Conformational Changes of Plasma Fibronectin Detected upon Adsorption to Solid Substrates: A Spin-Label Study†

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ABSTRACT: Changes in local environment of the free sulfhydryl groups in plasma fibronectin upon adsorption of the protein to polystyrene beads have been examined by electron spin resonance (ESR) spin-label spectroscopy. The two free sulfhydryl groups per subunit of plasma fibronectin were modified chemically with an [^{15}N, ^{2}H]maleimide spin-label. For soluble fibronectin, both free sulfhydryl groups are shown to be in confined environments as evidenced from the labeled protein exhibiting a strongly immobilized ESR spectrum as described previously using [^{14}N, ^{1}H]maleimide spin-labels [Lai, C.-S., & Tooney, N. M. (1984) *Arch. Biochem. Biophys. 228*, 465–473]. When the labeled protein was adsorbed to the beads, half of the strongly immobilized component was found to convert into a weakly immobilized component, a result indicating that one of the two labeled sites becomes exposed and exhibits a fast tumbling motion. Experiments conducted using various spin-labeled fibronectin fragments suggest that the newly exposed labeled site is located between the DNA-binding and the cell-binding regions of the molecule. The data obtained indicate that, upon adsorption to polystyrene beads, plasma fibronectin undergoes a conformational change through which the buried free sulfhydryl group near the cell-binding region of the molecule is exposed. This observation may have important implications regarding the expression of cell adhesive properties of the fibronectin molecule.

Plasma fibronectin (Fn), a glycoprotein present in blood plasma, has various biological activities including promoting cell adhesion, wound healing, and embryonic development (McDonagh, 1985). Many of these activities are expressed only when the protein is adsorbed onto a surface, either solid substrates in vitro or basement membranes in vivo (Akiyama & Yamada, 1987).

Polystyrene beads with defined sizes in the range of 0.1–2.0 μm are widely used solid substrates for in vitro studies of cell adhesive properties of plasma Fn (McAbee & Grinnell, 1983a; Schwarz & Juliano, 1984). It has been shown previously that plasma Fn coated on the surface of polystyrene beads binds to fibroblast cells at least 50 times stronger than does soluble Fn (Schwarz & Juliano, 1984). These observations suggest structural changes of the Fn molecule upon adsorption to a surface, although direct physical evidence for this hypothetical "surface activation" process is still lacking.

Human plasma Fn contains two free sulfhydryl groups per chain: one located between the DNA-binding and the cell-binding regions (Skorstengaard et al., 1986) (designated SH-1) and the other located in the carboxyl-terminal fibrin-binding region (Garcia-Pardo et al., 1985) (designated SH-2) (see Figure 1). Using electron spin resonance (ESR) spectroscopy and [^{14}N, ^{1}H]maleimide spin-labels, we have shown previously that these free sulfhydryl groups of plasma Fn are located in a cleft about 10.5 Å deep (Lai et al., 1984a). The use of ^{15}N, ^{2}H spin-labels has been shown to enhance spectral sensitivity and resolution because of the reduction of nuclear manifolds and of line overlap (Beth et al., 1981; Keith et al., 1974). In this paper, we have used an [^{15}N, ^{2}H]maleimide spin-label to modify the free sulfhydryl groups of plasma Fn and studied the effect of the surface binding on the local environments of these free sulfhydryl groups.

We demonstrate here that, upon surface binding, the SH-1 site is exposed and becomes very flexible while the SH-2 site remains buried; the results are consistent with our recent observation that SH-1 becomes titratable by DTNB upon binding of the protein to the polystyrene bead (Narasimhan et al., 1988). The significance of the findings in relation to the surface activation of plasma Fn is discussed.

Materials and Methods

Materials. Tris(hydroxymethyl)aminomethane (Tris) and phenylmethanesulfonyl fluoride (PMSF) were obtained from Sigma (St. Louis, MO). Polystyrene latex beads with amino or carboxyl groups on the surface were purchased from Polysciences (Warrington, PA); beads with diameters of 0.1 or 0.5 μm were used in this study. The stock concentration of the beads was 2.5% solids according to the manufacturer. The [^{15}N, ^{2}H]maleimide spin-label (see Figure 2 for chemical structure) was synthesized by Dr. Joy Joseph in our laboratory following the procedure of Griffith and McConnell (1966). The final product was pure without isomaleimide derivatives as demonstrated by infrared spectroscopy (Baratt et al., 1971). Plasma Fn was isolated from human plasma by using gelatin–Sepharose affinity chromatography (Engvall & Ruoslahti, 1977). The protein was essentially pure as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

