# Structure and Flexibility of Plasma Fibronectin in Solution: Electron Spin Resonance Spin-Label, Circular Dichroism, and Sedimentation Studies†

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ABSTRACT: Human plasma fibronectin has been investigated by electron spin resonance (ESR) spin-label methods in conjunction with circular dichroism (CD) and sedimentation techniques to investigate its structure and flexibility in solution. The buried sulfhydryl groups of fibronectin were modified with a maleimide spin-label [Lai, C.-S., & Tooney, N. M. (1984) *Arch. Biochem. Biophys. 228*, 465–473]. Both conventional and saturation transfer ESR spectra give a rotational correlation time of about (2–3) × $10^{-8}$ s for plasma fibronectin, a value that is at least 40 times faster than the rotational correlation time calculated from the minimal molecular dimensions. This argues that plasma fibronectin is not a compact, globular protein and suggests that the regions of ordered structural domains have a relatively high degree of independent mobility. ESR, CD, and sedimentation measurements showed that many structural features of plasma fibronectin remain unchanged when the pH is decreased from 7.4 to 3.0. On the other hand, ESR results indicate an unfolding of the protein molecule either at pH 11 or in 4 M urea solution. Similarly, the sedimentation coefficient decreases from about 13 to 8.4 S when the pH is raised to 10.8. At pH values above 11, the CD spectrum resembles a random coil; however, some ordered structure is retained either at pH 11 or in 4 M urea. It is likely that the sulfhydryl-containing regions of the molecule are more sensitive to urea or alkali than are portions of the molecule stabilized by intrachain disulfide bonds. Data obtained from all three experimental methods indicate that the protein is more expanded at physiological pH and high ionic strength (≥0.5) but retains its major regions of tertiary structure. These studies indicate that the correlation of ESR, CD, and ultracentrifugal methods provides an effective way to probe the structure, shape, and flexibility of protein molecules.

Plasma fibronectin is a high molecular weight glycoprotein present in blood plasma at levels of 300 mg/L (Mosesson & Umfleet, 1970). It is composed of two chains of about equal size linked near the carboxyl terminus by disulfide bridges. In vivo the protein appears to play important roles in blood coagulation, opsonization, differentiation, and embryonic development [see Hynes & Yamada (1982) and Yamada (1983) for reviews]. In vitro it mediates attachment and spreading of cultured mammalian cells on plastic or collagen-coated surfaces (Hughes et al., 1979; Lai et al., 1984).

The molecular shape of plasma fibronectin has been studied in solution by circular dichroism (CD), ultracentrifugation, and light scattering methods (Mosesson et al., 1975; Alexander et al., 1979; Odermatt et al., 1982; Williams et al., 1982). Studies by Alexander et al. (1979) and by Mosesson et al. (1975) showed that the molecule has an anomalous far-UV CD spectrum with little secondary structure. The sedimentation coefficient of the dimeric protien is 12–13 S at pH 7 and ionic strength 0.15 (Mosesson et al., 1975). The sedimentation constant fell linearly with increasing pH or ionic strength to a value of 8 at pH 11 with a modest change in CD spectra (Alexander et al., 1979). The molecule was therefore presumed to have both compact globular and flexible regions. The multidomain structure for plasma fibronectin as determined by CD and sedimentation methods is consistent with biochemical evidence that plasma fibronectin consists of separate protease-resistant functional domains [see Yamada (1983) for a review].

Previously we reported the selective modification of the free sulfhydryl groups of plasma fibronectin with a maleimide spin-label (Lai & Tooney, 1984). The electron spin resonance (ESR) spectrum of spin-labeled fibronectin showed that the labels are highly immobilized, suggesting that the free sulfhydryl groups of the protien are in small, confined environments. This modification of the free sulfhydryl groups in plasma fibronectin did not affect its structural integrity (CD and sedimentation studies) or biological activities (cell adhesion assay) (Lai & Tooney, 1984). The incorporated maleimide spin-label probe therefore serves as a reporter group to provide information on motional properties of fibronectin molecule in solution.

