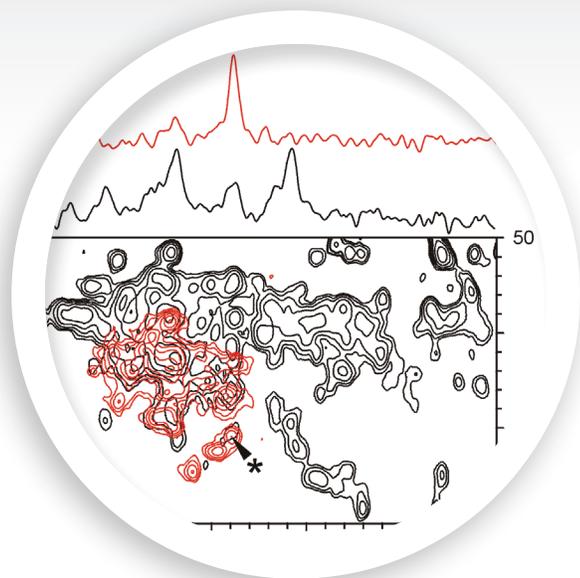




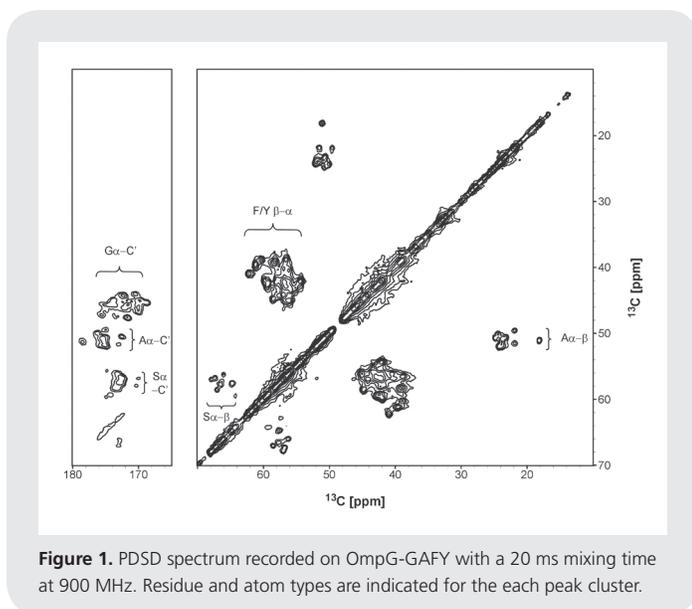
[2,3-¹³C]-Labeled Aromatic Residues as a Means to Improving Signal Intensities and Kick-Starting the Assignment of Membrane Proteins by Solid-State MAS-NMR

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Over the past few years solid-state MAS-NMR has rapidly been developing into a structure determination technique for biological macromolecules.¹⁻⁶ Its advantages include the ability to study membrane proteins in their native lipid environment,⁷⁻¹⁰ as well as making possible the study of non-soluble or non-crystallisable protein states, such as amyloid fibrils.¹¹⁻¹⁵ However, a prerequisite for structure determination is a high level of resonance assignment. There are numerous examples of small- and medium-sized proteins for which this has been possible,^{4,16-20} but for large membrane proteins, such as the 281-residue outer membrane protein G (OmpG)²¹⁻²² resonance assignment still remains a challenge. Signal overlap is an obvious problem, but fast longitudinal and transverse relaxation rates also contribute towards lower signal/noise ratios. Furthermore, membrane proteins can reduce the Q-factor of the coil, and the experimentalist is then left with the difficult task of balancing increased decoupling powers against possible sample heating which could lead to sample degradation. These problems result in lower signal intensities in proton-driven spin diffusion (PDS) spectra, however, they can be addressed using several different strategies, such as by improving coil design,²³⁻²⁵ by developing spectral editing pulse sequences,²⁶⁻²⁷ or by using novel labeling strategies.²⁸⁻²⁹ It is this last approach, using a novel labeling strategy, which is presented here in this application note.

