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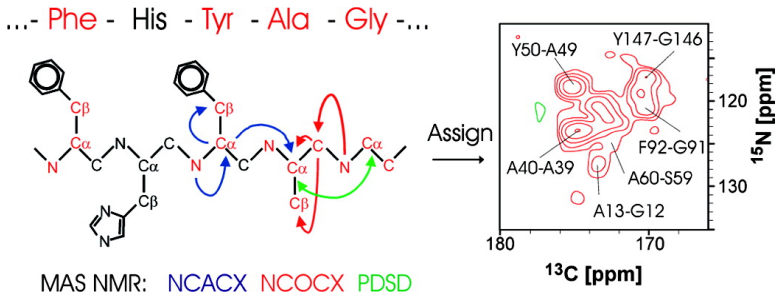

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[2,3-^{13}C]-labeling of Aromatic Residues—Getting a Head Start in the Magic-Angle-Spinning NMR Assignment of Membrane Proteins

Matthias Hiller,[†] Victoria A. Higman,[†] Stefan Jehle,[†] Barth-Jan van Rossum,[†] Werner Kühlbrandt,[‡] and Hartmut Oschkinat*,[†]

Leibniz-Institut für Molekulare Pharmakologie, Robert-Rössle-Strasse 10, 13125 Berlin, Germany, and Max-Planck-Institut für Biophysik, Max-von-Laue-Strasse 3, 60438 Frankfurt am Main, Germany

Received October 2, 2007; E-mail: oschkinat@fmp-berlin.de

In recent years solid-state magic-angle-spinning (MAS) NMR has developed into a structure determination technique for biological macromolecules.[1−2] It is particularly suited to the study of membrane proteins[3−4] and membrane protein-bound ligands[5] in near native conditions. However, a prerequisite to NMR studies, and the most time-consuming step, is always the assignment of resonances to specific nuclei within the protein. While near-full solid-state NMR assignments have been obtained for a number of small soluble proteins[1−2] this still remains a difficult task for larger membrane proteins, including for the 281-residue outer membrane protein G (OmpG) from *E.coli*.[6−8] Problems arise from signal overlap and fast longitudinal and transverse relaxation, caused in part by dynamics effects. In addition, membrane protein preparations tend to reduce the *Q*-factor of the coil, which can result in the need for a trade off between increased sample heating and insufficient compensation of dipolar interactions. Heating issues can to some extent be addressed using novel coil designs,[9] but most of the sources of fast relaxation are difficult to overcome and thus broad lines and reduced signal intensities in PDSD spectra are often unavoidable. Thus alongside spectral editing techniques[10] a reduced isotope labeling scheme which will decrease the number of observable nuclei is an indispensible method for improving spectral quality. While samples derived from the use of [1,3-^{13}C]- and [2-^{13}C]-labeled glycerol in the bacterial growth medium are useful for restraint generation at the structure calculation stage,[1] they are not sufficient for extensive assignment since the C′, Cα and Cβ sites are never simultaneously labeled. An alternative is to use samples in which labeling is only applied to certain amino acid types. However, it is necessary to restore the favorable cross-relaxation properties of the glycerol samples in order to obtain inter-residue cross-peaks which are important for successful sequential assignment. A suitable strategy for this might be to label several amino acids only at the N, Cα and Cβ positions (i.e., [^{15}N]- and [2,3-^{13}C]-labeling) while selecting other short amino acid types for full labeling. This will additionally significantly reduce overlap in NCO-type experiments[11] and aid unambiguous sequential assignment. In relation to aromatic residues, and in particular their Cβ signals, it is worth noting that they are often weak in carbon spectra of uniformly labeled samples. This situation can potentially be improved by reducing the number of carbon labels, for example, by incorporating [2,3-^{13}C]-labeled phenylalanines and tyrosines. This will remove the Cα−C′ and Cβ−Cγ *J*-couplings and ensures that the aromatic ring (which undergoes fast relaxation) does not act as a "sink", rapidly causing a loss of magnetization in the Phe and Tyr spin systems.

