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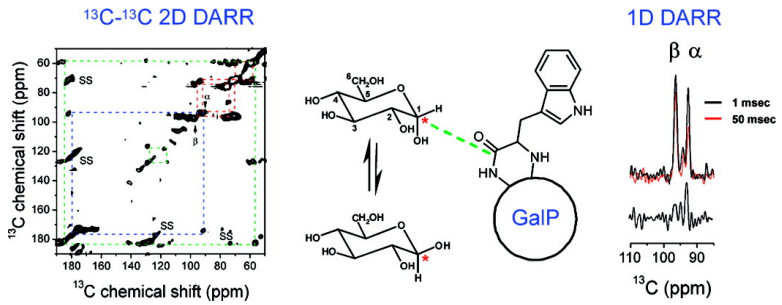


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# Solid-State NMR Spectroscopy Detects Interactions between Tryptophan Residues of the *E. coli* Sugar Transporter GalP and the α-Anomer of the D-Glucose Substrate

Simon G. Patching,[†] Peter J. F. Henderson,[†] Richard B. Herbert,[†] and David A. Middleton*[,‡]

*School of Biological Sciences, University of Liverpool, Crown Street, Liverpool L69 7ZB, United Kingdom, Astbury Centre for Structural Molecular Biology and Institute of Membrane and Systems Biology, University of Leeds, Leeds LS2 9JT, United Kingdom*

Received July 26, 2007; E-mail: d.a.middleton@liverpool.ac.uk

***Abstract:*** An experimental approach is described in which high resolution $^{13}C$ solid-state NMR (SSNMR) spectroscopy has been used to detect interactions between specific residues of membrane-embedded transport proteins and weakly binding noncovalent ligands. This procedure has provided insight into the binding site for the substrate D-glucose in the *Escherichia coli* sugar transport protein GalP. Cross-polarization magic-angle spinning (CP-MAS) SSNMR spectra of GalP in its natural membrane at 4 °C indicated that the α- and β-anomers of D-[1-$^{13}C$]glucose were bound by GalP with equal affinity and underwent fast exchange between the free and bound environments. Further experiments confirmed that by lowering the measurement temperature to −10 °C, peaks could be detected selectively from the substrate when restrained within the binding site. Dipolar-assisted rotational resonance (DARR) SSNMR experiments at −10 °C showed a selective interaction between the α-anomer of D-[1-$^{13}C$]glucose and $^{13}C$-labels within [$^{13}C$]tryptophan-labeled GalP, which places the carbon atom at C-1 in the α-anomer of D-glucose to within 6 Å of the carbonyl carbon of one or more tryptophan residues in the protein. No interaction was detected for the β-isomer. The role of tryptophan residues in substrate binding was investigated further in CP-MAS experiments to detect D-[1-$^{13}C$]glucose binding to the GalP mutants W371F and W395F before and after the addition of the inhibitor forskolin. The results suggest that both mutants bind D-glucose with similar affinities, but have different affinities for forskolin. This work highlights a useful general experimental strategy for probing the binding sites of membrane proteins, using methodology which overcomes the problems associated with the unfavorable dynamics of weak ligands.

## Introduction

Transport proteins are found in all living organisms, where they facilitate the movement of a diverse range of metabolites and other solutes across otherwise impermeable lipid membranes, thereby controlling the metabolic balance inside cells and organelles. The functions of transport proteins range from capturing nutrients for uptake into bacterial cells, which may be targets for developing antibacterial agents, to being the route of entry for drugs into human cells and tissues, where they exert their pharmacological effect;[1] they are also involved in ATP synthesis, environmental sensing, and the removal of toxins from cells.[2−4] There is limited knowledge about how membrane transport proteins recognize and interact with specific substrates, due to the difficulties of applying experimental techniques that usually provide high-resolution structural detail of soluble proteins, namely X-ray crystallography and solution NMR spectroscopy.

Mammalian transport proteins generally cannot be obtained in sufficient quantities to initiate structural studies, so transport proteins from bacteria, for which suitable expression systems are available, have to be used; the bacterial proteins chosen for study are therefore often homologous with important mammalian transporters,[5] as in this work (see below). Even with sufficient quantities of such proteins available, obtaining crystals with diffracting properties that provide high-resolution X-ray data of the membrane proteins is not straightforward, not least in the presence of a substrate. There are thousands of transport proteins (∼4−15% of the genomic complement in all organisms), and only very few high-resolution structures of such α-helical transport proteins (∼10) have been determined by X-ray crystallography.[6] Of these, only three have bound substrate or other ligand present.[7−9] All crystal structures have

[†] University of Leeds.
[‡] University of Liverpool.

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an additional limitation in that they only give a snapshot of the protein in one conformation and with the protein removed from native conditions. Transport proteins usually have $M_r$ values >45 000 with an α-helical secondary structure and, like most membrane proteins, must be in a lipid environment or in a detergent to retain their structure and function; these factors generally preclude solution-state NMR studies. Solid-state NMR spectroscopy, however, provides an attractive alternative to probe the structure of substrate binding by transport proteins, where interactions between isotopic labels (e.g., $^{13}C$) in the binding site of the protein and in a bound ligand can be measured with dipolar recoupling experiments[10] using the overexpressed protein in its native membrane environment.

Cross-polarization magic-angle spinning (CP-MAS) solid-state NMR has been used to provide quantitative information about ligand binding affinities for proteins embedded in natural lipid membranes.[11−13] The method exploits differences in molecular dynamics of the ligand in the free and bound environments—rapid and isotropic in the free state and slow and anisotropic in the bound state—to detect signals only from $^{13}C$-ligand associated with the membrane components. With appropriate control experiments using competitive ligands it is possible to assign peaks in the NMR spectrum to ligands or substrates bound to a specific protein. The first application of this approach to a transport protein was on the *Escherichia coli* galactose-$H^+$ symport protein, GalP,[14] a member of the major facilitator superfamily (MFS)[15] and, importantly, a structural and functional homologue of the GLUT family of human sugar transporters.[16] CP-MAS NMR spectroscopy was used to detect NMR signals for the binding of a $^{13}C$-labeled substrate, D-glucose, to the protein after its amplified expression in native *E. coli* inner membranes. A tight-binding inhibitor of the protein, forskolin, has also been used to confirm the specificity of the ligand−protein interaction.[17,18] The GalP protein, which has a molecular weight of 51 kDa and forms 12 putative transmembrane helices, is an ideal candidate for further exploratory solid-state NMR studies on transporters, since its expression can be amplified to 50−60% of total protein in native membranes,[19] important when strategies for isotopic labeling of the protein can compromise the level of expression.