It has been shown that samples produced using [1,3-¹³C]- and [2-¹³C]-labeled glycerol in the bacterial growth medium give rise to spectra with improved cross-relaxation properties and which prove very helpful at the restraint generation stage.¹ But assignment using solely these samples is not possible, since the C', C α and C β atoms are never simultaneously labeled. An alternative approach would be to use [1,2,3-¹³C]-, [1,2-¹³C]- and/or [2,3-¹³C]-labeled amino-acids. This would retain the favorable cross-relaxation properties of the glycerol samples by keeping the number of consecutively bonded labeled carbon atoms low, but would provide a more useful set of labels for assignment. The two former labeling patterns can easily be generated with uniformly labeled short amino acids such as alanine, serine, cysteine or glycine. Additional incorporation of other amino acids with [2,3-¹³C]-labeling would increase the number of detectable amino acids but reduce the overlap in NCO-type spectra,³⁰ thus providing scope for many unambiguous

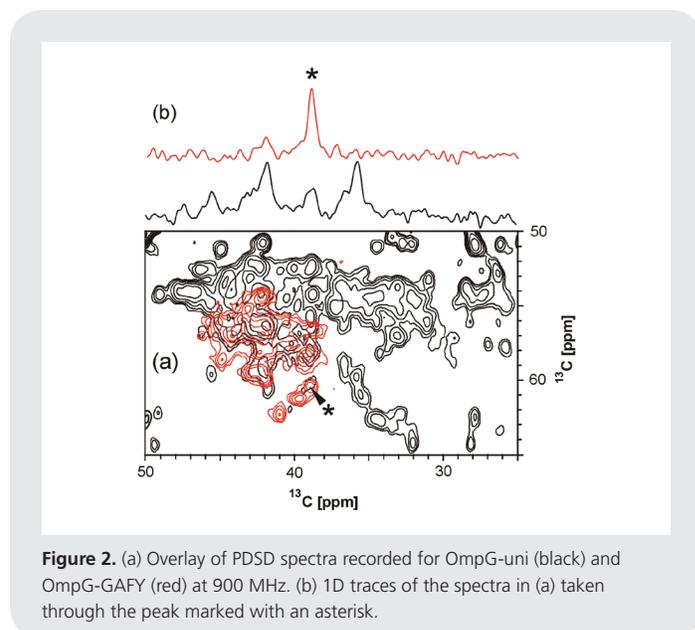


sequential assignments from these spectra. When considering which amino acid types to select for [2,3-¹³C]-labeling, it is worth noting that C β signals from aromatic amino acids are often very weak or not visible at all. [2,3-¹³C]-labeling aromatic amino acids would reduce J-couplings at the C α and C β positions, as well as ensure that the aromatic ring (which undergoes fast relaxation) does not draw away magnetization and effectively act as a magnetization “sink.” The [2,3-¹³C]-labeling would then not only aid assignment, but also improve the spectral quality and identification of aromatic spin systems.

This application note describes the use of a novel labeling strategy to increase the spectral quality and level of resonance assignment in MAS-NMR spectra of the biological macromolecule OmpG. Based on the protein’s sequence, a labeling scheme of fully [¹⁵N,¹³C]-labeled Ala and Gly and [2,3-¹³C,¹⁵N]-labeled Tyr and Phe was selected. The sample (hereafter referred to as OmpG-GAFY) was prepared using established protocols and incorporating [2,3-¹³C,¹⁵N]-Tyr (CIL #CNLM-7610), [2,3-¹³C,¹⁵N]-Phe (CIL #CNLM-7611), [U-¹³C₃,¹⁵N]-Ala (CIL #CNLM-534) and [U-¹³C₂,¹⁵N]-Gly (CIL #CNLM-1673).

Initial spectra showed that the ¹³C labels were incorporated as expected, with only minor contributions of labeled serine produced *in vivo* from the labeled glycine (Figure 1).

Improved signal intensities for [2,3-¹³C]-labeled aromatic residues. The gain in signal intensity for the Phe and Tyr residues was assessed by comparing spectra of OmpG-GAFY with those of uniformly labeled OmpG (OmpG-uni). Figure 2 shows that the number and intensity of aromatic C α /C β peaks has increased for the OmpG-GAFY sample. The intensity increases could come from the removal of J-couplings in OmpG-GAFY as well as from the fact that the aromatic ring is no longer able to act as a “magnetization sink.” The line widths were found to be similar for both samples which suggests that the latter process is the more dominant.



An alternative approach to improving the C α /C β cross-peaks of aromatic residues is the use of band-selective experiments. A DREAM spectrum^{31,32} of OmpG-uni has drastically improved signal intensities (by a factor of 10 or more), which are slightly higher than those in the OmpG-GAFY PDSB. A DREAM spectrum of OmpG-GAFY on the other hand only increases the signal intensity by a factor of two. This supports the fact that the isolation of the C α /C β spin system from the sidechain is the main source of signal enhancement when using [2,3-¹³C]-labeled residues.