We report here the solution properties of human plasma fibronectin as determined by ESR spin-label, CD, and sedimentation methods. The combination of these complimentary techniques permits a powerful approach to study factors affecting the structure and flexibility of plasma fibronectin in solution.

## Materials and Methods

*Materials.* 2-[[Tris(hydroxymethyl)methyl]amino]-ethanesulfonic acid (TES), tris(hydroxymethyl)aminomethane (Tris), 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), phenylmethanesulfonyl fluoride (PMSF), gelatin, and glycine were obtained from Sigma (St. Louis, MO). Gold-label reagent-grade urea and 4-maleimido-2,2,6,6-tetramethylpiperidinyl-1-oxy (maleimide spin-label) were purchased from Aldrich (Milwaukee, WI). Immersible CX-30 for concentrating protein solutions was obtained from Millipore (Bedford, MA).

Plasma fibronectin was isolated from human plasma by using gelatin–Sepharose 4B affinity chromatography as described previously (Engvall & Ruoslahti, 1977). Some purified plasma fibronectin prepared by using the same method was a gift of Dr. Gene Homandberg of Mount Sinai Medical Center, Milwaukee. Both preparations were essentially pure

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as determined by 7% sodium dodecyl sulfate (SDS)–polyacylamide gel electrophoresis.

*Spin-Labeling of Fibronectin.* Plasma fibronectin was modified with maleimide spin-label in the presence of 6 M urea essentially as described previously (Lai & Tooney, 1984). PMSF, a protease inhibitor ($1 \times 10^{-4}$ M) was added to all the solutions during protein purification and spin-labeling procedures.

*CD Measurements.* A Jasco 500A circular dichroism spectrometer with DP 501N data processor was used to record CD spectra. Sample temperature was controlled by using a Neslab Model RTE-5 refrigerated circulating bath and a 2-mm jacketed cell. Temperatures were monitored in the cell by using a YSI 43 electronic thermometer and probe. Ellipticity values were calculated by using a mean residue weight of 106 (average of two literature values: Mosesson et al., 1975; Alexander et al., 1979). Fibronectin samples were prepared in 0.02 M TES buffer solution containing 0.15 M NaCl, pH 7.4. TES, Tris, CAPS, and sodium phosphate were used as buffers, depending on the pH selected. Measurements carried out in the presence of urea were performed after dialyzing fibronectin for several hours against 0.02 M TES buffer, pH 7.4, containing 0.15 M NaCl and freshly deionized urea at the desired concentration.

*Ultracentrifuge Measurements.* A Spinco Model E analytical ultracentrifuge was used with double-sector cells to determine sedimentation coefficients.

*ESR Measurements.* ESR spectra were obtained with a Varian Century Line 9-GHz spectrometer equipped with a Varian variable-temperature accessory and a digital thermometer (Fluke 2100A). For conventional ESR, 100-kHz field modulation was used and the incident microwave power was 10 mW. The field sweep was 100 G and the modulation amplitude was 2.0 G. In some measurements, a 5-G modulation amplitude was employed to determine the maximum splitting. For saturation transfer ESR experiments, field modulation at 50 kHz with 90° out-of-phase detection at 100 kHz was used. In this $V'_2$ display, the field sweep was 100 G, the modulation amplitude 4.0 G, and the microwave power 63 mW.

The effective rotational correlation time in the fast tumbling time regime ($10^{-11}$–$10^{-9}$ s) was determined by using the equation given by Stone et al. (1965). $1/\tau = 3.6 \times 10^9/\Delta H_0[(H_0/H_{-1})^{1/2} - 1]$ s$^{-1}$ where $\Delta H_0$ is the peak-to-peak width of the central-field line in gauss and $H_0$ and $H_{-1}$ are peak-to-peak heights of the central-filed and high-field lines, respectively. The effective rotational correlation time in the slow tumbling regime ($10^{-9}$–$10^{-7}$ s) was estimated by comparing the conventional ESR spectra with simulated spectra (Freed, 1976). For saturation transfer ESR, Thomas et al.'s data were used to determine the effective rotational correlation time (Thomas et al., 1976).