Purification of Fn Fragments. The 34-, 185-, and 215-kDa fragments were purified following the method of Sekiguchi and Hakamori (1983), except that the separation of the 185-kDa fragment from the 215-kDa fragment was carried out at 22 °C by high-performance liquid chromatography using the Pharmacia (Uppsala, Sweden) FPLC (fast protein liquid chromatography) system equipped with a GP-250 gradient programmer as described previously (Narasimhan et al., 1988). Briefly, 0.5 mL of the fragment mixture (0.6 mg/mL) in buffer A (50 mM Tris and 0.5 mM EDTA, pH

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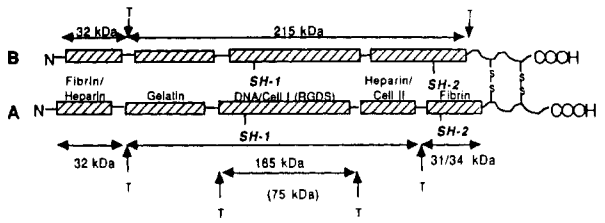

FIGURE 1: Schematic diagram depicting the trypsin cleavage sites (T) on the Fn molecule that gave rise to the fragments used in this study. The binding domains of Fn as well as the locations of the two free sulfhydryl groups, namely, SH-1 and SH-2, also are indicated (Narasimhan et al., 1988; McCarthy et al., 1986).

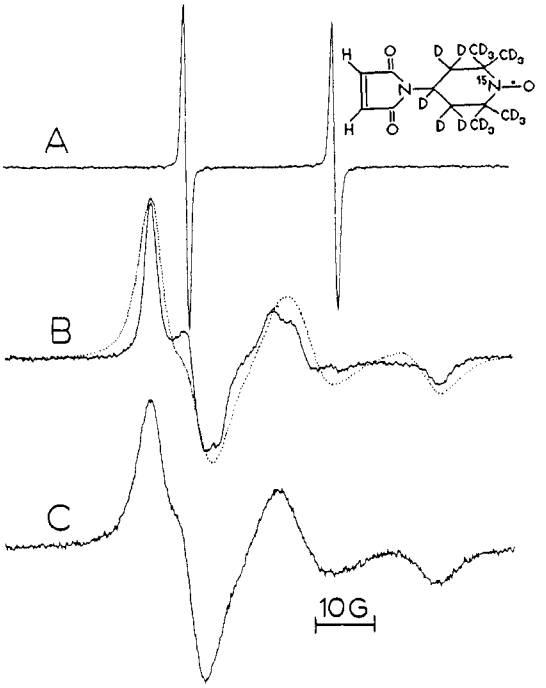

FIGURE 2: ESR spectra of the [^{15}N, ^{2}H]maleimide spin-label in various experimental conditions. (A) The free label (0.2 mM) in a buffer containing 20 mM Tris/150 mM NaCl, pH 7.4 at 22 °C. (B) Spin-labeled Fn (1.2 mg/mL) in the same buffer solution at 22 °C (solid line) and the computer-simulated nitroxide spectrum (dotted line); spectral parameters used for simulation are g_x = 2.00891, g_y = 2.00606, g_z = 2.00213, A_x = 10.56 G, A_y = 10.11 G, and A_z = 51.40 G (Pasenkiewicz-Gierula et al., 1983). Details of computer simulation are described under Materials and Methods. (C) The free label (0.25 mM) in the same buffer containing 50% glycerol; the spectrum was recorded at −33 °C. Note the spectral similarity between the simulated spectrum [dotted line in (B)] and spectrum C.