A major advantage of the OmpG-GAFY sample containing [2,3-¹³C]-labeled aromatic residues is thus the fact that peak intensities nearly reach those of an OmpG-uni band-selective experiment, even during broadbanded experiments. This becomes particularly important when trying to obtain long-range, as well as numerous sequential cross-peaks, which require long mixing times. Using DREAM or other band-selective pulse sequences for this purpose would be inadvisable because the intense and continued irradiation over a long mixing time would lead to increased sample heating and thus risk sample degradation. In addition, band-selective experiments have the disadvantage that only those cross-peaks selected for are visible. Several spectra would have to be recorded to obtain the information content of a single broadbanded experiment. Samples containing [2,3-¹³C]-labeled residues therefore offer an attractive way of improving signal intensities in broadbanded experiments which form an important part of the assignment and structure determination process of a protein.

Sequence-Specific Assignment

Initially, spin systems were identified using a 3D NCACX spectrum recorded using a 20 ms PDSB mixing time. Some overlap could not be resolved, but all alanines, 96% of the glycines and 85% of the aromatics were identified. Inter-residue connections were then identified using a variety of 2D and 3D spectra: 3D NCACX spectra with long mixing times (300-500 ms) contained N_i-C α -C α _i type

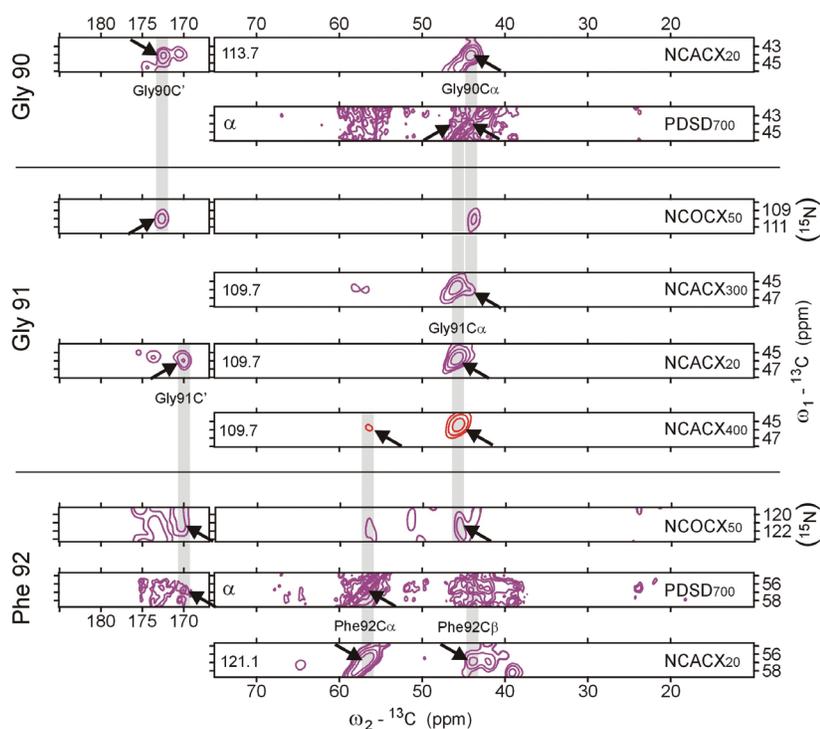


Figure 3. Spectra illustrating the assignment of the G90-G91-F92 motif in OmpG: Strips from 3D NCACX, 2D NCOCX and 2D PDS700 spectra are shown with the spectrum type and mixing time (in ms) indicated on the right. The ppm value of the 3D NCACX ^{15}N planes and carbon type of the PDS700 strips is indicated on the left. Purple spectra were recorded on OmpG-GAFY, red spectra on OmpG produced from $[2-^{13}\text{C}]$ -glycerol. Cross-peaks that define each residue and link neighboring ones are indicated by arrows and highlighted in gray. The carbonyl regions of the 50 ms NCOCX spectrum are replaced with a better quality 1ms REDOR spectrum.

