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# Resonance assignment of the outer membrane protein AlkL in lipid bilayers by proton-detected solid-state NMR

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## Abstract

Most commonly small outer membrane proteins, possessing between 8 and 12  $\beta$ -strands, are not involved in transport but fulfill diverse functions such as cell adhesion or binding of ligands. An intriguing exception are the 8-stranded  $\beta$ -barrel proteins of the OmpW family, which are implicated in the transport of small molecules. A representative example is AlkL from *Pseudomonas putida* *GPOI*, which functions as a passive importer of hydrophobic molecules. This role is of high interest with respect to both fundamental biological understanding and industrial applications in biocatalysis, since this protein is frequently utilized in biotransformation of alkanes. While the transport function of AlkL is generally accepted, a controversy in the transport mechanism still exists. In order to address this, we are pursuing a structural study of recombinantly produced AlkL reconstituted in lipid bilayers using solid-state NMR spectroscopy.

In this manuscript we present <sup>1</sup>H, <sup>13</sup>C and <sup>15</sup>N chemical shift assignments obtained via a suite of 3D experiments employing high magnetic fields (1 GHz and 800 MHz) and the latest magic-angle spinning (MAS) approaches at fast (60-111) kHz rates. We additionally analyze the secondary structure prediction in comparison with those of published structures of homologous proteins.

## Abbreviations

IMAC - immobilized metal affinity chromatography, EDTA - ethylenediaminetetraacetic acid, LDAO - lauryldimethylamine N-oxide, OG - n-octyl- $\beta$ -D-glucopyranoside, DMPC - 1,2-dimyristoyl-sn-glycero-3-phosphocholine, CP - cross polarization, INEPT - insensitive nuclei enhanced by polarization transfer, BASS-SD - band-selective spectral spin diffusion, TOCSY - total correlation spectroscopy, MAS - magic-angle spinning

## Keywords

$^1\text{H}$ -detected solid-state NMR, membrane proteins, lipid bilayers, alkane transport, beta-barrel

## Biological Context

Due to the ability to specifically degrade alkanes, the hydroxylase (Alk) system of bacteria such as *Pseudomonas putida* *GPO1* is of potential interest for the chemical industry in terms of biocatalysis and may even find applications in bioremediation of crude oil pollutions [1]. The catalytic complex is located at the cytoplasmic membrane consisting of AlkB, an integral-membrane non-heme diiron monooxygenase, the rubredoxin AlkG and the rubredoxin reductase AlkT [2], [3]. These proteins efficiently convert linear alkanes to primary alcohols when in isolation but showed only very limited turnover when engineered into *Escherichia coli* [4]. This suggests the crucial role of the outer membrane protein AlkL for uptake of medium to long chain alkanes, which are not capable to diffuse through the highly hydrophilic lipopolysaccharide layer at the outer membrane. Indeed, coexpression of AlkL significantly improves whole-cell based hydroxylation of various hydrophobic molecules [5]–[7].

AlkL is counted into the family of OmpW proteins and structures of homologs such as *E. coli* OmpW (sequence identity 24%) and *Pseudomonas aeruginosa* OprG (sequence identity 22%) have already been solved by X-ray crystallography and solution NMR spectroscopy [8]–[11]. Interestingly, the crystal and NMR structures show a consistent 8-stranded elliptical  $\beta$ -barrel but significant differences in the extracellular loops, namely  $\beta$ -sheets that extend beyond the hydrophobic membrane interior in crystals and long flexible loops in solution. Diffusion of small hydrophobic molecules through a lateral opening into the membrane was suggested as a transport mechanism of OmpW [8]. Due to the increased flexibility in solution, the previous NMR studies were not entirely supportive to this model. One might argue that loops are stabilized by crystal packing or that detergents destabilize the loop structure in solution, what remains evident is the importance of dynamics of the extracellular regions in OmpW proteins.