7.6) was injected onto an HR 5/10 Mono Q anion-exchange column equilibrated in buffer A. Fragments were eluted by an NaCl gradient (12.5 mM/min) at a flow rate of 1 mL/min. The fractions containing the pure 185-kDa fragment and the pure 215-kDa fragment were pooled separately, dialyzed against 20 mM Tris/150 mM NaCl, pH 7.4 (TBS), and then concentrated by using Aquacide III. The concentrations of plasma Fn and its fragments were estimated on the basis of extinction coefficients of 1.28 (intact Fn) (Mosesson et al., 1975), 1.0 (75 kDa), 1.22 (185 kDa), 1.21 (215 kDa), and 1.25 (34 kDa) mg mL^{-1} cm^{-1}, respectively; the extinction coefficients of the fragments were calculated from their respective amino acid compositions (Edelhoch, 1967). The concentrations of the fragments were also determined by using the Bio-Rad dye-binding assay with known concentrations of bovine serum albumin as a standard. For isolated fragments, the concentrations estimated by these two methods differed by about 10%. The 75-kDa and the C-terminal 31-kDa fragments of plasma Fn were kindly provided to us by Dr. James McCarthy of the University of Minnesota, Minneapolis. The origins of the fragments in plasma Fn used in this study are depicted in Figure 1.

SDS–Polyacrylamide Gel Electrophoresis (SDS–PAGE). SDS–PAGE was performed according to the method of Laemmli (1970) with 5, 8, or 12% gels depending upon the size of the fragment analyzed. Samples were reduced with 2% mercaptoethanol prior to being loaded on the gel. The apparent molecular weights of fragments were estimated by using the following proteins as markers: myosin, 200 000; *Escherichia coli* β-galactosidase, 116 250; rabbit muscle phosphorylase *b*, 97 400; bovine serum albumin, 66 000; hen egg white ovalbumin, 42 699; bovine carbonic anhydrase, 31 000; soybean trypsin inhibitor, 21 500; hen egg white lysozyme, 14 400.

Spin-Labeling of Fn and Its Fragments. Plasma Fn and its fragments were labeled with the [^{15}N, ^{2}H]maleimide spin-label, perdeuterated 1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl[^{15}N]maleimide, essentially as described by Lai and Tooney (1984); this procedure modifies selectively the free sulfhydryl groups on plasma Fn. Free sulfhydryl contents of Fn and fragments before and after spin-labeling were determined by Ellman's method as described previously (Narasimhan et al., 1988).

Preparations of Fn-Coated Beads. Fn-coated beads were prepared by the method of McAbee and Grinnell (1983a) with a slight modification: 0.2 mL of beads was incubated with Fn (0.5-mL final volume) at 37 °C for 10 min in a water bath with gentle shaking. After incubation, the mixture was washed with a buffer containing 20 mM Tris/150 mM NaCl, pH 7.4, by centrifugation. From the amount of unbound Fn in the supernatant, the amount bound to the beads was determined; Fn concentration was calculated by using the extinction coefficient at 280 nm (Mosesson et al., 1975). The Fn-coated beads remained well dispersed as examined under a microscope. Fn fragment coated beads were prepared in a similar manner. Spin-labeled Fn and spin-labeled fragment-coated beads were prepared as described above for unlabeled Fn and its fragments. No significant differences in binding affinity between spin-labeled and unlabeled Fn or fragments to polystyrene beads were detected.

ESR Measurements. ESR spectra were recorded with a Varian Century Line 9-GHz spectrometer equipped with a Varian variable-temperature accessory and a digital thermometer (Fluke 2100A). The field sweep was 100 G, and the incident microwave power was 10 mW. The modulation frequency was 100 kHz, and the modulation amplitude was 1.0 G. In some measurements, a 2.0-G modulation amplitude was used. All the spectra were recorded at 22 °C.

Simulations. The simulation program is a modified version of the program obtained from Dr. John R. Pilbrow, Monash University, Clayton, Victoria, Australia. A Monte Carlo method is used to test the goodness of spectral fitting (Pasenkiewicz-Gierula et al., 1987). Initial values of g_z and A_z are determined from the experimental spectra; g_x, g_y, A_x, and A_y are obtained after simulations of the near-rigid-limit spectra physical parameters. These are used to simulate the experimental spectra by using the program obtained from Dr. Jack Freed, Cornell University, to obtain rotational correlation times (Freed, 1976).

