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

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Supporting Information

ABSTRACT: The feasibility of using solid-state magic-angle-spinning NMR spectroscopy for in situ structural characterization of the LR11 (sorLA) transmembrane domain (TM) in native *Escherichia 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 the LR11 TM resonances. Approximately 50% of the LR11 TM residues were assigned by using ^{13}C PARIS data. These assignments allowed comparisons of the secondary structure of the LR11 TM in native membrane environments and commonly used membrane mimics (e.g., micelles). In situ spectroscopy bypasses several obstacles in the preparation of membrane proteins for structural analysis and offers the opportunity to investigate how membrane heterogeneity, bilayer asymmetry, chemical gradients, and macromolecular crowding affect the protein structure.

Integral membrane proteins reside in a complex lipid environment. The complexity of cellular membranes is reflected in their diverse lipid composition (>1000 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 are understood to be 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 have been 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 spectroscopy. Consequently, our structural knowledge of membrane proteins lags far behind that of soluble proteins, despite the fact that membrane proteins account for ∼30% of all proteins in the human genome, including biologically crucial molecules such as ion channels 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 recently been highlighted.[15−18] To date, most structural analyses have been carried out in detergent preparations, and only a few have been performed in synthetic lipid bilayers. Information about protein structure in biological environments is scarce.[19−22] Bacteriorhodopsin is the only protein that has been subjected to detailed in situ NMR structural characterizations in native purple membranes,[23−26] 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 in situ characterization of the transmembrane domain (TM) of a human protein, LR11/SorLA, in *Escherichia coli* membranes using solid-state magic-angle-spinning (MAS) NMR spectroscopy.

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.[30] The "amyloid hypothesis" suggests that the accumulation of amyloid-β peptides, proteolytic products of amyloid precursor protein (APP), is the primary cause of AD.[31,32] 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.[33−37] LR11 interacts directly with APP, regulating its

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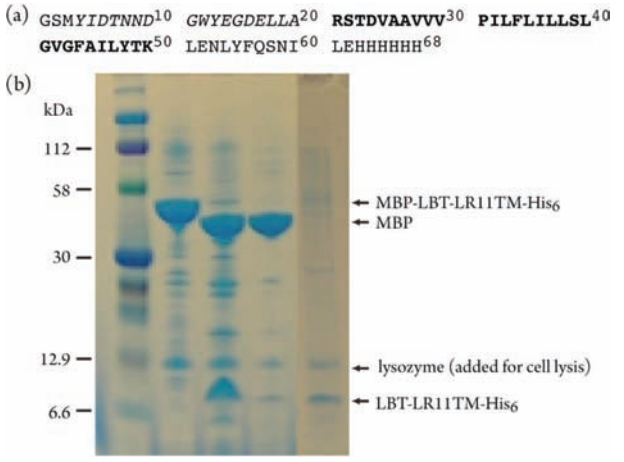

Figure 1. (a) Primary structure of LBT-LR11TM-His_6. The LR11 fragment is shown in bold, corresponding to residues 2132−2161 of the full-length protein. The lanthanide binding tag (LBT) is shown in italics. (b) SDS-PAGE results for the preparation of LR11 TM in native *E. coli* membranes. Lanes: 1, protein 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.

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.[33,38,39] The TMs 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 2132−2161; Figure 1a) in *E. coli*.[40] 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 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 comprises 70−80% of the total labeled proteins.

To examine sample homogeneity, spectral sensitivity and resolution, and the interference of background signals from *E. coli* proteins and lipids, ^{13}C MAS NMR spectra were acquired on a $^{13}C_{\alpha,\beta}$-alanine-enriched LR11 TM in isolated membranes. The phospholipid composition of *E. coli* from exponentially grown cultures is simple and includes mainly phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin.[41] Despite the fact that the lipids were not labeled in this preparation, their naturally abundant signals dominate the 1D spectrum collected with a single 90° pulse direct polarization (DP) experiment (Figure 2a, top). The resonances at ∼52 and 17 ppm are relatively sharp and likely come from the flexible lipid headgroups and methyl carbons, respectively, while the resonance at ∼31 ppm comes from the lipid methylene groups. The narrow resonances are effectively suppressed in the ^{1}H−^{13}C cross-polarization (CP) experiment, while the resonances from more rigid regions are greatly enhanced (Figure 2a, middle). The lipid ^{13}C signals can be further suppressed in the double-quantum-filtered CP (CP-DQF)[42] experiment, and thus, only resonances from $^{13}C_{\alpha,\beta}$-alanine-enriched proteins are detected (Figure 2a, bottom). These DP, CP, and CP-DQF spectra were collected in ∼45, 17, and 70 min, respectively, on a 600 MHz spectrometer, suggesting that the sensitivity is sufficient