Here we report the chemical shift assignments of AlkL in DMPC lipid bilayers obtained by  $^1\text{H}$ -detected solid-state NMR. The use of a fully protonated sample and magic-angle spinning above 100 kHz allowed the combined use of amide-proton and alpha-proton detected spectra for backbone assignment as well as extensive assignment of side-chain  $^{13}\text{C}$  and  $^1\text{H}$  resonances. The lipid environment closely resembles the native membrane and with this sample formulation we anticipate reliable insights into structure and function of AlkL.

## Materials and Methods

### Protein expression and purification; sample preparation

AlkL (28-230) from *P. putida* GPo1 (Uniprot Q00595) was expressed with a short C-terminal linker and a His-8 tag (in total 219 residues) and purified essentially as described previously [12]. Uniform  $^{13}\text{C}$  and  $^{15}\text{N}$  isotope labeling was achieved by the use of M9 minimal media containing 3 g/L  $^{13}\text{C}$ -glucose and 1 g/L  $^{15}\text{NH}_4\text{Cl}$ . Deuterated glucose and  $\text{D}_2\text{O}$  based media were used for  $^2\text{H}$ ,  $^{13}\text{C}$ ,  $^{15}\text{N}$ -labelled samples. AlkL was extracted from inclusion bodies and purified by IMAC in 50 mM Tris/HCl pH 8, 500 mM NaCl, 20-500 mM imidazole, 10% glycerol, 6 M guanidine HCl and refolded by drop-wise rapid dilution into 20 mM Tris/HCl pH 8, 1 mM EDTA, 1 M Urea, 2% LDAO. The refolded protein was further purified by anion exchange chromatography in 10 mM TRIS/HCl pH 8, 20-300 mM NaCl, 0.05% LDAO. The buffer was then exchanged to 20 mM phosphate pH 7, 2% OG using a desalting column. Protein concentrations were estimated based upon the absorption at 280 nm using a molar extinction coefficient of  $34380 \text{ M}^{-1}\text{cm}^{-1}$ . DMPC in 1% OG was added to achieve a protein to lipid ratio of 2:1, and the detergent was removed by dialysis against 20 mM phosphate buffer pH 7 using a 20 kDa cutoff membrane. After 2 days the white precipitate was collected by centrifugation, washed with buffer and packed into either 1.3 mm (deuterated sample) or 0.7 mm (protonated sample) MAS rotors using an ultracentrifuge packing device (Giotto Biotech).

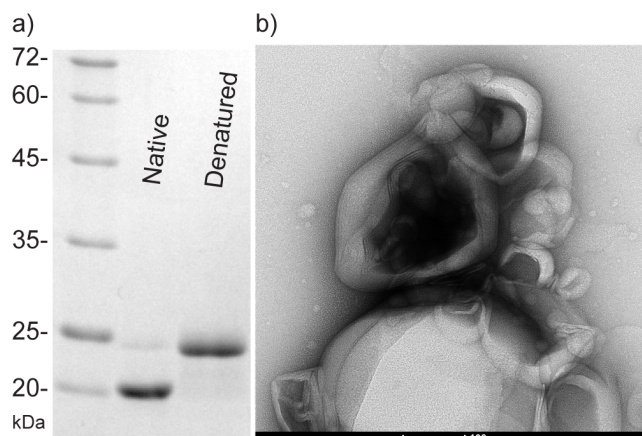
### Solid-state NMR spectroscopy

NMR spectra were recorded using a 0.7 mm triple-resonance ( $^1\text{H}$ ,  $^{13}\text{C}$ ,  $^{15}\text{N}$ ) probe on a 1000 MHz Bruker Avance III spectrometer or using a 1.3 mm triple-resonance ( $^1\text{H}$ ,  $^{13}\text{C}$ ,  $^{15}\text{N}$ ) probe on a 800 MHz spectrometer. All data were recorded at the MAS frequency of 111 kHz (0.7 mm probe) or 55 to 60 kHz (1.3 mm probe). The temperature of VT nitrogen gas was regulated to maintain a sample temperature of about 310 K. Backbone and sidechain resonance assignments were obtained from a set of three dimensional NMR spectra [13], [14]. Experimental details are given in table S1 and S2. Adamantane was used as the external reference. Spectra were processed using TopSpin 3.5 (Bruker Biospin) or NMRpipe [15] by zero filling to double the number of time increments, and apodization with a squared shifted cosine function (SSB 2 to 3). The direct dimension ( $^1\text{H}$ ) acquisition time was truncated to 8 ms. Spectral analysis and assignment was accomplished with CcpNmr Analysis [16] and Sparky [17]. The signal to noise ratio was estimated from a 1D hNH spectrum with 20 ms acquisition time, processed with 20 Hz exponential line broadening.

