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Review

New limits of sensitivity of site-directed spin labeling electron paramagnetic resonance for membrane proteins☆

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ABSTRACT

Site-directed spin labeling electron paramagnetic resonance is a biophysical technique based on the specific introduction of spin labels to one or more sites in diamagnetic proteins, which allows monitoring dynamics and water accessibility of the spin-labeled side chains, as well as nanometer distances between two (or more) labels. Key advantages of this technique to study membrane proteins are addressed, with focus on the recent developments which will expand the range of applicability. Comparison with other biophysical methods is provided to highlight the strength of EPR as complementary tool for structural biology. This article is part of a Special Issue entitled: Beyond the Structure-Function Horizon of Membrane Proteins edited by Ute Hellmich, Rupak Doshi and Benjamin McIlwain.

1. Introduction

All living organisms depend on membranes confining individual cells and the compartments/organelles within [1,2]. Thereby, approximately 30% of the human proteins are membrane-embedded or membrane-attached. Membrane proteins receive signals from outside the cell and translate them into intracellular action, enable a controlled exchange of material, information and energy across the membranes, as well as shape, stabilize, fuse or divide membranes, organelles and cells. However, compared to soluble proteins, our knowledge on the structure, function and interactions of membrane proteins in their native environment is still limited, as many experiments face technical limitations.

This review describes the defining aspects of site-directed spin labeling (SDSL) electron paramagnetic resonance (EPR) spectroscopy, a technique which can be applied without size or environment limitations to membrane proteins, providing with a high level of fidelity and sensitivity site-specific information about dynamics, water accessibility and relative location of spin-labeled probes covalently attached to proteins. In contrast to NMR, which shares with EPR the basic quantum mechanical description but relies on the detection of the complex interaction network of a variety of intrinsic nuclear spins isotopes (^{1}H, ^{2}H, ^{13}C, ^{14}N, ^{15}N, ^{31}P…), SDSL EPR requires in most cases the introduction of ad hoc spin labels carrying unpaired electrons. Naturally occurring electron spin centers also exist in proteins carrying metal cofactors, or if short-living radical species are created during a reaction, but these cases are not covered in this review. Site-specificity implies that one (or few) sites can be labeled and analyzed for each protein. This conveys both the major advantage of the technique in terms of specificity, sensitivity and negligible size/environment limitations, as well as its major disadvantage, i.e. it cannot provide protein structures at atomic resolution, but rather coarse-grained structural models.

SDSL EPR was introduced by the pioneering work of Hubbell in the 1990's (notably, one the first studied proteins was a membrane protein [3]) and it has now become a wide spread complementary tool in structural biology.

In the following, we describe the main information that can be obtained by SDSL EPR on membrane proteins, namely dynamics of the spin-labeled side chain, water/membrane accessibility and interspin distances. Breakthrough technical innovations will be highlighted in each paragraph, to provide a state-of-the-art answer to the question: “Can I use SDSL EPR to gain structural insights on my favorite membrane protein, and which information can I obtain?”. At the end, we will provide two examples of EPR studies from our lab performed on Bax, a propapoptotic human protein and on TM287/288, a bacterial ABC exporter.

2. The starting point: mutagenesis and labeling

First, we need to attach one (or few) spin label(s) to specific sites in a protein. The most common strategy is to utilize cysteines engineered at defined positions, and covalently attach a nitroxide label via different

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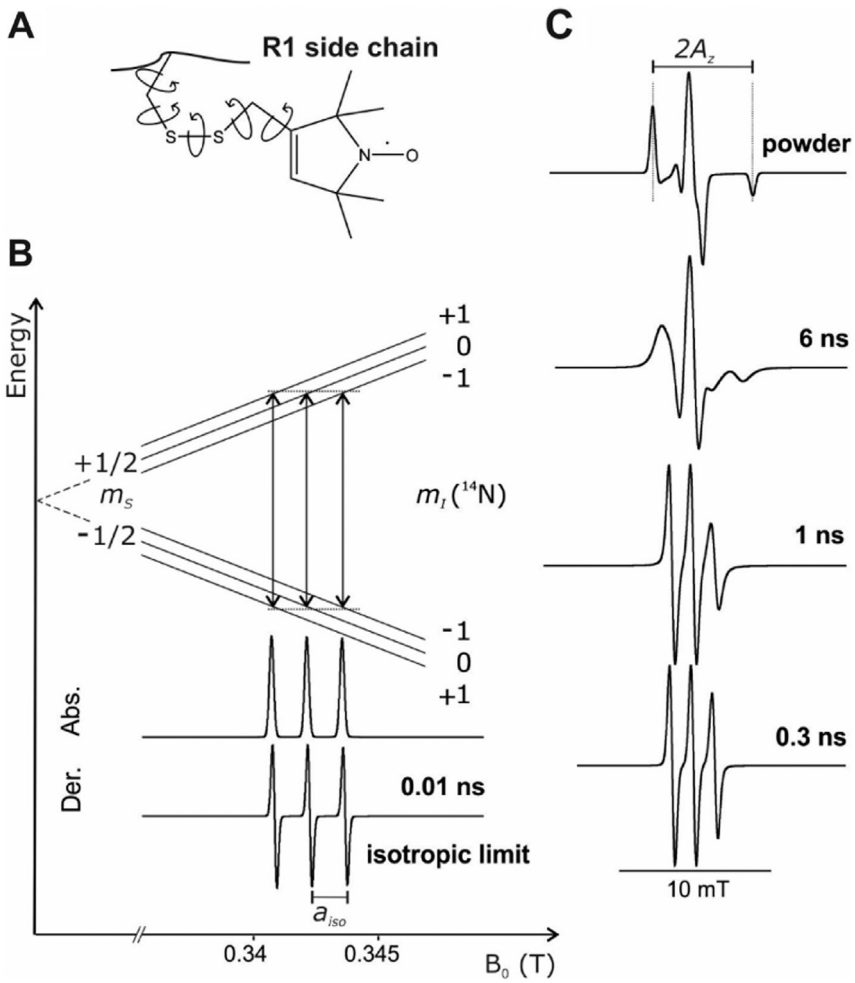