So as to address these issues we prepared a specifically labeled sample of OmpG which contains [^{15}N, 2,3-^{13}C]-labeled Phe and Tyr custom produced by Cambridge Isotope Laboratories (CIL). In addition, glycine and alanine were uniformly labeled since the OmpG sequence contains numerous sequential occurrences of Gly and Ala with Phe and Tyr. Gly and Ala also have the advantage that they are easily identified by their chemical shifts. The sample (hereafter referred to as OmpG-GAFY) was expressed, purified, refolded and reconstituted into 2D crystals following the protocols of Marley et al.[12] and Hiller et al.[3]

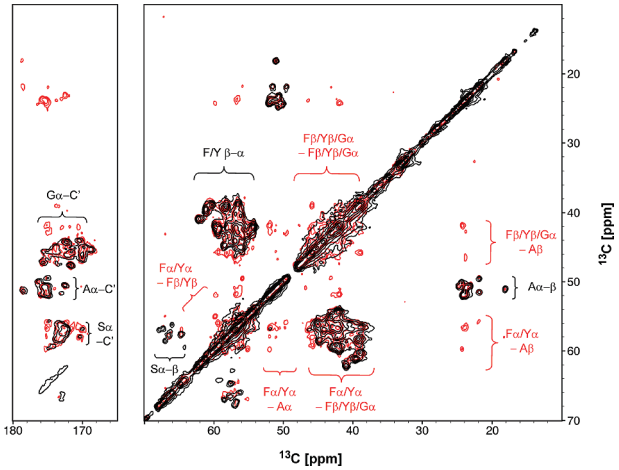

Figure 1. ^{13}C-^{13}C proton-driven spin diffusion spectrum of OmpG-GAFY recorded at 900 MHz with a 20 ms (black) and 700 ms (red) mixing time. Intra- (black) and inter-residual (red) cross-peak regions are indicated.

As can be seen in a ^{13}C−^{13}C proton-driven spin diffusion (PDSD) spectrum of OmpG-GAFY (Figure 1) Cα−Cβ signals for Phe, Tyr, and Ala and the Cα−C′ cross-peaks for Gly and Ala are visible. In addition, weak Ser Cα−Cβ and Cα−C′ peaks are also present. This is due to the *E.coli* metabolism which produces serine from glycine.[13] While there are still problems with overlap in the Phe/Tyr and Ala Cα−Cβ signal clusters, the spectrum is considerably less crowded than that of the uniformly labeled sample.[3] The Phe and Tyr Cα−Cβ cross-peaks are more intense and new peaks appear in the OmpG-GAFY spectrum compared to the uniformly labeled OmpG (OmpG-uni) spectrum (Figure 2a). The peak intensities improve more significantly than the line widths which suggests that the major cause of the poor spectral quality in the OmpG-uni spectrum is the ring system acting as a "magnetization sink". This is additionally supported by the fact that a ^{13}C−^{13}C DREAM experiment[14] optimized for the aromatic Cα/Cβ cross-peaks further increases the signal intensity, but to a far greater extent for OmpG-uni than for OmpG-GAFY (Figure 2b). Line widths are similar in all spectra and are comparable to those of peaks from other spin systems. The OmpG-GAFY sample with [2,3-^{13}C]-labeled aromatic residues thus has the major advantage that peak intensities are nearly those of an OmpG-uni band-selective experiment, even during

[†] Leibniz-Institut für Molekulare Pharmakologie.
[‡] Max-Planck-Institut für Biophysik.

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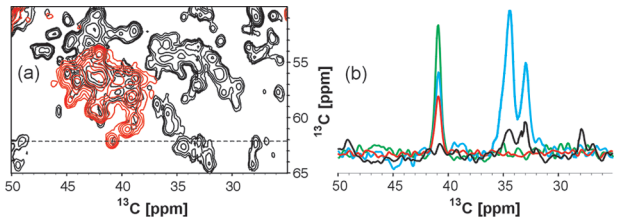

Figure 2. (a) Overlay of ^{13}C-^{13}C PDSD spectra (20 ms mixing time) recorded at 900 MHz on OmpG-uni (black) and OmpG-GAFY (red); (b) 1D traces of the spectra in panel a at the dotted line as well as from 2D ^{13}C-^{13}C DREAM spectra (1.5 ms, optimized for aromatic Cα/Cβ cross-peaks) recorded at 900 MHz on OmpG-uni (blue) and OmpG-GAFY (green).

broadbanded experiments. This is of particular importance for obtaining long-range and certain sequential cross-peaks which require long mixing times. This would not be possible with DREAM or other band-selective pulse sequences since the intense and continued irradiation over a long mixing time would lead to increased sample heating and thus sample degradation.