### Sequence alignment and secondary structure analysis

Sequence alignment was performed with the Clustal Omega [18] implementation on the Uniprot server using entries Q00595 (AlkL), P0A915 (OmpW) and Q9HWW1 (OprG). Secondary structure elements of AlkL were predicted from the assigned chemical shifts using TALOS-N [19]. Deposited chemical shifts of OmpW (BMRB Entry 19637) and OprG (BMRB Entry 25768) were reanalyzed using TALOS-N as well. Secondary structure elements of X-ray structures were directly transferred from the respective PDB entries (2F1T and 2X27)

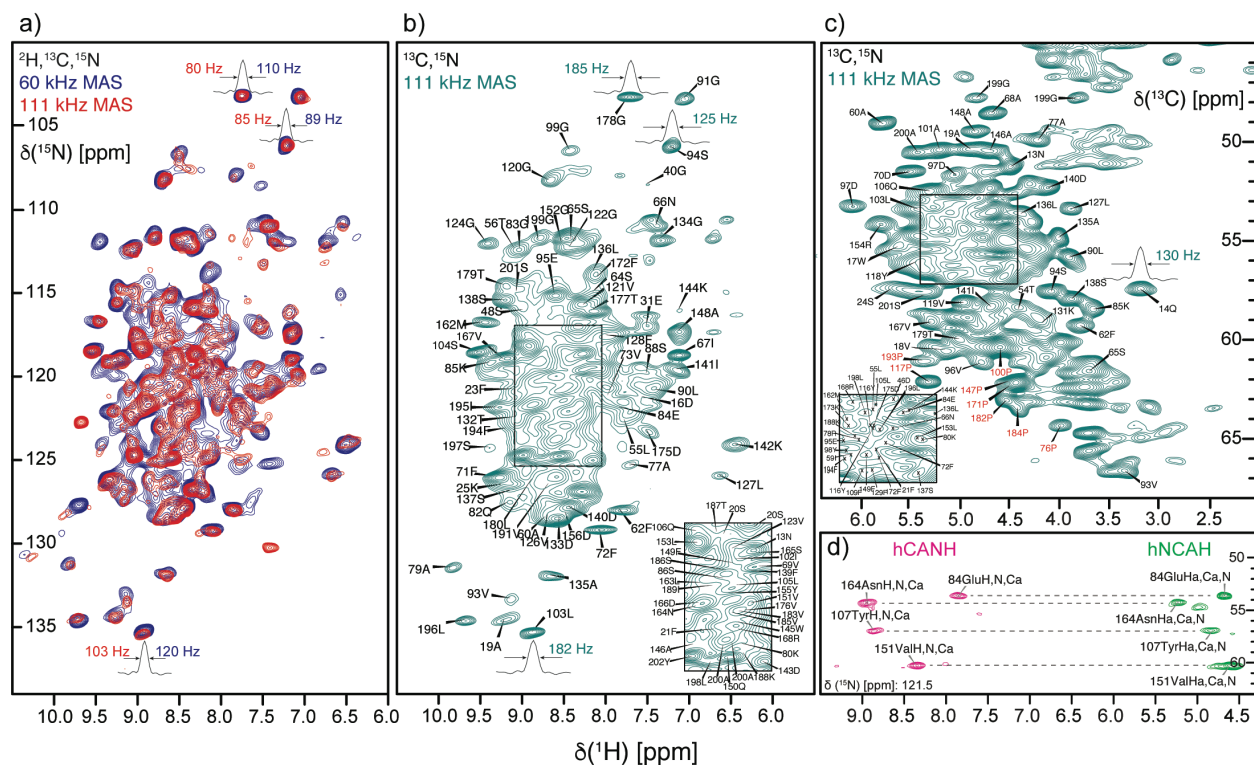
## Results



**Figure 1** a) SDS-PAGE analysis of purity and folding of AlkL. Folded (Native) sample was incubated with SDS-sample buffer at room temperature; the denatured sample was heated to 95 deg. C. b) Transmission electron micrographs of multilamellar vesicle preparations containing AlkL