Results and Discussion

Rotational Dynamics of Fibronectin in Solution. The ESR spectrum of the [^{15}N, ^{2}H]maleimide spin-label free in aqueous solution is shown in Figure 2A, which is characteristic of a

small nitroxide label tumbling rapidly in solution with an effective rotational correlation time, τ_c, of 7.0×10^{-11} s. When the label was covalently attached to plasma Fn, its motion was very restricted as evidenced by the resulting strongly immobilized spectrum as shown in Figure 2B (solid line). We have shown previously that under the labeling conditions used here only the two free sulfhydryl groups per chain are labeled by the maleimide spin-label and that both labeled sites are in a cleft about 10.5 Å deep (Lai & Tooney, 1984; Lai et al., 1984a). To further characterize the rotational dynamics of spin-labeled Fn in solution, attempts were made to simulate the spectrum of soluble spin-labeled Fn (Figure 2B, solid line) using Freed's slow-motion algorithms with the assumption that the labels on the protein undergo isotropic rotational diffusion. The result is shown in Figure 2B, dotted line. The disparity of the simulated spectrum (dotted line) from the experimental spectrum (solid line) in Figure 2B indicates that the bound labels on the protein molecule may not undergo isotropic rotational diffusion. The simulated spectrum, instead, closely resembles the spectrum obtained for the same label alone in 50% glycerol at −33 °C, as shown in Figure 2C, which presumably undergoes isotropic rotational motion under these experimental conditions. Thus, it is conceivable that plasma Fn in solution may undergo anisotropic rotation.

On the basis of spectral simulation, we estimated that the spectrum of spin-labeled Fn in solution exhibits a τ_c of 17 ns; this value, however, is only qualitative rather than quantitative. On the other hand, τ_c for a molecule in an isotropic solvent can be calculated by means of the Stokes–Einstein equation as

$$\tau_c = 4\pi\eta r^3/3kT$$

where r is the hydrodynamic radius, η is the solvent viscosity (0.01 P), and π, k, and T are the usual definitions. Assuming r to be 10 nm, τ_c for soluble Fn is found to be 1.0 μs which is 59 times slower than that estimated on the basis of the computer simulation; these values are refinements of our previous estimations (Lai & Tooney, 1984). Again, the data are consistent with the notion that soluble Fn is a loosely folded globular protein with a high degree of chain flexibility (Lai & Tooney, 1984; Alexander et al., 1979; Lai et al., 1984b).

Interaction of Plasma Fn with Polystyrene Beads. Interestingly, when spin-labeled Fn was adsorbed onto polystyrene beads, the strongly immobilized ESR spectrum (Figure 2B, solid line) was changed into a composite spectrum comprising a strongly immobilized component (S) and a weakly immobilized component (W) as shown in Figure 3A. The isotropic hyperfine constants of the W component in Figure 3A and of the spectrum for the label free in aqueous solution (Figure 2A) are 24.5 and 24.8 G, respectively; the smaller isotropic hyperfine constant of the former compared to that of the latter suggests that the vicinity of the exposed label is slightly hydrophobic. The reason, however, is not clear; this could be due to either the hydrophobic nature of the cleft in which the free sulfhydryl group is located or the close proximity of the label to the hydrophobic surface of the polystyrene bead or both. Nevertheless, the fact that the surface binding enhances profoundly the cell adhesion activity of the Fn molecule [see Akiyama and Yamada (1987) for a review] seems to rule out the possibility that the observed spectral change is simply a result of a surface-induced denaturation of the Fn molecule.

To further analyze the composite spectrum in Figure 3A, it is essential to separate the W component from the S component. We found that the spectrum obtained for the label free in a solution containing 25% ethanol and 37.5% glycerol at 6 °C (see Figure 3B) with a τ_c of about 0.5 ns matches the

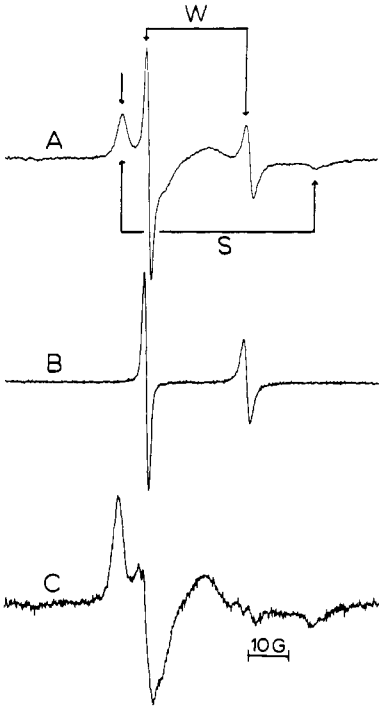

FIGURE 3: ESR spectra of spin-labeled plasma Fn on the bead. (A) Spin-labeled Fn (1.54 mg/mL) coated onto the polystyrene bead in a buffer containing 20 mM Tris/150 mM NaCl, pH 7.4 at 22 °C. (B) The free label (0.15 mM) in the same buffer as above except containing 25% ethanol and 37.5% glycerol; the spectrum was obtained at 6 °C. (C) The difference spectrum obtained by computer subtraction of spectrum B from spectrum A. The W and S components are as indicated.