The aim of the work here was to find conditions that would allow, for the first time, the deduction of constraints on the distances between specific residues in a membrane-embedded protein and a noncovalently bound ligand, by exploiting dipole−dipole interactions between $^{13}C$ labels placed in the GalP protein and in the bound substrate D-glucose. A critical element of this work is the use of two-dimensional solid-state NMR and dipolar recoupling methods to detect intermolecular interactions between glucose and tryptophan residues of GalP. Although the methodology has been used to probe the binding sites of ligands covalently bonded to membrane receptors (e.g., rhodopsin and the retinal chromophore),[20] these methods have not been applied to obtain constraints on the binding sites of noncovalent ligands within membrane-embedded proteins. It is especially challenging to acquire such constraints for low affinity substrates such as glucose, which has a dissociation constant in the millimolar range and must therefore be present in large excess over the protein to saturate the available binding sites. It is shown how measures can be taken to overcome the difficulties inherent in detecting specific protein−ligand interactions reliably without introducing complications from dynamics or from the high concentration of unbound ligand. The results provide important new constraints on the binding site for D-glucose within GalP.

## Experimental Section

**Cell Growth and Isotopic Labeling.** For the production of unlabeled GalP membranes, *E. coli* strain JM1100 with the plasmid pPER3 for expressing GalP was grown in minimal medium containing 28 mM D-glucose, as described previously,[19] in a 30-L fermenter (Applikon). For the production of uniformly $^{13}C$-labeled GalP membranes, the unlabeled D-glucose was substituted by $^{13}C_6$−D-glucose (99% $^{13}C$; Cambridge Isotope Laboratories) at a concentration of 18 mM in the same medium and growth was performed in batch culture (volumes of 500 mL in 2-L flasks); cells were grown at 37 °C typically over ∼24 h to a final $A_{600}$ of ∼1.5. For the production of [U-$^{13}C$, U-$^{15}N$]Trp-labeled GalP membranes, the tryptophan auxotrophic host strain CY15077 (*E. coli* genetic stock center, Yale University) transformed with the plasmid pPER3 for expressing GalP was grown in batch culture (volumes of 833 mL in 2-L flasks) in a modified M9 minimal medium that contained 11.1 mM D-glucose, 20 mg/L L-[U-$^{13}C$, U-$^{15}N$]tryptophan (99% $^{13}C/^{15}N$; Cambridge Isotope Laboratories) and all other unlabeled L-amino acids (Sigma) added in known quantities (alanine, arginine, glutamic acid, glutamine, glycine, and serine at 0.4 g/L; aspartic acid and methionine at 0.25 g/L; asparagine, histidine, isoleucine, leucine, lysine, proline, threonine, and tyrosine at 0.1 g/L; cysteine, phenylalanine and valine at 0.05 g/L); cells were grown at 37 °C typically over about 10 h to a final $A_{600}$ of ∼1.6.

**Membrane Preparation.** *E. coli* cells were disrupted using a French press (Sim Aminco) or by using a cell disruptor (Constant Cell Disruption Systems) followed by separation of inner/outer membranes by sucrose-density gradient ultracentrifugation.[19] The inner membrane fraction was washed three times by resuspension in 20 mM Tris-HCl buffer (pH 7.5) followed by ultracentrifugation, resuspension in the same buffer and storage at −80 °C after rapid freezing. The total protein concentration in the final suspensions was determined by the method of Schaffner and Weissmann[21] and the percentage of GalP content was estimated by densitometric analysis on the proteins resolved by SDS-PAGE and stained with Coomassie blue (Figure 1).

**NMR Sample Preparation.** NMR measurements were performed on *E. coli* inner membrane preparations that contained ∼5 mg (100 nmoles) GalP protein at a concentration of ∼1 mM. The membranes were suspended to 3 mL in 20 mM Tris-HCl buffer (pH 7.5) with the

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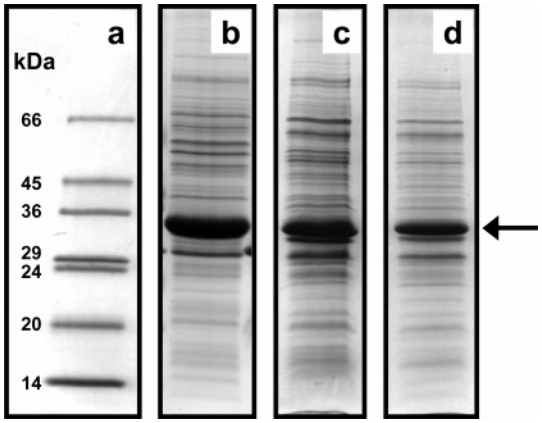


***Figure 1.*** Amplified expression of unlabeled and $^{13}$C-labeled GalP in native *E. coli* inner membranes. Coomassie-stained SDS-PAGE separations of MW marker proteins (a) and proteins in *E. coli* inner membrane preparations from cultures of JM110/pPER3 expressing unlabeled GalP (b) and uniformly $^{13}$C-labeled GalP (c) and from cultures of CY15077/pPER3 expressing [U-$^{13}$C, U-$^{15}$N]Trp-labeled GalP (d). The arrow indicates the position of GalP.

required concentration of D-[1-$^{13}$C]glucose or D-[U-$^{13}C_6$]glucose (99% $^{13}$C; Cambridge Isotope Laboratories) with brief vortexing to mix the sample, followed by incubation at 4 °C for 30 min. The membranes were collected by ultracentrifugation (100000 × *g*) and then packed into a MAS NMR sample rotor (4-mm external diameter) using a brief slow spin in a benchtop centrifuge. Additions of the unlabeled competing compounds D-glucose, D-galactose, and forskolin, were performed by adding a suitable volume of a more concentrated stock solution to the membrane suspension before the addition of the labeled substrate or to the membranes in the sample rotor with careful mixing, in each case followed by incubation at 4 °C for 30 min.

