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Potential artifacts in using a GST fusion protein system and spin labeling EPR methods to study protein-protein interactions

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Abstract

Site directed spin labeling EPR methods have been an important tool in studying protein-protein interactions. Labels are often attached to a cysteine residue side chain and spectra are acquired with and without binding partner(s) to provide information on the binding. This requires a knowledge of the location of the label, which is simplified if the label remains faithfully attached to the designated residue in the complex. We report a system where this is not the case because the label was extracted by dialysis-resistant glutathione molecules. Once this artifact is identified, spectral subtraction provides a simple solution for meaningful data interpretation.

Protein-protein interactions are crucial in almost all biological processes such as immune responses, cell signaling and regulation (1). Molecular level studies to identify the site(s) of interaction and to determine structures for mechanistic insights toward association affinity are key approaches in understanding these processes (1). Combining modern electron paramagnetic resonance (EPR) technologies and molecular biology methods to obtain recombinant proteins with specific sites for spin labeling provides powerful approaches to study molecular structures and interactions when either high-resolution NMR or X-ray techniques are difficult. Site-directed spin labeling EPR methods have been applied to study protein structures and protein-protein interactions in protein complexes (1), membrane proteins (2–4), amyloid peptides and fibrils (5–6). Typically, a recombinant protein with a cysteine residue at a designated position is labeled with a sulfhydryl reactive spin label molecule, and EPR spectra of the labeled protein are obtained under different conditions. For protein-protein interaction studies, the spectra are obtained in the absence and presence of non-labeled binding partner(s). The chemical environments and/or the dynamics of the bound spin label without and with the binding partners are compared. This experimental process is often repeated with the cysteine residue at different positions in the protein, a method sometimes called site directed spin labeling EPR (7). In some cases, a qualitative inspection of spectra of labeled proteins with different binding partners is sufficient to provide molecular information, as in allosteric connections involved in the activation process of G proteins (8) or in investigating local conformational changes of Band 3 protein around a residue whose mutation causes hemolytic disease (9). Otherwise, spectral parameters that reflect label mobility or environment are obtained quantitatively from the spectra (7,8,10). Either qualitative or quantitative interpretation of these spectra requires a knowledge of the location of the label, which is simplified if the label remains faithfully attached to the designated cysteine residue upon interaction with binding partner(s). However, if the location of the bound spin label changes

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upon binding the protein partners, the interpretation becomes complicated. We report a system here where the labels do not remain faithfully attached to the designated location, and we believe that this could potentially be a rather common situation when a glutathione S-transferase (GST)-fusion protein is used to prepare proteins used as binding partners. The cysteine-bound spin label was extracted by unsuspected glutathione (γ -glu-cys-gly, GSH) molecules in the unlabeled binding partner. We found that it is quite difficult to remove all GSH molecules in protein samples even for proteins without cysteine residues.

We have used GST-fusion protein protocols to prepare many cysteine site-specific α -spectrin model proteins (Sp α) (as described previously, for example, in refs. 11 & 12) for spin labeling by (1-oxy-2,2,5,5-tetramethyl-3-pyrrolyl-3-methyl) methanethiosulfonate (MTSSL), a widely used label (4,6,7). This labeling is reversible, and the label can be released with the addition of DTT (13). Generally, we were able to achieve one label per protein molecule (1.0 ± 0.2) for about 30 single cysteine proteins in this Sp α family. We used these spin labeled Sp α proteins to study their interaction with its binding partner Sp β . Sp β was also expressed as a GST-fusion protein, purified with affinity chromatography, which required an elution of the protein from the column with a buffer containing 5 mM GSH to release the GST-Sp β protein (12). Purified Sp β samples were dialyzed extensively to remove GSH molecules. We also prepared a cysteine-less Sp β variant, Sp $\beta\Delta$, with the two cysteine residues at 1962 and 2012 replaced by alanine. The spectrum of Sp α -16 (with a single labeled cysteine residue at position 16) (Fig 1A) changed substantially upon addition of Sp $\beta\Delta$ (Fig 1B). The signals in the complex became broader indicating a more immobilized spin label at position 16 in the presence of dialyzed Sp $\beta\Delta$. However, the spectrum of Sp α -16 in a complex with dialyzed Sp β exhibited two components (Fig 1C). One component appeared to be similar to the broad spectrum in (Fig 1B). The other component (about 6 % of total, see figure legend) consisted of rather sharp signals with a hyperfine splitting of about 32.2 G. As mentioned above, Sp β includes two native cysteine residues, and Sp $\beta\Delta$ has the two cysteine residues replaced by alanine residues. These alanine replacements did not seem to change the secondary structures, as indicated by circular dichroism data (not shown). Sp $\beta\Delta$ exhibited an association affinity with Sp α ($K_d = 0.5 \mu\text{M}$) that is similar to, or slightly higher than, that of Sp ($K_d = 1.1 \pm 0.1 \mu\text{M}$, ref. 12). The cysteine residue at position 2012 is in one of the two helices that have been suggested to interact with Sp α (14).

Thermo Finnigan LTQ FT hybrid linear ion trap - Fourier Transform ICR mass spectrometer (Thermo Fisher Scientific, MA) analysis of the Sp β sample used in EPR studies (in 5 mM phosphate buffer with 150 mM NaCl at pH 7.4) showed three components, with 52 % at 22,036.4 Da (consistent with the expected mass of 22,037 Da), 35 % at 22,345.0 Da, a mass of 308 Da higher than expected, and 14 % at 22,648.9 Da, a mass of 611.9 Da higher. The mass of protein with one GSH attached to its cysteine residue is expected to have an increased mass of 306.3 Da. So our extensively dialyzed Sp β sample did not remove all GSH, leaving about one third with one GSH and one tenth with two GSH molecules attached. The treatment of the extensively dialyzed Sp β with 0.1 mM dithiothreitol for 1 h at 37 °C followed by overnight dialysis removed some GSH, but not all; 25 % of it still had one GSH and only 75 % had no GSH, as indicated by mass spectrometry results.

