JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

COMMUNICATION

pubs.acs.org/JACS

Dynamic Nuclear Polarization-Enhanced Solid-State NMR of a ^{13}C-Labeled Signal Peptide Bound to Lipid-Reconstituted Sec Translocon

Lenica Reggie,[§,∥] Jakob J. Lopez,[§,∥] Ian Collinson,[‡] Clemens Glaubitz,[§] and Mark Lorch*[,†]

[†]Department of Chemistry, University of Hull, Hull HU6 7RX, United Kingdom

[‡]School of Biochemistry, University Walk, University of Bristol, Bristol BS8 1TD, United Kingdom

[§]Institute for Biophysical Chemistry and Centre for Biomolecular Magnetic Resonance (BMRZ), Goethe University, Max von Laue Str. 9, 60438 Frankfurt, Germany

Supporting Information

ABSTRACT: Dynamic nuclear polarization (DNP) has made it possible to record 2D double-quantum-filtered (DQF) solid-state NMR (ssNMR) spectra of a signal peptide bound to a lipid-reconstituted SecYEG translocon complex. The small quantity of peptide in the sample (~40 nmol) normally prohibits multidimensional ssNMR experiments. Such small amounts are not the exception, because for samples involving membrane proteins, most of the limited sample space is occupied by lipids. As a consequence, a conventional 2D DQF ssNMR spectrum with the sample used here would require many weeks if not months of measurement time. With the help of DNP, however, we were able to acquire such a 2D spectrum within 20 h. This development opens up new possibilities for membrane protein studies, particularly in the exploitation of high-resolution spectroscopy and the assignment of individual amino acid signals, in this case for a signal peptide bound to the translocon complex.

The signal enhancement in solid-state NMR spectroscopy (ssNMR) by dynamic nuclear polarization (DNP), as demonstrated by Griffin and co-workers,[1−4] has matured in recent years to such an extent that biological applications of NMR spectroscopy that have long been out of reach can now be envisaged. The most promising approach for ssNMR relies on the use of nitroxide-based polarizing agents and the cross effect or thermal mixing at low temperatures for efficient magnetization transfer from electrons to nuclei.[5]

The technique is now at a stage where it has been demonstrated to produce several hundred-fold enhancement of NMR signals in model systems.[5] Similar substantial enhancements have also been achieved with well-characterized test membrane proteins and amyloid fibrils.[2,6] If this enhancement in signals can be translated (even in part) to more challenging systems, then DNP may open the door to the study of biological samples that have intrinsically low signals and are currently inaccessible to conventional NMR methods. In this work, we applied DNP-enhanced magic-angle-spinning (MAS) NMR spectroscopy based on the cross effect utilizing the biradical TOTAPOL to a very challenging membrane protein translocation system.

The Sec translocon is a ubiquitous protein complex coordinating the transport of other proteins either across or into biological membranes. Protein substrates are recognized via an N-terminal signal sequence, the characteristics of which trigger the complex to translocate the protein across the membrane.[7] The structure of the SecY translocation complex activated by a signal sequence has been visualized by electron cryomicroscopy of 2D crystals.[8,9] MAS ssNMR could complement these data by providing high-resolution structural information and details on the contacts between the Sec complex and the preprotein peptide. However, the relative size of the peptide and the complex makes this an arduous task. The majority of the very limited space in the MAS rotor is taken up by the translocon complex and the surrounding lipids that constitute a membrane environment. Thus, the signal peptide makes up a very small fraction of the overall sample.

We reconstituted functional *Escherichia coli* SecYEG (over 600 amino acids) complexed with the 25 amino acid LamB signal peptide into *E. coli* lipid bilayers. The reconstitution method used here follows recently reported procedures that have been shown to produce a fully functional protein.[10,11] There is a single binding site for LamB (with $K_d = 10\ \mu M$[11]) within each complex, and excess peptide was removed by centrifugation and washing of the pellet. Therefore, the concentration of peptide could not exceed that of the SecYEG complex. The ligand was ^{13}C-labeled at residues Met-2, Leu-5, Ala-13, and Gly-17 (^{13}C-MLAG-LamB; the full sequence is given in the Supporting Information). For DNP, the sample was resuspended in 30:10:60 (w/w/w) glycerol/H_2O/D_2O with 20 mM TOTAPOL. This biradical polarization agent has been shown to facilitate excellent signal enhancement.[5] The sample was incubated at 20 °C for 2 h and then repelleted before being loaded into a 3.2 mm MAS rotor. The active rotor volume accommodated ~6 mg of SecYEG plus 6 mg of lipid, leaving only 40 nmol of binding sites for LamB (100 μg).

