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A simple guide to the structural study on membrane proteins in detergents using solution NMR

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Abstract NMR-based structural studies on membrane proteins are appreciated quite challenging due to various reasons, generally including the narrow dispersion of NMR spectra, the severe peak broadening, and the lack of long range NOEs. In spite of the poor biophysical properties, structural studies on membrane proteins have got to go on, considering their functional importance in biological systems. In this review, we provide a simple overview of the techniques generally used in structural studies of membrane proteins by solution NMR, with experimental examples of a helical membrane protein, caveolin 3. Detergent screening is usually employed as the first step and the selection of appropriate detergent is the most important for successful approach to membrane proteins. Various tools can then be applied as specialized NMR techniques in solution that include sample deteuration, amino-acid selective isotope labeling, residual dipolar coupling, and paramagnetic relaxation enhancement.

Keywords NMR, membrane protein, detergent screening, deuteration, amino-acid selective isotope labeling, paramagnetic relaxation enhancement, residual dipolar coupling

Introduction

Up to now, more than 10,000 protein structures have been determined and deposited. Subsequently, many protein structures have critically contributed to structure-based drug developments. For example, Brown and coworkers successfully developed an allosteric inhibitor of the interactions between Runx1 and core binding factor β (CBFβ), using the NMR structure of CBFβ[1,2]. It is now regarded that membrane proteins would represent more than 70% of all therapeutic targets[3,4]. Accordingly, drug developments are being frequently attempted using the membrane protein structures. In this context, membrane protein structure determination is appreciated to be critical for drug development. Unfortunately, however, the cumulative number of membrane protein structures is no more than 500. The first membrane protein structure was reported in 1985[5]. Since then, membrane protein structure determination gradually increased in early days, and nowadays it becomes rapidly increasing. Although membrane proteins are frequently investigated, their structural studies, particularly for NMR, are still very challenging. This review describes in outline specialized techniques to easily approach NMR study

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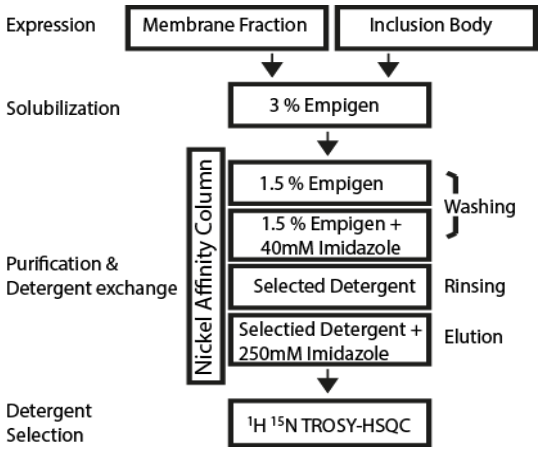

Figure 1. Scheme of detergent screening using the detergent exchange method in a column

of membrane proteins expressed in *E.coli* using detergent.

