Evidence that the two free sulfhydryl groups of plasma fibronectin are in different local environments

Saturation-recovery electron spin resonance study

Ching-San Lai, C. Narasimhan, and Jun-Jie Yin
National Biomedical Electron Spin Resonance Center, Department of Radiology, Medical College of Wisconsin, Milwaukee, Wisconsin 53226

ABSTRACT Human plasma fibronectin is a dimer consisting of two subunits; each contains two cryptic thiol groups that were selectively labeled with an $^{15}N,^{2}H$-maleimide spin label. Previous studies using conventional X-band electron spin resonance (ESR) methods showed that the spectrum of the labeled protein displays a single strongly immobilized component with an effective rotational correlation time of ~17 ns, suggesting that the physical environments of the two labeled sites per chain are indistinguishable. Here we have used saturation-recovery ESR to measure directly electron spin-lattice relaxation time (T_1) of the labeled protein in solution at 27°C. Interestingly, the time evolution of the signal was found to be biphasic, which was deconvoluted into two T_1 values of 1.37 and 4.53 μs. Thus, the two spin-labeled sulfhydryl sites of plasma fibronectin (Fn), being similar in rates of rotational diffusion, differ by a factor of 3.2 in T_1. Parallel experiments using various fibronectin fragments showed that the 1.37-μs component is associated with the label attached onto the thiol located in between the DNA-binding and the cell-binding domains, and the 4.53-μs component is associated with the label attached onto the thiol located within the carboxyl-terminal fibrin-binding domain. The data suggest that the saturation-recovery ESR is a useful method for differentiating multiple spin-labeled sites on macromolecules in which the labels undergo similar rates of rotational motion.

INTRODUCTION

Plasma fibronectin (Fn) is a glycoprotein present in blood plasma at ~0.3 mg per mL (1, 2). The protein is a dimer consisting of 240–250 kDa subunits. Each subunit is composed of a series of structural domains with binding affinities toward biomolecules like fibrin, collagen, heparin, DNA, and cell surface molecules. These binding activities relate closely to the functionalities of Fn in cell adhesion, wound healing, and phagocytosis.

Human plasma Fn contains two free sulfhydryl groups per chain; one located in a type-III homologous unit between the DNA-binding and the cell-binding domains (3) (designated SH-1; see Fig. 1), and the other situated in a type-III homologous unit that is part of the fibrin-binding domain near the carboxyl terminus (4) (designated SH-2; see Fig. 1). There is ample evidence that both SH-1 and SH-2 are buried in native Fn. For instance, they are not accessible to sulfhydryl reagents such as DTNB (5) or maleimide spin-label derivatives in the absence of chaotropic agents (6). Additionally, the solution properties of SH-1 and SH-2 seem to be indistinguishable by using DTNB-guanidine hydrochloride titration (7) or conventional electron spin resonance (ESR) line-shape analyses (6, 8).

Here, we report the use of saturation-recovery ESR (SR-ESR) methods to measure electron spin-lattice relaxation time (T_1) of a nitroxide spin-labeled maleimide covalently attached to the free sulfhydryl sites of human plasma Fn. The results show that T_1 mechanisms of the nitroxide moieties of the spin labels bound to the SH-1 sites and to the SH-2 sites of soluble plasma Fn are distinctively different. Solvent accessibility as well as relative hydrophobicity of the labeled sites that may account for the observed differences in T_1 are discussed.

Please send all correspondence to Ching-San Lai, Ph.D. at the National Biomedical ESR Center, Department of Radiology, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226. (414) 266-4051.