Different cysteine residues in a protein molecule may have different reactivities toward GSH. Since thiolates are more reactive than their protonated counterparts, the pKa of each thiolate plays an important role in determining the reactivity of GSH and cysteine side chain (15,16). We suggest that, when a spin labeled cysteine residue is near a bound or "trapped" GSH molecule, it is possible that the GSH extracts the spin label from the cysteine side chain, resulting in a spin labeled GSH, which exhibits sharp EPR signals, with a hyperfine separation of about 32.2 G (Fig 1D).

The extensively dialyzed Sp β sample was also introduced to another spin labeled Sp α , Sp α -49, with the labeled single cysteine residue at position 49 (Fig 1E), and the spectrum of Sp α this complex sample also exhibited a sharp signal component (about 5 % of total) (Fig 1G). The amount of sharp signal in a similarly prepared complex with labeled cysteine residue at position 25 was about 10 % of total (data now shown). When the extensively dialyzed Sp β was passed through a gel filtration column (Sephadex G-25, Pharmacia), and added to Sp α -49, the spectrum exhibited very little sharp signal (about 1.4 % of total) (Fig 1H), indicating only a very small amount of spin labeled GSH in the sample. Repeated gel filtration to remove excess spin labels in Sp α -16 (Fig 1A) or Sp α -49 (Fig 1E) also removed any residual GSH or labeled GSH in the Sp α alone samples.

Fig 1F shows the spectrum of Sp α -49 in a complex with Sp β Δ , the same physical sample used to obtain the spectrum shown in Fig 1B. A small amount of similar sharp signals (about 1.5 %), presumably from spin labeled GSH, was observed in this sample. Thus, even the cysteine-less Sp β Δ also contained some GSH molecules that were not removed via extensive dialysis. These GSH molecules were able to extract spin label from Sp α -49, but not from Sp α -16, to give observable sharp signals in the complexes of Sp α -49 and Sp β Δ .

In general, we have observed various amounts of sharp signals in complexes of Sp α /Sp β , with spin labels on Sp α at different positions, even when the same batch of Sp β was used, as shown here in Figs 1C and 1G presumably due to different accessibility or different pK_a values of the sulfhydryl-thiolate moiety in the cysteine residue(s).

In conclusion, we report a case where the location of some of the bound spin label in the protein changes upon binding its partner due to the presence of GSH, which is unexpectedly resistant to removal from our protein samples, even in the absence of cysteine residues. Not knowing the transfer of bound labels from the labeled protein to GSH molecules will complicate data analysis and interpretation. However, once this artifact is identified, spectral subtraction, to remove the spin labeled GSH signals, provides a simple solution for meaningful data interpretation.

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Abbreviations

DTT	dithiothreitol
EPR	electron paramagnetic resonance
GSH	reduced glutathione
GST	glutathione-S-transferase
K_d	equilibrium dissociation constant
MTSSL	

1-oxy-2,2,2,5,5-tetramethyl-3-pyrrolinyl-3-methyl) methanethiosulfonate

Sp α

erythroid alpha spectrin

Sp β

erythroid beta spectrin

Sp β Δ

cysteine-less erythroid beta spectrin

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Fig. 1.

The effect of GSH contamination on the EPR spectra of Sp α β complexes. Typical EPR spectrum (A) of Sp α -16 alone, with labeled cysteine residue at position 16, showing no sharp signals, (B) of Sp α -16 in a complex with dialyzed, cysteine-less Sp β Δ , showing no sharp signals, (C) of Sp α -16 in a complex with dialyzed Sp β , showing about 6 % of the sharp signal component, (D) of GSH deliberately labeled, (E) of Sp α -49 alone, with labeled cysteine residue at position 49, (F) of Sp α -49 in a complex with dialyzed cysteine-less Sp β Δ , showing about 1.5 % of the sharp signal component, (G) of Sp α -49 in a complex with dialyzed Sp β , showing about 5 % of the sharp signal component and (H) of Sp α -49 in a complex with Sp β that was dialyzed, followed by gel filtration column treatment, showing about 1.4 % of the sharp signal component. The concentrations for spin labeled Sp α -16 and Sp α -49 samples alone or in complexes ranged from 50 to 70 μ M. The spin label to protein ratios in both proteins were about 1. Since different Sp α β complexes exhibited different K_d values, different Sp β (or Sp β Δ) concentrations were used to ensure the formation of complexes in Sp α and Sp β mixtures. The concentrations for Sp β , and Sp β Δ used for Sp α -16/Sp β complexes (B & C) were about 120 μ M. The K_d for this complex was about 1.6 μ M. The concentrations for Sp β , and Sp β Δ used for Sp α -49/Sp β complexes (E, F & G) were about 275 μ M. The K_d for this complex was about 50 μ M. All EPR samples were in 40% glycerol. The EPR spectra (all 64 scans) were acquired on a Bruker EMX X-band spectrometer with an HS4021 cavity at 20 $^{\circ}$ C. The amount of sharp signals in the spectra indicated above was calculated from the intensity (double integration) of the computer-subtracted spectrum, using spin labeled GSH spectrum (D) to represent the sharp signal spectrum.