Spin label studies of sulfhydryl environment in plasma fibronectin

Ching-San Lai, Nancy M. Tooney* and Else G. Ankel

National Biomedical ESR Center, Department of Radiology, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226 and **Department of Medicine, University of Wisconsin Medical School, Milwaukee Clinical Campus, Milwaukee, WI 53201, USA*

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The local environment of the free sulfhydryl groups in plasma fibronectin has been investigated by ESR techniques using a series of maleimide spin labels, varying in chain length between the maleimide and nitroxide free radical groups. Chemical modification with these analogs does not affect either the CD spectra or the cell adhesion activity of the protein molecule. The ESR results show that the free sulfhydryl group of plasma fibronectin is in a cleft about 10.5 Å in length. The significance of this finding is discussed.

Plasma fibronectin *Spin label* *ESR* *Sulfhydryl group*

1. INTRODUCTION

Fibronectins are a family of glycoproteins normally present in blood and other body fluids [1,2]. Plasma fibronectin is composed of two chains with specific binding sites for a number of biologically important materials, including heparin, fibrin, collagen, actin, DNA, bacteria and cell surface. It is also required for the spreading of a variety of cells on solid substrata [3,4].

The dimeric fibronectin protein (M_r 440000) contains one to two free sulfhydryl groups per monomer which can be titrated by DTNB only when a chaotropic agent is present [5,6]. We have previously described the selective modification of these free sulfhydryl groups with a maleimide spin label and, based on the ESR observations have concluded that the free sulfhydryl group of plasma fibronectin is in a small, confined environment [7]. The insertion of maleimide spin labels onto the free sulfhydryl groups does not affect properties such as the CD spectrum or cell adhesion activity.

Here, we report a study on the use of a series of maleimide spin labels with different chain lengths between the maleimide and nitroxide groups to characterize the local environment of the free sulfhydryl groups in plasma fibronectin.

2. MATERIALS AND METHODS

Phenylmethylsulfonyl fluoride (PMSF), *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (Tes) were obtained from Sigma. A series of maleimide spin labels, 3-maleimido-PROXYL (I), 3-(maleimidomethyl)PROXYL (II), 3-(2-maleimidoethylcarbamoyl)PROXYL (III), 3-(3-maleimidopropylcarbamoyl)PROXYL (IV) and 3-[2-(2-maleimidoethoxy)ethylcarbamoyl]PROXYL (V) (see fig.1 for chemical structures) were obtained from Aldrich. Plasma fibronectin isolated from human plasma using gelatin–Sepharose 4B affinity chromatography was provided by Dr Gene

* Present address: Department of Chemistry, Polytechnic Institute of New York, Brooklyn, NY 11201, USA

Abbreviations: DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); PROXYL, 2,2,5,5-tetramethyl-1-pyrrolidinyloxy; TEMPO, 2,2,6,6-tetramethyl-1-piperidinyloxy

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Homandberg of Mount Sinai Medical Center, Milwaukee. The preparations were at least 95% pure as determined by 7% SDS-polyacrylamide gel electrophoresis.

Plasma fibronectin was chemically modified with maleimide spin label in the presence of 6 M urea exactly as in [7]. PMSF, a protease inhibitor (1×10^{-4} M) was added to all of the buffer solutions during protein purification and spin labeling procedures.

The potential effects of spin label modification on the structure of plasma fibronectin were determined by CD spectroscopy as in [7]. The cell adhesion assay essentially as in [4] was used to evaluate the biological activities of maleimide spin-labeled plasma fibronectins.

All ESR spectra were recorded with a Varian Century Line 9 GHz spectrometer operating at 9.1 GHz. The instrumental settings were: field sweep, 100 G; modulation amplitude, 2.0 G; microwave power, 10 mW. The cavity temperature was 22 ± 1°C.

The effective rotational correlation time in the slow tumbling regime (10^{-9}–10^{-7} s) was estimated by comparing the experimental ESR spectra with simulated spectra [8]. The effective rotational correlation time in the fast tumbling time regime (10^{-11}–10^{-9} s) was determined using the equation given in [9]:

$$[1/\tau = 3.6 \times 10^9/\Delta H_o[(H_o/H_{-1})^{1/2} - 1]\ s^{-1}$$

where ΔH_o is the peak-to-peak width of the central field line (in G) and H_o and H_{-1} are peak-to-peak heights of the central-field and high-field lines, respectively.