MATERIALS AND METHODS

Materials

Tris(hydroxymethyl)aminomethane (Tris) and phenylmethanesulfonyl fluoride (PMSF) were obtained from Sigma Chemical Co. (St. Louis, MO). Aquacide III was obtained from Calbiochem Behring Corp. (San Diego, CA). Potassium ferricyanide was from Fisher Scientific Co., Allied Corp. (Itasca, IL). The $^{14}N,^{1}H$-maleimide spin label was purchased from Aldrich Chemical Co. (Milwaukee, WI). The $^{15}N,^{2}H$-maleimide spin label was synthesized by Dr. Joy Joseph in our laboratory after the procedure of Griffith and McConnell (9). The final product was pure without isomaleimide derivatives as demonstrated by infrared spectroscopy (10).

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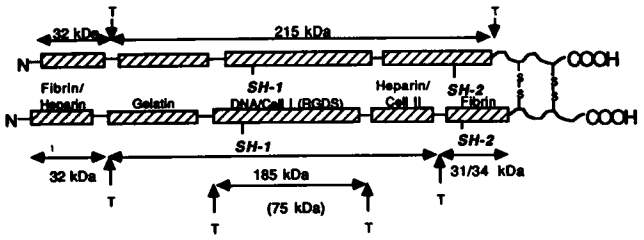

FIGURE 1 Schematic diagram depicting the trypsin cleavage sites (T) on the Fn molecule that gave rise to the fragments used in this study. The binding domains of Fn as well as the locations of the two free sulfhydryl groups, namely, SH-1 and SH-2, are also indicated.

Plasma Fn was isolated from human plasma using gelatin-Sepharose affinity chromatography (11). The protein was essentially pure as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The fragments of Fn were prepared as described previously (12).

Labeling of plasma fibronectin with maleimide spin labels

The free sulfhydryl groups of plasma Fn and its fragments were selectively labeled with the $^{15}N,^{2}H$-maleimide spin label essentially as described by Lai and Tooney (6).

ESR measurements

(a) Conventional ESR

Conventional ESR spectra were recorded with a Century-Line 9-GHz spectrometer equipped with a variable temperature controller accessory (Varian Associates, Inc., Palo Alta, CA) and a digital thermometer (model 2100A; John Fluke Manufacturing Co., Seattle, WA). 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. All spectra were recorded at 22°C.

(b) Saturation-recovery ESR (SR-ESR)

The SR-ESR spectrometer equipped with a loop-gap resonator used here has been described previously (13, 14). A field-effect transistor (FET) microwave with the time response limit of 0.1 μs was used. In order to minimize the distortion of the transient signal, a high order-low pass filter (25-MHz cut off) was fixed at the input to the analogue to digital converter. Typically 20,000 decays per second were acquired with 512 data points on each decay. The total accumulation time was 10–20 min. Aperture intervals were 120 ns for intact Fn and 60 ns for its fragments.

The SR-ESR technique is based principally on the use of an intense microwave power to saturate a spin population having certain distributions with respect to the magnetic field. After the saturation is relieved, the time evolution of the signal recovery is characterized by spin-lattice relaxation time (T_1), assuming that saturation transfer due to spectral diffusion is negligible, which is the case in this work when pulse duration is greater than spectral diffusion.

The SR-ESR experiments were performed as described by Yin et al. (14, and references therein). Samples were placed in 0.6-mm diameter (inner diameter) capillaries made from methylpentene polymers (TPX). A flow of temperature-regulated nitrogen gas over the capillary was used to remove oxygen. The theoretical models were compared with experimental saturation recovery curves using the damped-least-squares method. In essence, it utilizes the Gauss-Newton minimization procedure to which a scalar factor is included; this helps to "damp" the process toward the minimum and assures the convergence in the iterative steps. The curve-fitting program was run on an IBM PC/AT computer. All experiments were carried out at 27°C in triplicates.

RESULTS

Saturation-recovery ESR measurement

The conventional X-band ESR spectrum of the $^{15}N,^{2}H$-maleimide spin-labeled plasma Fn in solution at 22°C displayed a single strongly immobilized component with an effective rotational correlation time of 17 ns as shown in Fig. 2 *A*. This suggests that the physical environments of the two spin-label sites in the protein molecule are similar and are indistinguishable by conventional X-band ESR methods (6). This suggestion is also consistent with our previous study in which we showed that both free sulfhydryl groups per chain of plasma Fn are probably situated in a cleft-like structure ~10.5 Å in depth (8).

