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Conformational change in thrombospondin induced by removal of bound Ca^{2+}

A spin label approach

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The effect of removal of Ca^{2+} bound to thrombospondin (TSP) on the protein structure in solution has been investigated using ESR spin-label techniques. A maleimide spin label was selectively attached to the free thiol group presumably near the carboxyl-terminal domain in which Ca^{2+}-binding sites are situated. The ESR spectra of spin-labeled TSP showed that the bound label undergoes a relatively fast rotational motion with an effective rotational correlation time in the nanosecond time regimes. Removal of bound Ca^{2+} in TSP by dialyzing spin-labeled TSP from a Ca^{2+}-containing buffer into an EDTA-containing buffer resulted in an increase in the mobility of the bound label by a factor of 2.3. The data suggest that EDTA chelation of bound Ca^{2+} in TSP induces a conformational change of TSP at least near the site of spin labeling.

Thrombospondin; ESR; Spin labeling; Protein conformation

1. INTRODUCTION

TSP is a large adhesive glycoprotein found in the α-granules of platelets [1] and in tissues [2]. Various investigations have suggested that TSP contains discrete functional domains which interact with various biological substrates [1,3]. TSP also contains at least twelve Ca^{2+}-binding sites with a dissociation constant of 50–120 μM [4,5]; the Ca^{2+}-binding domain of TSP appears to have some sequence homology with calmodulin [3]. The native trimer of TSP is composed of disulfide-linked monomers each with an asymmetric dumbbell shape consisting of a large C-terminal and a small N-terminal globular domain connected by an extended strand [1,3,5–7].

TSP contains one free thiol group per chain [8] which is located near the carboxyl terminus [1]. Here, we have modified this thiol group selectively with a maleimide spin label, and followed the changes in molecular motion in this localized environment using ESR spectroscopy.

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Abbreviations: TSP, thrombospondin; PMSF, phenylmethylsulfonyl fluoride; Proxyl, 2,2,5,5-tetramethyl-1-pyrrolidinyloxyl; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Tempo, 2,2,6,6-tetramethyl-1-piperidinooxyl; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

2. EXPERIMENTAL

2.1. *Materials*

TSP was purified from human platelets according to [9]. Purified TSP (about 0.25 mg/ml) was stored at −70°C in a buffer containing 10 mM Tris, 0.15 M NaCl, 0.1 mM $CaCl_2$, pH 7.4. Prior to use, the frozen protein solution was thawed in a water bath at 37°C. PMSF in ethanol was added to the protein solution to 0.1 mM; the final ethanol concentration was less than 1%. 3-(2-Maleimidoethylcarbamoyl)-Proxyl, a maleimide spin label, was obtained from Aldrich (Milwaukee, WI). 4-Maleimido-Tempo was synthesized by Dr Joy Joseph in our laboratory. Disposable G-25 gel filtration columns were from Pharmacia (Uppsala). Spectrapor dialysis tubing (Fisher Scien-

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tific, M_r cut-off 3500) was used in dialysis. Electrophoresis supplies were from Bio-Rad (Richmond, CA). All other chemicals were from Sigma (St. Louis, MO).

2.2. *Spin labeling*

Spin labeling of TSP was carried out according to Lai and Tooney [10], except that treatment of the protein with urea was not necessary for labeling the free thiols in TSP. Briefly, TSP (0.25 mg/ml) was incubated with the maleimide spin label at a molar stoichiometry of 1:60 (protein/probe) for 2 h at 30°C with gentle shaking. The reaction mixture was then dialyzed extensively against 10 mM Tris, 150 mM NaCl, pH 7.4, containing either 0.1 mM $CaCl_2$ or 1 mM EDTA at 4°C. Residual unreacted label was further removed by passing the spin-labeled TSP solution through a Pharmacia G-25 column pre-equilibrated in the appropriate buffer.

2.3. *ESR spectroscopy*

Spectra were obtained using a Varian Century-Line spectrometer, operating at a frequency of 9 GHz with 20 mW microwave power incident upon a rectangular TE_{102} cavity at 22°C. The modulation amplitude was 1 G, the modulation frequency 100 kHz, and the field sweep 100 G.

