STUDIA UBB CHEMIA, LVIII, 2, 2013 (p. 61 – 69)
(RECOMMENDED CITATION)

SITE DIRECTED SPIN LABELING OF HEMERYTHRIN AND HEMOGLOBIN

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ABSTRACT. Site directed spin labeling in combination with electron paramagnetic resonance spectroscopy has become a very effective tool for studying the dynamics and structure of biomolecules in their native environment. This work presents the basics of site directed spin labeling and provides our results obtained in spin labeling hemerythrin and hemoglobin using methanethiosulfonate spin label. Best fit magnetic and libration parameters are obtained by simulation of EPR spectra.

Keywords: *site-directed spin labeling, EPR, proteins, nitroxides*

INTRODUCTION

The concept of site-directed spin labeling (SDSL) in combination with electron paramagnetic resonance (EPR) spectroscopy was developed by Wayne L. Hubbell and coworkers [1, 2]. Often conventional spectroscopic techniques are insufficient for studying structural properties of biomolecules, and EPR spectroscopy has distinguished itself by offering the possibility of studying the behavior of proteins in their native-like environment. The technique is available for studying soluble biomolecules such as proteins and nucleic acids, regardless of the size or complexity of the system [3, 4, 5, 6]. Although the majority of proteins are EPR-silent, SDSL and EPR can be used together, often in conjunction with site directed mutagenesis for creating a specific attachment point for the attachment of a nitroxide spin label, or, in cases where multiple such points already exist, for selecting only one of those for SDSL. Cysteine is a convenient aminoacid to use as target for SDSL, as its thiol group will react with the functional groups of the spin label; methanethiosulfonate, maleimide and iodoacetamide, creating a covalent bond.

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The nitroxide spin labels contain the nitroxyl radical (N-O) incorporated in a heterocyclic ring and the unpaired electron localized mostly on the N-O bond. Once attached to the protein their motion is dominated by their environment and the proteins backbone motion. The nitroxide spin label is influenced by his environment, surrounding structures and the motion of the protein, which is reflected in the EPR spectra of the spin label [7, 8].

Among the long list of available spin labels the (1-oxyl-2,2,5,5-tetramethylpyroline-3methyl) methanethiosulfonate spin label (MTSSL) [9] is the most often used, because its small molecular volume and flexibility, due to the link between the piperidine-oxyl group and the protein backbone, minimizing the disturbance of protein folding. After labeling the attached side chain is abbreviated as R1. Although their length, 5-8Å depending on the conformation, MTSSL side chains don't influence or disrupt the structural and functional properties of the protein [10].

The free electron situated on the nitroxyl radical has a strong dipolar interaction with the nitrogen nucleus, due to the nitrogen's nuclear spin state of I=1, the EPR spectral line shape will be formed by three lines each arising from one of the three quantum states of the nitrogen nucleus. The electron is also sensible to the anisotropic environment of the chemical bond. These anisotropies of the surrounding interactions make the label sensitive to its motion.

Continuous wave (cw) EPR spectroscopy of the spin labeled systems gives information about side chain mobility, solvent accessibility, polarity of the spin labels environment and distance between two paramagnetic centers [11].

Side chain mobility is a term used to describe the effect of motional rate, anisotropy and reorientational motion of the spin label on the EPR spectra [12]. At room temperature the EPR spectrum is particularly sensitive to the reorientational motion of the side chain because of the partial motional averaging of the anisotropic components of the g- and hyperfine tensors [10, 13, 14]. When exposed to a motionally less restrictive environment (e.g., water), the nitroxide will gain a faster rotational correlation time, in the ns range. In this case the three-peaked EPR spectra will look sharper, with a small central line width (ΔH_0) and small hyperfine splitting. In case of mobility restriction of the spin label (e.g., due to higher viscosity solvents or buried location of the side chain), the central line width and also the hyperfine splitting will increase and the first and last peaks intensity will also decrease [4, 15].

This paper reports the procedure and our results obtained in site directed spin labeling of *Phascolopsis gouldii* (Peanut worm) hemerythrin (Hr) and *Bos Taurus* (Bovine) hemoglobin (Hb).

RESULTS AND DISCUSSION

In Figure 1 the EPR spectrum of the MTS spin label free in water is presented, where the spin label moves faster and has a fast rotational correlation time of 0.79 ns. The second EPR spectrum represents also unbound MTS spin label, but now in a more viscous environment - 85% glycerol in water. The high viscosity of the glycerol determines the spin label to move slower obtaining a rotational correlation time of 2.12 ns. The difference is noticeable on the spectral lines, and the first and third peak have decreased in the case of 85% glycerol, when the label became more immobile; also, the central line width changes from ΔH_0=1.17 G in water, to 2.05 G in the glycerol/water mixture.