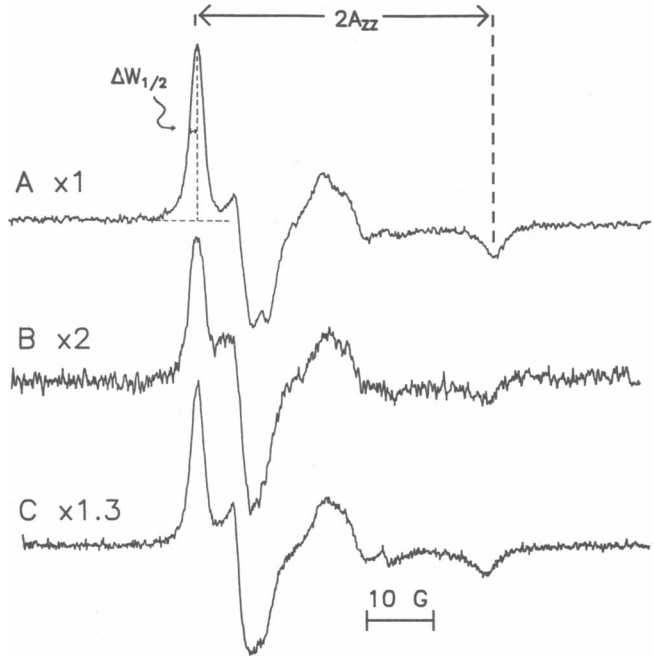

FIGURE 2 ESR spectra of $^{15}N,^{2}H$-maleimide spin-labeled Fn in 20 mM Tris, 150 mM NaCl, pH 7.4 at 22°C. (*A*) control, (*B*) in the presence of 30 mM potassium ferricyanide, and (*C*) in the presence of 0.5 M NaCl. Other experimental conditions were described in Methods. The concentrations of the protein used were 1.1 mg/mL in *A* and *B*, and 1.0 mg/mL in *C*.

To further characterize the labeled sites, we have carried out SR-ESR experiments to measure electron spin-lattice relaxation time, T_1, of the nitroxide moieties of the spin labels bound to the free sulfhydryl sites in the protein molecule. We reasoned that if both free sulfhydryl sites in the protein are located in an identical environment, the saturation-recovery signal of the labeled protein should yield a single exponential with a single T_1 value. On the other hand, if they are not situated in an identical environment, the time evolution of the recovery signal may be a double exponential consisting of two T_1 values. The results in Fig. 3 showed that the saturation-recovery signal of the labeled protein was not a single exponential, but a double exponential, which was deconvoluted into two T_1 values of 1.37 and 4.53 μs, respectively. Control experiments using the $^{15}N,^{2}H$-maleimide spin label free in solution at 27°C showed that the time evolution of the signal recovery was a single exponential with a T_1 value of 1.25 μs (Table 1).

To ascertain whether the two different T_1 values arise from the two different labeling sites in the protein molecule, we prepared spin-labeled Fn fragments that contain only SH-1 or SH-2 or both SH-1 and SH-2 (see Fig. 1) and measured the saturation recoveries of these labeled fragments. The conventional X-band spectra of the spin-labeled isolated Fn fragments resembled that of the labeled Fn (data not shown), which is consistent with the notion that the isolated domains of Fn retain their proper foldings and functions (15). The SR-ESR data of spin-labeled Fn and its fragments are summarized in Table 1 in which the spin-labeled 185-kD fragment containing the SH-1 site was shown to exhibit a single T_1 value of 2.70 μs, and the spin-labeled 34-kD fragment containing the SH-2 site displayed a single T_1 value of 4.61 μs. On the other hand, the spin-labeled 215-kD fragment that contains both SH-1 and SH-2 gave rise to two T_1 values of 1.49 and 4.43 μs, respectively. It is thus probable that the 1.37-μs (fast) component of intact Fn is associated with the nitroxide bound to the SH-1 site, whereas the 4.53-μs (slow) component is associated with the nitroxide bound to the SH-2 site, Table 1. The T_1 values between these two labeled sulfhydryl sites thus differ by a factor of 3.2.