## Results

The ESR spectrum of plasma fibronectin modified with maleimide spin-label, as shown in Figure 1, spectrum 1, has previously been characterized (Lai & Tooney, 1984). Only the free sulfhydryl groups of plasma fibronectin were found to be modified by the label under the experimental conditions with a stoichiometry of 2.8 labels to one dimeric protein molecule. The rotational correlation time estimated from the ESR spectrum of spin-labeled fibronectin at 21 °C was about $2 \times 10^{-8}$ s (Lai & Tooney, 1984). Figure 2 shows the saturation transfer ESR spectrum of spin-labeled fibronectin at 22 °C. By comparison with the calibrated ST-ESR spectra provided by Thomas et al. (1976), it resembles a spectrum with

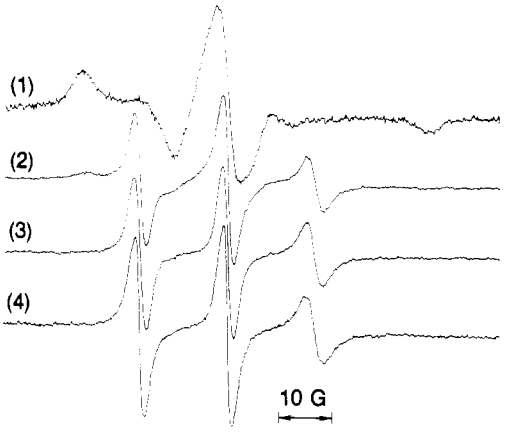


FIGURE 1: Effects of urea on the ESR spectrum of plasma fibronectin modified with maleimide spin-label. The fibronectin concentration was about $5.2 \times 10^{-6}$ M in 0.02 M TES buffer containing 0.15 M NaCl, pH 7.4. (1) Control; (2) 2 M urea; (3) 4 M urea; (4) 6 M urea. The control spectrum shows a strongly immobilized signal that is converted into a weakly immobilized signal with increasing urea concentration, indicating that the molecule becomes unfolded.

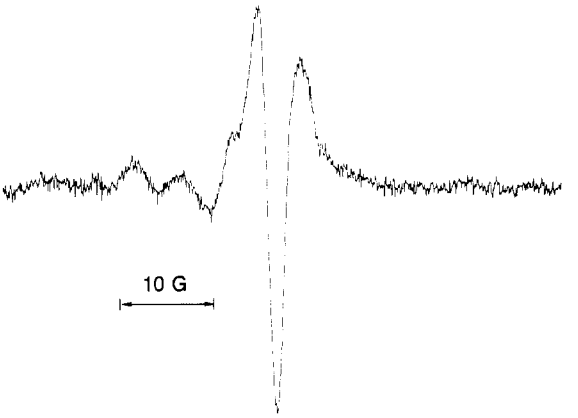


FIGURE 2: Saturation transfer ESR spectrum of plasma fibronectin modified with maleimide spin-label. The protein concentration was about $5.0 \times 10^{-6}$ M in the buffer described in Figure 1. Measurements were carried out at ambient temperature.

a rotational correlation time of about (2–3) $\times 10^{-8}$ s, which is in good agreement with the results obtained from conventional ESR studies (Lai & Tooney, 1984). Assuming a rigid, globular shape for plasma fibronectin with a Stokes radius of 10 nm, the rotational correlation time calculated from the Stokes–Einstein equation was about $7.8 \times 10^{-7}$ s (Lai & Tooney, 1984). The factor of at least 40 difference in rotational correlation time between experimental and calculated values indicates that plasma fibronectin tumbles about 40 times faster than the estimated value based on its minimal molecular dimensions. This suggests that plasma fibronectin is not a rigid, globular protein and has a significant degree of chain flexibility.