**NMR Methods.** Experiments at 4 °C were performed with a Varian InfinityPlus 300 spectrometer using conditions as described previously.[18] All other experiments (at 5, 0, and −10 °C) were performed using a Bruker Avance 400 spectrometer equipped with a double-resonance solid-state MAS probe tuned to 100.13 MHz for $^{13}$C and 400.1 MHz for $^{1}$H. The temperature of the sample was estimated by observing changes in $^{13}$C line shapes around the phase transitions of d-camphor (occurring at −28.5 °C) and pivalic acid (occurring at 7.1 °C) as described elsewhere.[22] Under CP-MAS conditions the actual sample temperatures were found to be ∼1 °C higher than the measured temperature owing to heating by sample spinning and high-power irradiation. All temperatures quoted are the measured temperatures. Sample spinning rates were maintained automatically to within ± 1 Hz. CP-MAS experiments employed proton fields of 63 kHz for cross-polarization over a 2-ms contact time and 85 kHz for proton decoupling during the acquisition period. Two-dimensional dipolar-assisted rotational resonance (DARR) NMR spectra[23] were recorded with 64 hypercomplex points in the indirect dimension with a mixing time of 50 ms during which the proton field was adjusted to the spinning frequency of 5 kHz. For one-dimensional DARR experiments, the polarization of the carbonyl spins was selectively inverted using a DANTE pulse train and a nonselective 4.5 $\mu$s pulse was applied to the $^{13}$C magnetization to store it longitudinally after the contact time for a mixing period $t_m$ of 1 ms or 50 ms. Protons were irradiated at a field of 5 kHz during $t_m$, and the $^{13}$C signal was detected after applying a 4.5 $\mu$s read-out pulse. One-dimensional experiments were performed with block averaging, interleaved between the long and short mixing times, to minimize intensity differences in the spectra arising from tuning drift during the long acquisition times.

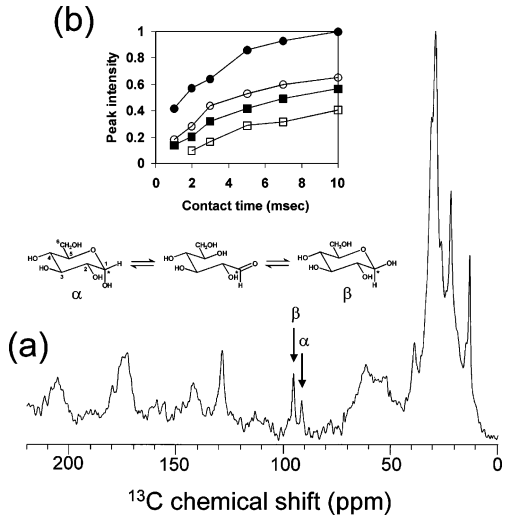


***Figure 2.*** Detection of D-glucose binding to GalP in *E. coli* membranes using $^{13}$C CP-MAS NMR. (a) A $^{13}$C CP-MAS NMR spectrum of unlabeled GalP membranes containing 10 mM D-[1-$^{13}$C]glucose, indicating the characteristic peaks from the α and $\beta$ anomers of the sugar at 96 and 92 ppm and showing the structures for the C-1 isomers of D-[1-$^{13}$C]glucose. The spectrum was obtained at 75 MHz for $^{13}$C using a CP contact time of 3 ms with the sample spinning at 2.4 kHz and at a temperature of 4 °C. (b) CP intensity profiles for the $\beta$ (circles) and α (squares) signals of D-[1-$^{13}$C]glucose at concentrations of 10 mM (black) and 5 mM (open) in spectra of unlabeled GalP membranes as in (a) recorded over a range of contact times.

2D C−H DIPSHIFT spectra were recorded using the constant time experiment described elsewhere,[24] at a spinning rate ($\nu_r$) of 4260 Hz. The indirect dimension of the 2D spectra was constructed from a series of 13 FIDs as follows. Each FID was recorded after allowing the $^{13}$C magnetization to evolve under the influence of C−H dipolar interactions for a period *t*, where $0 \le t < 1/\nu_r$. Proton−proton interactions were eliminated using frequency switched Lee−Goldberg homonuclear decoupling during the evolution period. Period *t* was incremented 12 times by 19.6 $\mu$s to generate the 13 FIDs. These were Fourier transformed into 1024 points and the intensity values at each equivalent point in the 13 spectra were normalized to the value at $t = 0$ and corrected for $T_2$ relaxation by multiplication of each point by a factor $\exp(t/T_2)$, substituting a value of $T_2$ such that the intensity values at $t = 0$ and $t = 12$ were equal to 1. The 13 spectra ($S_n$) were then replicated and concatenated ($S_1$, $S_2$ ... $S_{13}$, $S_1$, $S_2$ ... $S_{13}$, etc), to give a 1024 × 128 matrix, which was Fourier transformed in the indirect dimension to produce the pseudo-two-dimensional spectra, with the $^{13}$C chemical shift information in the direct dimension and C−H dipolar spectrum in the indirect dimension.

## Results

**Detection of D-Glucose Bound to GalP.** In aqueous solution, D-glucose exists as a mixture of two isomers that differ in their configuration at C-1; these interconvert via an open-chain form, which is only present in minute quantities. At equilibrium, the α and $\beta$ isomers have a ratio of 0.36:0.63,[25] where the hydroxyl group at C-1 in the $\beta$-isomer occupies the preferred equatorial position on the sugar ring. Equilibration of D-[1-$^{13}$C]glucose with GalP membranes (Figure 1b) produces signals in $^{13}$C CP-MAS NMR spectra at 96 and 92 ppm, which represent the $\beta$ and α isomers, respectively (Figure 2a). In membranes containing 10 mM D-[1-$^{13}$C]glucose and at a temperature of 4 °C, the observable signals are completely displaced by the addition of

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100 mM unlabeled D-glucose or D-galactose or by the addition of the inhibitor forskolin (2-fold excess over concentration of GalP)[17,18] and, therefore, represent the specific binding of D-[1-$^{13}$C]glucose to GalP. The intensities of the two signals have a ratio of 0.32:0.68 in the spectrum shown (Figure 2a) and are similar to that found in solution. In spectra recorded with a range of contact times, the $\alpha/\beta$ ratio remains relatively constant and suggests that the isomers bind with similar affinities[12] (Figure 2b). The shapes of the CP profiles are consistent with relatively weak binding (a Kd of millimolar or greater), since maximum peak intensities have apparently not been reached at a contact time of 10 ms.