peaks (where j is a neighboring residue or one close in space to i), PDS700 spectra (400-700ms) gave rise to $\text{C}\alpha$ - $\text{C}\alpha$, $\text{C}\alpha$ - $\text{C}\beta$ and $\text{C}\beta$ - $\text{C}\beta$ peaks, and a 2D NCOCX spectrum (50 ms) and a REDOR spectrum (1 ms) provided unambiguous inter-residue correlations of the type $\text{N}_i\text{-C}'_{i-1}$, $\text{N}_i\text{-C}'_{i-1}\text{-C}\alpha_{i-1}$ and $\text{N}_i\text{-C}'_{i-1}\text{-C}\beta_{i-1}$. Starting points generally came from the PDS700 and NCOCX/REDOR spectra. The NCACX spectra could then be used to identify further atoms in each spin system and link the other spectra. Figure 3 illustrates the assignment for the GGF motif. Further 2D PDS700-DARR, 3D NCACX and 3D NCOCX spectra were recorded for OmpG samples made using $[1,3-^{13}\text{C}]$ and $[2-^{13}\text{C}]$ -labeled glycerol¹. The $[2-^{13}\text{C}]$ -glycerol NCACX spectrum was used to distinguish between Phe and Tyr residues on the basis of N-C α -C γ peaks. Overall, these spectra were used to corroborate and extend the assignments obtained from the OmpG-GAFY sample leading to the identification of 45 sequence-specific assignments.

Conclusions

The GAFY labeling scheme which incorporates small uniformly labeled amino acids and $[2,3-^{13}\text{C}]$ -labeled aromatic residues has provided a highly successful starting point for the sequence-specific assignment of the large membrane protein OmpG. The main advantage of the labeling scheme is its composition of a low number of small, isolated spin systems (pairs and triplets of sequentially labeled carbon atoms). This limits transfer of magnetization into the sidechain resulting in enhanced spectral quality. In addition, the number of inter-residue cross-peaks is significantly increased which is important both for the assignment and structure calculation stages. The use of a mixture of $[2,3-^{13}\text{C}]$ -

and short U- $[^{13}\text{C}]$ -labeled amino acids has eased the interpretation of NCO- and NCA-type of spectra. In addition, this labeling scheme has the advantage compared to the $[1,3-^{13}\text{C}]$ - and $[2-^{13}\text{C}]$ -glycerol ones, that there is a direct connection between the $\text{C}\alpha$ and $\text{C}\beta$ chemical shifts by which the spin systems can be identified. Furthermore, $[2,3-^{13}\text{C}]$ -labeling provides the spectral quality of band-selective experiments while allowing broadband experiments to be recorded, thus improving and increasing the information content of spectra.

Acknowledgments

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Additional products of interest

