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Liquid state DNP for water accessibility measurements on spin-labeled membrane proteins at physiological temperatures

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ABSTRACT

We demonstrate the application of continuous wave dynamic nuclear polarization (DNP) at 0.35 T for site-specific water accessibility studies on spin-labeled membrane proteins at concentrations in the 10–100 μM range. The DNP effects at such low concentrations are weak and the experimentally achievable dynamic nuclear polarizations can be below the equilibrium polarization. This sensitivity problem is solved with an optimized home-built DNP probe head consisting of a dielectric microwave resonator and a saddle coil as close as possible to the sample. The performance of the probe head is demonstrated with both a modified pulsed EPR spectrometer and a dedicated CW EPR spectrometer equipped with a commercial NMR console. In comparison to a commercial pulsed ENDOR resonator, the home-built resonator has an FID detection sensitivity improvement of 2.15 and an electron spin excitation field improvement of 1.2. The reproducibility of the DNP results is tested on the water soluble maltose binding protein MalE of the ABC maltose importer, where we determine a net standard deviation of 9% in the primary DNP data in the concentration range between 10 and 100 μM. DNP parameters are measured in a spin-labeled membrane protein, namely the vitamin B_{12} importer BtuCD in both detergent-solubilized and reconstituted states. The data obtained in different nucleotide states in the presence and absence of binding protein BtuF reveal the applicability of this technique to qualitatively extract water accessibility changes between different conformations by the ratio of primary DNP parameters ϵ. The ϵ-ratio unveils the physiologically relevant transmembrane communication in the transporter in terms of changes in water accessibility at the cytoplasmic gate of the protein induced by both BtuF binding at the periplasmic region of the transporter and ATP binding at the cytoplasmic nucleotide binding domains.

1. Introduction

The combination of site directed spin labeling (SDSL) with nitroxide-based spin probes and electron paramagnetic resonance (EPR) gives site-specific structural information on water soluble and membrane proteins [1]. In particular, this technique is very sensitive to the dynamics of the spin-labeled side chain and of the backbone to which the label is attached to and provides accurate interspin distances between two spin-labeled sites. Both types of information are valuable to characterize protein structures and structural transitions. In addition, insights into the nitroxide micro-environment in terms of water accessibility can be obtained using continuous wave (CW) and pulsed EPR methods both at room and cryogenic temperatures. The three main methods applied to spin-labeled proteins to extract water accessibility towards the nitroxide are briefly described in the following. The quantification of the 'water accessibility' is method-specific and is based on different nitroxide parameters correlated to the presence of water around the label.

First, a change in water accessibility can be detected with CW EPR at temperatures below 180 K at X-or W-band frequencies. In fact, a change in polarity (electric field along the NO bond) and proticity (H-bond formation to NO) in the nitroxide micro-environment expresses as a change in the hyperfine tensor component A_{zz} and in the g-tensor component g_{xx} [2–5]. This method requires cryogenic temperatures and the combined effects of water molecules, charged or protonated groups and hydrogen bond donors constitute the overall water accessibility parameter.

Second, an indirect water accessibility parameter is the nitroxide relaxation enhancement induced by water soluble paramagnetic quenchers (e.g. NiEDDA or CrOX) measured at room temperature via CW power saturation methods [6] or via saturation recovery techniques [7]. While this approach reveals proximity between the aqueous phase and the nitroxide, the charge and size of the paramagnetic quenchers may lead to different quencher accessibility compared to water accessibility for spin-labeled side chains close to charged surfaces or in narrow translocation channels.

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Third, water accessibility can be characterized via the hyperfine splitting caused by nearby deuterated water molecules with electron spin echo envelope modulation (ESEEM) [8,9]. This technique has the advantage to provide a parameter that is more directly related to the local concentration of (deuterated) water molecules in close proximity to the nitroxide, however it requires cryogenic temperatures.

With these three methods it was possible for example to map the water permeability profile of the proton translocation channel in bacteriorhodopsin [10], to unveil the molecular architecture and the mechanism of the activation gating in the potassium channel KcsA [11,12] and to quantify the average number of water molecules at specific spin-labeled sites in LHCII [13], respectively.

To overcome some limitations of the above mentioned techniques, we demonstrate here the use of dynamic nuclear polarization (DNP) for direct determination of water accessibility towards spin-labeled side chains at physiological temperatures. The DNP mechanism relevant for water accessibility is the Overhauser effect [14]. In Overhauser DNP, saturated electron spins transfer polarization to the nucleus by coupled electron-nucleus cross-relaxation. Because the gyromagnetic ratio of an unpaired electron (γ_S) is-658 times higher than that of a proton (γ_I), the nuclear equilibrium polarization can be enhanced drastically upon electron saturation. Due to the high measurement sensitivity achievable with DNP, several applications in nuclear magnetic resonance (NMR) and magnetic resonance imaging (MRI) are emerging [15,16].

For a systematic treatment, a DNP parameter ϵ can be defined as [17]

$$\epsilon = \xi s f \frac{\gamma_S}{\gamma_I} \tag{1}$$

where ξ is the coupling factor, s is the saturation factor and f the leakage factor. The DNP in units of the equilibrium polarization P_0 therefore reads $P = 1 + \epsilon$, which is often referred to as the DNP enhancement.