Detergent screening for NMR study

Detergents perform a key role in membrane protein structure determination. The amphipathic feature (a hydrophilic head group with a hydrophobic tail) of detergent molecules enables them to extract integral membrane proteins (IMPs) from membrane and/or inclusion body fractions produced by cell disruption. Newly developed materials such like bicelle[6], nano-disk[7] have been recently used for membrane protein structure determination using NMR. Detergents, however, have an advantage of easy approach over the newly developed materials. Structure and dynamics of IMPs are closely linked to the properties of the surrounding membrane environment[8]. Therefore, protein-specific approaches to choose a suitable detergent are essential for yielding favorable conditions for NMR. The physico-chemical properties of individual detergents used for membrane protein structure study can be gleaned from Table 1. Selected detergents can be introduced to a protein via a detergent exchange step in a column. An alternative method called reconstitution can be employed for introducing detergents to a protein, which is not demonstrated in this review. The scheme of detergent exchange is represented in Figure 1. Preparation of a fusion construct with either an N- or a C-terminal His-tag is a prerequisite for the detergent exchange method. Membrane protein can be recovered from both the membrane and inclusion bodies fractions in the typical *E.coli* expression systems. Inclusion bodies can be isolated from crude lysate via centrifugation at 15,000× g for 30 min, while membrane fraction can be isolated from remaining supernatant via ultracentrifugation at 90,000× g for 2-3 hrs. Inclusion bodies and cell membrane fraction can be solubilized in 3% Empigen (Calbiochem) in Buffer A (40mM HEPES, pH 7.5, 300mM NaCl, 0.5mM DTT). Other detergents can then replace Empigen, depending on specific characteristics of the protein. In detail, solubilized inclusion bodies or cell membrane extracts are loaded onto Ni-NTA resin (Qiagen). The resin is then washed with six column volumes of Buffer A containing 1.5% Empigen followed by an additional washing with a six column volumes of Buffer A containing 1.5% Empigen, 40-50 mM imidazole and 0.5 mM DTT. Subsequent process of detergent exchange can be completed with rinsing the resin with 10 column volumes of rinse buffer (20 mM sodium phosphate, selected detergent, 0.5 mM DTT, pH 7.2). Final NMR sample is pooled by eluting the detergent-protein complexes from the resin, using the buffer containing 250mM imidazole, pH 7.2, 0.5mM DTT and the selected detergent. The 2D [^{1}H, ^{15}N]TROSY-HSQC spectra can be used to evaluate the sample properties in each detergent, which is vital for selecting the appropriate detergent. The 2D [^{1}H, ^{15}N]TROSY-HSQC pulse sequence optimized by Weigelt[9] is recommendable for enhanced sensitivity and filtering out of the considerable ^{1}H signals from imidazole as well as detergents. Measurement at high temperature such 45 °C usually gives a better spectrum. Total number of peaks and the number of tryptophan side-chain peaks are important criterions for evaluation of sample properties. For an experimental example, shown in figure 2 are the NMR spectra of a helical membrane protein, caveolin 3, measured in different detergents. The spectral quality represented by the number of peaks observed

greatly varied depending on the detergent used, which finally suggested the LPPG as the most suitable detergent for the NMR-based structural study of caveolin 3.

Useful NMR techniques for structural study of membrane proteins

Protein deuteration

Deuteration (^{2}H labeling) of protein sample is usually favored for membrane proteins and/or large proteins at early stage of structural study using NMR[23]. Perdeuteration, in particular, gives benefits as follows: (1) strong reduction of external contributions to dipole-dipole relaxation (2) removal of internal contributions for $^{13}CH_n$ (3) increasing in T2 and T1 of $^{13}CH_n$ and ^1H_N (4) removal of *J* coupling between protons. Although not as much as perdeuteration, random fractional deuteration is also beneficial for increasing T2 and T1 of ^1H_N and reducing dipole-dipole interaction of Hα. Thus, the resolution and sensitivity of NMR spectrum can be dramatically enhanced by the sample deuteration. In our experience with caveolin 3 in LPPG detergent complexes, we have observed a significant spectral improvement in the 3D ^{15}N-edited NOESY spectrum by the random fractional deuteration of the protein. For the 3D experiments involving ^{13}C with directly attached protons, the introduction of perdeuteration during the protein expression would be essential.

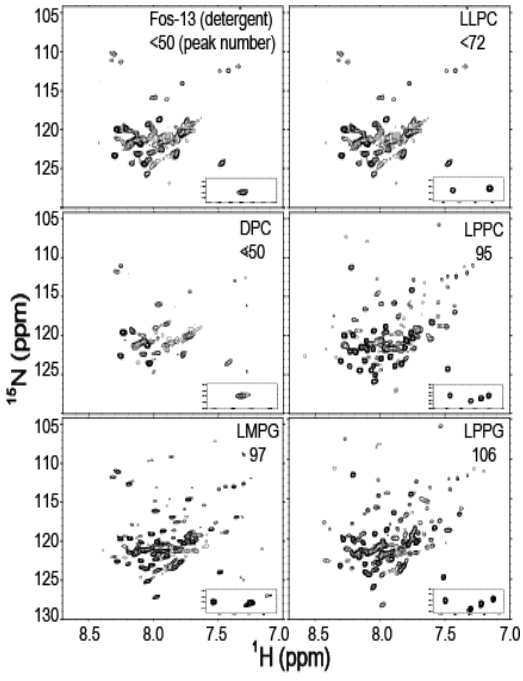

Figure 2. Comparison of the [^{1}H, ^{15}N]TROSY-HSQC spectra of caveolin 3 in various detergent micelles. Detergent name and the number of observed peaks are presented in each spectrum. All spectra were recorded at 318K on a Bruker 800 Mhz NMR machine.