2.4. *Calculation of effective rotational correlation time (τ_2)*

The τ_2 values of the protein-bound spin label were calculated from the ESR spectrum using the motional narrowing formalisms [11–13]. The linear and quadratic terms for τ_2 (in seconds) are calculated by:

$$\tau_2 = 6.5 \times 10^{-10}\, \Delta H(0)[(h_0/h_{-1})^{1/2} - (h_0/h_{+1})^{1/2}]\ \text{s} \quad \text{(linear term)} \quad (1)$$

$$\tau_2 = 6.5 \times 10^{-10}\, \Delta H(0)[h_0/h_{-1})^{1/2} + (h_0/h_{+1})^{1/2} - 2]\ \text{s} \quad \text{(quadratic term)} \quad (2)$$

where $\Delta H(0)$ is the peak-to-peak linewidth of the central field line (in G), h_0, h_{-1} and h_{+1} being the respective first-derivative resonances of the central, high- and low-field peaks.

2.5. *Other methods*

The free sulfhydryl content of TSP was assayed using the DTNB assay of Ellman [14] with an extinction coefficient of $1.36 \times 10^{-4}\ M^{-1} \cdot cm^{-1}$ at 412 nm for the dinitrophenol anion generated. The intactness of TSP before and after spin labeling was examined by SDS-PAGE as described by Laemmli [15].

3. RESULTS AND DISCUSSION

The motion of a spin label in solution in fast tumbling time regimes (10^{-9}–10^{-11} s) can be characterized by the relative signal intensities of its ESR spectrum [11–13]. For example, the ESR spectrum of the maleimide spin label free in solution, as shown in fig.1A, exhibits three sharp and relatively symmetric lines, which is indicative of a fast tumbling motion. The motion of the label covalently bound to TSP in a Ca^{2+}-containing buffer was relatively restricted compared to that free in solution as demonstrated by the resulting spectrum in fig.1C which is characteristic of a partially immobilized spectrum. Interestingly, when Ca^{2+} was removed from TSP by EDTA chelation, the motion of the bound label was increased as shown in fig.1B. This process was reversible.

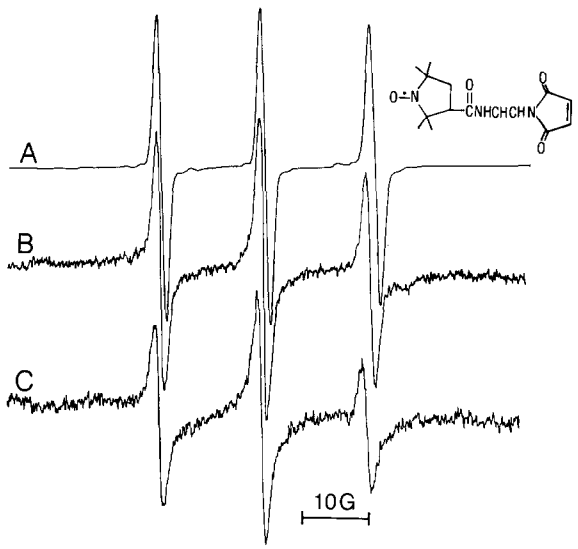

Fig.1. ESR spectra of maleimide spin-labeled thrombospondin. (A) 10 μM maleimide spin label (structure shown above) in 10 mM Tris, 150 mM NaCl, 0.1 mM $CaCl_2$, pH 7.4. (B) Spin-labeled TSP (0.6 μM) in 10 mM Tris, 150 mM NaCl, pH 7.4, with 1 mM EDTA. (C) The same sample as in (B) was dialyzed into 10 mM Tris, 150 mM NaCl, pH 7.4, containing 0.1 mM $CaCl_2$. Receiver gain in (B,C) was 25-times that in (A).

Based on an isotropic hyperfine constant of 16.1 G and the accessibility of the labeling site to the spin-label reagent in the absence of denaturants, it is probable that the nitroxide moiety of the label bound to the protein is in direct contact with aqueous solution.