*Effects of Urea.* The free sulfhydryl groups of plasma fibronectin are buried and can be titrated with DTNB only in the presence of 6 M urea or 3 M guanidine (Smith et al., 1982). Previously we showed that 6 M urea was necessary for the modification of plasma fibronectin with maleimide spin-label (Lai & Tooney, 1984). These findings imply that urea unfolds the protein molecule to expose the buried free sulfhydryl groups. Figure 1 demonstrates that this in fact is the case. The ESR spectrum of spin-labeled fibronectin measured in the absence of urea (Figure 1, spectrum 1) showed that the sites of labeling were strongly immobilized, suggesting that the sulfhydryl groups are in small, confined environments

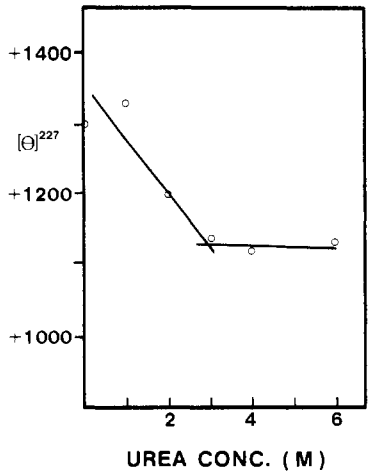


FIGURE 3: Effects of urea on the far-UV positive ellipticity band of fibronectin. Protein concentrations were in the range of 0.3–1 mg/mL. Measurements were carried out in a 0.5 mm path length cell at ambient temperature.

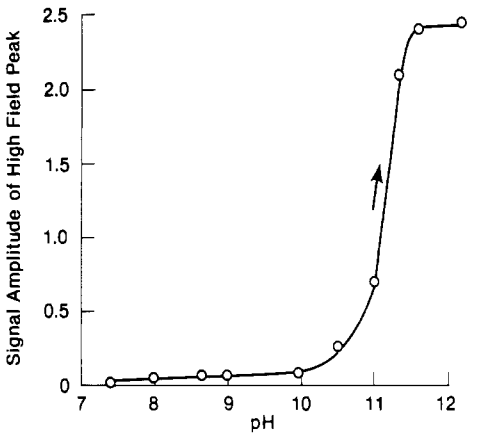


FIGURE 4: Effects of pH on the signal amplitude of the high-field peak of the ESR spectra of plasma fibronectin modified with maleimide spin-label. Plasma fibronectin modified with maleimide spin-label in 0.02 M TES–0.02 M CAPS buffer and 0.15 M NaCl, pH 7.4, was titrated to alkaline pH by dropwise additions of 1.0 N NaOH. The ESR measurements were carried out at the pH points as indicated on the abscissa.

or simply buried inside the molecule. The addition of 2 M urea converted the strongly immobilized component into a weakly immobilized component (Figure 1, spectrum 2), suggesting the exposure of buried sulfhydryl groups due to protein unfolding. This process reached a plateau at 4 M urea (Figure 1, spectrum 3).

The rotational correlation time of spin-labeled fibronectin estimated from the spectrum in 6 M urea (Figure 1, spectrum 4) was about $5.2 \times 10^{-10}$ s based on the ESR line narrowing formula (Stone et al., 1965). The factor of nearly 40 increase from $(2–3) \times 10^{-8}$ to $5.2 \times 10^{-10}$ s in rotational correlation time due to urea effects clearly demonstrated that urea unfolds plasma fibronectin and thus exposes the buried sulfhydryl groups.

Mosesson et al. (1975) reported that the sedimentation coefficient of fibronectin drops from 13.5 to 5.6 S in 0.1 M Tris–phosphate buffer, pH 7.0, ionic strength 0.18 in the presence of 7 M urea, indicating an unfolding of the molecule. The effects of increasing urea concentration on the amplitude of the 227-nm CD band of unmodified fibronectin are shown in Figure 3. (The presence of urea made it difficult to carry out measurements below 220 nm). The ellipticity decreases from about +1300 to nearly +1100 between 3 and 4 M urea concentration. Little change is seen when urea concentration is increased from 3 to 6 M (Figure 3). However, urea at concentrations up to 6 M does not completely denature fibronectin as measured by changes in the 227-nm band. The decrease is only in the range of 15–20%. It is likely that both urea unfolding and disruption of disulfide bridges are required to cause major perturbation of the near-UV CD spectrum and of the far-UV CD spectrum.