3. RESULTS AND DISCUSSION

When the maleimide spin labels I–V (fig.1) were covalently bound to plasma fibronectin, the ESR spectra as shown in fig.2 were obtained. Previously we demonstrated that under the experimental conditions only free sulfhydryl groups are labeled by a maleimide spin label analog with a stoichiometry of 2.8 labels to one dimeric protein [7]. The incorporation of spin label I into the fibronectin molecule gives rise to a broad and asymmetric ESR spectrum with a maximum splitting value of 67 G at 22°C (fig.2). This spectrum is essentially identical to that reported previously using maleimido-

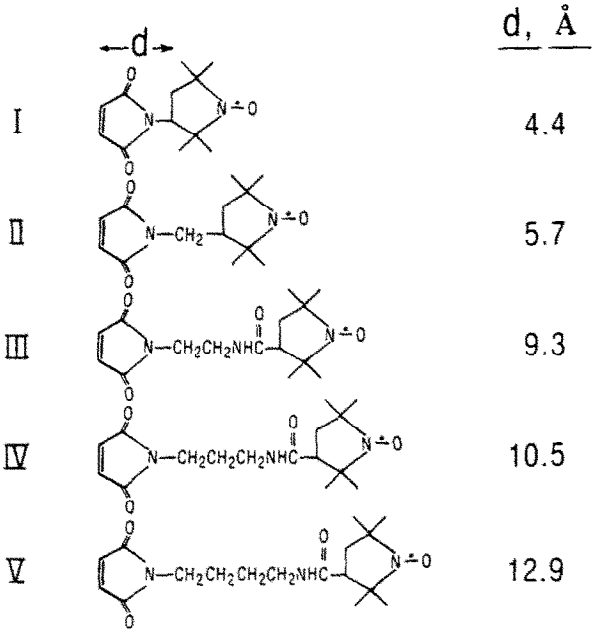

Fig.1. Chemical structures and chain lengths of maleimide spin labels.

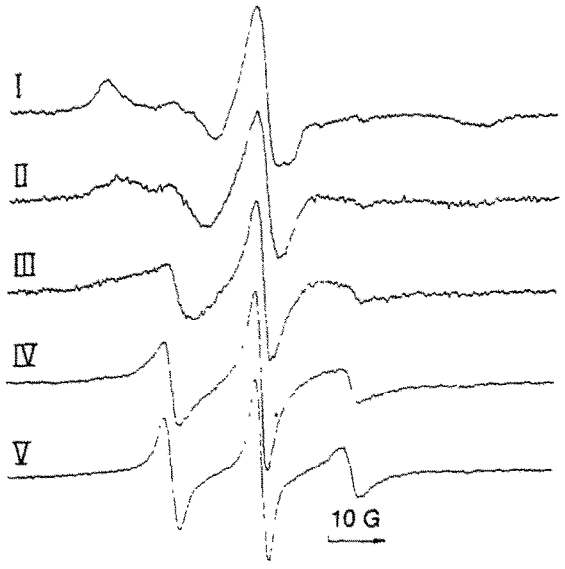

Fig.2. ESR spectra of maleimide spin-labeled plasma fibronectin. I–V correspond to the spin labels in fig.1. Protein concentrations were 3.0×10^{-6} M (I), 1.2×10^{-6} M (II), 0.9×10^{-6} M (III), 1.6×10^{-6} M (IV) and 1.5×10^{-6} M (V) in 0.01 M Tes buffer containing 0.15 M NaCl and 10^{-4} M PMSF (pH 7.4). Receiver gain: (I) 6.3×10^4, (II) 1×10^5, (III) 1×10^5, (IV) 5×10^4 and (V) 4×10^4.

TEMPO [7]. This suggests that there is no difference between maleimide-PROXYL (spin label I) and maleimide-TEMPO for modification of free sulfhydryl groups in plasma fibronectin. Since the spectrum of spin label I in fig.2 resembles the rigid limit spectrum, it is likely that the nitroxide group of spin label I is strongly immobilized and that the label is rigidly bound to fibronectin molecule.