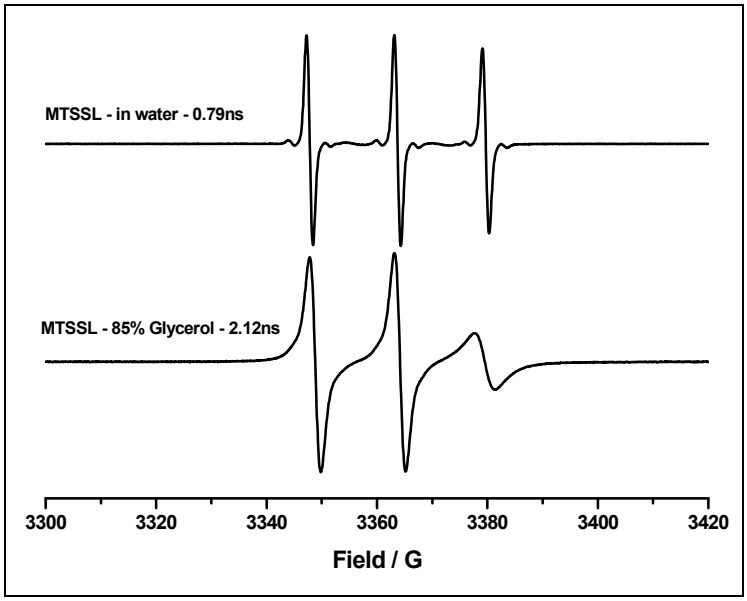

Figure 1. EPR spectra of MTSSL in water and glycerol-water solution.

Hemerythrin is a respiratory protein extracted from the blood of the Peanut worm; the protein is responsible for the oxygen transport in the organism by using a non-heme di-iron site [16, 17, 18]. Hr is a relatively large protein with a homooctameric structure and 108 kDa mass. The subunits are consistent of a four-helix bundle protein backbone, with 114 aminoacids. Every subunit is identical and contains a native cysteine at the 51st position, allowing one to spin label the protein at this selected site; Figure 2 illustrates the position of this cysteine within the monomer.

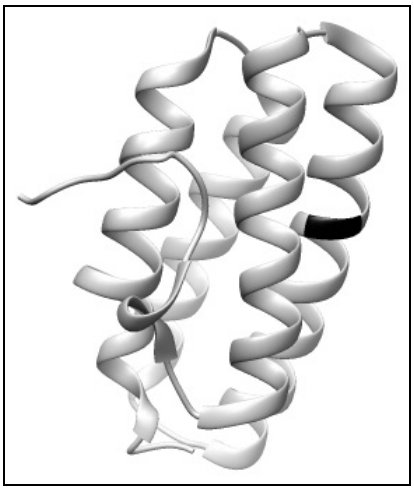

Figure 2. Hemerythrin subunit A with native cysteine at the 51stposition (black)

For a next step the labeling protocol was also applied to bovine hemoglobin. This type of hemoglobin is slightly different from the human one, in that it has only native cysteine in the *beta* (β) subunit at the 92nd position, the protein is composed from two *alpha* subunits and two *beta* subunits, containing in total 2 cysteines available for labeling. After labeling we abbreviated the sample Hb92R1. Figure 3 illustrates the beta subunit of the Hb containing the native cysteine in blacked.

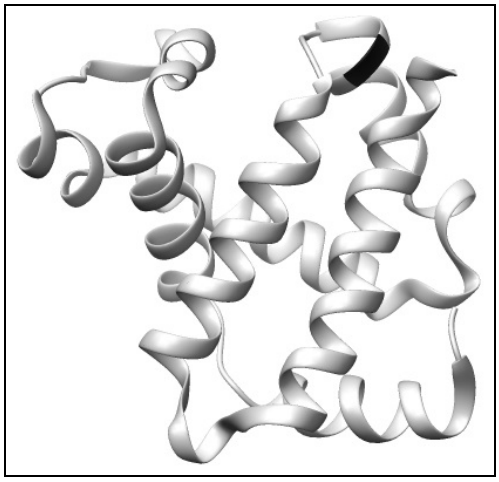

Figure 3. Representation of the Hb beta subunit with native cysteine colored in black.