Table I: Summary of A_z Values for the Spectra of Spin-Labeled Fn and Its Fragments

sample	A_z (G)[a] in solution	A_z (G)[a] on beads[b]
intact Fn	45.9 ± 0.2	45.2 ± 0.2
215 kDa	43.2 ± 0.1	46.7 ± 0.9
185 kDa	43.3 ± 0.7	45.4 ± 0.7
75 kDa	42.8 ± 0.3	46.2 ± 0.1
31 kDa	44.0 ± 0.1	45.4 ± 0.1

[a] A_z, the maximum splitting value, was measured as the separation between the extreme low- and high-field peak positions of the strongly immobilized ESR spectra as shown in Figure 3A in which the two arrows were used to connect these two peaks (the S component). [b] 0.1-μm carboxy beads.

W component in Figure 3A. Computer subtraction was carried out to subtract the spectrum in Figure 3B from the composite spectrum in Figure 3A, and the resulting difference spectrum is shown in Figure 3C, which is the remaining S component of the spectrum for the labeled protein adsorbed onto the bead. The spectral characteristics of the remaining S component (Figure 3C) are similar to, but not identical with, those for the labeled protein in solution (Figure 2B). For example, as shown in Table I, the A_z value of the former is smaller than that of the latter by 0.7 G, suggesting that the local environment of the remaining S component becomes even more flexible upon surface binding. Thus, the effect of the surface binding on Fn conformation appears to extend to various regions of the protein molecule.

Double integration of the spectra showed that the relative signal intensities of the spectra for the labeled Fn bound to the bead (Figure 3A) and for the label free in a mixture of aqueous and ethanolic solution (Figure 3B) were 1.98 and 1.0, respectively. This indicates that when the labeled protein was coated onto the bead, about half of the labeled sites was

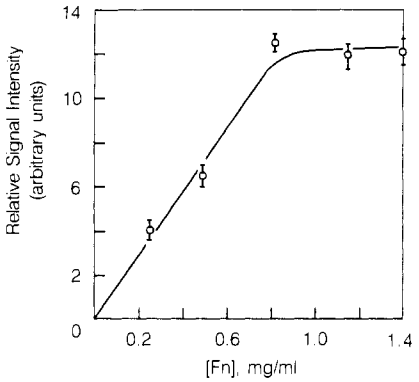

FIGURE 4: Titration profile of spin-labeled Fn bound to polystyrene beads. The relative signal intensities of bound spin-labeled Fn were plotted against various concentrations of added spin-labeled Fn; the number of beads was kept constant at 9.1×10^{12}. The Fn-coated bead was prepared as described under Materials and Methods. After washing to remove unbound protein, Fn-coated beads were placed in a 50-μL ESR flat cell. The relative signal intensities were obtained by measuring the peak height of the low-field strongly immobilized component (see the arrow shown in Figure 3A).

converted from an S component into a W component, which is in accord with our previous observations that, upon binding to polystyrene beads, 0.87 ± 0.05 sulfhydryl group per chain becomes titratable by DTNB (Narasimhan et al., 1988). Accurate quantitation of the remaining S component (Figure 3C) was not possible because of its poor signal-to-noise ratio.

Titration of spin-labeled Fn bound to the beads was shown in Figure 4 in which the relative signal intensities of bound Fn were plotted as a function of the concentration of spin-labeled Fn. As shown in Figure 4, the binding of spin-labeled Fn to the beads is saturable; this process, however, is not reversible as evidenced from our inability to remove the bound protein using a variety of denaturants including SDS, urea, and guanidine hydrochloride as described previously by other investigators (Grinnell & Feld, 1981; Iwamoto et al., 1985; Jonsson et al., 1982). To ensure direct contact of spin-labeled plasma Fn with the surface, the concentrations of the labeled protein used in all ESR experiments reported in this study were within the initial linear region in Figure 4.

Identification of the Labeled Sites. Since Fn contains two free sulfhydryl groups per chain (see Figure 1 for their relative locations in the molecule), the question arises as to whether SH-1 or SH-2 is responsible for the appearance of the W component upon adsorption of the labeled protein onto the bead as shown in Figure 3A.