The assignment of OmpG-GAFY was achieved by a combinatorial use of PDSD, NCACX, NCOCX, and REDOR spectra. The 3D NCACX spectrum with 20 ms PDSD mixing contains strong intraresidual peaks and was used initially to identify the N, Cα, Cβ, and C′ chemical shifts for each of the spin systems. Some overlap could not be resolved, but all alanines, 96% of the glycines, and 85% of the aromatics were identified. Sequential inter-residue correlations involving Ala and Gly could be derived from the NCOCX and REDOR spectra, as these provide links from N_i to C'_{i-1}, $C\alpha_{i-1}$, and $C\beta_{i-1}$. Both sequential and long-range links involving all types of labeled residue were observed in the 3D NCACX and 2D ^{13}C-^{13}C PDSD spectra recorded with long PDSD mixing times (300−700 ms). For an unambiguous identification of the GAFY motifs, either the NCOCX or PDSD spectra (with long mixing times) served as a starting point, the NCACX spectra then helped to identify further atoms within each spin system and often served as a link between the other spectra (a figure illustrating the assignment of the AYY motif is provided in the Supporting Information).

Since OmpG is an antiparallel β-sheet protein[6−8] and some interstrand Cα−Cα or Cα−Cβ distances are of comparable length to the sequential Cα−Cβ distances, such correlations are also observable and will be highly valuable at a structure calculation stage. Further help and confirmation of assignments was provided by NCACX and NCOCX spectra of OmpG expressed in media containing [2-^{13}C]- and [1,3-^{13}C]-glycerol as the sole carbon source.[1] In many cases it was possible to distinguish between Phe and Tyr residues on the basis of their Cγ resonances in the [2-^{13}C]-glycerol 100 ms 3D NCACX spectrum. These spectra also allowed several motifs to be extended beyond the GAFY residues. Thus on the basis of the GAFY labeling scheme sequence specific assignments of at least two resonances could be made for a total of 45 residues (assigned spectra and a resonance list are available in the Supporting Information).

The GAFY labeling scheme has thus provided a highly successful starting point for the assignment of the large membrane protein OmpG. Advantages of the labeling scheme lie in the fact that it is composed of a low number of small, isolated spin systems (pairs and triplets of sequentially labeled carbon atoms). Transfer of magnetization into the side chain is thus eliminated and spectral quality enhanced. Furthermore, the number of inter-residue cross-peaks is significantly increased which is important both for assignment and structure calculation. The use of a mixture of [2,3-^{13}C]- and short U-[^{13}C]-labeled amino acids has eased the interpretation of NCO- and NCA-type spectra. In addition, it has the advantage compared to the [1,3-^{13}C]- and [2-^{13}C]-glycerol labeling schemes that there is a direct connection between the Cα and Cβ chemical shifts by which to identify the spin systems. Furthermore, [2,3-^{13}C]-labeling provides the spectral quality of band-selective experiments while allowing broadband experiments to be recorded.

Another strategy for the labeling of membrane proteins is reverse labeling in which certain amino acid types can be suppressed using unlabeled amino acids.[15,16] Hong and Jakes combine this with the use of [2-^{13}C]-glycerol[15] which provides samples which are good for collecting structural restraints, but less advantageous for assignment. The labeling scheme presented here should be applicable and invaluable to a wide variety of large membrane proteins, enabling assignment of this important class of proteins in a first step toward their structural characterization.

