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In Situ Structural Characterization of a Recombinant Protein in Native *Escherichia coli* Membranes with Solid-State MAS NMR

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Abstract

The feasibility of using solid-state MAS NMR for *in situ* structural characterization of the LR11 (sorLA) transmembrane domain in native *Escherichia coli* (*E. coli*) membranes is presented. LR11 interacts with the human amyloid precursor protein (APP), a central player in the pathology of Alzheimer's disease. The background signals from *E. coli* lipids and membrane proteins had only minor effects on LR11 TM resonances. Approximately 50% of the LR11 TM residues were assigned by using ¹³C PARIS data. These assignments allow comparisons of the secondary structure of LR11 TM in native membrane environments and in commonly used membrane mimics (e.g. micelles). *In situ* spectroscopy bypasses several obstacles in the preparation of membrane proteins for structural analysis, and offers an opportunity to investigate the consequences of membrane heterogeneity, bilayer asymmetry, chemical gradients, and macromolecular crowding on the protein structure.

Integral membrane proteins reside in a complex lipid environment. The complexity of cellular membranes is reflected in their diverse lipid composition (>1,000 different lipid species), lateral heterogeneity (e.g. lipid rafts, lipid microdomains), transbilayer asymmetry, chemical and electrical gradients, dynamics, and shapes.^{1–5} Membranes are no longer viewed as simple passive barriers that separate cells from their environments, but rather as active participants in important biological processes such as intracellular signal transduction, protein localization and protein trafficking.^{6–8} Biological membranes are crowded and contain as much protein as they do lipid.^{9,10} The implications of this intramolecular crowding are increasingly recognized.^{11–14} Although the unique lipid environment is a major determinant of membrane protein conformation and function, this environment is incompatible with the conventional methods of X-ray crystallography and solution NMR. Consequently, our structural knowledge of membrane proteins lags far behind that of soluble proteins, despite the fact that membrane proteins account for approximately 30% of all proteins in the human genome, including biologically crucial molecules such as ion channels

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SUPPORTING INFORMATION. Sample preparation, experimental details, chemical shifts of LR11 TM in *E. coli* membranes and DPC micelles, complete references 10, 34 and 38. This material is available free of charge via Internet at <http://pubs.acs.org>.

and G-protein coupled receptors. As of April 2011, there are only ~280 unique membrane protein structures in the Protein Data Bank, mostly from prokaryotes.

The importance of membrane mimetic environments in supporting the native structure, dynamics and function of membrane proteins has been highlighted recently.^{23–26} So far, most structural analyses were carried out in detergent preparations and only a few in synthetic lipid bilayers. Information about protein structure in biological environments is scarce.^{15–18} Bacteriorhodopsin is the only protein that has been subjected to *in situ* detailed NMR structural characterizations in native purple membranes,^{19–22} thanks to its natural abundance. Recent developments in the condensed Single-Protein-Production (cSPP) system have allowed the detection of membrane proteins without purification.^{27–29} Here, we demonstrate the feasibility of characterizing the transmembrane domain (TM) of a human protein, LR11/SorLA, *in situ* in *E. coli* membranes by using solid-state magic-angle spinning (MAS) NMR.

LR11 is a recently identified type I transmembrane protein involved in the development of Alzheimer's disease (AD). AD causes a gradual loss of memory and general cognitive decline. It is the most common form of dementia in the elderly and currently affects more than 5.4 million Americans (<http://www.alz.org>). The “amyloid hypothesis” suggests that the accumulation of A β peptides, proteolytic products of amyloid precursor protein (APP), is the primary cause of AD.^{30,31} APP is a type I transmembrane protein and is continuously sorted through multiple subcellular organelles (e.g. *trans*-Golgi network, plasma membrane and endosome). Its aberrant intracellular trafficking is linked to the development of AD.

LR11 has emerged as a critical regulator for APP transport and processing.^{32–36} LR11 interacts directly with APP, regulating its subcellular localization. Variants of LR11 are associated with AD, and the expression of LR11 is dramatically decreased in the brains of patients suffering from sporadic AD.^{32,37,38} The transmembrane domains of LR11s from mammals are highly conserved and share >95% sequence identity, pointing to their potential functional significance. Using a new MBP-fusion expression vector, we produced human LR11 TM (residues 2132 to 2161, Figure 1a) in *E. coli*.³⁹ The recombinant protein is expressed in the membranes at a much higher level relative to the background of *E. coli* membrane proteins, as shown in Figure 1b. We have developed a protocol to cleave MBP at the native membrane surface and have obtained LR11 TM in *E. coli* membranes through ultracentrifugation and buffer washes. The SDS-PAGE result for the prepared sample is also shown in Figure 1b. LR11 TM consists of 70~80% of total labeled proteins.