CLM-1397	$2-^{13}\text{C}$ Glycerol
CLM-1857-K	$1,3-^{13}\text{C}_2$ Glycerol

References

1. Castellani, F.; van Rossum, B.; Diehl, A.; Schubert, M.; Rehbein, K.; Oschkinat, H. **2002**. *Nature*, *420*, 98-102.
2. Seidel, K.; Etzkorn, M.; Heise, H.; Becker, S.; Baldus, M. **2005**. *Chem Biochem*, *6*, 1638-1647.
3. Zech, S.G.; Wand, A.J.; McDermott, A.E. **2005**. *J Am Chem Soc*, *127*, 8618-8626.
4. Lange, A.; Becker, S.; Giller, K.; Pongs, O.; Baldus, M. **2005**. *Angew Chem Int Ed Engl*, *44*, 2089-2092.
5. Loquet, A.; Bardiaux, B.; Gardiennet, C.; Blanchet, C.; Baldus, M.; Nilges, M.; Malliavin, T.; Böckmann, A. **2008**. *J Am Chem Soc*, *130*, 3579-3589.
6. Franks, W.T.; Wylie, B.J.; Frericks Schmidt, H.L.; Nieuwkoop, A.J.; Mayrhofer, R.M.; Shah, G.J.; Graesser, D.T.; Rienstra, C.M. **2008**. *Proc Nat Ac Sci*, *105*, 4621-4626.
7. Hiller, M.; Krabben, L.; Vinothkumar, K. R.; Castellani, F.; van Rossum, B.J.; Kühlbrandt, W.; Oschkinat, H. **2005**. *Chem Biochem*, *6*, 1679-1684.
8. Lorch, M.; Fahem, S.; Kaiser, C.; Weber, I.; Mason, A.J.; Bowie, J.U.; Glaubitz, C. **2005**. *Chem Biochem*, *6*, 1693-1700.
9. Andronesi, O.C.; Becker, S.; Seidel, K.; Heise, H.; Young, H.S.; Baldus, M. **2005**. *J Am Chem Soc*, *127*, 12965-12974.
10. Schneider, R.; Ader, C.; Lange, A.; Gilelr, K.; Hornig, S.; Pongs, O.; Becker, S.; Baldus, M. **2008**. *J Am Chem Soc*, *130*, 7427-7435.
11. Petkova, A.T.; Ishii, Y.; Balbach, J.J.; Antzutkin, O.N.; Leapman, R.D.; Delaglio, F.; Tycko, R. **2002**. *Proc Nat Ac Sc*, *99*, 16742-16747.
12. Jaroniec, C.P.; MacPhee, C.E.; Bajaj, V.S.; McMahon, M.T.; Dobson, C.M.; Griffin, R.G. **2004**. *Proc Nat Ac Sci*, *101*, 711-716.
13. Heise, H.; Hoyer, W.; Becker, S.; Andronesi, O.C.; Riedel, D.; Baldus, M. **2005**. *Proc Nat A Sci*, *102*, 15871-15876.
14. Ferguson, N.; Becker, J.; Tidow, H.; Tremmel, S.; Sharpe, T.D.; Krause, G.; Flinders, J.; Petrovich, M.; Berriman, J.; Oschkinat, H.; Fersht, A.R. **2006**. *Proc Nat Ac Sci*, *103*, 16248-16253.
15. Wasmer, C.; Lange, A.; Van Melckebeke, H.; Siemer, A.B.; Riek, R.; Meier, B.H. **2008**. *Science*, *319*, 1523-1526.
16. Pauli, J.; Baldus, M.; van Rossum, B.; de Groot, H.; Oschkinat, H. **2001**. *Chem Biochem*, *2*, 272-281.
17. Böckmann, A.; Lange, A.; Galinier, A.; Luca, S.; Giraud, N.; Juy, M.; Heise, H.; Montserret, R.; Penin, F.; Baldus, M. **2003**. *J Biomol NMR*, *27*, 323-339.
18. Igumenova, T.I.; McDermott, A.E.; Zilm, K.W.; Martin, R.W.; Paulson, E.K.; Wand, A.J. **2004**. *J Am Chem Soc*, *126*, 6720-6727.
19. Goldbourt, A.; Gross, B.J.; Day, L.A.; McDermott, A.E. **2007**. *J Am Chem Soc*, *129*, 2338-2344.
20. Franks, W.T.; Zhou, D.H.; Wylie, B.J.; Money, B.G.; Graesser, D.T.; Frericks, H.L.; Sahota, G.; Rienstra, C.M. **2005**. *J Am Chem Soc*, *127*, 12291-12305.
21. Fajardo, D.A.; Cheung, J.; Ito, C.; Sugawara, E.; Nikaido, H.; Misra, R. **1998**. *J Bacteriol*, *180*, 4452-4459.
22. Misra, R.; Benson, S.A. **1989**. *J Bacteriol*, *171*, 4105-4111.
23. Stringer, J.A.; Bronnimann, C.E.; Mullen, C.G.; Zhou, D.H.H.; Stellfox, S.A.; Li, Y.; Williams, E.H.; Rienstra, C.M. **2005**. *J Magn Reson*, *173*, 40-48.
24. Doty, F.D.; Kulkarni, J.; Turner, C.; Entzminger, G.; Bielecki, A. **2006**. *J Magn Reson*, *182*, 239-253.
25. Gor'kov, P.L.; Chekmenev, E.Y.; Li, C.G.; Cotten, M.; Buffy, J.J.; Traaseth, N.J.; Veglia, G.; Brey, W.W. **2007**. *J Magn Reson*, *185*, 77-93.
26. Jehle, S.; Hiller, M.; Rehbein, K.; Diehl, A.; Oschkinat, H.; van Rossum, B.J. **2006**. *J Biomol NMR*, *36*, 169-177.
27. Jehle, S.; Rehbein, K.; Diehl, A.; van Rossum, B.J. **2006**. *J Magn Reson*, *183*, 324-328.
28. Hong, M.; Jakes, K. **1999**. *J Biomol NMR*, *14*, 71-74.
29. Etzkorn, M.; Martell, S.; Andronesi, O.C.; Seidel, K.; Engelhard, M.; Baldus, M. **2007**. *Angew Chem Int Ed Engl*, *46*, 459-462.
30. Baldus, M.; Petkova, A.T.; Herzfeld, J.; Griffin, R.G. **1998**. *Mol Phys*, *95*, 1197-1207.
31. Verel, R.; Baldus, M.; Ernst, M.; Meier, B.H. **1998**. *Chem Phys Lett*, *287*, 421-428.
32. Verel, R.; Ernst, M.; Meier, B.H. **2001**. *J Magn Reson*, *150*, 81-99.