AlkL was expressed by *E. coli* into inclusion bodies and purified under denaturing conditions followed by refolding into detergent micelles. Denaturation ensured the efficient back exchange of amide protons for the deuterated samples. The refolding efficiency was evaluated from the heat-modifiability of AlkL in SDS-PAGE (Figure 1a). Folded AlkL is denatured inefficiently by SDS at room temperature and migrates differently than the unfolded protein. We estimated the refolding efficiency to >90%. The yield of  $^{13}\text{C}$ ,  $^{15}\text{N}$  and  $^2\text{H}$ ,  $^{13}\text{C}$ ,  $^{15}\text{N}$ -labeled protein after purification and refolding was about 20 and 10 mg per liter of culture, respectively. Several types of lipids and different lipid to protein ratios were tested initially with  $^{15}\text{N}$ -labeled AlkL as described in [20]. Lipids were saturated with OG detergent, initially forming mixed micelles. Removal of OG by dialysis resulted in the formation of multilamellar vesicles, which appear as a white precipitate. A transmission electron micrograph of the final NMR sample is shown in Figure 1b. The precipitated vesicles were ultracentrifuged into MAS NMR rotors.

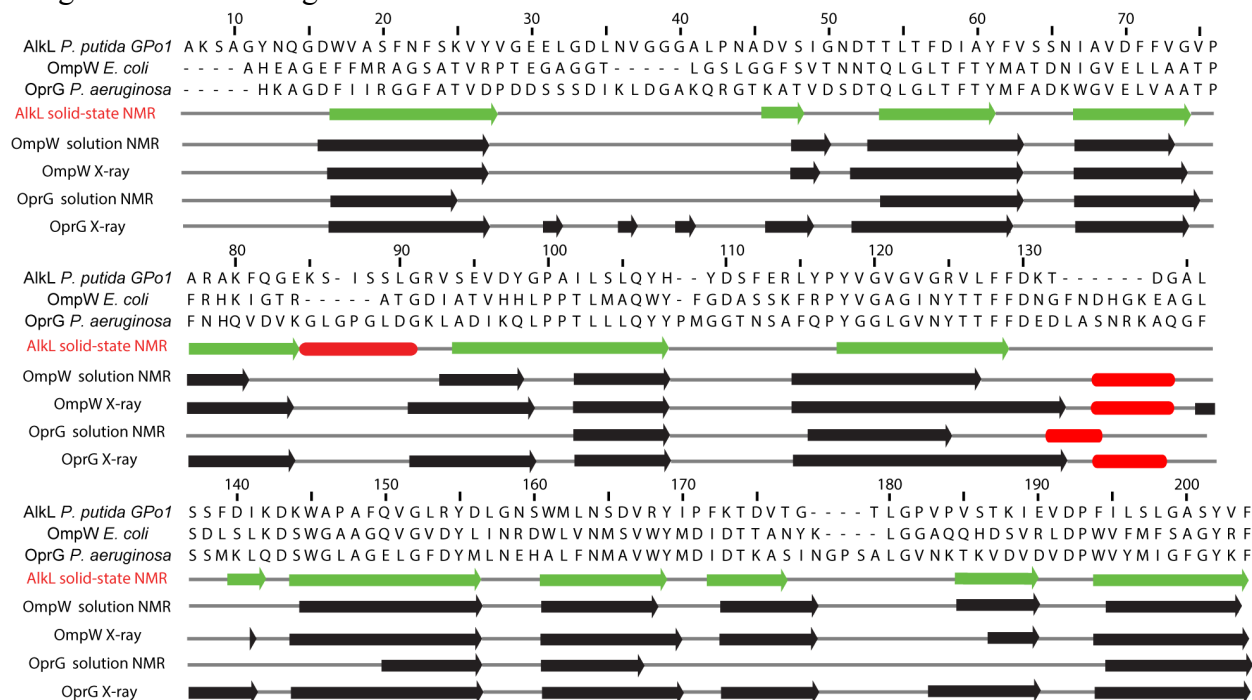
The 2D  $^1\text{H}$ - $^{15}\text{N}$  spectrum of deuterated and protonated AlkL are shown in Figure 2a,b. The protonated sample showed an average  $^1\text{H}$  linewidth of 150 Hz at 111 kHz MAS, the deuterated sample 90 Hz at 111 kHz MAS and 120 Hz at 60 kHz MAS. As concluded elsewhere before, deuteration is still beneficial for  $^1\text{H}$  linewidth and coherence lifetimes, even at >100 kHz MAS [21], [22]. Even though the fully protonated sample showed a lower resolution most assignments were made on this sample. While a deuterated samples only permits the classic set of  $^1\text{H}^{\text{N}}$ -detected triple-correlation spectra for backbone sequential assignment, it was useful in assigning loop residues for which peak intensities were lower. Fully protonated samples allow recording of an additional set of  $^1\text{H}\alpha$ -detected spectra, which exploit the favorable resolution of the 2D  $\text{C}\alpha$ - $\text{H}\alpha$  fingerprint. The  $^{13}\text{C}\alpha$  region of 2D  $^1\text{H}$ - $^{13}\text{C}$  correlation spectra of AlkL, reconstituted in DMPC, is shown in Figure 2c.  $\alpha$ -protons also display  $^1\text{H}$  linewidth of approximately 150 Hz, which is in line with non-crystalline membrane protein samples studied previously and indicative for high sample homogeneity [23], [24]. The decent signal to noise ratio ( $\text{S/N}/\sqrt{t} = 24.7$ ) and long refocused