DNP-enhanced ^{1}H−^{13}C cross-polarization (CP) experiments with MAS were carried out at 100 K using a 393 MHz/259 GHz spectrometer equipped with a high-power gyrotron as the microwave source.

The ^{13}C CP-MAS spectrum of ^{13}C-MLAG-LamB complexed with SecYEG contains contributions from the labeled ligand as well as from the natural-abundance background from the protein, lipids, and glycerol (Figure 1 a,b). The two large peaks at 63 and

Received: October 5, 2011
Published: November 01, 2011

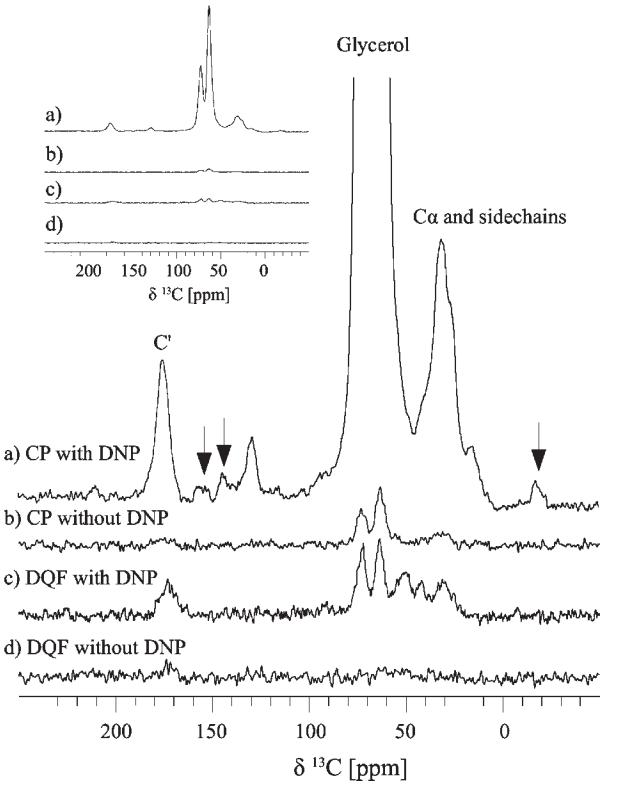

Figure 1. DNP−MAS NMR spectra of uniformly labeled ^{13}C-MLAG-LamB bound to SecYEG (40 nmol) complex reconstituted into *E. coli* lipids (protein/lipid molar ratio = 1:50) and then resuspended in 20 mM TOTAPOL and 30:10:60 (w/w/w) glycerol/H_2O/D_2O. Data were recorded at 100 K with a MAS spin rate of 8 kHz using a 393 MHz/259 GHz DNP NMR spectrometer. The ^{13}C CP MAS spectra (a) with and (b) without microwave irradiation show a 32-fold DNP enhancement for glycerol and lipid/protein resonances. Spectra were acquired with 32 scans. POST-C7 DQ filtering removed most of the natural-abundance background (c, d). The C′, Cα, and side-chain regions of the spectra are labeled, as are the peaks arising from glycerol; arrows indicate spinning side bands. The inset shows all of the spectra scaled to full intensity.

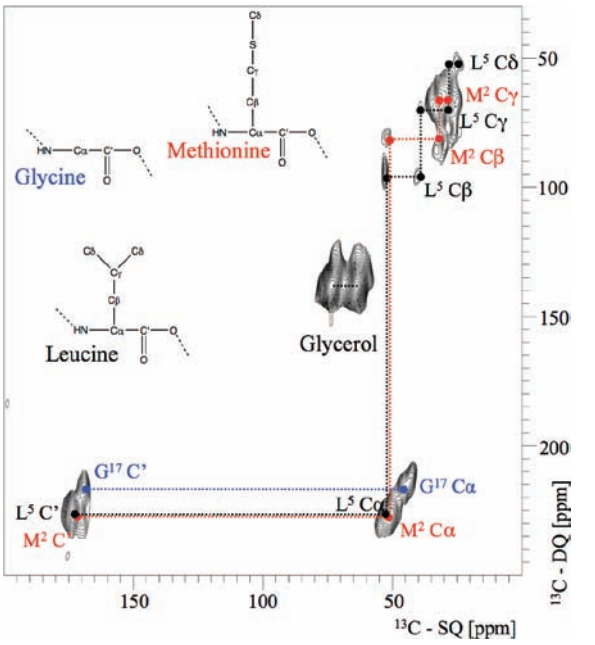

Figure 2. DNP-enhanced ^{13}C-DQSQ correlation spectrum of uniformly labeled ^{13}C-MLAG-LamB bound to SecYEG (40 nmol) complex reconstituted into *E. coli* lipids (protein/lipid molar ratio = 1:50) and then resuspended in 20 mM TOTAPOL and 30:10:60 (w/w/w) glycerol/H_2O/D_2O. The experiments were carried out at 100 K using a sample spinning rate of 8 kHz, 1024 acquisitions, and 96 increments. The measurement time was ~20 h. The peptide line widths were ~400 Hz, comparable to those reported for ^{13}C-labeled neuropeptides bound to GPCRs.[13,14] These measurements produce chemical shift information in the direct dimension and bond connectivities in the indirect dimension. This allows "assignment walks", which are further assisted by the fact that the sum of the frequencies from coupled nuclei in the direct dimension is equal to their frequencies in the indirect dimension. Thus, each amino acid type produces a characteristic spectral fingerprint.[13,14]