**Conditions for Detecting Substrate−Protein Dipolar Interactions.** NMR measurements of dipole interactions between nuclear spins provide information about interatomic distances that are potentially valuable as structural constraints on the locations of ligands bound to receptors. The results of the CP-MAS experiments at 4 °C described above indicate that D-[1-$^{13}$C]glucose exchanges rapidly between free and bound environments, which would hamper the detection of weak dipole−dipole interactions between $^{13}$C labels incorporated into the protein and those in the bound substrate. Ideally, conditions must be found in which the substrate could be restrained in the binding site while minimizing any residual molecular dynamics that could scale or abolish the observed dipolar interactions. This can be achieved in principle by reducing the measurement temperature to below 0 °C, but, by doing so, an important consideration must be addressed. Above freezing, the NMR signal from the substrate in the CP-MAS experiment arises because the restraint of the substrate within the binding site provides suitable conditions for generating observable $^{13}$C magnetization by cross-polarization. The magnetization is then transferred with the substrate as it dissociates from the protein and is observed as peaks in the spectrum after Fourier transformation of the signal.[12−14] The large excess of substrate molecules that do not bind during the cross-polarization contact time are not detected because their rapid isotropic reorientation prevents cross-polarization from occurring. At extreme low temperatures, all of the substrate molecules, in the aqueous environment or elsewhere, including those bound by GalP, are equally rigid and the peaks in the spectrum represent the entire substrate distribution. Clearly, D-[1-$^{13}$C]glucose molecules associated peripherally with GalP and other $^{13}$C-labeled components of the membrane could give rise to observable dipolar interactions that could be interpreted incorrectly as interactions between substrate and GalP. The temperature should thus be low enough to keep in place the D-[1-$^{13}$C]glucose that is specifically bound by GalP, but not so low as to introduce contributions to the spectrum from free D-glucose or from substrate in undefined sites in addition to substrate bound to GalP. In practice, spectra of D-[1-$^{13}$C]glucose and GalP should be collected at a range of temperatures alongside control experiments carried out after adding a competitive ligand that displaces D-glucose from its specific binding site. The control experiment confirms whether the peaks detected represent only the substrate in the specific GalP binding site or also represent GalP in other environments.

In Figure 3a, $^{13}$C CP-MAS spectra are shown of *E. coli* membranes with amplified expression of GalP in the presence of 50 mM D-[1-$^{13}$C]glucose. As the temperature was lowered from 5 °C the peak intensities from the α and $\beta$ forms of D-[1-$^{13}$C]glucose at 96 and 92 ppm progressively grew in intensity and at −10 °C peaks were clearly visible after accumulating for less than 2 h. The GalP inhibitor forskolin was added to the membranes to identify whether the peaks at −10 °C arose entirely from D-[1-$^{13}$C]glucose bound specifically to GalP or whether the signal originated from nonspecifically bound substrate and/or from substrate immobilized in the frozen aqueous phase (Figure 3b).

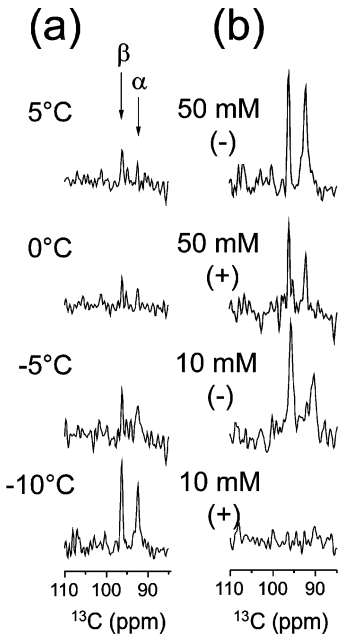


***Figure 3.*** Conditions for the detection of dipolar interactions between D-glucose and GalP in *E. coli* membranes using $^{13}$C CP-MAS NMR. Spectra were obtained at 100.13 MHz for $^{13}$C using a CP contact time of 2 ms with the sample spinning at 4 kHz. Spectra of membranes containing 50 mM D-[1-$^{13}$C]glucose show characteristic peaks from the α and $\beta$ anomers of the sugar at 96 and 92 ppm, which become more pronounced as the temperature is lowered from 5 °C to −10 °C (a). At −10 °C the fraction of signal attributable to D-[1-$^{13}$C]glucose bound to GalP was assessed by the addition of the competitive ligand forskolin to a final concentration of 2 mM (b). At 50 mM D-[1-$^{13}$C]glucose (top two spectra) the peak intensities for the α and $\beta$ anomers in the absence of forskolin (−) were reduced by approximately 50−60% after addition of the competing ligand (+). At 10 mM D-[1-$^{13}$C]glucose (bottom two spectra) the peaks were completely abolished in the presence of forskolin (+). Spectra were obtained by accumulating 6144 scans.

The concentration of forskolin (2 mM) was high enough in principle to exclude completely D-[1-$^{13}$C]glucose from the binding site of GalP, but only 50−60% of the signal from 50 mM D-[1-$^{13}$C]glucose was eliminated suggesting that a large proportion of the signal arose from nonspecifically bound, or free but frozen, substrate at −10 °C. The signals were reduced to a similar extent when 500 mM unlabeled D-glucose or D-galactose was used to compete 50 mM D-[1-$^{13}$C]glucose from separate samples under the same conditions (not shown). In a similar experiment with 10 mM D-[1-$^{13}$C]glucose, the addition of forskolin completely abolished the observable signals from the labeled sugar, indicating that at this concentration the peaks at 96 and 92 ppm could be attributed exclusively to specifically bound substrate. All further experiments were therefore carried out at this lower concentration of D-[1-$^{13}$C]-glucose.

**Mobility of Glucose in the Binding Site.** Next, the molecular mobility of the two anomers of D-[1-$^{13}$C]glucose within the GalP