For a quantification of the successfully bonded spin label to the protein, spin labeling efficiency, the EPR spectra of precisely prepared 100μM MTSSL sample were recorded as control. The double integral area of the EPR spectra of the MTSSL control sample can be used as a reference for the spin quantity corresponding to 100 μM spin units. The concentration of the protein was determined by UV-vis spectroscopy, via the absorption at 330 nm in the case of the Hr and at 430 in case of Hb.

From the double integral area of the EPR spectra of the samples the concentration of spins in the samples could then be determined – which, reported to the protein concentration and taking into account the number of cysteines available per monomer allows one to calculate the spin labeling efficiency.

For the hemerythrin sample prepared without reducing with DTT the labeling efficiency was 82%, whereas for the other sample, in which preparation included the DTT reducing step, a 91% labeling efficiency was attained. The nearly 10% difference observed by using DTT is a considerable advantage and improvement to the procedure. In the case of the hemoglobin the obtained spin labeling efficiency was 97%. Overall, these are very good yields. Indeed, both of the proteins have their native cysteine in a location easily accessible for the spin label; this is known to be one of the major influencing factor in spin labeling.

Upon analysis of the EPR spectra, the g- and hyperfine tensor values for the samples and the rotational correlation time of the spin label along with the component fraction were calculated. Using the simulation software for nitroxide spin labels Multicomponent EPR 495 developed by Christian Altenbach, we simulated the EPR spectra of the Hr with 3 components. The idea in a simulation is to use the minimum number of components possible in a simulation and obtain the best result. In the case of the Hb the simulation was done with 2 components.

Table 1. Best fit simulation parameters for spin labeled hemerythrin EPR spectra

Comp	**Fraction** (%)	$\mathbf{A_{xx}}$ (G)	$\mathbf{A_{yy}}$ (G)	$\mathbf{A_{zz}}$ (G)	$\mathbf{g_{xx}}$	$\mathbf{g_{yy}}$	$\mathbf{g_{zz}}$	**Corr. Time ns**
I	68.34	7.35	7.65	33.02	2.0087	2.0067	2.0032	4.26
II	22.57	8.25	8.55	32.05	2.0085	2.0065	2.0045	3.5
III	9.09	8.55	8.85	31.56	2.0085	2.0065	2.0041	1.16

A possible practical explanation for the three Hr components would be that the largest fraction represents the Hr octameric structure, the smallest fraction would represent the monomeric state of the protein and the third component would represent a multimeric structure of the protein.

As seen in Table 1 the small fractioned component is the fastest one; the explanation that this component represents the monomeric state in the protein fits perfectly, because of the large possible mobility of the spin label on the single subunit, due to his outer side position on the helix of the subunit. This mobility is limited when we talk about the octameric form since in that case the spin label is not at the surface anymore - it is between two subunits, and this fact is observed also in the rotational correlation time.

Table 2 contains the data obtained from simulations on the hemoglobin spectra. The mobility of this protein is quite different from the Hr; the protein is tighter, smaller and more restrictive for the spin label movement as the Hr. In this case the label is not pointing towards the outside as in the case of the Hr subunits, but is rather trapped inside the beta unit; this explains the slower rotational correlation times.

Table 2. Best fit simulation parameters for spin labeled hemoglobin EPR spectra

Comp	**Fraction** (%)	$\mathbf{A_{xx}}$ (G)	$\mathbf{A_{yy}}$ (G)	$\mathbf{A_{zz}}$ (G)	$\mathbf{g_{xx}}$	$\mathbf{g_{yy}}$	$\mathbf{g_{zz}}$	**Corr. Time ns**
I	21.21	9.83	10.13	33.68	2.0070	2.0050	2.0039	3
II	78.79	5.9	6.2	34.76	2.0080	2.0060	2.0022	4.73

By looking at the spectra of the two proteins on the Figure 4 one can clearly see the differences in the shape. The simulation data shows that the Hr is more mobile, and this is noticeable in the EPR spectra as well, with the first peak more intense and sharp. In the Hb spectra, this component is shifted to the left and has less sharpness and intensity. In the case of Hb, due to the tightness of the system, the label is not as mobile as in the case of the Hr, this is reflected in the spectra as well as in the correlation times.