*Effects of pH.* Plasma fibronectin modified with maleimide spin-label at pH 7.4 was dialyzed against buffer solutions at physiological ionic strength ranging from pH 3 to pH 11. At neutral pH, the ESR spectrum showed that the labels on the protein were highly immobilized. At pH 3.1, the rotational motion of the protein resembles that of the protein at neutral pH. At alkaline pH, however, the strongly immobilized ESR spectrum was converted into a weakly immobilized spectrum. At pH 10.9, nearly all strongly immobilized components were changed into a weakly immobilized component, similar to the spectrum shown in Figure 1, spectrum 4. A plot of signal amplitude of the high-field peak vs. pH, Figure 4, revealed a midpoint pH of protein unfolding at about pH 11.2. The data suggest that plasma fibronectin appears to be unfolded above pH 11. Table I shows the effect on sedimentation coefficient of raising the pH from 7.4 to 10.8 at near-physiological ionic strength. The values of 13.0 and 12.7 S observed at 20 °C for pH 7.4 and pH 9.2 buffers, respectively, are not significantly different. However, when the pH is raised to 10.8, the sedimentation coefficient decreases to 8.4 and the peak becomes hypersharp. We were not able to extend these measurements to higher pH values. In contrast, lowering the pH to 3.1 at an ionic strength of 0.15 (see Table I) had a more modest effect on the $s_{20,w}$, reducing it to 10.3. The shape of the far-UV CD spectrum is markedly affected by raising the pH. Figure 5a shows spectra obtained at pH 7.4 (curve 1) and pH 12 (curve 2) at an ionic strength of about 0.17. At pH 12, the prominent band at 227 nm is completely abolished. Figure 5b shows that the ellipticity changes from pH 7.4 to about pH 10.8 are relatively small, but a dramatic change occurs when the pH is increased from 10.8 to 12. A "break" in the shape occurs near pH 11.5. Similar results are observed for ellipticity changes at 212 nm in that almost no change occurs until a pH of above 11 is reached (Figure 5c). As pH is increased from 11 to 12, the negative ellipticity of this band nearly doubles in magnitude. These data suggest that the conformation of the molecule is relatively unperturbed until pH is raised to above 11; then the spectrum rapidly changes to qualitatively resemble a random coil. We have noted that samples taken to pH 10.8 and then dialyzed back into pH 7.4 buffer regenerate the original pH 7.4 spectrum. However, irreversible effects were observed above pH 11.

Table I: Effects of Solution Conditions on Sedimentation Coefficients[a]

| buffer | pH | ionic strength | $s_{20,w}$ |
|---|---|---|---|
| 0.15 M NaCl, 0.02 M TES | 7.4 | 0.17 | 13.0 |
| 0.15 M NaCl, 0.02 M HOAc | 3.1 | 0.15 | 10.3 |
| 0.15 M NaCl, 0.02 M Tris | 9.2 | 0.17 | 12.7 |
| 0.15 M NaCl, 0.02 M CAPS | 10.8 | 0.17 | 8.4 |
| 0.75 M NaCl, 0.02 M TES | 7.4 | 0.78 | 8.1 |

[a] Fibronectin samples were prepared in 0.02 M TES buffer solution containing 0.15 M NaCl, pH 7.4, and dialyzed against different buffer solutions, depending on the pH selected, for 4 h prior to measurement at ambient temperature.