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Supporting Information Available: Details of sample preparation and NMR experiments; a list of resonance assignments; figures of assigned spectra, the AYY motif assignment, and the [1,3-^{13}C]- and [2-^{13}C]-glycerol labeling patterns. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Castellani, F.; van Rossum, B.; Diehl, A.; Schubert, M.; Rehbein, K.; Oschkinat, H. *Nature* **2002**, *420*, 98−102.
(2) Seidel, K.; Etzkorn, M.; Heise, H.; Becker, S.; Baldus, M. *Chem. Biochem.* **2005**, *6*, 1638−1647. Zech, S. G.; Wand, A. J.; McDermott, A. E. *J. Am. Chem. Soc.* **2005**, *127*, 8618−8626.
(3) Hiller, M.; Krabben, L.; Vinothkumar, K. R.; Castellani, F.; van Rossum, B. J.; Kühlbrandt, W.; Oschkinat, H. *Chem. Biochem.* **2005**, *6*, 1679−1684.
(4) Andronesi, O. C.; Pfeifer, J. R.; Al Momani, L.; Ozdirekcan, S.; Rijkers, D. T. S.; Angerstein, B.; Luca, S.; Koert, U.; Killian, J. A.; Baldus, M. *J. Biomol. NMR* **2004**, *30*, 253−265. Lorch, M.; Fahem, S.; Kaiser, C.; Weber, I.; Mason, A. J.; Bowie, J. U.; Glaubitz, C. *Chem. Biochem.* **2005**, *6*, 1693−1700. Frericks, H. L.; Zhou, D. H.; Yap, L. L.; Gennis, R. B.; Rienstra, C. M. *J. Biomol. NMR* **2006**, *36*, 55−71.
(5) Lange, A.; Giller, K.; Hornig, S.; Martin-Eauclaire, M. F.; Pongs, O.; Becker, S.; Baldus, M. *Nature* **2006**, *440*, 959−962. Krabben, L.; van Rossum, B. J.; Castellani, F.; Bocharov, E.; Schulga, A. A.; Arseniev, A. S.; Weise, C.; Hucho, F.; Oschkinat, H. *FEBS Lett.* **2004**, *564*, 319−324.
(6) Fajardo, D. A.; Cheung, J.; Ito, C.; Sugawara, E.; Nikaido, H.; Misra, R. *J. Bacteriol.* **1998**, *180*, 4452−4459. Misra, R.; Benson, S. A. *J. Bacteriol.* **1989**, *171*, 4105−4111.
(7) Yildiz, O.; Vinothkumar, K. R.; Goswami, P.; Kühlbrandt, W. *EMBO J.* **2006**, *25*, 3702−3713. Subbarao, G. V.; van den Berg, B. *J. Mol. Biol.* **2006**, *360*, 750−759.
(8) Liang, B.; Tamm, L. K. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 16140−16145.
(9) Stringer, J. A.; Bronnimann, C. E.; Mullen, C. G.; Zhou, D. H. H.; Stellfox, S. A.; Li, Y.; Williams, E. H.; Rienstra, C. M. *J. Magn. Reson.* **2005**, *173*, 40−48. Doty, F. D.; Kulkarni, J.; Turner, C.; Entzminger, G.; Bielecki, A. *J. Magn. Reson.* **2006**, *182*, 239−253. Gor'kov, P. L.; Chekmenev, E. Y.; Li, C. G.; Cotten, M.; Buffy, J. J.; Traaseth, N. J.; Veglia, G.; Brey, W. W. *J. Magn. Reson.* **2007**, *185*, 77−93.
(10) Jehle, S.; Hiller, M.; Rehbein, K.; Diehl, A.; Oschkinat, H.; van Rossum, B. J. *J. Biomol. NMR* **2006**, *36*, 169−177. Jehle, S.; Rehbein, K.; Diehl, A.; van Rossum, B. J. *J. Magn. Reson.* **2006**, *183*, 324−328.
(11) Baldus, M.; Petkova, A. T.; Herzfeld, J.; Griffin, R. G. *Mol. Phys.* **1998**, *95*, 1197−1207.
(12) Marley, J.; Lu, M.; Bracken, C. *J. Biomol. NMR* **2001**, *20*, 71−75.
(13) Michal, G. *Biochemical Pathways*; Spektrum Akademischer Verlag: Heidelberg, Berlin, 1999.
(14) Verel, R.; Baldus, M.; Ernst, M.; Meier, B. H. *Chem. Phys. Lett.* **1998**, *287*, 421−428. Verel, R.; Ernst, M.; Meier, B. H. *J. Magn. Reson.* **2001**, *150*, 81−99.
(15) Hong, M.; Jakes, K. *J. Biomol. NMR* **1999**, *14*, 71−74.
(16) Etzkorn, M.; Martell, S.; Andronesi, O. C.; Seidel, K.; Engelhard, M.; Baldus, M. *Angew. Chem., Int. Ed.* **2007**, *46*, 459−462.

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