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Low ^{13}C-Background for NMR-Based Studies of Ligand Binding Using ^{13}C-Depleted Glucose as Carbon Source for Microbial Growth: ^{13}C-Labeled Glucose and ^{13}C-Forskolin Binding to the Galactose-H$^+$ Symport Protein GalP in *Escherichia coli*

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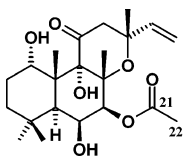
We describe how the proportion of ^{13}C in bacterial membranes can be reduced by growth on ^{13}C-depleted glucose and how this expedites the detection of ligand binding to membrane proteins.

The hydrophobic membrane bilayer surrounding living cells is inherently impermeable to the great majority of hydrophilic solutes required for cell nutrition and to many of the waste products and/or toxins that must be excreted.[1] Consequently, the membrane contains a variety of proteins that each aid the translocation of specific substrate(s) through the membrane. The structure−activity relationships of these proteins have proved difficult to elucidate: they are of low natural abundance in the membrane, they are very hydrophobic and refractory to aqueous isolation methods, and even when purified in nondenaturing detergents, they are very difficult to crystallize to employ X-ray or electron diffraction for determination of structure. We have addressed these problems; first we devised strategies for amplifying expression of typical membrane transport proteins in the experimentally tractable bacterium *Escherichia coli*,[2] and second, we have helped develop solid-state magic angle spinning (SS MAS) NMR methods to measure binding of ^{13}C-ligands to the overexpressed proteins.[3] An important advantage of these methods is the use of biological membranes containing overexpressed transport proteins in their active natural state and without the need for their purification. However, in the case of many ^{13}C-labeled ligands, substantial natural abundance ^{13}C resonances in proteins and lipids in the membrane obscure the signal from the added labeled ligand. An example of this is the binding of the well-characterized inhibitor, forskolin **1**, to the *E. coli* galactose-H$^+$ symport protein GalP. GalP is the bacterial homologue of the human GLUT1 glucose transport protein,[4] and forskolin **1** is a highly specific inhibitor of both proteins.[5] Using membranes containing GalP, C-1 of the substrate glucose **2** resonates in a clear region of the natural abundance ^{13}C spectrum (at ca. 90 ppm).[3a,c] Easily, the most accessible labeling site in the inhibitor forskolin **1** is C-22 (21 ppm),[3c] but forskolin that is labeled at this position gives an NMR signal which overlaps with the natural abundance methyl signals from the protein and lipids and is swamped by them.



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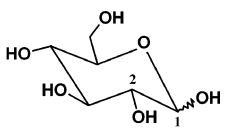

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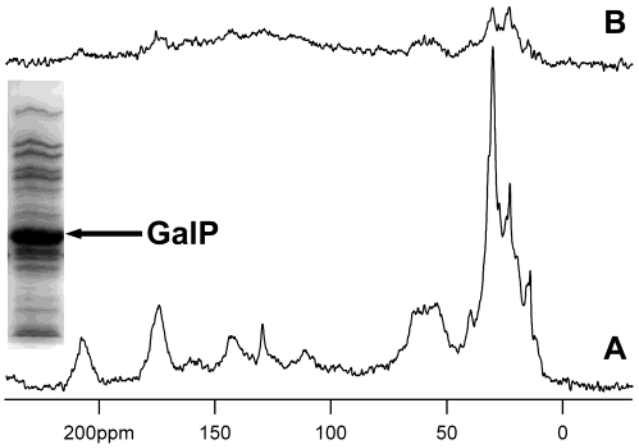

Figure 1. ^{13}C CP-MAS NMR spectra at 75 MHz of membranes containing 300 nmol GalP. (A) Natural abundance membranes, (B) ^{13}C-depleted membranes. Inset is an SDS-PAGE separation of proteins in the ^{13}C-depleted membranes, showing the overexpressed (ca. 40%) GalP protein.

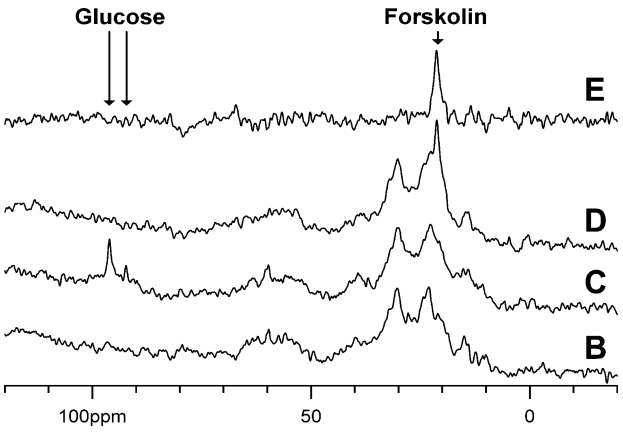

Figure 2. ^{13}C CP-MAS NMR spectra at 75 MHz of GalP membranes. (B) ^{13}C-depleted membranes as in Figure 1B, (C) ^{13}C-depleted membranes + 30 μmol (10 mM) [1-^{13}C]-D-glucose [Cambridge Isotope Laboratories], (D) as C + 500 nmol (167 μM) [22-^{13}C]forskolin [preparation as ref 3c], (E) spectrum D minus spectrum B.