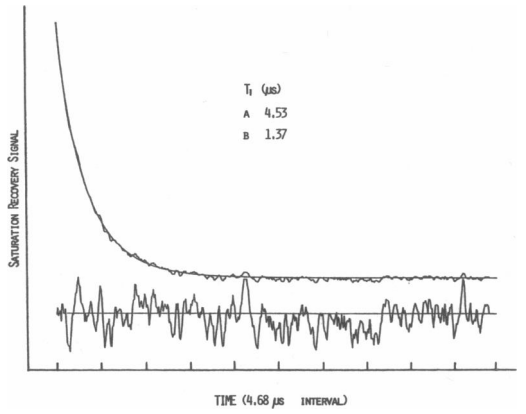

FIGURE 3 Time evolution of signal recovery for $^{15}N,^{2}H$-maleimide spin-labeled Fn (45 μM) in 20 mM Tris, 150 mM NaCl, pH 7.4 at 27°C. Simulated (*smooth line*) and the experimental curves are superimposed. The difference between the simulated and experimental curves (multiplied by a factor of 7.5) is shown across the bottom of the figure.

TABLE 1 T_1 values of spin-labeled fibronectin and its fragments

Sample	T_1*	
	μs	
	(1)	(2)
Intact Fn	1.37 ± 0.02‡	4.53 ± 0.01
34 kD	—	4.61 ± 0.12
185 kD	2.70 ± 0.04	—
215 kD	1.49 ± 0.14	4.43 ± 0.01
Maleimide spin label	1.25 ± 0.01	

*SR-ESR measurements of T_1 were as described in Methods.
‡Data presented are averages of at least three independent measurements.

Spin-label—spin-probe method

An additional method to evaluate the heterogeneity of spin-labeled sites on macromolecules is the use of paramagnetic broadening agents such as potassium ferricyanide (16). This agent is known to broaden the ESR lines of spin labels through either dipole-dipole and/or Heisenberg exchange mechanisms, depending on the interaction distances between these two interacting paramagnetic species (17, 18).

Addition of 30 mM potassium ferricyanide induced the line broadening of the spectrum of spin-labeled Fn, causing a reduction of signal intensity, and concomitant with a decrease in the value of A_{zz} by 0.7 G as shown in Fig. 2 *B*. Thus, potassium ferricyanide appears to exert a dual effect on the spectrum of the labeled Fn. The effect on the signal reduction and line broadening can be ascribed to magnetic interactions (rather than chemical reactions) between potassium ferricyanide and spin labels, inasmuch as both reduced signal and line broadening were found to be fully recovered upon removal of potassium ferricyanide by applying the treated sample to a G-25 column (data not shown). The observed decrease in A_{zz} suggests an increase in probe mobility in the presence of potassium ferricyanide. Because the hydrodynamic properties of Fn are known to be affected by high ionic strength (19), it is likely that the observed decrease in A_{zz} could be a result of ionic strength effects exerted by potassium ferricyanide. This in fact is the case. As shown

in Fig. 2 *C*, addition of 0.5 M NaCl reduced the A_{zz} value to the same extent as that exerted by 30 mM ferricyanide, but without an effect on the line widths. Interestingly, similar experiments using the spin-labeled 185-kD and 34-kD fragments showed that addition of NaCl has no effect on either the line widths or the A_{zz} value (data not shown). The increase in probe mobility exerted by salt is therefore due perhaps to the disruption of ionic interactions between two subunits in intact Fn (20), rather than a direct effect of salt on the local environments of the labeled sites.