**Figure 2** a) 2D  $^1\text{H}$ - $^{15}\text{N}$  correlation spectra (hNH) of  $^2\text{H}$  (100%  $^1\text{H}^{\text{N}}$ ),  $^{13}\text{C}$ ,  $^{15}\text{N}$ -labeled AlkL in DMPC vesicles recorded on a sample packed in a 1.3 mm rotor with 60 kHz MAS (blue) or in a 0.7 mm rotor with 111 kHz MAS on a 1 GHz spectrometer. b) hNH spectrum of fully protonated  $^{13}\text{C}$ ,  $^{15}\text{N}$ -labeled AlkL recorded with 111 kHz MAS. c)  $^{13}\text{C}\alpha$ - $^1\text{H}\alpha$  region of 2D  $^1\text{H}$ - $^{13}\text{C}$  correlation spectrum (hCH) of fully protonated  $^{13}\text{C}$ ,  $^{15}\text{N}$  labeled AlkL recorded with 111 kHz MAS. d) Slice of two 3D  $^1\text{H}$ - $^{13}\text{C}$ - $^{15}\text{N}$  correlation spectra (hCANH and hNCAH) at the  $^{15}\text{N}$  frequency of 121.5 ppm. The spectra were recorded simultaneously on fully protonated AlkL. Sequence specific resonance assignments are annotated on peaks in panel b, c and d.