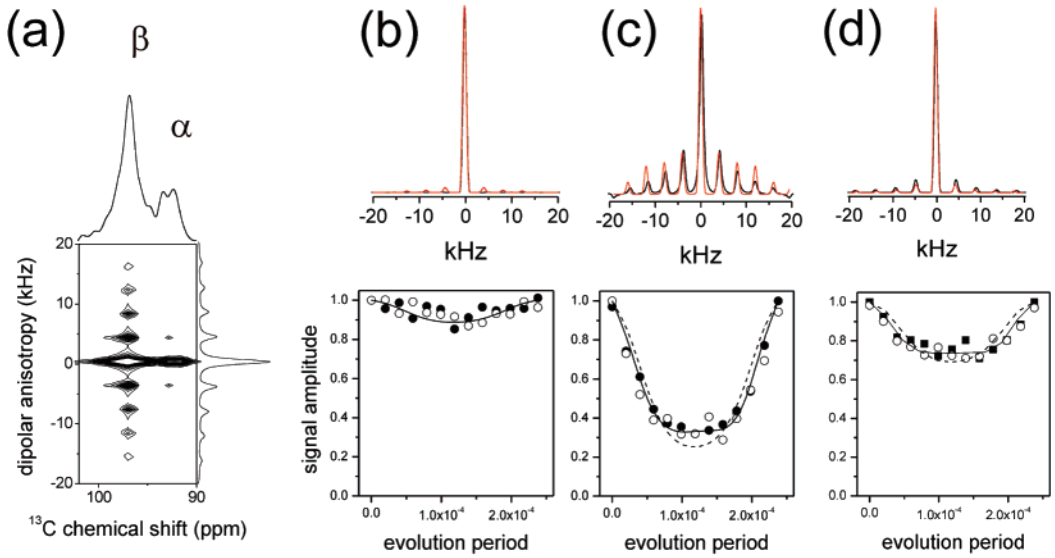


***Figure 4.*** $^{1}H-^{13}C$ dipolar anisotropy measurements reflecting the dynamics of D-glucose at the C-1 position in the presence of GalP. Dipolar sideband patterns for D-[1-$^{13}$C]glucose were obtained by slicing through the indirect dimension of a $^{1}H-^{13}C$ 2D-DIPSHIFT spectrum at 92 and 96 ppm (a). In b−d (top) dipolar sideband patterns for 10 mM D-[1-$^{13}$C]glucose at 5 °C (b) and −10 °C (c) and for 50 mM D-[1-$^{13}$C]glucose at −10 °C (d) were constructed for both the α-anomer (black) and *β*-anomer (red). Below each pair of sideband patterns are shown the corresponding raw time-domain data for the α-anomer (solid circles) and *β*-anomer (open circles) from which the sideband patterns above them were generated. The curves show the normalized signal amplitudes for C-1 of D-[1-$^{13}$C]glucose at evolution periods of up to one cycle of sample rotation. Amplitude curves were calculated from apparent dipolar anisotropy ($\delta_{app}$) values as described in the main text. Solid lines show curves in closest agreement with the experimental data, corresponding to $d_{app}$ values of 1800 Hz (b), a 4:1 combination of $\delta_{app}$ values of 12 400 and 0 Hz (c) and a 3:7 combination of $\delta_{app}$ values of 12 400 and 0 Hz (d). Dashed lines show the best fitting curves for single $\delta_{app}$ values of 8500 Hz (c) and 5000 Hz (d).

membranes was examined by measuring the $^{13}C-^{1}H$ dipolar anisotropy at C-1 of the substrate. The dipolar anisotropy is sensitive to dynamics in the $10^4-10^5$ $s^{-1}$ range and thus provides a measure of the degree of motional restraint of D-glucose in the GalP binding site at different temperatures. DIPSHIFT spectra[24] were obtained in which the normal $^{13}C$ spectrum is projected in the direct dimension and the $^{13}C-^{1}H$ dipolar sideband pattern for the substrate was obtained from the indirect dimension by slicing vertically through appropriate regions of the spectrum (Figure 4a). Calculations indicate that with increasing substrate mobility the central peak will grow in intensity relative to the sideband intensities and the frequency range spanned by the sidebands will diminish.

The dipolar sideband pattern for 10 mM D-[1-$^{13}$C]glucose in the GalP membranes at 5 °C shows only a central peak for the *β*-anomer (Figure 4b, top), consistent with the substrate being highly dynamic and undergoing rapid exchange between free and bound environments. The dipolar sideband pattern for the same sample at −10 °C (Figure 4c, top) indicates that the apparent $^{13}C-^{1}H$ dipolar anisotropy increases markedly at the lower temperature, indicating a reduction in the mobility of the substrate. The sideband pattern spans the wide frequency range (>30 kHz) expected for a dipolar anisotropy that is unaveraged by molecular dynamics, but the central peak is slightly more intense than predicted from ideal calculations. Similarly, the dipolar spectrum of GalP membranes with 50 mM D-[1-$^{13}$C]-glucose also shows sidebands over a wide frequency range, but the central line now dominates the spectrum (Figure 4d, top). The nonideal intensity of the central band for the substrate at −10 °C suggests that the signal may also have an additional contribution from D-glucose in a more mobile environment, but the central peak intensity can also be affected by processing artefacts. Crucially, at each temperature and substrate concentration, the sideband patterns were very similar for the α- and *β*-anomers, indicating that the dynamics of both species were very similar in the GalP membranes. This information is important for interpreting experiments aimed at detecting interactions between GalP and the two substrate isomers, as will be shown later in this paper.

Clues about the nonideal line-shapes in the sideband patterns were provided by examining the raw data collected in the DIPSHIFT experiment, which consists of a set of signal amplitudes for C-1 of D-glucose over one cycle of sample rotation (Figure 4b−d, bottom). In principle, order parameters *S* for D-glucose in the GalP membranes can be determined by comparing the signal amplitudes with numerically simulated curves calculated for apparent $^{13}C-^{1}H$ anisotropy values ($\delta_{app}$) reflecting the degree of motional averaging. The order parameter is defined as $S = \delta_{app}/\delta_{CH}$, where $\delta_{CH}$ is the anisotropy for a rigid solid (12 400 Hz) and *S* can take values of between zero (representing isotropic disorder) and 1 (corresponding to a rigid solid). As expected for the highly mobile substrate at 5 °C, amplitudes showed little modulation and the best fitting curve was consistent with an *S* value of 0.16 for the α- and *β*-isomers. For the same sample at −10 °C, the closest fitting calculated curve (for a single $\delta_{app}$ value of 8500 Hz) deviated significantly from the experimental data at the first and last time points of the sample rotation cycle and in the center of the evolution period (Figure 4c, bottom; solid line). A much closer fit was achieved with an average curve generated for $\delta_{app}$ values of 12 400 and 0 Hz combined in a 4:1 ratio (Figure 4c, bottom; dashed line). In other words, the data for 10 mM D-glucose at −10 °C are consistent with 80% of the substrate being immobilized, with the remaining 20% in a highly mobile environment. Similarly, the data for 50 mM substrate at −10 °C are consistent with only 30% of the substrate being restrained and the remainder being highly mobile (Figure 4d, bottom). The less mobile population of substrate is probably bound to GalP and

the more dynamic species could be interacting with other, undefined sites, perhaps at the membrane−water interface, which clearly becomes more dominant at higher substrate concentrations.

