Practical 1

Continuous-wave EPR spectroscopy at room temperature

Site-directed spin labeling and applications to protein dynamics studies.

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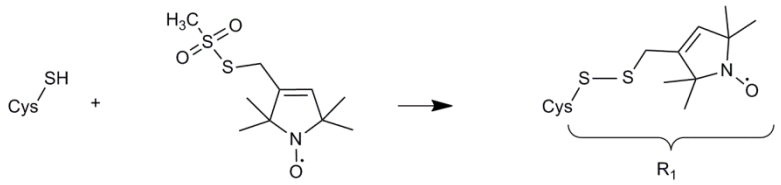
Monitoring dynamics, structural transitions and protein-protein interactions is of crucial interest and in particular, information has to be obtained under physiological and functional conditions affording essential knowledge on functional related conformational changes. Site-Directed Spin Labeling (SDSL) combined to Electron Paramagnetic Resonance (EPR) has emerged as a powerful technique to gain structural and dynamic information on many biological systems.[1–3] The chemical and spectroscopic properties of the spin label itself, and the strategy used for its introduction into the protein under study, are central aspects of SDSL.[4–7] Classical SDSL is based on the insertion of a paramagnetic label (a nitroxide radical, RNO•) at a selected site of a protein and its subsequent analysis by EPR spectroscopy. For proteins, MTSL (1-oxyl-2,2,5,5-tetramethyl-3-pyrroline-3-methyl methane thiosulfonate) is mostly used; it reacts with the SH-group of cysteine residues to form a covalent disulfide bridge leading to a R1 chain (scheme 1). The label reacts with either native cysteine residue or cysteines introduced by site-directed mutagenesis. The presence of the label does not usually affect the protein properties thanks to its rather small size which is comparable to that of a tryptophane residue (7 Å).



Scheme 1: Labeling reaction scheme for cysteine residues using methanethiosulfonate spin label (MTSL).

As a model of protein-protein interaction, we will use the well-described NarJ, a chaperone protein ensuring folding and assembly of the membrane-bound respiratory nitrate reductase complex, NarGHI,[8,9] member of a large group of molybdenum containing enzymes.[10] A first interaction site between NarJ and NarGHI has been identified: NarJ binds to the N-terminus of the NarG subunit of the nitrate reductase. In particular, the NarJ binding site on NarG was shown to be restricted to the first 15 residues (NarG(1-15)), without any influence on

the binding properties. [Lorenzi et al PlosOne2012] In this practical, we will use the NarJ/NarG(1-15)peptide complex as a working model.

1. Materials

Prepare all solutions using ultrapure MilliQ water (prepared by purifying deionized water to attain a resistivity of 18 MOhms.cm at 25°C) and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing waste materials. The NarG(1–15) peptide was chemically synthesized and purified by Synprosis (Marseille, France) as previously reported[11].

Spin labeling

1. Single-cysteine mutated protein NarJ (C207, E119C).

2. Appropriate buffer without any reductant. Here for the labeling of NarJ, the buffer is : 10 mM 50 mM Tris-HCl pH8 100 mM NaCl.

3. 1.5 mL and 0.5 mL Eppendorfs.

4. Dithiotreitol solution (DTT), Sigma-Aldrich.

5. Spin label: 1-oxyl-2,2,5,5-tetramethyl-3-pyrroline-3-methylmethane-thiosulfonate (MTSL) (Toronto Research Chemicals Inc.). A solution of known concentration of MTSL will be made in acetonitrile and aliquots will be kept frozen in liquid nitrogen.

6. Two PD-10 Desalting Columns containing 8.3 mL of SedaphexTM G-25 Medium (GE Healthcare).

7. Vivaspin 4 mL concentrators (Sartorius Stedim Biotech) with appropriate cut-off (10 KDa).

8. UV-vis absorption spectrometer.

EPR spectroscopy

1. Bruker Magnettech MS 5000 operating at 9.9 GHz.

2. Quartz capillary of 1.0-1.1 mm inner diameter for a sample volume of 30 µL-40 µL.

3. 100 µL syringe (SGE or Hamilton) equipped with a 20 cm-long needle.