*Effects of Ionic Strength.* When plasma fibronectin modified with maleimide spin-label was dialyzed against buffer solution at pH 7.4 of varying ionic strengths, the maximum splitting in gauss of the ESR spectrum of spin-labeled fibro-

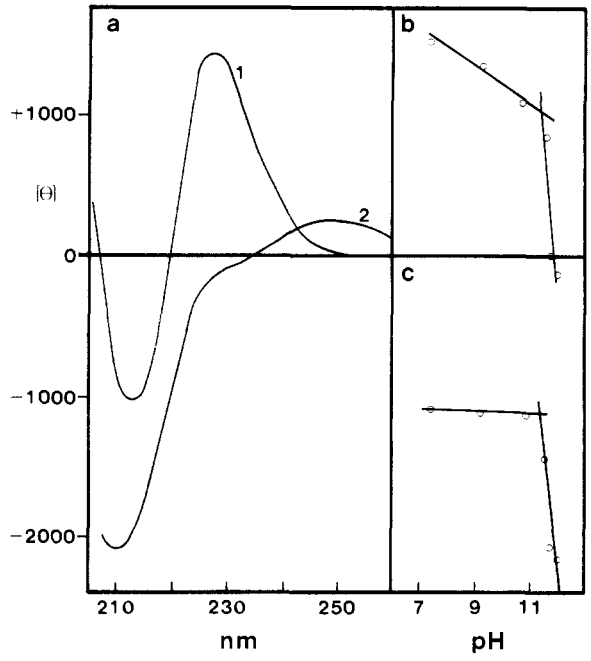


FIGURE 5: Effects of pH on the far-UV CD spectrum of fibronectin. Conditions as described in Figure 3 except that plasma fibronectin was in 0.02 M TES–0.02 M CAPS buffer and 0.15 M NaCl, pH 7.4, and was titrated to alkaline pH by dropwise additions of 1.0 N NaOH. Panel a: curve 1, spectrum measured at pH 7.4; curve 2, spectrum measured at pH 12. Panel b: ordinate the same as in panel a; change in ellipticity at 227 nm as a function of pH. Panel c: ordinate the same as in panel a; change in ellipticity of the 212-nm band as a function of pH.

nectin was found to decrease with increasing ionic strength; the maximum splitting changed from 66.5 to 65.3 G when the salt concentration changed from 0.15 to 0.75 M. However, there is no appearance of a weakly immobilized component, in contrast to the changes observed upon raising the pH.

High ionic strength conditions also have a modest effect on the sedimentation coefficient (see Table I). For example, in 0.02 M TES buffer, pH 7.4, raising the salt concentration from 0.15 to 0.75 M NaCl results in a reduction of the $S_{20,w}$ value from about 13 to 8.1 S. This difference may indicate a change in molecular shape or hydration. In a preliminary CD experiment (data not shown), the sample in high-salt conditions had an ellipticity value of about +1570, similar to that seen at physiological ionic strength at low temperature. That is, the effects of high pH and high salt on the far-UV CD spectrum differ.

## Discussion

Human plasma fibronectin appears to contain one to two free sulfhydryl groups per monomer in two different sites, one being about 170 kilodaltons from the amino terminus and the other in the carboxyl-terminal, fibrin-binding domain (McDonald et al., 1980; Wagner & Hynes, 1980; Smith et al., 1982; Sekiguchi & Hakomari, 1982). The function of these free sulfhydryls in the protein is still not certain (Yamada, 1983). Recent amino acid sequence work indicates that plasma fibronectin contains three different types (I–III) of internal homology (Pierschbacher et al., 1982; Peterson et al., 1983). Vibe-Pederson et al. (1982) reported a type III homologous sequence containing a free sulfhydryl group in a 170-kilodalton fragment isolated from bovine plasma fibronectin and Schwarzbauer et al. (1983) found a type III homologous sequence containing a free sulfhydryl group near the C-terminal region of rat fibronectin. It is therefore likely that both free sulfhydryls are in type III homology regions.