To quantify the changes in molecular motion upon EDTA chelation of Ca^{2+} in spin-labeled TSP, we have calculated the effective rotational correlation time, τ_2, based on the line-narrowing formalisms [11–13]. The data are summarized in table 1. Upon removal of Ca^{2+} bound in TSP by EDTA chelation, τ_2 increases by a factor of 2.3 (table 1). The increase in mobility of the bound label suggests a local conformational change of TSP, perhaps due to partial unfolding of the local protein chain near the site of spin labeling.

Table 1

Effective rotational correlation times of spin-labeled thrombospondin

Systems	Effective τ_2 ($\times 10^{10}$ s)[a]		
	(*A*) Linear	(*B*) Quadratic	*B/A*
10 μM maleimide spin label + 0.1 mM $CaCl_2$[b]	0.70 ± 0.03[c]	0.76 ± 0.08	1.09
Spin-labeled TSP + 1 mM EDTA	1.70 ± 0.68	3.30 ± 0.61	1.94
Spin-labeled TSP + 0.1 mM $CaCl_2$	3.90 ± 1.30	7.60 ± 0.70	1.95

[a] The linear and quadratic terms are defined in section 2
[b] The buffer used comprised 10 mM Tris, 0.15 M NaCl, pH 7.4, containing either 0.1 mM $CaCl_2$, or 1 mM EDTA
[c] Values reported are means ± SD of 3 independent experiments

For a spin label undergoing rapid isotropic rotational diffusion, the ratio of linear and quadratic values estimated from eqns 1 and 2 should be near unity [11–13] as demonstrated in table 1 for the maleimide spin label free in solution. The deviation of the ratio from unity is indicative of the label exhibiting an anisotropic rotational diffusion in solution, i.e. rotation along one axis of the molecule is more free than along the other axis. It is worthy of note that the ratios of linear and quadratic values of effective τ_2 for the spectra of spin-labeled TSP are close to 2 whether or not Ca^{2+} is present (table 1). This suggests that the label bound to TSP undergoes an anisotropic rotational diffusion which may be due to the elongated shape of TSP in solution [16].

To determine whether other maleimide spin labels bound to TSP would report the similar change in TSP conformation, we then modified the free thiols of the protein with the spin label 4-maleimido-Tempo in which the nitroxide reporter group is closer to the maleimide moiety than it is with 3-(2-maleimidoethylcarbamoyl)-Proxyl. Hence, upon covalently binding to TSP, the nitroxide in the former should bind closer to the protein surface. The same trend in the data was observed: the effective τ_2 of the bound label was again 2–3-times faster in Ca^{2+}-depleted TSP (not shown).

Although the site to which the spin label attached was not determined directly in this work, two lines of evidence suggest that residue 974 of TSP was labeled by the spin label reagent. (i) Based on the primary sequence of TSP as analyzed by Frazier [1], this cysteine residue is not involved in disulfide formation so that it is a free thiol group. (ii) Preliminary studies by Skorstengaard and Mosher (unpublished) showed that residue 974 could be alkylated by iodo[^{14}C]acetate. In addition, to ascertain whether the spin-label reagents modified selectively the free thiols in TSP, we measured DTNB-titratable thiol groups before and after spin labeling. The results showed that less than 0.1 reactive thiol group per subunit remained on spin-labeled TSP.

In summary, we conclude that the spin label bound near the carboxyl-terminal domain of TSP is sensitive to conformational changes of the protein induced by EDTA chelation of bound Ca^{2+}. The partial unfolding of Ca^{2+}-depleted TSP observed via ESR spectroscopy is in good agreement with previous circular dichroism [4] and electron microscopic observations [7,17]. Thus, whether immobilized on a surface or in solution, TSP appears to undergo a conformational change when Ca^{2+} is removed. The large carboxyl-terminal globular domain contains the platelet and cell binding regions of TSP [1]. The Ca^{2+} sensitivity of the three-dimensional structure of this domain suggests a regulatory role for Ca^{2+} in the platelet aggregation functions of TSP. This could be further investigated by examining spin-labeled TSP in the presence of platelets and other biological substrates.

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