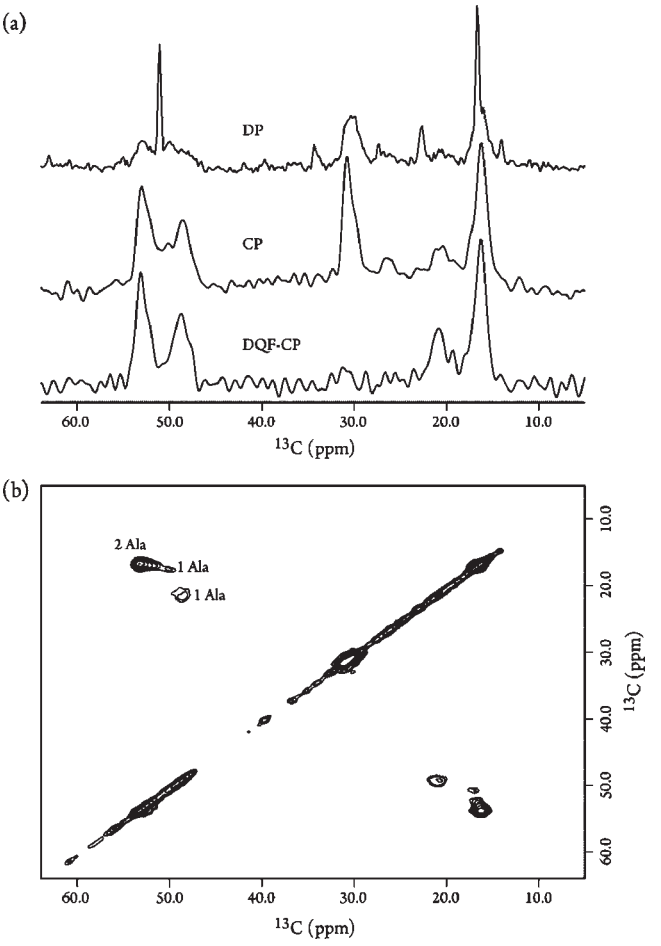

Figure 2. ^{13}C MAS NMR spectra of $^{13}C_{\alpha,\beta}$-Ala-enriched LR11 TM in native *E. coli* membranes recorded at 305 K on a Bruker 600 MHz spectrometer using a home-built low-E 3.2 mm probe. The spinning rate was 10 kHz. (a) 1D spectra recorded using DP, CP, and CP-DQF polarization schemes with 512, 512, and 2048 scans and 5, 2, and 2 s recycle delays, respectively. (b) 2D ^{13}C−^{13}C PARIS spectrum collected with a 20 ms mixing time, 9.2 and 7.0 ms acquisition times for the direct and indirect dimensions, a 1.5 s recycle delay, and 512 scans per t_1 point.

for multidimensional NMR experiments to improve the spectral resolution.

A 2D ^{13}C−^{13}C PARIS[43] spectrum is shown in Figure 2b. The resonance line width is ∼1.0 ppm, which is typical for noncrystalline samples and indicates good homogeneity of the preparation. Two well-resolved and two partially overlapped cross-peaks can be seen in the Ala C_α−C_β chemical shift region, as expected for the four Ala residues in the protein sequence and consistent with the resonances arising from the 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.

To pursue resonance assignments and validate the secondary structure of the TM, 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-space dipolar couplings, so at a short mixing time of 5 ms, most of the cross-peaks result from directly bonded ^{13}C sites (Figure 3a). Even though ^{13}C-enriched lipids are also present, the lipid resonances do not interfere with the protein resonances because