If the bound protein could be recovered from the bead surface, the most direct approach to assign the position of the newly exposed free sulfhydryl group would have been first to label the free sulfhydryl groups with a radiolabeled sulfhydryl reagent and then identify the labeled fragment using a gel electrophoresis technique. However, the tight binding of the protein to polystyrene beads precludes such a possibility. To answer the question on which one of the two thiol groups is exposed upon adsorption, we have prepared spin-labeled Fn fragments including 215, 185, 75, and 31 kDa and studied their free sulfhydryl environment(s) upon interactions with polystyrene beads using ESR spectroscopy.

Panel A in Figure 5 shows the ESR spectra of spin-labeled Fn fragments of 215, 185, 75, and 31 kDa in solution. Partial exposure of the labeled site(s) on the fragments is noticeable as indicated by the appearance of the W component in all the spin-labeled fragments in solution (panel A in Figure 5). Since the procedure for labeling the fragments with the maleimide spin-label was identical with that for labeling the intact

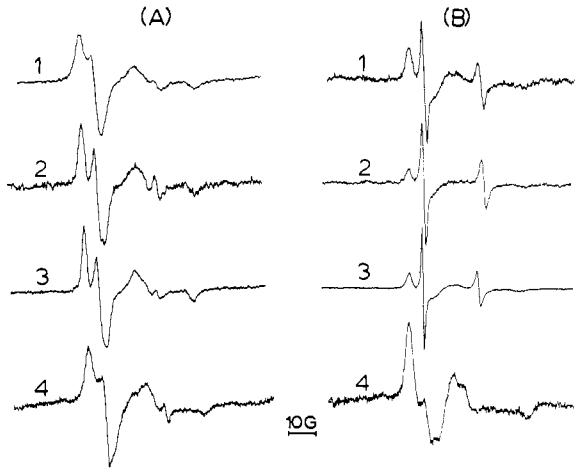

FIGURE 5: ESR spectra of spin-labeled Fn fragments in solution and on beads. Fn fragments were prepared, modified with the spin-label reagent, and adsorbed on polystyrene beads as described under Materials and Methods. (A) In solution: (1) 215 kDa (0.83 mg/mL); (2) 185 kDa (0.42 mg/mL); (3) 75 kDa (0.9 mg/mL); (4) 31 kDa (0.37 mg/mL). (B) On beads: (1) 215 kDa (0.68 mg/mL); (2) 185 kDa (0.45 mg/mL); (3) 75 kDa (0.87 mg/mL); (4) 31 kDa (0.20 mg/mL). The spectra were obtained as described under Materials and Methods.

molecule, the reason for the presence of the small proportion of the W component in the spin-labeled fragments may be attributed at least in part to the partial unfolding of the fragments under these experimental conditions. Nevertheless, it is of interest that the spectra for the spin-labeled 185- and 75-kDa fragments, each of which contains only an SH-1 site, have a higher percentage of the W component (between 10 and 20% of the total signal intensity), while the spectra for the spin-labeled 215-kDa fragment containing both SH-1 and SH-2, and the 31-kDa fragment containing only SH-2, show a smaller percentage of the W component (<5%). This seems to suggest that the SH-1 site in the isolated fragments is partially unfolded. However, apart from the differences in the extent of the W component, the spectral characteristics of all spin-labeled fragments in solution including the A_z values (Table I) and their line shapes are remarkably similar, suggesting that the bound labels in various fragments undergo similar rates of rotational motion. Since the fragments differ greatly in size ranging from 215 to 31 kDa, the similarity in rotational motion for all labeled fragments examined here suggests weak domain–domain interactions within the 215-kDa fragment region. If there were strong interactions between various regions within the 215-kDa fragment, the A_z values would have been decreased with the decrease in the fragment size. Additionally, for the labeled fragments in solution, the spectra showed that the labels bound to the fragments remain strongly immobilized, suggesting that isolated binding domains of Fn retain their compactness and tight folding (Skorstengaard et al., 1986) and that the local environments of the labels on SH-1 and SH-2 are similar and indistinguishable by ESR methods as suggested previously (Lai et al., 1984a; Lai & Tooney, 1984).