73 ppm arise from the glycerol, which was added as a cryoprotectant. The remaining signals arise from the lipids and proteins. Under microwave irradiation, ~32-fold DNP signal enhancement for all resonances was observed (Figure 1a). Because of the size of SecYEG and quantity of lipids in the sample, the ^{13}C natural-abundance background was ~6 times larger than the ^{13}C signal expected from ^{13}C-MLAG-LamB. We therefore applied CP followed by dipolar double-quantum (DQ) filtering using POST-C7 to suppress the natural-abundance contribution (Figure 1c,d). The use of DQ filtering comes at the cost of 60% of the signal intensity, pushing the already strained boundaries of sensitivity beyond reasonable limits (Figure 1d). However, when the DQ-filtered (DQF) measurements were conducted under microwave irradiation, good-quality DQF spectra were obtained after 512 scans from just 40 nmol of ^{13}C-MLAG-LamB (Figure 1c).

To resolve all of the resonances of ^{13}C-MLAG-LamB, 2D DQ dipolar INADEQUATE correlation spectroscopy[12] (DQSQ) was used (Figure 2). The length of the DQ excitation time was set to filter mainly through-bond connectivities. This allowed intraresidue assignment walks to identify amino acid types through their characteristic spectral fingerprints, and consequently, individual resonances could be assigned. The LamB labeling scheme used here contained only four different residues, which simplified the sequence-specific assignment. This approach has been used in the past for the study of small ^{13}C-labeled neuropeptides bound to G-protein coupled receptors (GPCRs).[13,14] Our study represents a comparable but rather more challenging situation because of the limited signal-to-noise ratio. From the spectrum shown in Figure 2, the ^{13}C resonances of Met-2, Leu-5, and Gly-17 could be identified. Signals for the natural-abundance background from the glycerol could also be seen. Signals from Ala-13 were not resolved, despite being apparent in the lyophilized sample (see Supplementary Figure 1 in the Supporting Information). The Ala-13 C′−Cα resonances may be part of the C′−Cα cross-peaks assigned to Leu-5 and Met-2 at 175 and 52 ppm. However, there is no apparent Cα-Cβ correlation for alanine (expected at 19 and 50−55 ppm in the direct dimension and ~70 ppm in the indirect dimension according to the Biological Magnetic Resonance Databank[15]). The lack of signal from Ala-13 could be due to its being inaccessible to the TOTAPOL polarization agent. Recent evidence suggests that Ala-13 is located deep within the membrane,[11] potentially giving weight to this interpretation of the data. However, further analysis revealed that a conventional DARR ^{13}C−^{13}C correlation spectrum (collected at 190 K without DNP) of the same sample also failed to find any signals from Ala-13 (data not shown). Therefore, it seems more likely that the lack of signals is the result of altered Ala-13

methyl group rotation at 100 K, which interferes with decoupling and/or elongated longitudinal relaxation.[16]

The chemical shifts of amino acids' C′ and Cα resonances are heavily dependent on the secondary structure in which they are found. Hence, the "secondary chemical shifts" (i.e., the differences between shifts of proteins in the folded and unfolded states) can lead to backbone torsion angle restraints that can be used in structure calculations.[17] This strategy has been used to great effect in the past for peptide ligands bound to membrane proteins.[13,14] The chemical shifts of the resolved resonances in uniformly labeled ^{13}C-MLAG-LamB suggest that the peptide is in an α-helical conformation, consistent with its structure when bound to the partner ATPase SecA,[18] the translocon partner of the SecY complex.

The spectra presented here demonstrate that data on otherwise intractable biological systems can be obtained with the use of DNP. The line shapes and line widths of the cross-peaks observed in the 2D DQSQ spectrum of ^{13}C-MLAG-LamB complexed with SecYEG are similar to those reported in other low-temperature studies and are caused by freezing-induced structural heterogeneity.[13,14,19]

An expansion of this study is underway with the addition of a number of peptides with different labeling schemes for coverage of the entire LamB signal sequence. When these are assigned, the backbone structure can be determined using less than a week of DNP−NMR measurement time. In contrast, conventional NMR spectroscopy would require several months to collect the same quantity and quality of data.