transverse coherence lifetimes ( $T_2^*$ ) of 3.6 ms ( $^1\text{H}^{\text{N}}$ ), 18 ms ( $^{15}\text{N}$ ), 12.2 ms ( $^{13}\text{C}\alpha$ ) and 17 ms ( $^{13}\text{C}'$ ) obtained for the sample at 110 kHz allowed the acquisition of an extensive set of three dimensional spectra. The combination of amide and  $\alpha$ -proton detected spectra proved to be very powerful for sequence specific resonance assignment. While in the classic amide-based backbone walk both the  $^1\text{H}^{\text{N}}$  and  $^{15}\text{N}$  chemical shift of a pre/succeeding residue needs to be identified, the  $^1\text{H}\alpha$  detected spectra add the sequential  $^{15}\text{N}$  chemical shift, facilitating the linkage dramatically. The strategy relies on the identification of  $^1\text{H}^{\text{N}}$ ,  $^1\text{H}\alpha$ ,  $^{13}\text{C}\alpha$  and  $^{15}\text{N}$  resonances of individual spin-systems (i.e. intraresidue correlations) which is, fortunately, often straightforward, using the hCANH and hNCAH spectra as demonstrated in Figure 2d. The spectra in Figure 2d were acquired simultaneously to improve sensitivity and maximize consistency of chemical shifts [25], [26]. The succeeding  $^{15}\text{N}(i+1)$  chemical shift can then be found in the hNcoCAH spectrum and adjacent residues are simply linked over the  $^{13}\text{C}\alpha$  resonance. Additional  $^{13}\text{C}'$  and  $^{13}\text{C}\beta$  correlations resolved most of remaining ambiguities, finally resulting in backbone assignments of 172 (out of 219) residues, corresponding to 79% total and more than 90% of the detectable residues. An overview of assigned heteronuclei is provided in figure S2. Most unassigned residues are located at the N- and C-termini and in the region between residues 33 and 53. These fragments likely show increased

dynamics and are therefore undetectable in the CP-based MAS spectra. In general, the highest peak intensity was observed in the transmembrane  $\beta$ -strands, while the loop regions were more challenging to assign due to lower signal intensity. The automated assignment algorithm FLYA was employed to jointly analyze the large set of correlation spectra and already identified the transmembrane  $\beta$ -strands. Manual analysis was necessary to extend these assignments into loop residues. High sensitivity data, recorded on a deuterated sample in a larger 1.3 mm rotor, was particularly useful to add assignments to loop 1 (residues 22-47), a region of general low peak intensity, and to define the  $\beta$ -strand edges. All obtained resonance assignments are annotated in Figure 2b and c. Note the assigned proline residues, marked in red in Figure 2c, that are unavailable from amide proton based assignment spectra. A representative strip plot for the assignments of residues 83 to 87 is shown in Figure S1. The amide  $^1\text{H}^{\text{N}}$  and  $^{15}\text{N}$  assignments were further cross-checked using a 3D NNH spectrum based on proton-proton BASS-SD mixing [27]. We found that for  $\beta$ -strands, the transfer to neighboring amide protons was particularly efficient using this technique. The assigned  $^{13}\text{C}\alpha$ - $^1\text{H}\alpha$  pairs additionally provided a starting point to the analysis of a 3D CCH-TOCSY and a 4D HCCH-TOCSY spectra, which allowed the assignment of the majority of the sidechain aliphatic  $^{13}\text{C}$  and  $^1\text{H}$  resonances [14]. We did not record additional spectra to assign the aromatic ring resonances.



**Figure 3:** Sequence alignment and secondary structure analysis of the proteins AlkL, OmpW and OprG. Backbone torsion angles were calculated from NMR chemical shifts using TALOS-N or directly extracted from PDB files for X-ray crystal structures.  $\beta$ -strands are plotted as green or black arrows, and  $\alpha$ -helices as red rounded rectangles.

The degree of protein folding in the extracellular domain of OmpW proteins is highly dependent on the experimental conditions and showed discrepancies between previously published X-ray and solution NMR studies of OmpW and OprG. Analysis of the chemical shifts towards the secondary structure of AlkL revealed 13  $\beta$ -strands and one short  $\alpha$ -helix (Figure 3). The positions of the  $\beta$ -

strands are in good agreement with the X-ray structures of OmpW and OprG, proving that extensive folding of the extracellular domain is present in our DMPC preparation. The helix in AlkL is located in a different region compared to the other two proteins.

In conclusion, we have found experimental conditions that are close to the native environment of this outer membrane protein, and importantly also favor to a protein fold close the one previously observed via X-ray crystallography. We anticipate to gain new insight into the transport mechanism of the protein by applying further NMR measurements sensitive to dynamics and interactions with small molecules.

### Accession Number

<sup>1</sup>H, <sup>13</sup>C and <sup>15</sup>N backbone and side-chain chemical shifts of the outer membrane protein AlkL have been deposited in the BioMagResBank (<http://www.bmrb.wisc.edu>) under the accession number 34365.

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