In a novel approach to overcome, or at least minimize, this problem, we have grown the *E. coli* cells that overproduce the GalP transport protein on ^{13}C-depleted D-glucose (^{13}C ≤ 0.07%—compare with 1.1% natural abundance) in a minimal salts medium. As illustrated above this strategy demonstrably reduced signals due to natural abundance ^{13}C (Figure 1) and substantially improved the detection of labeled inhibitor bound to the protein (Figure 2).

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The GalP protein was overexpressed in 3 L of minimal salts medium[2] supplemented with 18 mM ^{13}C-depleted D-glucose (Spectra Stable Isotopes, Spectra Gases Inc., Columbia, MD 21045) in a 5-L Bioflo 3000 fermenter aerated at 10 L/min with mixing at 500 rpm. Unavoidably, some L-histidine (80 mg/L), thymine (20 mg/L), and thiamine (0.1 mg/L) containing normal levels of ^{13}C had to be included, together with carry-over (3.2%) of normal ^{13}C-containing constituents of the rich medium (2X tryptone/yeast extract) used for inoculum growth. After 24 h of growth to an $A_{680\ nm} = 1.34$, the cells were harvested (15 g wet weight) and a preparation made of inner membranes.[2] ESI mass spectrometry confirmed the low level of ^{13}C-isotope in the glucose to be $\leq$0.07%.

MAS SS NMR spectra were generated by means of cross polarization (CP). In ^{13}C CP-MAS NMR, matched Hartman−Hahn spin lock fields allow transfer of magnetization from ^{1}H spins to ^{13}C spins via the dipolar coupling between them.[6] The weaker the coupling the slower the transfer. For rigid solids dipolar coupling is strong and CP transfer is efficient. It can produce gains in signal-to-noise ratios over simple directly excited ^{13}C spectra since ^{1}H nuclei have higher equilibrium magnetization and typically relax faster. In solution the dipolar coupling is averaged to zero by molecular tumbling, and thus no significant transfer occurs. Thus, CP may be used to edit spectra from small molecules to display only those that are immobilized by binding.[3]

A ^{13}C CP-MAS NMR spectrum was obtained for normal inner membranes of *E. coli* and compared with an identically acquired spectrum obtained from membranes of the bacteria grown on ^{13}C-depleted glucose (Figure 1, A and B, respectively). A marked reduction in background, by a factor of between 5 and 8, was observed for the latter, most significantly in the region 0−70 ppm but elsewhere too, notably in the carbonyl region.

As previously found[3a] in membranes without ^{13}C-depletion, the substrate [1-^{13}C]-D-glucose **2** was observed bound to GalP in ^{13}C-depleted membranes, giving rise to two signals (at 96.0 and 92.3 ppm) for the β and α anomers of the sugar (Figure 2C, compared to the same system without added glucose, Figure 2B). Substrate binding studies on GalP are much facilitated by use of forskolin **1**, which is a tight binding inhibitor for this protein.[5] The expected quantitative displacement of bound [1-^{13}C]-D-glucose **2** by forskolin **1** (Figure 2D), unambiguously confirmed that in the ^{13}C-depleted membranes *normal specific* binding to the sugar transport site(s) of GalP was being observed.[3a]

Previously, [22-^{13}C]forskolin **1** when bound to GalP gave a ^{13}C signal that was only detected with difficulty.[3c] Nonetheless, use of [21,22-$^{13}C_2$]forskolin **1** in a double quantum filtration (DQF) experiment allowed clear observation of bound forskolin at −35 °C. But under these conditions the predominant signal obtained for the substrate [1,2-$^{13}C_2$]-D-glucose **2** arose from sugar immobilized in solid water. This temperature was necessary for the satisfactory observation of the forskolin resonance, but unfortunately, it prevented any exploration of the relationships associated with the simultaneous binding of substrate and inhibitor. The efficiency of DQF experiments is also less than 100%, leading to reduced signal intensity.[7]

With ^{13}C-depleted membranes, the NMR signal for [22-^{13}C]-forskolin **1** can be clearly observed (Figure 2D), particularly in the difference spectrum (Figure 2E). The reduced background (Figure 2B =1B) means that the exact line shape observed in this trace is of reduced significance. The clear, relative enhancement of the signal due to the inhibitor **1** that is shown in Figure 2E now permits quantitative studies on the mechanistic relationship between bound sugar substrates and the inhibitor forskolin. Importantly for further NMR studies of binding, *both sugar substrate and inhibitor can be observed simultaneously* in these membranes. For forskolin tight binding is observed, while for glucose significant exchange takes place during the time scale of the NMR experiment.