The modification of buried sulfhydryl groups with maleimide spin-label gives rise to an ESR spectrum showing strong immobilization of the sites of labeling as indicated by maximum spitting values of 66.5 G at 22 °C (Figure 1, spectrum 1). This suggests the presence of regions of compact globular structure in the protein. Both conventional (Figure 1, spectrum 1) and saturation transfer (Figure 2) ESR studies show a rotational correlation time of about (2–3) $\times 10^{-8}$ s for spin-labeled plasm fibronectin; in contrast, the rotational correlation time calculated from the minimal dimensions of the protein is about $7.8 \times 10^{-7}$ s. The difference between experimental and calculated values suggests that plasma fibronectin under physiological conditions is not a rigid, globular protein but rather is loosely folded (Lai & Tooney, 1984).

Urea apparently disrupts some of the ordered structure of the protein and exposes the buried sulfhydryl groups (Figure 1). ESR measurements (Figure 1) show a significant change in the observed rotational correlation time from (2–3) $\times 10^{-8}$ to about $5 \times 10^{-10}$ s. This change (a factor of 40) is consistent with the notion that fibronectin, at least near the sites of spin-labeling, is completely unfolded in the presence of urea. Urea effects are reversible. The CD changes are also consistent with a loss of ordered structure (see Figure 3). Over the urea concentration range employed, however, the molecule does not become completely unfolded, in that the 227-nm band does not vanish in the presence of 6 M urea. Plasma fibronectin appears to contain both disulfide-rich domains in amino and carboxyl termini and a disulfide-free domain in the central region [see Yamada (1983) for a review]. It is likely that both amino- and carboxyl-terminal portions stabilized by disulfide bonds are resistant to urea denaturation, whereas the central region of the molecule is sensitive to urea denaturation because of the lack of disulfide bonds. The difference between ESR and CD results on urea sensitivity could, therefore, be due to the fact that ESR reports only on the local environment of the sulfhydryl-containing domains in the central region of the molecule, and CD is sensitive to the entire molecule. The remaining amplitude of the 227-nm band in the presence of 6 M urea may represent contributions from the structure of amino and carboxyl termini resistant to urea denaturation. This suggestin is in agreement with the observations made by Markovic et al. (1983).

The pH effects on ESR and CD spectra are irreversible, in that dialysis back to pH 7.4 after exposure of protein samples to pH 11 or greater does not regenerate the ESR or CD spectra originally measured at pH 7.4. Although CD spectra at pH 12 are consistent with a random coil, unfolding of the protein molecule at pH 12 and above is complicated by the fact that disulfide bonds present in plasma fibronectin can be disrupted, resulting in irreversible changes (Tanford, 1968). Caution should be used in exposing plasma fibronectin to alkaline pH because of irreversible conformational changes at high pH. (A commercially available human fibronectin is prepared in CAPS buffer, pH 11 (Collaborative Research, Lexington, MA). The biological relevance of observed pH effects is not known at the present time.

Ionic strength affects the ESR spectrum and sedimentation coefficient of fibronectin. The major change in sedimentation coefficient is consistent with a possible change in shape or hydration (Alexander et al., 1979). The effect of ionic strength on the ESR spectrum is less dramatic than the effect of pH or urea in that the maximum splitting is decreased only from about 66.5 to 65.3 G when ionic strength is raised to 0.75 M NaCl. There is no appearance of a weakly immobilized signal, suggesting that there is no major change in rotational corre-

lation times or unfolding of the protein molecule at high ionic strength. This suggests that at physiological pH and high ionic strength, the protein is expanded but retains its major regions of tertiary structure. We have found that high ionic strength has no effect on the maximum splitting values of the ESR spectrum of spin-labeled 140-kilodalton fragment (the central region of the molecule) (unpublished results). It is conceivable that the interdomain regions rather than the domains per se are affected by high ionic strength.

Fibronectin has binding affinities for a number of biological macromolecules [for reviews, see Hynes & Yamada (1982) and Yamada (1983)]. The shape changes we observe at high ionic strength may be related to functional changes induced in the molecule when fibronectin interacts with heparin. For example, Jilek & Hörmann (1979) have shown that the polyanion heparin promotes the binding of fibronectin to native and denatured collagen. The partial unfolding induced by high salt could expose binding sites on fibronectin for other macromolecules such as collagen.

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