When spin-labeled Fn fragments were adsorbed onto the beads, the spectra changed drastically as shown in panel B of Figure 5. The spectrum of the spin-labeled 215-kDa fragment changed in a manner similar to that of the labeled intact molecule; about half of the S component was converted into the W component. On the other hand, while the majority of the signal amplitude in the spectra of spin-labeled 185-kDa and 75-kDa fragments, each of which contains only SH-1, was converted from the S component into the W component, no

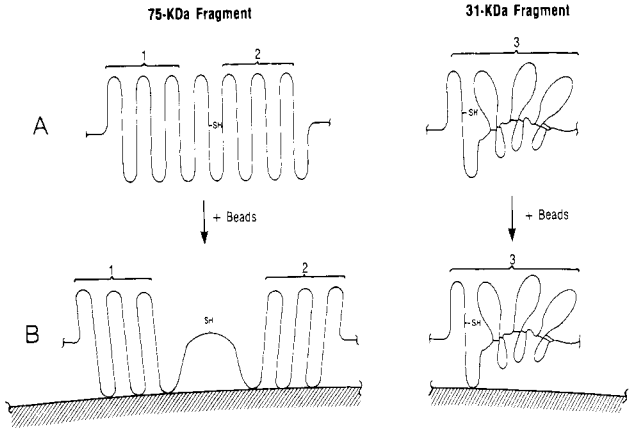

FIGURE 6: Schematic model for the two free sulfhydryl environments of plasma Fn in solution (A) and on the surface (B). (1) DNA-binding region; (2) cell-binding region; (3) carboxyl-terminal 31-kDa fibrin-binding region (Petersen & Skorstengaard, 1985).

such spectral conversion was seen for the spin-labeled 31-kDa fragment which contains only SH-2. It is thus probable that the label on the SH-1 site is responsible for the observed spectral conversion as shown in Figure 3A for spin-labeled intact plasma Fn adsorbed on the beads. (Exact quantitation of the percentage of the W and S components in some of these spectra, however, was rather difficult because of the poor signal-to-noise ratios.) The presence of the remaining minor S components in the spectra for the spin-labeled 75-kDa and 185-kDa fragments upon binding to the bead suggests that not all SH-1 sites in these two fragments are exposed upon binding to the bead. Perhaps the fragmentation generates new sites for the surface interaction which are absent in intact Fn, and/or the interactions of the isolated fragments with the surface are slightly different from those of the intact molecule. It is, however, of interest to note that when adsorbed to the bead, the A_z values of all spin-labeled fragments were increased to a value similar to that of intact Fn (Table I), suggesting a further immobilization of fragments coated onto the bead. A schematic diagram shown in Figure 6 illustrates the differential exposure of the free sulfhydryl groups in plasma Fn upon adsorption of the protein to solid substrates.

As discussed previously, the differential exposure of the free sulfhydryl groups in plasma Fn upon adsorption to solid substrates may be related to the positions of the free sulfhydryl groups in the protein molecule (Narasimhan et al., 1988). Although SH-1 and SH-2 are both located in type III homologous units, SH-1 is situated in an interdomainal type III unit between the DNA-binding and the cell-binding domains, while SH-2 is located in a type III unit which is part of the fibrin-binding domain (Skorstengaard et al., 1986; Kornblihtt et al., 1985). It is known that the DNA-binding, cell-binding, and fibrin-binding domains are tightly folded, stable structures and are resistant to further proteolytic digestion and that the interdomainal region is more flexible and more susceptible to proteolytic attack (Skorstengaard et al., 1986). This may explain why upon surface binding, SH-1 located in an interdomainal region is exposed, while SH-2 located within a binding domain is not.

Polystyrene beads used in this study consist mainly of phenyl groups which are highly hydrophobic. It is expected that the driving force for the interactions between plasma Fn or its fragments with the surface of polystyrene beads must be hydrophobic in nature. The fact that all fragments examined in this study bind to the bead suggests that plasma Fn contains various hydrophobic regions capable of interacting directly with the hydrophobic surfaces. Using hydrophobic column chromatography, Morgenthaler suggested previously that plasma Fn possesses at least one hydrophobic binding site (Morgenthaler, 1982). It is likely that the hydrophobic binding sites responsible for the surface binding are present in intact Fn as well as in isolated fragments, although the possibility that some of the hydrophobic regions may be exposed only after proteolysis cannot be ruled out at present. Judging from the drastic structural change in SH-1 site upon surface binding, we speculate that at least one hydrophobic site may be located near the DNA-binding and the cell-binding regions.