Selective isotope labeling

NMR assignments of helical membrane proteins usually suffer from a narrow dispersion and overall overlapping of peaks in the spectra. Amino-acid selective isotope labeling can aid in sequence-specific resonance assignments in the crowd regions of 2D and 3D NMR spectra. Several techniques are used to achieve the selective labeling, including the cell free protein synthesis[24], *in vivo* labelling using auxotrophic strains[16], specific inhibition of an amino acid synthesis[25], and complex media formulations. Cell free protein synthesis, which is not a cell-based expression system, is a particularly useful tool for producing cytotoxic proteins and offers a quite freely labeled pattern. However, it also has disadvantages such as low yields of protein production and expensive and labor-sensitive processes. *In vivo* labeling is simple in principle but has a disadvantage of additional labels in undesired amino acids, which is called the isotope scrambling or dilution. The isotope scrambling is usually tolerable for the amino acids Ala, Arg, Asn, Cys, His, Ile, Lys, Met, Pro and Trp, which are end-products in the metabolic pathways, whereas Asp, Glu and Gln, which occupy an uppermost or intermediate position in the metabolic pathway, are highly prone to the isotope scrambling. The remaining amino acids (Gly, Phe, Leu, ser, Thr, Tyr and Val) show a weak to medium tendency towards the isotope scrambling. The isotope label scrambling and dilution in the cells can be minimized by using auxotrophic cells, which lack the synthesis enzyme producing a specific amino acid, or by incorporating

Table 1: Physico-chemical properties of representative detergents used in structural study of membrane proteins

Detergent (abbreviation)	Ionic property	FW (Da)	cmc (mM)	Aggregation number
n-decylphosphocholine (FC-10)	zwitterionic	323	11[a]	24[a], 45-53[10]
n-dodecylphosphocholine (FC-12)	zwitterionic	351	1.5[a]	54[a], 70-80[11]
n-decyl-*â*-D-maltoside (DM)	non-ionic	483	1.8[a]	69[a], 82-90[10]
n-dodecyl-*â*-D-maltoside (DDM)	non-ionic	511	0.17[a]	140[12], 78-149[a]
n-octyl-*â*-D-glucoside (OG) (50 mM)	non-ionic	292	18[13]-3[14]	87[15], 27-100[16]
n-nonyl-*â*-D-glucoside (NG) (10 mM)	non-ionic	306	6.5[a]	133[a]
n-decyl-*â*-D-glucoside (DG)	non-ionic	320	2.2[17]	200-400[18]
1,2-dihexanoyl-*sn*glycerophosphocholine(DHPC)	zwitterionic	507	14-15[19]	27[20], 35[19]
1-myristoyl-2-hydroxy-*sn*-glycero-3-[phospho-(1'-*rac*-glycerol)] (LMPG)	ionic	478	0.16[21]	55[22]
1-palmitoyl-2-hydroxy-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (LPPG)	ionic	507	0.018[21]	125[20]
3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) (25 mM)	zwitterionic	615	8.0[22]	10[22]

[a]Anatrace, Inc.; Formula weight of a detergent monomer (FW), critical micelle concentration (cmc) and aggregation number were taken from the literature.

specific inhibitors of a certain amino acid synthesis enzyme in the bio-synthetic pathway. Thus, the amino-acid selective labeling *in vivo* combined with modern NMR techniques is effectively employed as an essential tool for resolving resonance assignments in the crowd region. Recently, advanced methods for an efficient and simple isotope labeling were also developed using the common prototrophic *E coli* strains[26,27]

Application of residual dipolar coupling (RDC)