To examine sample homogeneity, spectral sensitivity and resolution, and the interference of background signals from *E. coli* proteins and lipids, ¹³C MAS NMR spectra were acquired on a ¹³C _{α,β} -Alanine enriched LR11 TM in isolated membranes. The phospholipid composition of *E. coli* from exponentially grown cultures is simple, mainly phosphatidylethanolamine, phosphatidylglycerol and cardiolipin.⁴⁰ Despite the fact that lipids are not labeled in this preparation, their naturally abundant signals overwhelm the 1D spectrum collected with a single 90°-pulse direct polarization (DP) experiment (Figure 2a, top). The resonances at ~17 and 52 ppm are relatively sharp and likely come from the flexible lipid headgroups and methyl carbons, while the resonance at ~31 ppm comes from the lipid methylene groups. The narrow resonances are effectively suppressed in the ¹H-¹³C cross polarization (CP) experiment, while the resonances from more rigid regions are greatly enhanced (Figure 2a, middle). The lipid ¹³C signals can be further suppressed in the double-quantum filter (CP-DQF)⁴¹ experiment and thus resonances solely from ¹³C _{α,β} -Alanine enriched proteins are detected (Figure 2a, bottom). These DP, CP and CPDQF spectra were collected in ~45, 17 and 70 minutes, respectively, on a 600 MHz spectrometer, suggesting

that the sensitivity is sufficient for multidimensional NMR experiments to improve spectral resolution.

A 2D ^{13}C - ^{13}C PARIS⁴² spectrum is shown in Figure 2b. The resonance line-width is ~ 1.0 ppm which is typical for non-crystalline samples and indicates good homogeneity of the preparation. Two well-resolved and two partially overlapped cross peaks are observed in the Ala C_α - C_β chemical shift region as expected from the four Ala residues in the protein sequence, consistent with the resonances arising from LR11 TM. One cross peak at 50.1 and 17.6 ppm is much weaker and has a slow PARIS buildup. This can be tentatively attributed to the relatively flexible residue of Ala20 at the N-terminus of the TM domain.

To pursue resonance assignments and validate the secondary structure of the TM domain, 2D ^{13}C PARIS spectra with various mixing times were collected on a uniformly ^{13}C , ^{15}N enriched sample. The ^{13}C - ^{13}C correlations are generated by through-spaced dipolar couplings, thus at a short mixing time of 5 ms most of the cross peaks result from directly bonded ^{13}C sites (Figure 3a). Despite having lipids that are also ^{13}C enriched, lipid resonances do not interfere with the protein resonances because CP and dipolar-coupling mediated magnetization transfer select against relatively flexible lipid resonances. The Ala C_α - C_β cross peak region, highlighted in the blue box, is identical to the above spectrum from the $^{13}\text{C}_{\alpha,\beta}$ -Ala labeled sample. Based on their characteristic chemical shifts and spin systems, the resonances of Ile, Ser, Val, Leu, and Gly are easily identified. The cross peak at 37.1 and 61.1 ppm was assigned to C_β - C_α of Phe, with the aid of its connectivity to resonances in the aromatic region (data not shown). The cross peak at 26.4 and 47.8 ppm was attributed to C_γ - C_δ of Pro based on its unique chemical shifts and connectivity at a longer mixing time (see below). Thus, all amino acid residue types of the LR 11 TM domain were readily identified. The C_α and C_β chemical shifts of Ile at 63.9 and 35.8 ppm, Leu at 56.2 and 40.0 ppm, Phe at 60.3 and 37.1 ppm, and Val at 64.3 and 29.7 ppm are indicative of an α -helical backbone conformation.⁴³ For helical membrane proteins, the overlapping cross peaks of the same amino acid type are common. Resonances from some of the tag residues are also detected (e.g. the cross peak at 53.6 and 40 ppm is likely from Asp and/or Asn), but they generally show a signature of chemical exchange broadening due to the relative flexibility of the tags.

Figures 3b and 3c show 2D ^{13}C - ^{13}C PARIS spectra acquired with mixing times of 20 and 100 ms, respectively. The long mixing times permit magnetization transfers between ^{13}C spins, separated by multiple bonds or from different residues, and provide connectivity for resonance assignment. Starting from the cross peak of Pro C_γ - C_δ , the cross peaks of C_β - C_δ and C_δ - C_α were identified in Figure 3b. Since there is only one Pro residue in the LR11 TM sequence, the C_α and C_β chemical shifts for residue Pro31 were obtained. Ile32 was subsequently assigned based on its C_γ connectivity to the C_β of Pro31 in Figure 3c. Six PARIS cross peaks at 64.6, 60.4, 56.2, 40.7, 29.6, and 21.9 ppm were observed for Gly at 46.1 ppm with 100 ms mixing time, and they were assigned to C_α of Val, Phe, and Leu, and C_β of Leu, Val, and Ala, respectively, based on the amino acid type information in Figure 3a. Furthermore, a cross peak between Phe C_α at 61.1 ppm and Ala C_α at 48.4 ppm was observed. These connectivities were mapped to the LGVGFA fragment in the TM sequence. In addition, several cross peaks between Leu and Ile and between Phe and Leu were identified, but could not be unambiguously assigned to specific sites without additional data. Most of the unassigned peaks in Figure 3a come from residues of LBT and His tags, and a few of them might be *E. coli* background signals. From ^{13}C - ^{13}C PARIS data, we have readily assigned 12 out of 23 residues of the LR11 TM and their chemical shifts are listed in Table S1. All assigned residues show characteristic secondary shifts of an α -helix and are in agreement with the secondary shifts⁴⁴ of LR11 TM in DPC micelles (listed in Table S1) except for residue Ala45. This residue is near the C-terminus of the predicted TM domain

