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Lipid−Protein Nanodiscs as Reference Medium in Detergent Screening for High-Resolution NMR Studies of Integral Membrane Proteins

Zakhar O. Shenkarev,*,† Ekaterina N. Lyukmanova,† Alexander S. Paramonov,† Lyudmila N. Shingarova,† Vladimir V. Chupin,† Mikhail P. Kirpichnikov,† Marcel J. J. Blommers,‡ and Alexander S. Arseniev*,†

Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, 16/10 Miklukho-Maklaya str., 117997 Moscow, Russia and Novartis Institutes for BioMedical Research, CH-4002 Basel, Switzerland

Received November 17, 2009; E-mail: zh@nmr.ru; aars@nmr.ru

NMR spectroscopy is one of the well established techniques to study the structure and dynamics of integral membrane proteins (IMPs) in solution.[1] The development of relaxation optimized TROSY experiments[2] and sophisticated isotope labeling schemes significantly extend the applicability of solution-state NMR methods for helical IMPs.[3] Because these proteins cannot be measured in a bilayer, a suitable detergent-based membrane mimetic should be chosen to study the protein by solution state NMR. In the selected mimetic, the native structure of the protein should be conserved.[4] The screening of detergents can be guided by functional tests such as ligand binding or enzymatic activities,[4b] but if direct functional tests are not available, it is a risky assumption that an NMR spectrum of good resolution represents the conformation of the IMP in the bilayer membrane. Two spectroscopic criteria are employed most frequently for selection of a membrane mimetic: (1) CD spectroscopy is used for the estimation of secondary structure and (2) 2D ^{1}H,^{15}N-correlation spectra (HSQC or TROSY) are used for the assessment of the quality of NMR spectra.

In the present communication, we present a novel method for detergent screening that can be used in case the functional state of the IMP cannot be easily tested. This method relies on the fact that the chemical shift is very sensitive to structural changes. Therefore, the 2D ^{1}H,^{15}N-correlation spectrum can be considered as a fingerprint of the IMP spatial structure. We propose to use the 2D ^{1}H,^{15}N-fingerprint of the protein, measured in a bilayer membrane model system, as a reference in the screening of the conventional membrane mimetics (micelles or small bicelles) more suitable for detailed structural investigations. As a reference medium we propose to use lipid−protein nanodiscs (LPNs), also known as rHDL particles.[5] Each discoid LPN (10 nm × 4 nm) represents a patch of lipid bilayer (∼160 lipid molecules) stabilized by two copies of special membrane scaffold protein (MSP). The bilayer of LPN can stabilize the functionally active state of a variety of polytopic helical IMPs.[5b] The possibility of obtaining the ^{1}H,^{15}N-fingerprints of IMPs incorporated into LPNs was recently suggested.[6a,b,d]

The utility of the proposed approach was verified using the isolated voltage-sensing domain (VSD) of the K+ KvAP channel from archaeon *Aeropyrum pernix*.[7] The VSD contains four transmembrane (TM) helices (S1−S4, 150 a.a.) which in a full-length KvAP channel are extended by the pore domain formed by S5−S6 helices (Figure S1). As a part of the tetrameric KvAP channel, each of the four VSDs is responsible for the reception of the TM potential,[7] but this functionality cannot be tested for the isolated domain. On the other hand, it has been shown that the VSD reconstituted into a bilayer of liposomes adopts a spatial structure very similar to the structure of the domain in full-length functional KvAP channel.[7c]