NMR experiments on membrane proteins, particularly with helical ones, face several difficulties. A representative one is the limited set of long-range distance restraints that are typically derived from the nuclear Overhauser enhancement (NOE) data. However, in case of membrane proteins with detergents and/or lipids, the observed resonances from the membrane mimetic systems (micelles or bicelles) used can interrupt the analysis of NOEs between protons directly attached to carbon. Although those interruptions can be overcome by using deuterated membrane components, it is quite costly and sometimes not commercially available. In such a case, alternative method to replace or complement the long-range distance restraints is required for structural determination of membrane proteins. Residual dipolar coupling (RDC) provides a great source of orientational restraints, including both the short- and long-range information. In contrast to NOEs that provide distance restraints between the two atoms spatially close to each other, RDCs can provide overall distance information through the angular constraints formed by the vectors connecting two atoms within a tensor axis system. Strained polyacrylamide gel is typically used to produce a tensor axis system for RDC measurement of

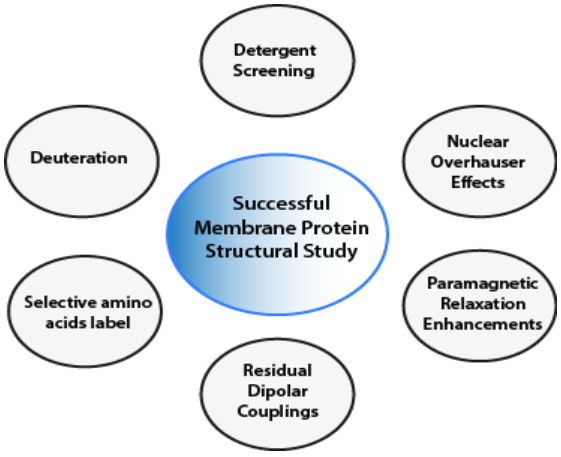

Figure 3. Diagram for successful membrane protein structural study

membrane proteins. Besides polyacrylamide, newly developed methods were recently reported using the DNA-nanotube[28] and the lanthanide ion bound to a small metal chelator[29]. Typically, ^{1}D(N–HN) couplings measured via ARTSY (intensity difference-based) or IPAP-HSQC (chemical shift difference-based) is most frequently used for membrane protein structural studies. Other couplings such as ^{1}D(Hα–Cα), ^{1}D(N–C′), ^{1}D(C′–Cα) and ^{2}D(HN–C′) are also valuable to obtain useful information.

Paramagnetic relaxation enhancement (PRE) measurement

Additionally to RDC, the paramagnetic relaxation enhancement (PRE) represents another useful tool for long-range distance information in the structural study of membrane proteins[30]. Chemical PRE probe can be divided into two classes: (1) nitroxide stable radicals (N-O$^{\bullet}$) and (2) paramagnetic metal ions (such as Mn^{2+} or Gd^{2+}) with a tightly bound chelator (such as EDTA or DTPA). These chemical probes are covalently attached usually to the solvent-exposed cysteine residues. Cysteine is most frequently used as the conjugation site for the site-specific incorporation of the paramagnetic probe, where the conjugation can be easily achieved by a disulfide bond formation[31]. The PRE arising from magnetic dipolar interactions between the unpaired electron of paramagnetic probe (such as a nitroxide spin-label) and its counteracting nucleus has been proved to effectively provide structural information, on the basis of the relationship between the $\langle r^{-6} \rangle$ distance and the PRE Γ_2 rate measured as $\Gamma_2 = R_2^{para} - R_2^{dia}$, where R_2^{para} and R_2^{dia} are the transverse relaxation rates in the paramagnetic and diamagnetic states, respectively. The MTSL compounds derived from methanethiosulfonate are very frequently used for the PRE experiments. A simple way to get PRE data is to compare the resonance intensities in the HSQC or TROSY-HSQC spectra of the MTSL-labeled protein having the attached radical between in quenched and unquenched states. Quenching of PRE probe can be easily achieved by addition of excess ascorbic acid to the sample. Obtained intensity ratio from the two spectra can be converted into a distance restraint[32].

Concluding remarks

In summary, although the NMR-based structural study of membrane proteins is highly tricky and still challenging, many advanced tools have been developed to aid in structure determination. First of all, incorporating the protein into a suitable membrane mimetics is the most important to take an optimized condition for the structural study. In addition, protein deuteration, amino-acid selective isotope labeling, and application of RDC and/or PRE are essential tools to investigate the membrane protein structure (Figure 3). We expect that this review to glance at the essential techniques could provide a simple guide to an efficient structural study of membrane proteins in solution, using NMR.

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