and resides in the membrane-solution interface region, where there are substantial differences between bilayers and micelles and where structural discrepancy likely occurs.

Our studies have demonstrated the feasibility of *in situ* detection of the human LR11 TM domain in native *E. coli* membranes. The spectral sensitivity and resolution are adequate for a structural analysis of this small protein. Signals from lipids and membrane proteins of *E. coli* are minimally interfered with the detection of LR11 TM resonances. By using ^{13}C - ^{13}C homonuclear correlation experiments, we have assigned ~50% of TM residues. Their secondary chemical shifts are consistent with expected values for an α -helix conformation. Most of the unassigned residues are Leu and Val because of their high abundance in the sequence. We expect that the spectral resolution can be improved further by using multidimensional heteronuclear correlation experiments and advanced enrichment strategies.⁴⁵⁻⁵¹

Although the composition of *E. coli* membranes differs from that of human cells, *in situ* detection eliminates the use of detergents for extraction, purification and reconstitution of recombinant membrane proteins. Moreover, our approach offers an opportunity to validate and refine membrane protein structures in a native environment and investigate the consequences of membrane heterogeneity, bilayer asymmetry, chemical gradients, and macromolecular crowding on the protein structure, characteristics which cannot be addressed in studies using detergent micelles and synthetic lipid bilayers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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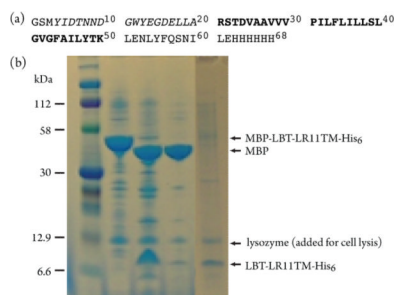


Figure 1.

(a) Primary structure of the LBT-LR11TM-His6. The LR11 fragment is shown in bold, corresponding to residues 2132 to 2161 of the full-length protein. The LBT (lanthanide binding tag) is shown in italics. (b) SDS-PAGE results for the preparation of LR11 TM in native *E. coli* membranes. Lanes: 1, protein marker; 2, isolated *E. coli* membrane fraction; 3, thrombin cleavage of the sample in lane 2; 4, buffer washes of the sample in lane 3; 5, prepared membrane fraction for NMR experiments.

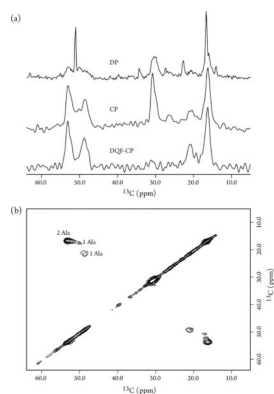


Figure 2. ^{13}C MAS spectra of $^{13}\text{C}_{\alpha,\beta}$ -Ala enriched LR11 TM in native *E. coli* membranes recorded at 305 K on a Bruker 600 MHz spectrometer using a home-built low-E 3.2 mm probe. The spinning rate was 10 kHz. (a) 1D spectra recorded using DP, CP and CP-DQF polarization schemes with 512, 512 and 2048 scans, 5, 2 and 2s recycle delays, respectively. (b) A 2D ^{13}C - ^{13}C PARIS spectrum collected with 20 ms mixing time, 9.2 and 7.0 ms acquisition time for direct and indirect dimensions, 1.5 s recycle delay and 512 scans per t1 point.

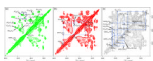


Figure 3. 2D ^{13}C - ^{13}C PARIS spectra of ^{13}C , ^{15}N uniformly enriched LR11 TM in native *E. coli* membranes recorded for resonance assignment with (a) 5 ms mixing time and 96 scans per t1 point; (b) 20 ms mixing time and 112 scans per t1 point; (c) 100 ms mixing time and 128 scans per t1 point. The acquisition times for direct and indirect dimensions were 10.3 and 4.8 ms, and the recycle delay was 1.5 s.