It is clear from Figure 1B (=2B) that ^{13}C-depleted membranes allow detection of labels over the entire ^{13}C spectral range, whereas previously high backgrounds limited the regions of the spectrum where enriched signals could be observed.

The spectra shown here are for a sample which was prepared from ^{13}C-depleted glucose, but the sample is inevitably contaminated with materials containing normal levels of ^{13}C in the inoculum, and in added auxotrophic requirements. From the unused nonmembrane fractions of the *E. coli* we can now prepare ^{13}C-depleted materials for future use in the growth experiments, thus diminishing further the ^{13}C-background. Now that the binding of forskolin is measurable in unfrozen samples, methods for quantitating velocity and equilibrium constants can be applied.

In conclusion: obtrusive ^{13}C-backgrounds can be a problem in ^{13}C NMR-based studies of ligand binding to membrane transport proteins in their natural state in native membranes. This is largely solved for the bacterial galactose-H^+ symport protein GalP by growing the producing organism *E. coli* on ^{13}C-depleted glucose ($^{13}C \leq 0.07\%$) as the main carbon source. GalP is a paradigm for transport proteins found in organisms from cyanobacteria, eubacteria, parasitic protozoa, fungi, and plants to animals, including mammals such as humans.[4,8] Not only will this ^{13}C-depletion method have widespread applicability in NMR studies of ligand binding in transport systems, but also of any membrane protein[9] that can be overexpressed in a bacterial host grown in a suitable medium. Applications in high through-put screening of drugs for particular targets are envisaged.

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Supporting Information Available: Details of NMR spectroscopy (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Henderson, P. J. F. In *Transporters Factsbook*; Griffith, J. K., Sansom, C. E., Eds.; Academic Press: London, 1997; pp 3−29.
(2) Ward, A.; Sanderson, N. M.; O'Reilly, J.; Rutherford, N. G.; Poolman, B.; Henderson, P. J. F. In *Membrane Transport: A Practical Approach*; Baldwin, S. A., Ed.; Oxford University Press: UK, 2000; Chapter 6, pp 141−166.
(3) (a) Spooner, P. J. R.; Rutherford, N. G.; Watts, A.; Henderson, P. J. F. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 3877−3881. (b) Spooner, P. J. R.; O'Reilly, W. J.; Homans, S. W.; Rutherford, N. G.; Henderson, P. J. F.; Watts, A. *Biophys. J.* **1998**, *75*, 2794−2800. (c) Appleyard, A. N.; Herbert, R. B.; Henderson, P. J. F.; Watts, A.; Spooner, P. J. R. *Biochim. Biophys. Acta* **2000**, *1509*, 55−64. (d) Patching, S. G.; Brough, A. R.; Herbert, R. B.; Henderson, P. J. F.; Middleton, D. A. *J. Am. Chem. Soc.* **2003**. In press.
(4) Griffith, J. K.; Sansom, C. E. In *Transporters Factsbook;* Griffith, J. K., Sansom, C. E., Eds.; Academic Press: London, 1997; pp 262−287.
(5) Martin, G. E. M.; Seamon, K. B.; Brown, F. M.; Shanahan, M. F.; Roberts, P. E.; Henderson, P. J. F. *J. Biol. Chem.* **1994**, *269*, 24870−24877; Martin, G. E.; Rutherford, N. G.; Henderson, P. J. F.; Walmsley, A. R. *Biochem. J.* **1995**, *308*, 261−268.
(6) Yannoni, C. S. *Acc. Chem. Res.* **1982**, *15*, 201−208.
(7) Shaka, A. K.; Freeman, R. *J. Magn. Reson.* **1983**, *51*, 169−173.
(8) Pao, S. S.; Paulsen, I. T.; Saier, M. H. *Microbiol. Mol. Biol. Rev.* **1998**, *62*, 1−32.
(9) Watts, A. *Curr. Opin. Biotechnol.* **1999**, *10*, 48−53.

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