4. Sucrose (Sigma-Aldrich).

2. Protocol

Spin labeling

A critical point for achieving high labeling yields is the reduction of cysteine residues prior to incubation with the nitroxide reagent (MTSL). Incubation of the protein with an appropriate reducing agent, such as DTT, is thus carried out as the first step and a gel filtration onto a desalting column is done to remove excess of reducing agent. Once DTT has been removed, it is recommended to immediately proceed to spin labeling to avoid re-oxidation of cysteines. Figure 1 illustrates the protocol of the labeling experiment. Fractions containing the labeled protein are pooled and concentrated using an appropriate concentrator. An EPR spectrum of this concentrated pool is then recorded.

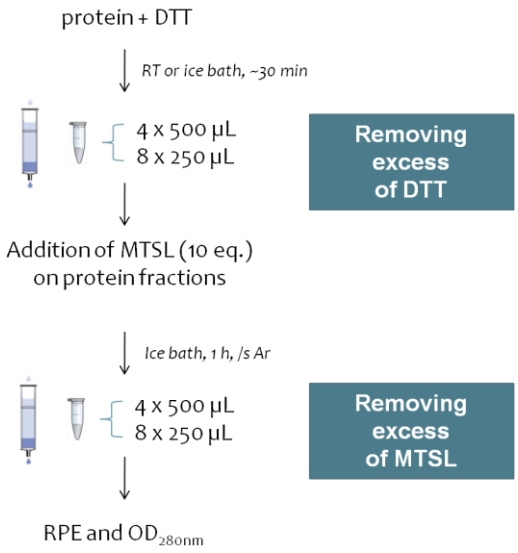
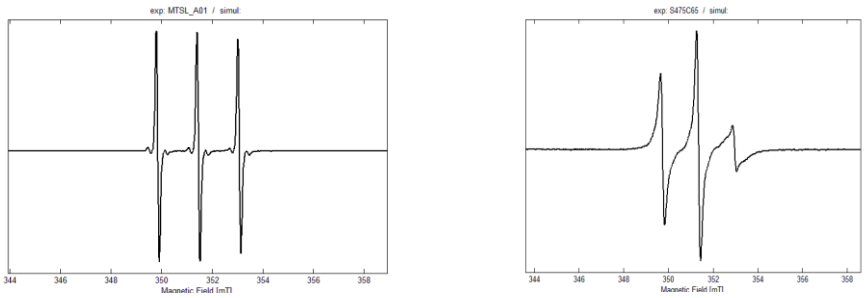


Figure 1: Labeling experiment of a protein using methanethiosulfonate spin label (MTSL).

EPR spectroscopy

The analysis of the nitroxide spin probe mobility is based on the analysis of the EPR spectral shape recorded at room temperature.[12] It is thus crucial to record spectra without distorting the EPR spectral shape and with an optimized signal to noise ratio (SNR). Two major parameters have to be carefully adjusted to avoid signal distortions: the microwave power (*P*) and the modulation amplitude of the magnetic field (*MA*).



A B

Figure 2: Room temperature CW EPR spectra of A) MTSL in H_2O and B) an example of a labelled protein with MTSL.

Spin quantification

Spin concentration of the labeled protein (C_{spin}) is obtained by comparing the double integration of the EPR signal of the labeled protein (I) with the double integration (I_{std}) of the EPR signal of a standard solution of known concentration (C_{std}) (ideally a 100µM stable nitroxide radical solution).

Spin concentration C_{spin} is obtained using the following equation:

$$C_{spin} = C_{std} \frac{I}{I_{std}} \frac{RG_{std}}{RG} \frac{MA_{std}}{MA} \sqrt{\frac{P_{std}}{P}} \frac{NA_{std}}{NA}$$

where I is the result of the double integration of the signal of the labeled protein, RG the receiver gain, MA the modulation amplitude of the magnetic field, P the microwave power, and NA the number of accumulations. The subscript *std* corresponds to the standard solution.

The labeling yield is obtained by dividing the spin concentration (C_{spin}) by the protein concentration (deduced from the absorbance at 280 nm). Labeling yields typically range from 50 to 100%, reflecting the accessibility of cysteine residues.

3. References

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