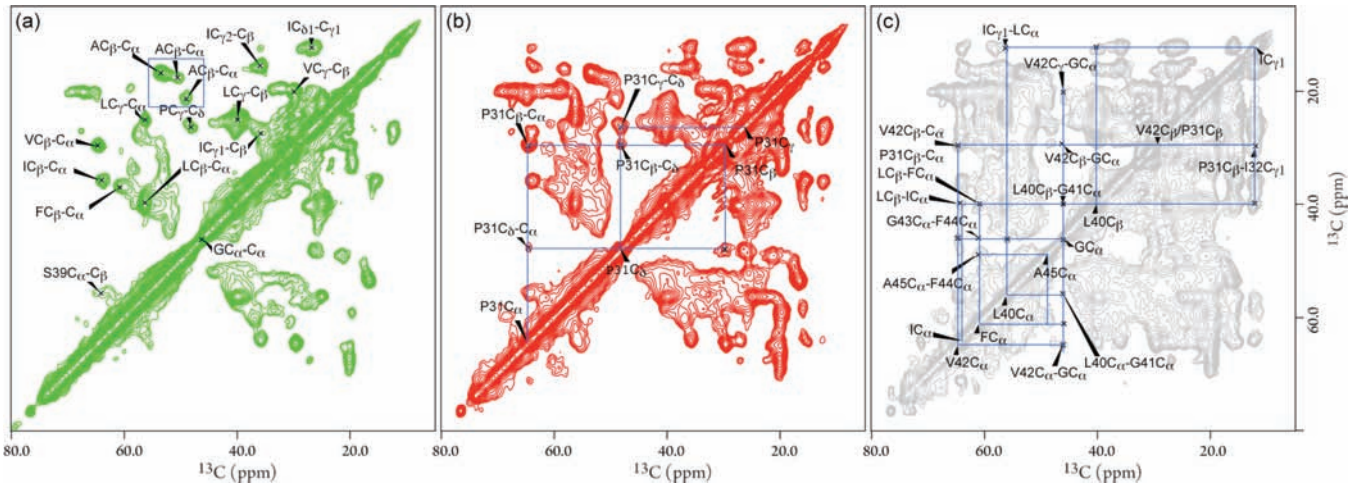

Figure 3. 2D $^{13}C-^{13}C$ PARIS spectra of uniformly $^{13}C,^{15}N$-enriched LR11 TM in native *E. coli* membranes recorded for resonance assignment with (a) a 5 ms mixing time and 96 scans per t_1 point, (b) a 20 ms mixing time and 112 scans per t_1 point, and (c) a 100 ms mixing time and 128 scans per t_1 point. The acquisition times for the direct and indirect dimensions were 10.3 and 4.8 ms, and the recycle delay was 1.5 s.

CP- and dipolar-coupling-mediated magnetization transfer select against relatively flexible lipid resonances. The Ala $C_\alpha-C_\beta$ cross-peak region (highlighted in the blue box in Figure 3a) is identical to the above spectrum from the $^{13}C_{\alpha,\beta}$-Ala-labeled sample. On the basis of 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 $C_\beta-C_\alpha$ 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 $C_\gamma-C_\delta$ of Pro on the basis of its unique chemical shifts and connectivity at a longer mixing time (see below). Thus, all of the amino acid residue types of the LR 11 TM 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.[44] For helical membrane proteins, overlapping cross-peaks of the same amino acid type are common. Resonances from some of the tag residues were also detected (e.g., the cross-peak at 53.6 and 40 ppm is likely due to Asp and/or Asn), but they generally showed a signature of chemical exchange broadening due to the relative flexibility of the tags.

Figure 3b,c shows 2D $^{13}C-^{13}C$ PARIS spectra acquired with mixing times of 20 and 100 ms, respectively. The longer mixing times permit magnetization transfers between ^{13}C spins that are separated by multiple bonds or come from different residues, providing connectivity for resonance assignments. Starting from the cross-peak of Pro $C_\gamma-C_\delta$, the cross-peaks of $C_\beta-C_\delta$ and $C_\delta-C_\alpha$ were identified (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 on the basis of its C_γ connectivity to C_β of Pro31 (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 the 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, on the basis of 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 the LBT and His tags, and a few of them might be *E. coli* background signals. From the $^{13}C-^{13}C$ PARIS data, we readily assigned 12 of the 23 residues of the LR11 TM, and their chemical shifts are listed in Table S1 in the Supporting Information. All of the assigned residues show characteristic secondary shifts of an α-helix and are in agreement with the secondary shifts[45] of the LR11 TM in DPC micelles (also listed in Table S1), except for residue Ala45. This residue is near the C-terminus of the predicted TM and resides in the membrane−solution interface region, where there are substantial differences between bilayers and micelles and where structural discrepancies likely occur.

Our studies have demonstrated the feasibility of in situ detection of the human LR11 TM 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* provided minimal interference with the detection of LR11 TM resonances. By using $^{13}C-^{13}C$ homonuclear correlation experiments, we have assigned ~50% of the TM residues. Their secondary chemical shifts are consistent with the values expected 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 further improved by using multidimensional heteronuclear correlation experiments and advanced enrichment strategies.[46−52]

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 how the protein structure is affected by membrane heterogeneity, bilayer asymmetry, chemical gradients, and macromolecular crowding, which are characteristics that cannot be addressed in studies using detergent micelles and synthetic lipid bilayers.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Sample preparation; experimental details; chemical shifts of the LR11 TM in *E. coli* membranes and

DPC micelles; and complete refs 10, 35, and 39. This material is available free of charge via the Internet at http://pubs.acs.org.

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