The plots of the linewidth changes as a function of concentrations of potassium ferricyanide for spin-labeled Fn and its fragments are depicted in Fig. 4. For intact Fn, it clearly shows a biphasic curve with a discontinuity ~50 mM. Similar plot for the spin-labeled Fn fragment, either the 185-kD fragment containing the SH-1 site or the 34-kD fragment containing the SH-2 site, yielded only a single straight line, respectively. Quantitatively, the effectiveness of line broadening can be expressed by the broadening factor, which is defined as the value of the line broadening (in gauss) divided by the concentration of the paramagnetic ion (in millimolar) (21). The values of the broadening factor for potassium ferricyanide in spin-labeled intact Fn and its fragments are summarized in Table 2 in which one broadening factor of intact Fn is shown to resemble that for the 185 kD, and the other is close to that for the 34 kD. The biphasic behavior of intact Fn as shown in Fig. 4 could then be due to different accessibilities of the SH-1 and SH-2 sites toward potassium ferricyanide; the initial slope is associated with the titration of the SH-1 site, and the later slope is associated with the titration of the SH-2 site. The small difference between the values of intact Fn and isolated fragments may be attributed to a slight increase in the accessibility of potassium ferricyanide to sulfhydryl sites upon fragmentation. Additionally, the value of the broadening factor for the maleimide spin label free in solution is ~3 to 13 times greater than those for the labeled intact Fn (Table 2). This is as expected because while the label free in solution is fully accessible to potassium ferricyanide, the label bound to the protein is less accessible to the agent due to the cryptic nature of sulfhydryl sites in the protein (5, 6).

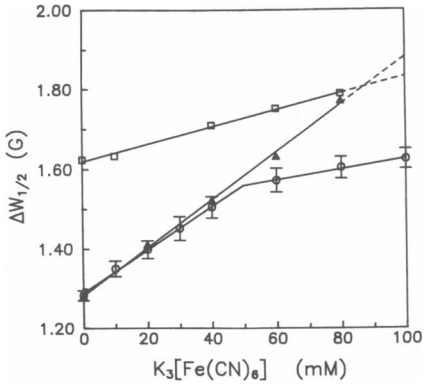

FIGURE 4 Effects of potassium ferricyanide on the spectral linewidth of $^{15}N,^{2}H$-maleimide spin-labeled Fn and its fragments in 20 mM Tris, 150 mM NaCl, pH 7.4 at 22°C. $\Delta W_{1/2}$ is defined as the half-width at the half-height of the low-field peak as indicated in Fig. 2. -O-, intact Fn (2.5 μM); -▲-, 185-kDa fragment (3.8 μM); -□-, 34-kDa fragment (6.2 μM). In these experiments, while varying the concentrations of potassium ferricyanide, the protein concentrations were kept constant.

TABLE 2 The broadening effects of potassium ferricyanide on the spectral lines of spin-labeled fibronectin and its fragments*

Sample	Broadening factor‡ (G/mM) × 10^3	
	(1)	(2)
Intact Fn	5.5 (first slope)	1.4 (second slope)
185 kD	6.3	—
34 kD	—	2.1
Maleimide spin label	18.0	—

*Data presented here are calculated based on the results presented in Figure 4.

‡The broadening factor is defined as the value of the line broadening (in gauss) divided by the concentration of the paramagnetic ion (in millimolar) (21).

DISCUSSION

Here, SR-ESR has been applied to definitively show that the single strongly immobilized spectrum of the maleimide spin-labeled intact Fn in solution obtained by using conventional X-band ESR methods can be separated into two distinct components with T_1's of 1.37 and 4.53 μs, respectively. Subsequent work using spin-labeled Fn fragments revealed that the 1.37-μs component is associated with the label on SH-1 and the 4.53-μs component is associated with the label on SH-2. Thus, the two labeled sites, which are indistinguishable by spectral lineshapes, were found to differ profoundly in relaxation processes.