The coupling factor ξ describes the net cross-relaxation efficiency between one saturated electron spin and one proton spin. In liquids, ξ thus depends on the relative electron–proton dynamics stochastically modulating the dipolar coupling at timescales of the electron Larmor frequency. In the narrowing regime at low fields, ξ can reach its theoretical maximum value of 0.5. For moderate fields at X-band frequencies, the coupling factor is sensitive to changes in the picosecond correlation times of translational hydration dynamics [18]. At high fields, fast sub-picosecond dynamics are studied [19].

The saturation factor s describes to which extent the electron spectrum is saturated and reaches 1 if all spins are saturated. To remove the dependency on the excitation power, it is common practice to extrapolate the saturation factor to infinite microwave powers [20]. The maximum saturation factor $s_{\max}$ then reveals how saturated electron spins are coupled amongst distinct hyperfine lines. Such coupling is mediated by Heisenberg exchange at high spin label concentration or by fast nitrogen nuclear relaxation for spin labels characterized by slow reorientational motion [21]. The factor $s_{\max}$ approaches 1 for a broad range of rotational correlation times of nitroxide probes ($\tau_c \approx 10^{-9} - 10^{-6}$ s) [18], which are typically observed for spin labels attached to membrane proteins.

The leakage factor f describes the exchange between protons in close proximity of the spin label (local water) and the bulk proton reservoir in the sample (bulk water). This factor is given by

$$f = 1 - \frac{T_1}{T_{10}} \tag{2}$$

where the relaxation enhancement T_1/T_{10} of the proton spins is the ratio of longitudinal relaxation times in the presence (T_1) and absence (T_{10}) of the electron spins. The leakage factor therefore depends on electron spin concentration. In a heterogeneous environment, such as a spin label in an isolated shell of water, the leakage factor also encodes long range accessibility to the spin label by bulk water. The DNP parameter ϵ therefore contains 'water accessibility' information in terms of local dynamics of water-label collisions (ξ) and in terms of exchange between local water and bulk water (f).

Up to date, biochemical DNP studies focused on monitoring protein aggregation [22] or hydration dynamics in synthetic soft matter [23–26] and water soluble proteins [27,28]. The hydration dynamics studies made use of the theoretical force-free hard-sphere model [29] to extract absolute values of water diffusivity. In the approach presented here, we make use of the primary DNP data (ϵ) to extract relative changes of water accessibility at specific sites in membrane proteins. The aim of this work is to show that it is possible to directly obtain coarse-grained water accessibility to follow conformational changes in spin-labeled membrane proteins at concentrations in the 10–100 μM range. For this purpose, we present the performance of an optimized DNP probe head which can be used in a modified pulse EPR spectrometer or in a dedicated CW EPR spectrometer equipped with a commercial NMR console. The probe head is tested on TEMPOL stock solutions and on a water-soluble protein.

DNP is then used to extract water accessibility changes on a membrane protein during its conformational transitions both in detergent and liposomes. The membrane protein investigated is the vitamin B_{12} importer BtuCD in different nucleotide states in the presence and absence of its binding protein BtuF. The water accessibility changes obtained by direct comparison of the primary DNP data agree with the polarity data obtained by low temperature CW EPR and complement the analysis of the BtuF-and nucleotide-induced conformational changes of the translocation gates of the transporter previously obtained by double electron electron resonance (DEER) [30]. DNP is shown here to be a powerful new tool to study conformational changes in membrane proteins which involve changes in the spin label's water accessibility.

2. Instrumentation and sample preparation

2.1. Sample preparation

TEMPOL was purchased from Sigma. The 100 μM solution was prepared from a 100 mM stock solution in milli-Q water.

The cysteine residue replacing MalE-T36 was introduced by site-directed mutagenesis into the pAL60 plasmid using Stratagenes Quik change mutagenesis kit according to the manufacturers instructions. His6-MalE (wild type and cysteine mutant) was purified from the cytosolic fraction of *Escherichia coli* strain JM109 according to [31]. The cysteine mutant of MalE (20 μM protein concentration) was spin labeled with a 10-fold molar excess of MTSSL ((1-oxyl-2,2,5,5,-tetramethyl-d-3-pyrroline-3-methyl) methanethiosulfonate, Toronto research chemicals, North York, Canada) and incubated 1 h at 4 °C under gentle shaking. Excess of MTSSL was removed with a PD10 column and the labeled protein was concentrated to 110 μM. Protein concentration was determined by UV and spin labeling efficiency was calculated based on second integral analysis of the room temperature EPR spectra and found to be 90%.

Cysteine mutations in BtuCD at positions 141 and 168 were introduced on a 'cys-less' plasmid and the BtuCD mutants were overexpressed in *E. coli* BL-21-(DE3) Gold cells and purified according to [30]. The protein was desalted with a HiPrep desalting column (GE Healthcare) into 50 mM Tris–HCl (pH 7.5) containing 0.1% LDAO (lauryldimethylamine-N-oxide, Anatrace) and 500 mM