**Detection of D-Glucose Interactions with Tryptophan Residues in GalP.** The experiments above established that the spectra of GalP in native membranes with 10 mM D-[1-$^{13}$C]-glucose at −10 °C exhibit signature peaks for the α- and β-anomers that correspond to substrate immobilized within the binding site of GalP. These conditions are therefore suitable for detecting dipolar interactions between amino acid residues in GalP and the bound substrate. A two-dimensional DARR NMR experiment[23,20] was used with the aim of detecting $^{13}$C−$^{13}$C dipolar interactions between 10 mM D-glucose and GalP in native membranes at −10 °C. Ideally, cross-peaks are observed when dipolar interactions occur between the substrate and any $^{13}$C labels within the protein that are situated approximately 6 Å or less from the label in the ligand, although interactions over longer distances can in principle be detected because of spin-diffusion mediated relay effects. The DARR experiment was selected because it is has been shown to detect weak $^{13}$C−$^{13}$C couplings in the presence of stronger couplings (e.g., between the bonded $^{13}$C pairs of uniformly labeled amino acids) more effectively than other dipolar recoupling methods such as radiofrequency-driven recoupling (RFDR).[20]

Earlier work examining the transport properties of various GalP mutants suggested that tryptophan residues may be situated close to the substrate binding site.[26,27] We therefore prepared membranes containing GalP with all tryptophans uniformly $^{13}$C- and $^{15}$N-labeled ([U-$^{15}$N,$^{13}$C-Trp]GalP) and two-dimensional DARR NMR was used to detect interactions between the substrate and any of the twelve native tryptophan residues. The labeling strategy achieved exclusive incorporation of labels into the tryptophan residues in GalP; this has been demonstrated in both solution- and solid-state NMR spectra of the purified protein (not shown). The spectra of these membranes containing D-[U-$^{13}$C]glucose (Figure 5a) show cross-peaks for the intramolecular dipolar coupling networks within the tryptophan and D-glucose spin systems (green and red lines, respectively). The spectrum also shows a rather strong cross-peak between the C-1 resonance of the D-glucose α-anomer at 92 ppm and resonances at around 175 ppm, consistent with an intermolecular $^{13}$C−$^{13}$C dipolar interaction between the substrate and tryptophan carbonyl groups. It appears that there are no cross-peaks representing couplings between the β-anomer of D-glucose and tryptophan carbonyl groups. Further cross-peaks occur between resonances at 60 and 75 ppm, which may also reflect D-glucose-GalP couplings, although this cannot be confirmed because the D-glucose C-6 and tryptophan Cα resonances overlap at 60 ppm. Surprisingly, no cross-peaks occur from D-glucose to the tryptophan indole rings, although this may simply be a consequence of the poor signal-to-noise observed in the aromatic region of the spectrum.

The intermolecular cross-peak in Figure 5a could arise from interactions between D-glucose and tryptophan residues in membrane proteins other than GalP, which constitute about 40−50% of the total protein mass in the membranes. A control

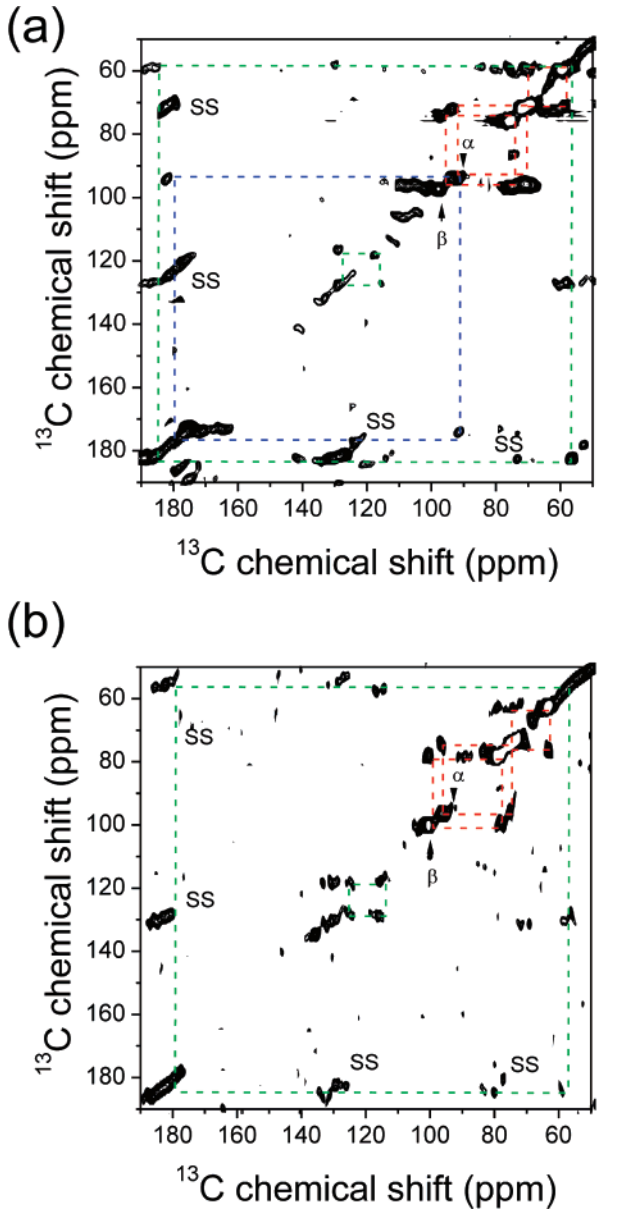


***Figure 5.*** Detection of interactions between $^{13}$C labeled D-glucose and $^{13}$C-labeled GalP (approximately 1 mM) in native membranes at −10 °C using two-dimensional (2D) DARR. A 2D DARR spectrum of [U-$^{15}$N,$^{13}$C-Trp]-GalP in native membranes with 10 mM D-[U-$^{13}$C]glucose exhibits cross-peaks consistent with $^{13}$C−$^{13}$C dipolar interactions within the Trp residues of GalP (green lines), glucose−glucose interactions (red lines) and glucose−Trp interactions (blue lines) (a). Spinning sidebands are denoted "SS". A 2D DARR spectrum of [U-$^{15}$N,$^{13}$C-Trp]GalP in native membranes with 50 mM D-[U-$^{13}$C]glucose added after prior addition of 2 mM unlabeled forskolin to the membranes does not show cross-peaks consistent with glucose-Trp interactions (b).

experiment was therefore conducted in which [U-$^{15}$N,$^{13}$C-Trp]-GalP membranes were treated with forskolin to saturate all of the available glucose binding sites in GalP, and then a high concentration of D-[U-$^{13}$C]glucose (50 mM) was added to promote substrate interactions with other nonspecific sites. The two-dimensional DARR spectrum showed similar intramolecular cross-peaks to those in Figure 5a, but did not show cross-peaks between C-1 of glucose and any of the tryptophan resonances (Figure 5b), reinforcing our conclusion that D-glucose−tryptophan couplings only occur only when the substrate binds specifically to GalP.

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(27) Walmsley, A. R.; Martin, G. E. M.; Henderson, P. J. F. *J. Biol. Chem.* **1994**, *269*, 17009−17019.