Fig. 1. Nitroxide probes and dynamics encoded in the spectral lineshape. **A**) Scheme of an MTSL probe bound to a cysteine residue. The five rotatable bonds are highlighted. B) Energy scheme showing the origin of the three-line spectrum for a nitroxide in the isotropic fast motion limit. The rotational correlation time used for the spectral simulation is 10 ps (simulation done with Easyspin [16]). The upper spectrum is the X-band absorption, the bottom one is the derivative. CW EPR spectra are usually detected via lock-in detection, therefore they are presented as derivative spectra. C) Example of X-band spectra with different rotational correlation times (written in the figure legend). The first is the 'powder' spectrum in the absence of molecular motion (powder or frozen samples); the distance between the low and high field peak is the $2A_z$ hyperfine component. The other spectra show the effects of rotational motion on the spectral features. Faster motions are characterized by narrower lines and higher intensity of the third line with respect to the central one.

functional groups [4]. Therefore, it follows that the membrane protein of interest should ideally contain no natural cysteines, or eventually only few which are not accessible to the label. In general, natural cysteines are removed and, if this is not impairing protein function and structure, one can start to introduce one cysteine at a time. If functionally relevant cysteines are present, one can try to circumvent the problem by producing a protein incorporating unnatural amino acids which can either react specifically with a functionalized nitroxide probe or directly carry a nitroxide label (see for example [5,6]), which for membrane proteins remains a challenging task, because it requires much more effort than conventional recombinant protein purification methods. Otherwise, an alternative possibility is to attach a nitroxide spin probe to a tyrosine residue, which was shown to be effective and specific [7].

The most common spin label for cysteines is a methanethiosulfonate functionalized nitroxide radical (MTSL, (1-Oxyl-2,2,5,5-tetramethyl-Δ3-pyrroline-3-methyl) methanethiosulfonate), which carries a stable spin ½ delocalized in the N-O bond (Fig. 1A) and targets engineered cysteine residues in proteins with high specificity. However, the labeling efficiency (how many cysteines carry the nitroxide label) is site-specific, and it can reach 100% for exposed sites, but can also be close to 0% for sites buried in the protein interior. Thus, pre-knowledge of the possible water exposure of the engineered site favours the success of the SDSL EPR study. Due to its high specificity for cysteines and good degree of labeling, MTSL is the right choice if one wants to extract information on the dynamics of a protein site at physiological temperature. This spin label is inert at most positions and a large amount of data exist in literature, which facilitates data analysis and comparison. Due to the diamagnetic nature of the non-labeled proteins, the EPR signal arises only from the fraction of labeled variants in the ensemble. Therefore, for mobility and water accessibility studies, low labeling efficiencies are not detrimental for data analysis, but only if it is possible to ascertain the correct fold and functionality of the spin-labeled fraction in the ensemble. In case the labile nature of the S-S bond between the cysteine and the label causes problems, other types of labels functionalized with maleimido or iodacetamido groups are commercially available, which are linked to the protein via a more stable S-C bond. If reducing environments cannot be avoided, a series of sterically protected nitroxide labels are also available (for a recent review on nitroxide labels see [8]).

Besides nitroxide probes, many other spin labels were recently introduced, especially for interspin distance measurements. Examples are: functionalized chelators of paramagnetic lanthanides (Gd^{III}, spin 7/2) reacting with cysteines (for a review see [9]); carbon-based radicals (trityl, spin ½) [10] targeting cysteines; metals such as copper (Cu^{II}, spin ½) which specifically bind to engineered high-affinity histidine binding sites in proteins [11]. Despite specific advantages, which will be introduced later, the latter probes are spectroscopically orthogonal to nitroxides, meaning that they can be distinguished by spectral absorption, relaxation times, detection schemes, etc. Therefore, if protein oligomers or multiprotein complexes are labeled with distinct probes, simultaneous extraction of interspin distances between different pairs in the same sample is possible (e.g. Gd-Gd, Gd-nitroxide, Cu-nitroxide, etc.). In the last few years, several new spectroscopically orthogonal spin label pairs and distance measurements were reported (see paragraph "Interspin distances" below). The biggest disadvantage of the Gd-based and trityl labels is that they are bulkier than nitroxide probes and labeling site must be chosen with care to avoid interference in protein

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