■ ASSOCIATED CONTENT

Supporting Information. Experimental procedures and setup and a supporting figure and table. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author
M.lorch@hull.ac.uk

Author Contributions
‖These authors contributed equally.

■ ACKNOWLEDGMENT

The work presented here was supported by grants from EU-NMR, EAST-NMR, SFB 807 Membrane Transport, and BBSRC (Grant BB/F007248/1).We thank Dr. Vasyl Denysenkov and Dr. Jörn Plackmeyer from the Institute for Theoretical and Physical Chemistry (Goethe University) for invaluable help with the gyrotron and probe head and for providing TOTAPOL.

■ REFERENCES

(1) Herzfeld, J.; Mak-Jurkauskas, M. L.; Bajaj, V. S.; Hornstein, M. K.; Belenky, M.; Griffin, R. G. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 883–888.

(2) Griffin, R. G.; Maly, T.; Andreas, L. B.; Smith, A. A. *Phys. Chem. Chem. Phys.* **2010**, *12*, 5872–5878.

(3) Griffin, R. G.; Bajaj, V. S.; Mak-Jurkauskas, M. L.; Belenky, M.; Herzfeld, J. *J. Magn. Reson.* **2010**, *202*, 9–13.

(4) Warschawski, D. E.; Arnold, A. A.; Beaugrand, M.; Gravel, A.; Chartrand, E.; Marcotte, I. *Biochim. Biophys. Acta* **2011**, *1808*, 1957–1974.

(5) Maly, T.; Debelouchina, G. T.; Bajaj, V. S.; Hu, K. N.; Joo, C. G.; Mak-Jurkauskas, M. L.; Sirigiri, J. R.; van der Wel, P. C. A.; Herzfeld, J.; Temkin, R. J.; Griffin, R. G. *J. Chem. Phys.* **2008**, *128*, No. 052211.

(6) Griffin, R. G.; Debelouchina, G. T.; Bayro, M. J.; van der Wel, P. C. A.; Caporini, M. A.; Barnes, A. B.; Rosay, M.; Maas, W. E. *Phys. Chem. Chem. Phys.* **2010**, *12*, 5911–5919.

(7) Driessen, A. J. M.; Kusters, I. *Cell. Mol. Life Sci.* **2011**, *68*, 2053–2066.

(8) Economou, A.; Gouridis, G.; Karamanou, S.; Gelis, I.; Kalodimos, C. G. *Nature* **2009**, *462*, 363–U132.

(9) Frauenfeld, J.; Gumbart, J.; van der Sluis, E. O.; Funes, S.; Gartmann, M.; Beatrix, B.; Mielke, T.; Berninghausen, O.; Becker, T.; Schulten, K.; Beckmann, R. *Nat. Struct. Mol. Biol.* **2011**, *18*, 614–621.

(10) Deville, K.; Gold, V. A. M.; Robson, A.; Whitehouse, S.; Sessions, R. B.; Baldwin, S. A.; Radford, S. E.; Collinson, I. *J. Biol. Chem.* **2011**, *286*, 4659–4669.

(11) Hizlan, D.; Robson, A.; Whitehouse, S.; Gold, V. A.; Vonck, J.; Mills, D. J.; Kühlbrandt, W.; Collinson, I. *Cell Rep.*, in press.

(12) Hong, M. *J. Magn. Reson.* **1999**, *136*, 86–91.

(13) Luca, S.; White, J. F.; Sohal, A. K.; Filippov, D. V.; van Boom, J. H.; Grisshammer, R.; Baldus, M. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 10706–10711.

(14) Lopez, J. J.; Shukla, A. K.; Reinhart, C.; Schwalbe, H.; Michel, H.; Glaubitz, C. *Angew. Chem., Int. Ed.* **2008**, *47*, 1668–1671.

(15) Biological Magnetic Resonance Data Bank. http://www.bmrb.wisc.edu (accessed Oct 5, 2011).

(16) Andrew, E. R.; Hinshaw, W. S.; Hutchins, M. G.; Sjoblom, R. O. I.; Canepa, P. C. *Mol. Phys.* **1976**, *32*, 795–806.

(17) Spera, S.; Bax, A. *J. Am. Chem. Soc.* **1991**, *113*, 5490–5492.

(18) Gelis, I.; Bonvin, A. M. J. J.; Keramisanou, D.; Koukaki, M.; Gouridis, G.; Karamanou, S.; Economou, A.; Kalodimos, C. G. *Cell* **2007**, *131*, 756–769.

(19) Hong, M.; Su, Y. C. *J. Phys. Chem. B* **2011**, *115*, 10758–10767.