether the donor (the P probe) experiences the presence of one or two different acceptors (the C probe) or vice versa by lifetime analyses. The other approach would be to use monomeric Fn, retaining the same folding and intactness as in the native Fn (Robinson & Hermans, 1984), to repeat the above experiments.

In summary, we demonstrate in this study that the combination of selective labeling techniques and fluorescence energy transfer methods is a useful approach to elucidate the spatial relationships of various structural domains and, thus, the high-order structure of this large glycoprotein. This type of information should be of importance in elucidating the three-dimensional structure of this important protein.

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Effect of Methylamine on the Reaction of α_2-Macroglobulin with Enzymes†

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ABSTRACT: The kinetics of reaction of α_2-macroglobulin (α_2M) with thrombin and with trypsin were studied in the presence and absence of methylamine. The rate of enzyme-induced thiol release was found to be the same whether or not amine was present. The result suggests that covalent bond formation and enzyme-catalyzed amine incorporation proceed via a common (enzyme-dependent) rate-determining step. The reaction of lysyl-modified enzymes (which show poor covalent binding with α_2M) was similarly unaffected by amine, indicating that enzyme-catalyzed steps were also rate determining for hydrolysis of the thiol ester. The products of the reactions were analyzed by native and denaturing gel electrophoresis. Methylamine did not affect the total binding of enzyme to α_2M but did cause a substantial decrease in covalent binding. Surprisingly, not all covalent complexes were affected by the presence of amine: complexes in which enzyme was covalently bound to one half-molecule increased compared to the reaction with no amine; complexes in which two half-molecules are cross-linked by two bonds to a single enzyme were substantially reduced, however. The results are consistent with a mechanism of reaction in which an enzyme-dependent step is rate determining. This step is accompanied by activation of two thiol esters. One of these reacts immediately with the bound enzyme (or may be hydrolyzed if the enzyme amine groups are blocked). The other activated center is capable of reaction with external nucleophiles such as methylamine.

The reaction of methylamine with the plasma proteinase inhibitor α_2-macroglobulin (α_2M)[1] has provided a useful tool for the understanding of the mode of action of the inhibitor with enzymes. It is now understood that methylamine inactivates α_2M by competing with the lysyl amino groups of enzymes. Binding of enzymes to α_2M is associated with the activation of internal Cys-Glu thiol esters which react with the enzyme amino groups. Although covalent binding is not required for the reaction of enzymes with α_2M, a substantial number of new Glu-Lys cross-links are formed [for reviews, see Roberts (1986) and Sottrup-Jensen (1989)]. The potential for reaction is great, and unusual complexes can form in which more than one subunit of α_2M is bound via Lys–Glu bonds to the enzyme (Wang et al., 1983, 1984). In addition to providing a clue to the identity of the site of nucleophilic reaction, methylamine may provide information about the dynamics of the enzyme reaction since it has been observed that, in the presence of enzymes, the rate of reaction is greatly enhanced. This activation of α_2M by enzymes can, in fact, allow incorporation of other amines and even other proteins (Sottrup-Jensen et al., 1981; Salvesen et al., 1981). Sottrup-Jensen et al. (1981) made the surprising observation, however, that the total enzyme incorporation was not substantially reduced when methylamine was present. They found that covalent binding was about 50% reduced, although it has been reported that covalent binding can be almost completely eliminated by treatment of α_2M with methylamine in the presence of chymotrypsin (Crews et al., 1988). To further investigate this process, we studied the effect of methylamine on the kinetics of appearance of thiols during the reaction of enzymes with α_2M. We found that the rate of thiol generation was the same in the enzyme-catalyzed methylamine incorporation as in normal reaction with enzyme, indicating that the two processes share a rate-determining step. Product distribution indicated that amine competed only with the enzyme amino group that formed the second covalent bond to α_2M.

Materials and Methods

Human α_2M was prepared by a combination of zinc chelate chromatography (Virca et al., 1978) and chromatography on Cibacron Blue–Sepharose (Kurecki et al., 1979) as described previously (Feinman et al., 1985). Human α-thrombin was the generous gift of Dr. John W. Fenton II of the New York State Department of Health, Albany, NY. Trypsin (type II) and sodium dodecyl sulfate ($NaDodSO_4$) were from Sigma. All other reagents were the highest grade commercially available. [^{14}C]Methylamine specially purified by distillation was purchased from New England Nuclear.

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[1] Abbreviations: α_2M, α_2-macroglobulin; $NaDodSO_4$, sodium dodecyl sulfate; TCA, trichloroacetic acid; DFP, diisopropyl fluorophosphate; *cis*-DDP, *cis*-dichlorodiammineplatinum(II); 4-PDS, 4,4′-dithiodipyridine.

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