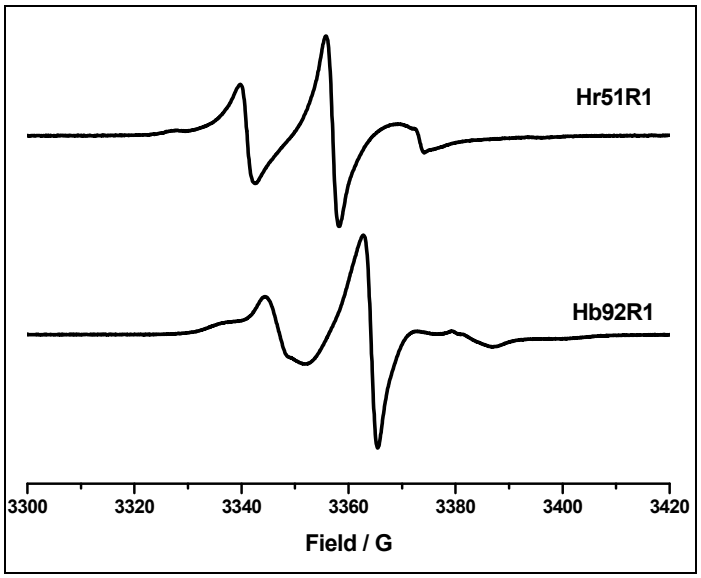

Figure 4. EPR spectra of the spin labeled hemerythrin (top) and spin labeled Hemoglobin (bottom).

CONCLUSIONS

By analysis of EPR spectra of nitroxide radical motion within a labelled protein one can obtain the main dynamic parameters of protein domains and characterizes protein conformation and any changes that may occur in different environments, too. Proof of concept data are shown here for hemerythrin for the first time, in comparison with the more often-studied hemoglobin.

EXPERIMENTAL SECTION

The EPR measurements were carried out on a Bruker EMX EPR spectrometer with continuous wave at X-band (9 GHz), equipped with a Bruker liquid nitrogen temperature controller. The spectra were recorded at room temperature with a microwave frequency of 9.45 GHz, microwave power of 4 mW, modulation frequency of 100 kHz, modulation amplitude of 1 G and microwave attenuation of 17 dB. The samples were measured in quartz capillary tubes containing 15 µL of sample.

The MTS spin label was inquired from Enzo Life Sciences and was dissolved in DMSO (100 mM, stock solution).

Simulation of the EPR spectra of labelled proteins, was performed by Multi-Component EPR Fitting v2 version 495 program, a LabVIEW software, developed by Dr. Christian Altenbach (University of California, Los Angeles, California, *https://sites.google.com/site/altenbach/labview-programs/epr-programs/multicomponent)*

For the purpose of spin labeling, *Phascolopsis gouldii* Hr, purified as previously described [19], was first suspended in PBS buffer and a final concentration of 10 mM DTT was added to the solution. The mixture was incubated and constantly shaken at 4^{o}C for 2 hours. To obtain a good spin labeling efficiency, by preventing the reduction of the disulfide bridge between the cysteine and the side chain, the DTT was removed by washing from the system using a 10kDa Millipore filter in a Beckman J21B centrifuge. The sample was centrifuged 6 times at 4^{o}C, 5000 rpm for 30 minutes. After each step the flow-trough was checked by UV-vis spectroscopy for traces of DTT. After the complete removal of DTT from the system the MTSSL spin label was introduced into the sample with a 10 times excess to each monomer. A second sample of Hr was also prepared, where the DTT part was altogether omitted, on the grounds that Hr does not contain disulfide bonds – and the role of DTT in principle would be precisely to cleave disulfide bonds in order to liberate the thiols for labeling. The two samples were incubated overnight at 4^{o}C.

The next step was to remove the unbound excess spin label from the sample and to determine the efficiency of the spin labeling. The two samples were washed out of the excess spin label by using 10 kDa Millipore filters and were centrifuged at 5000 rpm and 4^{o}C for 5 times. After each step of washing the flow-trough was checked for remnant spin labels by EPR spectroscopy. After the 4th step the flow-through EPR spectra showed no traces of spin label. This was done to both of the samples in the same conditions. The obtained sample after labeling is abbreviated as Hr51R1, 51 representing the 51st position of the subunit where the spin label is bound and R1 is the name of the side chain after labeling, as mentioned before.

ACKNOWLEDGMENTS

IMT acknowledges the financial support of the Sectorial Operational Programme for Human Resources Development 2007-2013, co-financed by the European Social Fund, under the project number POSDRU/107/ 1.5/S/76841 with the title „*Modern Doctoral Studies: Internationalization and Interdisciplinarity*”.

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