To obtain reference ^{1}H,^{15}N-fingerprints, the VSD was extracted from *E. coli* membranes using mild nonionic detergent (DDM). Subsequently the protein was transferred into LPNs containing DMPC, DMPG, or a mixture of POPC/DOPG (3:1) lipids (see Supporting Information). This procedure resembles previously used methods for reconstitution of the VSD in liposomes[7c] by which its spatial structure is conserved. The ^{1}H,^{15}N-TROSY spectra of VSD/LPN complexes are of sufficient quality to serve as a reference for the proposed screening (Figures 1A and S3). The obtained fingerprints were very similar, indicating that charge and saturation of lipid molecules have no big impact on the structure of the domain. All fingerprints reveal the presence of μs−ms conformational fluctuations within the VSD. However, these fluctuations, which become apparent via selective broadening of cross-peaks, were less pronounced in LPNs containing anionic lipids. Interestingly, the membrane of *Aeropyrum pernix*, the natural environment of the KvAP channel, also contains a large amount of anionic phospholipids.[8]

The combined 1D ^{1}H NMR and amino acid analysis of VSD/LPN/DMPG complexes revealed that each complex consists of one VSD, two MSP, and ∼100 lipid molecules. The translational diffusion coefficient (D_T 0.64 × 10^{-10} m^2/s, at 30 °C) of DMPG molecules and CSA/dipolar cross correlation rate (η_{XY} 59 s^{-1}, at 45 °C and 700 MHz, corresponding to $\tau_R \approx 52$ ns) of VSD ^{15}N nuclei match for reorientation of a spherical particle with a hydrodynamic Stokes radius (R_H) ≈ 43 and 45 Å, respectively. The estimated R_H values are in good agreement with the diameter of the complex measured by size-exclusion chromatography (∼9.5 nm, Figure S2). In conclusion, the VSD is well embedded into the LPN and reorients with it as one monomeric particle.

The ^{1}H,^{15}N-fingerprint of the VSD observed in DMPG nanodiscs was used as a reference for screening among the set of conventional detergent-based membrane mimetics and low-polarity organic solvent (TFE). In micelles of anionic detergents (SDS, LMPG, LPPG) and in TFE solution, the ^{1}H,^{15}N-HSQC spectra of VSD (Figures 1DE and S3) were significantly different from the reference. These spectra were characterized by a mild dispersion of backbone ^{1}H^{N} signals (7 to 9 ppm) typical for helical proteins without pronounced helix−helix interactions and almost identical intensities of cross peaks, indicating uniform dynamic properties within the different parts of the protein. In contrast, the ^{1}H,^{15}N-HSQC spectra of VSD measured in zwitterionic DPC micelles and DMPC/DHPC (1:4) bicelles (Figures 1B, S3) demonstrated a much larger ^{1}H^{N} dispersion (7 to 11 ppm). These spectra were much closer to the reference fingerprint and display similar positions of characteristic downfield signals and exchange broadening for the same set of VSD resonances. The ^{1}H,^{15}N-HSQC spectra of the

† Russian Academy of Sciences.
‡ Novartis Institutes for BioMedical Research.

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VSD measured in weakly cationic LDAO micelles were also very close to the reference fingerprint, but the degree of signal broadening was significantly smaller (Figures 1C and S3). Furthermore, the observed fast aggregation of VSD indicated instability of domain structure in LDAO micelles.

We conclude, based on the comparison of spectra with reference spectra of the IMP embedded in LPN, that the VSD conserves its "membrane-like" spatial structure only in the environments based on the zwitterionic detergents. This conclusion could not be drawn from an analysis of CD spectra (Figure 1F). They represent an almost identical secondary structure of the VSD in all tested detergent-based membrane mimetics (helical content ~60%) and significantly increased helicity (~80%) in TFE solution. This clearly shows the limitation of CD spectroscopy and the advantage of the here proposed use of 2D NMR.