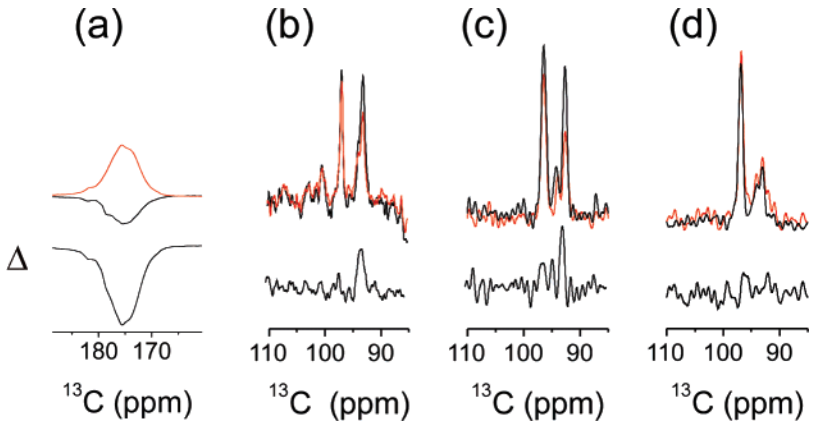


***Figure 6.*** Detection of interactions between $^{13}$C-labeled D-glucose and the carbonyl groups of $^{13}$C-labeled GalP (approximately 1 mM) in native membranes at −10 °C using one-dimensional (1D) DARR. 1D $^{13}$C DARR NMR spectra of GalP in native membranes containing 10 mM D-[1-$^{13}$C]glucose were obtained by inverting the protein carbonyl magnetization (resonating at ∼175 ppm) (a) and observing the effect on the D-glucose peak intensities (b−d) after mixing times of 1 ms (black) and 50 ms (red). Difference spectra were obtained by subtraction of the 50 ms spectrum from the 1 ms spectrum (Δ). Spectra of D-[1-$^{13}$C]glucose in the presence of [U-$^{15}$N,$^{13}$C]GalP (b) and [U-$^{15}$N,$^{13}$C-Trp]GalP (c) both show a reduction in peak intensity for the α-isomer of D-glucose at the longer mixing time, consistent with coupling between $^{13}$C at the 1-position of D-glucose and labeled carbonyl groups in the protein. No peaks occur within the region 90−98 ppm for either [U-$^{15}$N,$^{13}$C]GalP or [U-$^{15}$N,$^{13}$C-Trp]GalP in the absence of D-[1-$^{13}$C]glucose (data not presented). The DARR spectra of membranes containing D-[1-$^{13}$C]glucose and unlabeled GalP (d) do not show any change in either peak intensity for D-glucose at the longer mixing time. The sample spinning rate was 5 kHz and 1D spectra were obtained by accumulating 20 000−30 000 block-averaged scans with a recycle delay of 1 s.

A one-dimensional variant of the DARR experiment was employed to investigate further whether selective interactions occur between the GalP tryptophan carbonyls and the α-isomer of bound D-glucose. To detect dipolar interactions in a one-dimensional experiment, the polarization of the carbonyl $^{13}$C spins from the protein was selectively inverted and dipolar-mediated magnetization exchange between the carbonyls and the C-1 carbon of D-[1-$^{13}$C]glucose was monitored after a short mixing period $t_m$ (1 ms) and again after a long mixing period (50 ms). The experiment was first carried out on D-[1-$^{13}$C]-glucose bound by uniformly $^{13}$C-labeled GalP in *E. coli* membranes at −10 °C. The carbonyl peak at 175 ppm remained inverted after the short mixing period but became positive after the longer mixing period, which is diagnostic of dipolar exchange from the carbonyl spins to other labeled sites within the membrane sample (Figure 6a). A simultaneous decrease in the peak intensities at 96 and 92 ppm at the longer mixing period would indicate that magnetization exchanged between the carbonyl and the α- and β-isomers of D-glucose. After a mixing period of 50 ms the peak at 92 ppm for the α-anomer diminished in intensity relative to its intensity after 1 ms, but the intensity of the peak at 96 ppm for the β-anomer did not change (Figure 6b). The same experiment on D-[1-$^{13}$C]glucose in [U-$^{15}$N,$^{13}$C-Trp]GalP membranes showed a similar selective decrease in the peak for the α-anomer (Figure 6c). Earlier experiments (Figure 4c) indicate that this selective effect is unlikely to arise from differences in the dynamics of the two substrate isomers at −10 °C. These observations, taken together with the two-dimensional DARR spectrum, suggest that D-glucose in the α-conformation might be situated in the binding pocket with C-1 orientated toward carbonyl groups, specifically from tryptophans, whereas the β-anomer is oriented such that the $^{13}$C label faces away from the protein. In the spectrum for D-[1-$^{13}$C]glucose bound to unlabeled GalP (Figure 6d), neither of the peaks for the sugar change in intensity at the longer mixing time, consistent with the low probability of magnetization exchange from the natural abundance $^{13}$C spins in the protein to the labeled substrate.

**D-Glucose-Binding by Tryptophan Mutants of GalP.** Earlier results had shown that separate mutation of tryptophan residues 371 and 395 to phenylalanine produces a 10−20 fold reduction in the affinity of energized transport of D-galactose by GalP compared with the wild-type protein.[26] Cytochalasin B, like forskolin, is a tight-binding inhibitor of GalP. By competitive displacement of cytochalasin B−binding by GalP, the same work also suggested that the W371F mutation produces a 15-fold reduction in the affinity of the inward-facing conformation of the protein for binding D-galactose under non-energized conditions. In the same experiment, the W395F mutation had no significant effect on the affinity for D-galactose binding compared with the wild-type protein. Moreover, while the W371F mutation was shown to have little effect on forskolin-binding by the inward-facing conformation of the protein, the W395F mutation had a more significant effect (0.8- and 2.6-fold reductions, respectively, compared with wild-type).[26] On the basis of the above observations, it was reasonable to suggest, therefore, that one or both of the tryptophan residues 371 and 395 are situated close to the substrate binding site of GalP. In the current work, binding of D-[1-$^{13}$C]glucose by the two (unlabeled) tryptophan mutants was monitored by $^{13}$C CP-MAS NMR at 4 °C to investigate whether W371 and W395 are necessary for substrate binding (Figure 7). In both of the mutant membranes, the peaks for the α- and β-anomers of D-[1-$^{13}$C]glucose at a concentration of 10 mM and at two different contact times (2 and 10 ms) were of similar intensity to the peaks observed in the spectra of wild-type GalP membranes. This suggests that it is not essential for residues 371 and 395 to be tryptophan residues in order for the protein to retain its direct binding activity of D-glucose, which, in the case of W371F, differs from its ability to compete with cytochalasin B-binding by D-galactose. Consistent with previously described results,[26] the two mutants appeared to have different affinities for the inhibitor forskolin (Figure 7). The peaks for D-[1-$^{13}$C]-glucose-binding by the W371F mutant were almost completely displaced by addition of a 2-fold excess of forskolin, similar to that for the wild-type protein. However, the same concentration