There are two prevailing models for explaining the function of the fibronectin cell-binding domain: one stresses the role of the conformation of the Arg-Gly-Asp cell-binding sequence (Pierschbacher & Ruoslahti, 1987), and the other postulates two distinct binding sites (Akiyama & Yamada, 1987). Recently, using site-directed mutagenesis techniques, Obara et al. (1988) demonstrated that there are two separate, synergistic sites existed within the 75-kDa fragment of the cell-binding domain of human Fn: one being the Arg-Gly-Asp sequence and the other probably located in the region between type III units 7 and 8 of the Fn molecule. It is worthy of note that SH-1 (residue 1201) is located in type III unit 7 (residues 1142–1234) of the molecule (McDonagh, 1985). On the basis of the observed increase in exposure and flexibility of the SH-1 site upon surface binding, we postulate that type III unit 7 undergoes a drastic conformational change upon adsorption to a surface (see Figure 6), which may be at least in part the structural basis for the surface activation of the Fn molecule.

In summary, we demonstrate in this paper that the surface binding induces a conformational change of plasma Fn as indicated by the exposure of buried SH-1. This conclusion is consistent with our previous observations that SH-1 becomes accessible to the DTNB reagent upon adsorption of plasma Fn to polystyrene beads (Narasimhan et al., 1988). Thus, both chemical reactivity and the physical environment of SH-1 in plasma Fn are changed drastically upon adsorption of the protein to the surface, while those for SH-2 remain relatively unchanged.

The function of the free sulfhydryl groups in Fn is not known at present. We have shown previously that the free sulfhydryl groups in Fn are not required for initial cell attachment and spreading (Lai & Tooney, 1984). It is tempting to speculate that owing to its increased accessibility upon surface binding, the exposed SH-1 may serve as an additional cell-binding site within the 75-kDa fragment through disulfide formation with other cell surface molecules at later stages after initial cell attachment.

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Ionic Strength Dependence of Cytochrome *c* Structure and Trp-59 H/D Exchange from Ultraviolet Resonance Raman Spectroscopy†

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ABSTRACT: Ultraviolet resonance Raman spectra are reported for cytochrome *c* (cyt *c*) in Fe^{II} and Fe^{III} oxidation states at low (0.005 M) and high (0.9-1.5 M) ionic strength. With 200-nm excitation the amide band intensities are shown to remain constant, establishing that redox state and ionic strength have no influence on the α-helical content. The tyrosine 830/850-cm^{-1} doublet, however, shows a loss in 830-cm^{-1} intensity at $I = 0.005$ M for the Fe^{III} protein, suggesting a weakening or a loss of H-bonding from an internal tyrosine, probably Tyr-48, which is H-bonded to a heme propionate group in cyt *c* crystals. Excitation profiles of tryptophan peak at ~229 nm for both Fe^{II} and Fe^{III} forms of cyt *c*, but at ~218 nm for aqueous tryptophan. The ~2200-cm^{-1} red shift of the resonant electronic transition is attributed to the Trp-59 residue being buried and H-bonded. Consistent with this Trp environment, the H-bond-sensitive 877-cm^{-1} Trp band is strong and sharp, and the 1357/1341-cm^{-1} doublet has a large intensity ratio, ~1.5, for both Fe^{II} and Fe^{III} cyt *c*. The 877-cm^{-1}-band frequency shifts to 860 cm^{-1} when the Trp indole proton is replaced by a deuteron. This band was used to show that Trp H/D exchange in D_2O is much faster for Fe^{III} than Fe^{II} cyt *c*. The half-time for exchange at room temperature is estimated to be ~30 and ~5 h, respectively, for Fe^{II} and Fe^{III} when examined at $I = 0.005$. Increasing the ionic strength to 1.5 M, however, raises the half-time to ~30 h for Fe^{III} cyt *c* and to a much larger value for the Fe^{II} cyt *c*. This variation in the protein dynamics is consistent with recent evidence that the radius of gyration of the Fe^{III} protein increases with decreasing ionic strength (Trewhella et al., 1988).

It is now well recognized that proteins are not rigid arrays of atoms whose positions are frozen at their crystallographically derived coordinates. Proteins may undergo a variety of internal motions of a character that is not always evident from an examination of the crystal structure. A clear illustration is offered by cytochrome *c* (cyt *c*), whose crystallographically determined tertiary structure is essentially the same for the

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