The insertion of a single methylene group between the maleimide and nitroxide groups (see spin label II in fig.1) decreases the maximum splitting value from 67 to 64 G (fig.2), indicating an increase in motional freedom of the nitroxide group of spin label II bound to fibronectin molecule. As the chain length progressively increases (spin labels III–V in fig.2) the spectrum becomes narrower and more symmetrical, suggesting that the nitroxide group emerges from a restricted environment to an environment where it rotates relatively freely. This result is consistent with the notion that the sulfhydryl group of plasma fibronectin is in a cleft-like environment.

A plot of the effective rotational correlation times of the spectra in fig.2 *vs* the chain length reveals a break at 10.5 Å as shown in fig.3. The distance corresponding to this break has been interpreted as the minimum depth of the cleft

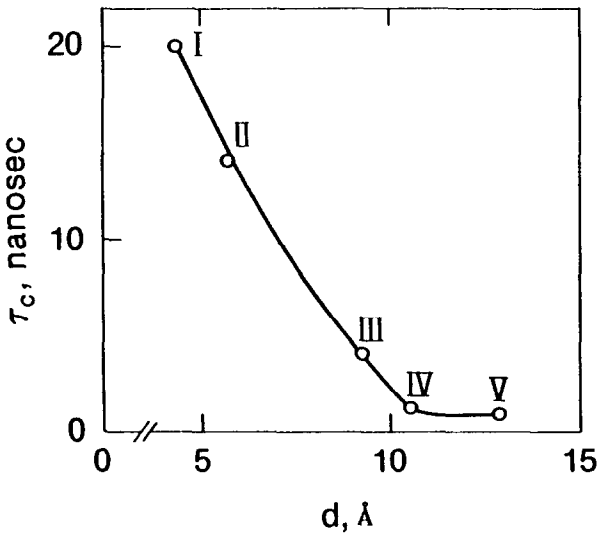

Fig.3. Rotational correlation times of the ESR spectra of maleimide spin-labeled plasma fibronectin *vs* the chain lengths, *d*, of the spin labels. The data points, indicated by I–V, correspond to the spin labels in fig.1.

[10–13]. The sulfhydryl group of fibronectin is therefore probably in a cleft about 10.5 Å deep. A schematic diagram of this sulfhydryl containing cleft is proposed in fig.4.

Plasma fibronectin is one of the essential components in serum that are required for cell spreading. Recently we developed a cell adhesion assay based on the quantitation of spreading of Chinese hamster ovary (CHO) cells on fibronectin-coated microcarriers [4]. The promotion of 50% of cell spreading on plasma fibronectin-coated microcarriers requires about 3.4×10^8 fibronectin molecules per bead. Using such an assay, we found that all spin-labeled fibronectins (I–V in fig.2) promoted the spreading of CHO cells as effectively as the control protein (not shown). The cell spreading activity of plasma fibronectin is apparently not affected by the introduction of maleimide spin label regardless of chain lengths. We also carried out CD studies to determine the effects of spin label modification on the secondary and tertiary structure of fibronectin molecules and found that the insertion of spin labels I–V had no measurable effect on both near- and far-UV CD spectra (not shown). This result is consistent with our previous CD observations on spin-labeled and control fibronectins [7]. It is concluded that the functional and structural integrity of the fibronectin molecule is preserved under the conditions used in this study.

Human plasma fibronectin contains one to two sulfhydryl groups per monomer which are located

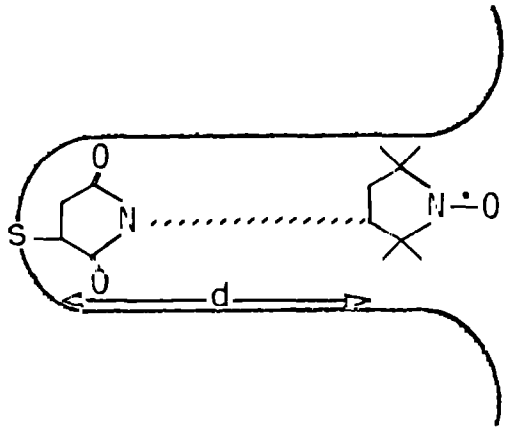

Fig.4. Schematic diagram of the local environment of free sulfhydryl group in plasma fibronectin.