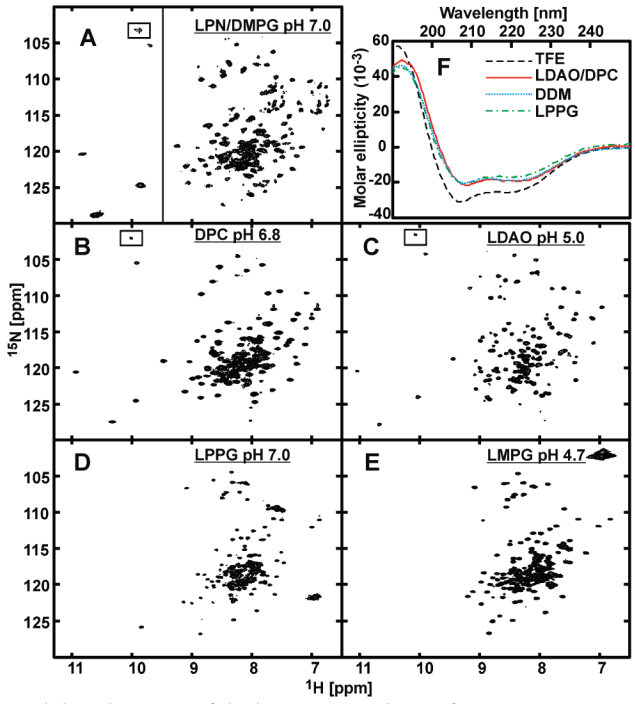

Figure 1. (A) Reference TROSY spectrum of VSD in LPNs. The downfield region of the spectrum is processed with a strong exponential window function and drawn with decreased threshold (B−E) NMR screening of membrane mimetics. The boxed cross-peaks are folded in the ^{15}N dimension. (F) CD spectra of VSD in different membrane mimetics.

A further round of the fine-tuning of the VSD solubilization medium revealed the superior properties of DPC/LDAO (2:1) mixed micelles, in terms of preservation of LPN fingerprint, long-time sample stability, and exchange broadening (Figure S3). The detailed NMR investigation of VSD solubilized in this medium[9] revealed a spatial structure in solution that is very similar to the crystal structure of the isolated domain[7a] as well as the EPR derived structure in a full-length active channel solubilized in liposomes at zero TM potential.[7b] The analysis of NMR relaxation data[9] complements the crystal structure with information on the conformational dynamics of the VSD that may play a functional role during voltage gating. The obtained results confirm the correct choice of membrane mimetic for NMR studies and validate the applicability of LPNs as a "reference" medium.

The inability of anionic detergents to provide a native-like environment of the VSD can be explained by the analysis of its spatial structure. The four-helical bundle of VSD is stabilized by electrostatic interactions between the charged side chains.[7a,9] Negatively charged head groups of detergents in a highly dynamic anionic micelle may compete with these salt bridges. This does not take place in anionic bilayers where the semirigid two-dimensional lipid matrix prevents contacts between lipid headgroups and charged side chains in the middle of the TM helices. Interestingly, similar observations were made in the recent study of the bacterial multidrug resistance transporter (Smr).[4b] It was found that, although LMPG and LPPG provided superior NMR spectra and long-time stability, these detergents were unable to maintain the native structure and functional activity of the protein.[4b]

The novel method described here is the first successful example of application of LPNs for high-resolution NMR study of helical IMPs. The previous examples involved small peripherally bound helical peptide Aam-I;[6a,b] two helical IMPs, full-length K+ channel KcSA[6b] and one-TM CD4 fragment[6c] for which only mobile solvent-exposed domains were observed in ^{1}H,^{15}N- and ^{1}H,^{13}C-correlation spectra;[6b,c] and the β-barrel mitochondrial outer membrane protein VDAC, for which a remarkably well resolved ^{1}H,^{15}N-fingerprint could be measured.[6d] Although in the present work the nanodiscs play only a supplementary role, the good signal-to-signal correspondence between VSD spectra measured in LPNs/DMPG and in DPC/LDAO micelles (Figure S4) suggests the feasibility of NMR structural studies of integral membrane proteins incorporated into the bilayer of lipid−protein nanodisc particles.

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Supporting Information Available: Sample preparation and analysis, NMR spectra of VSD in different media. This material is available free of charge via the Internet at http://pubs.acs.org.

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