Since 1974, SR-ESR has been used to measure T_1's of the nitroxide in various biological systems, particularly in biomembranes (14, and references therein; 22). However, so far, there are only two published reports on its applications to spin-labeled macromolecular systems (13, 23). Previous approach to determine T_1 was to use continuous wave saturation (24, and references herein). Experimental factors that might affect T_1 of the nitroxide in aqueous solution include probe motions, polarity, oxygen, and paramagnetic ions. Among them, the effects of oxygen and paramagnetic ions on T_1 are well described

(17, 18, 25). Of particular relevance to the interpretation of the present results are the effects of probe motions and polarity on T_1 of the nitroxide, because oxygen and paramagnetic ions are absent during SR-ESR measurement. Huisjen and Hyde (23) showed that T_1 of tanol in sec-butyl benzene was invariant between rotational correlation times of 10^{-5} to 10^{-7} s. Similar observation was made by Fajer et al. (13); they described a weak dependence of T_1 on rotational motions of spin-labeled hemoglobin in solution between rotational correlation times of 10^{-4} to 10^{-8} s. On the other hand, so far, little is known regarding the effect of polarity on T_1 of the nitroxide.

Although both SH-1 and SH-2 are situated in a type-III homologous sequence, and are buried from the protein surface, we showed previously that upon adsorption to a surface, SH-1 becomes fully exposed to the solvent while SH-2 remains buried (12). The ease of SH-1 exposure suggests that the local environment of SH-1 may be relatively hydrophilic compared with that of SH-2. We speculate that the observed difference in T_1 between these two labeled sites may be a result of the difference in solvent accessibility. In accord with this notion, T_1 of the nitroxide on the SH-1 site resembles T_1 of the same spin label free in aqueous solution (Table 1); the latter presumably is fully exposed to solvent molecules, implying that the nitroxide moiety of the label of SH-1 is more accessible by water than that on SH-2. Nevertheless, the reason why the T_1 value of the spin-labeled 185-kD fragment containing only the SH-1 site is about a factor of 2 longer than that of the same site in intact Fn (Table 1) is not known. It is possible that deletion of both carboxyl and amino terminal regions from intact Fn may affect the structure of the central region in which the SH-1 site is situated.

It is known that Heisenberg exchange between oxygen and spin labels shortens the T_1 of spin labels (25) and that a hydrophobic locale contains more oxygen than the hydrophilic counterpart in membranes (26). One possible experiment to verify the claim of a difference in hydrophobicity between SH-1 and SH-2 would be to measure the saturation recovery of the labeled samples in the presence of air. However, the poor signal-to-noise due to the presence of oxygen precluded such an experiment.

Other evidence to support the suggestion that the SH-1 site is more hydrophilic than the SH-2 site comes from the results obtained using potassium ferricyanide as a broadening agent (Fig. 4 and Table 2), in which we showed that the nitroxide on the SH-1 site is more accessible to this paramagnetic ion than that on the SH-2 site. However, attempts using positively charged paramagnetic ions such as Cu^{2+} and Ni^{2+} failed because of the occurrence of protein as well as ion aggregations at high concentrations of these ions at physiological pH. Therefore, the possibility that the observed differential effect of potassium ferricyanide on the spectra of the labeled SH-1 and SH-2 sites is due to charge differences in the vicinity of the labeled sites cannot be ruled out at present. On the other hand, the line broadening effect of a paramagnetic ion on the spectrum of the spin label arises from Heisenberg exchange and dipole-dipole interactions; both presumably contribute to the observed effects reported in the present work. Separation of these two contributions may require the use of multifrequency pulse ESR methods inasmuch as Heisenberg exchange is known to be frequency independent. This warrants further investigation.

In summary, the results clearly demonstrate that SR-ESR is a useful method for separation of multiple spin-labeled sites in macromolecules in which the labels exhibit similar rates of rotational motion. We therefore caution the interpretation of spin-label data of single-site labeling based solely on observed single component spectra obtained from conventional X-band ESR methods.

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