at two different sites; one being about 170 kDa from the amino terminus and the other in the carboxyl-terminal fibrin binding domain [5,14,15]. Modification of these free sulfhydryl groups with various chain lengths of maleimide spin label, namely, spin labels I–V in fig.1, revealed only a single component ESR spectrum (fig.2), argues that these two free sulfhydryl groups are in similar environments.

Three different types (I–III) of internal homology have been found in plasma fibronectin (review [1]). Authors in [16] reported a type III homologous sequence containing a free sulfhydryl group in a 170-kDa fragment isolated from bovine plasma fibronectin. Recently, authors in [17] also found a type III homologous sequence containing a free sulfhydryl group near the C-terminal region of rat fibronectin. These results are consistent with earlier data on the location of free sulfhydryl groups in fibronectin [5,14,15]. It is therefore likely that both free sulfhydryls are in type III homology regions, thus having similar environments.

The presence of the free sulfhydryls in a crevice about 10 Å deep is not unique to plasma fibronectin. It has been reported that the free sulfhydryl of plasma albumin is in a crevice approx. 10 Å in depth [12,13]. The sulfhydryl in plasma albumin has been suggested to be involved as a catalyst in the formation of a disulfide interchange isomer [18,19]. The function of the free sulfhydryl groups of plasma fibronectin remains a problem for further investigation.

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REFERENCES

[1] Yamada, K.M. (1983) Annu. Rev. Biochem. 52, 761–799.
[2] Hynes, R.O. and Yamada, K.M. (1982) J. All. Biol. 95, 369–377.
[3] Grinnell, F., Hays, D.G. and Minter, D. (1977) Exp. Cell Res. 110, 175–190.
[4] Lai, C.-S., Ankel, E.G. and Hopwood, L.E. (1984) Exp. Cell Res. 150, 77–83.
[5] Smith, D.E., Mosher, D.F., Johnson, R.B. and Furcht, L.T. (1982) J. Biol. Chem. 257, 5831–5838.
[6] Mosher, D.F. and Johnson, R.B. (1983) J. Biol. Chem. 258, 6595–6601.
[7] Lai, C.-S. and Tooney, N.M. (1984) Arch. Biochem. Biophys. 228, 465–473.
[8] Freed, J.H. (1976) in: Spin Labeling Theory and Applications (Berliner, L.J. ed.) vol.1, pp.562–563, Academic Press, New York.
[9] Stone, T.J., Buchman, T., Nordis, P.L. and McConnell, H.M. (1965) Proc. Natl. Acad. Sci. USA 54, 1010–1017.
[10] Hsia, J.C. and Piette, L.H. (1969) Arch. Biochem. Biophys. 129, 296–307.
[11] Chignell, C.F. and Starkweather, D.K. and Erlich, R.H. (1972) Biochim. Biophys. Acta 271, 6–15.
[12] Cornell, C.N. and Kaplan, L.J. (1978) Biochemistry 17, 1750–1754.
[13] Hull, H.H., Chang, R. and Kaplan, L.J. (1975) Biochim. Biophys. Acta 400, 132–136.
[14] Haynshi, M. and Yamada, K.M. (1983) J. Biol. Chem. 259, 3332–3340.
[15] Wagner, D.D. and Hynes, R.O. (1980) J. Biol. Chem. 255, 4304–4312.
[16] Vibe-Pedersen, K., Sabl, P., Skorstengaard, K. and Petersen, T.E. (1982) FEBS Lett. 142, 27–30.
[17] Schwarzbauer, J.E., Tamkun, J.W., Lemischka, I.R. and Hynes, R.O. (1983) Cell 35, 421–431.
[18] Nikkel, H.J. and Foster, J.F. (1971) Biochemistry 10, 4479–4486.
[19] Stroupe, S.D. and Foster, J.F. (1973) Biochemistry 12, 3824–3830.