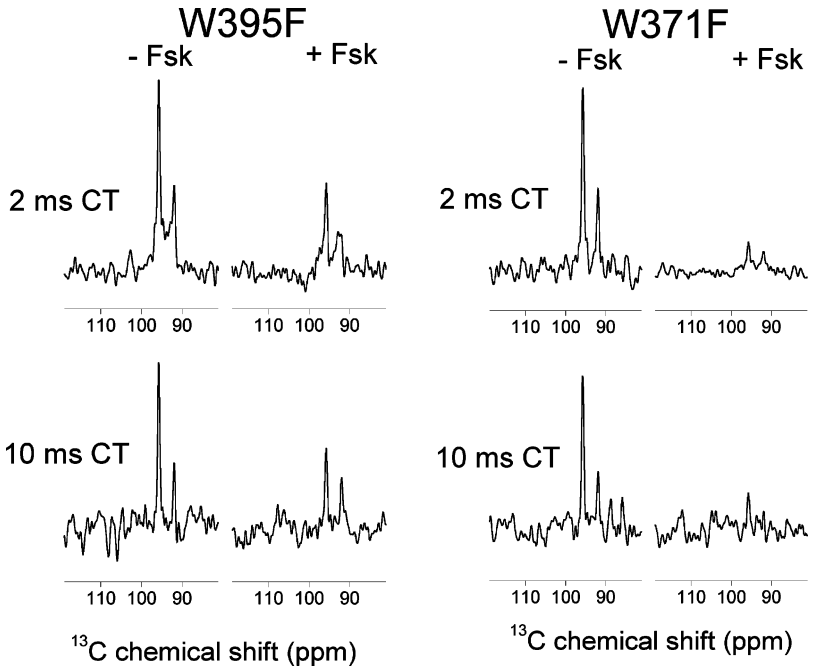


***Figure 7.*** Detection of D-[1-$^{13}$C]glucose binding to W395F and W371F mutants of GalP in *E. coli* membranes using $^{13}$C CP-MAS NMR. Spectra were obtained at 100.13 MHz for $^{13}$C with the sample spinning at 4 kHz and a temperature of 4 °C. Spectra were obtained for GalP mutants in unlabeled membranes with 10 mM D-[1-$^{13}$C]glucose in the absence of forskolin (−Fsk) and after the addition of forskolin to a concentration of 2 mM (+Fsk) at contact times (CT) of 2 and 10 ms. Each spectrum was obtained by accumulating 6144 scans.

of forskolin produced only an ∼50% reduction in peak intensities for D-[1-$^{13}$C]glucose-binding by the W395F mutant, which further suggests that the tryptophan residue at this position in wild-type GalP is involved in the binding of forskolin.

## Discussion and Conclusions

This work has demonstrated how, by carefully selecting suitable conditions and concentrations, and with appropriate control experiments, it is possible to detect interactions between specific amino acids of membrane-embedded proteins and weakly binding noncovalent ligands. By establishing conditions in which peaks in the NMR spectrum could be assigned exclusively to the D-glucose substrate restrained in the binding site of GalP, a DARR experiment was used to detect a selective interaction between the α-anomer of D-[1-$^{13}$C]glucose and $^{13}$C-labeled tryptophan residues in GalP with amplified expression in native membranes.

The results suggest that the position of the α-anomer of D-glucose in the binding site of GalP puts C-1 of the sugar to within a distance of 6 Å from the carbonyl carbon of one or more tryptophan residues in the protein. Conversely, the position of the $\beta$ anomer in the binding site puts C-1 in the sugar too far away from the $^{13}$C labeling in tryptophan residues to enable the detection of a DARR interaction. Two tryptophan residues in GalP (371 and 395) have been shown to be involved in the transport of substrate by the protein, and in the case of Trp371, also involved in substrate-binding.[26] It is very likely that one or both of these residues are in sufficiently close proximity to the bound sugar to provide the observed DARR interaction with the α-anomer of the sugar.

A further indication that C-1 in the α-anomer of D-glucose is involved in close interactions with the protein is provided by an observed splitting of the sugar signal at 92 ppm, for example, see Figure 4, parts c and d; this splitting has routinely been observed in spectra recorded at −10 °C, but not at higher temperatures. A possible explanation for the splitting of the signal for the α-anomer is an observation of the sugar binding at two different sites in the protein; however, the addition of forskolin always displaces both of the signals for the α-anomer (and the signal for the $\beta$-anomer) of the sugar. A more likely explanation is that the sugar is being observed in a single binding site with the protein in different conformations, for example, inward and outward facing. The lower temperature may be sufficient to slow down mobility in the system to allow "capture" of sugar binding to an alternative and less preferred conformation of the protein. The α-anomer may be in such close proximity to a functional group in the protein that this change in conformation of the protein imparts a small chemical shift change to the signal for the α-anomer, resulting in the observed splitting. A possible approach to discriminate binding of the sugar to different conformations of the protein would be to observe the binding for a labeled sugar locked in the α-configuration. This has been considered, but based on fluorescence quenching studies,[27] the locked glucose analogue 1-*O*-methyl-α-D-glucose is bound by GalP with a significantly reduced affinity (Kd 111 mM) compared with D-glucose. Use of such high concentrations of sugar would produce significant nonspecific signals. Interestingly, the effect of using the sugar locked in the $\beta$-configuration, 1-*O*-methyl-$\beta$-D-glucose, does not have such an effect on the affinity of binding (Kd 5.9 mM). No splitting of the signal for the $\beta$-anomer of the sugar has been observed.

The detection of coupling between $^{13}$C-labeling in GalP and bound D-[1-$^{13}$C] glucose using a one-dimensional DARR experiment is the first observation of a direct dipolar interaction between a transport protein and a ligand by NMR spectroscopy. The approach presented has the advantage of retaining the protein in its native membranes and will allow the first steps in

testing the validity of crystal structures or computer-generated models for liganded transport protein binding sites. With appropriate adaptation of concentrations and temperatures, this approach can be used in principle for studying many membrane proteins and their ligands, but it is critical to carry out careful control experiments using unlabeled displacing ligands when protein expression levels are low or if the ligands have a propensity to bind nonspecifically. By using a combination of ligands and amino acids with $^{13}C$ labels at specific positions and site-directed mutagenesis, the detected interaction could be made specific between single positions in the ligand and in single amino acid residues, therefore providing a map of the protein−ligand interaction. Our strategy for specific isotopic labeling of tryptophan residues in GalP also makes accessible other opportunities for probing residue-specific NMR interactions in the protein.

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**Supporting Information Available:** Complete ref 4. This material is available free of charge via the Internet at http://pubs.acs.org.

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