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# Selective NMR observation of inhibitor and sugar binding to the galactose-$H^+$ symport protein GalP, of *Escherichia coli*

Antony N. Appleyard [b], Richard B. Herbert [b], Peter J.F. Henderson [b], Anthony Watts [a], Paul J.R. Spooner [a,*]

[a] *Biomembrane Structure Unit, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK*
[b] *Astbury Centre for Structural Molecular Biology, University of Leeds, Leeds LS2 9JT, UK*

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**Abstract**

The binding of the transport inhibitor forskolin, synthetically labelled with $^{13}C$, to the galactose-$H^+$ symport protein GalP, overexpressed in its native inner membranes from *Escherichia coli*, was studied using cross-polarization magic angle spinning $^{13}C$ NMR. $^{13}C$-Labelled D-galactose and D-glucose were displaced from GalP with the singly labelled [7-$OCO^{13}CH_3$]forskolin and were not bound to any alternative site within the protein, demonstrating that any multiple sugar binding sites are not simultaneously accessible to these sugars and the inhibitor within GalP. The observation of singly $^{13}C$-labelled forskolin was hampered by interference from natural abundance $^{13}C$ in the membranes and so the effectiveness of double-quantum filtration was assessed for the exclusive detection of $^{13}C$ spin pairs in sugar (D-[1,2-$^{13}C_2$]glucose) and inhibitor ([7-$O^{13}CO^{13}CH_3$]forskolin) bound to the GalP protein. The solid state NMR methodology was not effective in creating double-quantum selection of ligand bound with membranes in the 'fluid' state (approx. 2°C) but could be applied in a straightforward way to systems that were kept frozen. At −35°C, double-quantum filtration detected unbound sugar that was incorporated into ice structure within the sample, and was not distinguished from protein-bound sugar. However, the method detected doubly labelled forskolin that is selectively bound only to the transport system under these conditions and provided very effective suppression of interference from natural abundance $^{13}C$ background. These results indicate that solid state NMR methods can be used to resolve selectively the interactions of more hydrophobic ligands in the binding sites of target proteins. 

*Keywords:* Membrane transport protein; Ligand binding; $^{13}C$ magic angle spinning nuclear magnetic resonance; Double-quantum filter

## 1. Introduction

A substantial portion (3–12%) of the open reading frames in known bacterial genomes encode for membrane transport proteins [1]; such proteins fulfil vital functions in all living cells through the acquisition of nutrients and the excretion of wastes/toxins. In spite of their importance, for a variety of reasons including the difficulty in crystallization for study by X-ray or electron diffraction, the 3-D structures of rather few integral membrane proteins have been defined [2]. The best crystallographic data on a membrane transporter protein have recently been reported at 7 Å resolution [3]. The data resolve well features of 12 transmembrane helices but yield no details on the

Abbreviations: CP, cross-polarization; MAS, magic angle spinning; DQ, double-quantum

* Corresponding author. Fax: +44 (1865) 275234;
E-mail: spooner@bioch.ox.ac.uk

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substrate binding site. Over recent years, novel solid state NMR methods have been developed to provide structural details of membrane proteins [4,5]. Following earlier work with sugar substrates [6,7], the current study shows that these NMR methods can be applied to elucidate the characteristics of inhibitor binding to sugar transport proteins embedded in native membranes of *Escherichia coli*.

The galactose-$H^+$ symport protein from *E. coli*, GalP, can be overexpressed to 50–60% of total inner membrane protein [8] and so is eminently suitable for structural studies by NMR and other biophysical methods. GalP is predicted to adopt a 6+6 helix topology in common with many transport systems [9], and is closely related to other transport proteins in bacteria and many higher organisms, including the glucose transporter, GLUT1, in man [10]. Forskolin and cytochalasin B are known inhibitors of sugar transport in GalP and GLUT1 [8,11], binding at least 100 times more tightly than sugars. These inhibitors are predicted to bind at the cytoplasmic face of GalP [11,12] and for the current study, $^{13}$C-labelled forskolin is used in combination with the $^{13}$C-labelled sugars to determine whether any alternative sugar binding sites could be demonstrated for the transportable ligands.

Direct CP MAS NMR observations used in this study can require measurement over extended times due to the large interference from natural abundance $^{13}$C in the NMR spectra, which in turn can prevent any quantitative treatment of the data. This situation illustrates well the general difficulties that are encountered when attempting to resolve ligand interactions with a specific binding environment in highly complex and heterogeneous macromolecular systems such as natural membranes. Solid state NMR approaches have been developed to circumvent this problem, firstly by introducing pairs of isotope labels into the ligand and then by selecting these isolated pairs of spins as part of the NMR pulse scheme. This selection was demonstrated successfully (in a model compound) using adjacent *heteronuclei* in the TEDOR (Transferred-Echo Double Resonance) version [13] of the conventional REDOR (Rotational Echo DOuble Resonance) [14] experiment from which a precise distance can then be determined to a third *heteronucleus* within the system.

The practical difficulty of introducing a particular combination of *heteronuclear* spins into complex systems, and then effectively manipulating all of these spins in the solid state NMR experiment has led to a greater number of techniques designed to select adjacent or close *homonuclear* spin pairs (e.g. $^{13}$C-$^{13}$C), normally from exciting their exclusive multiple-quantum states. The task of devising methods for recovering the *homonuclear* dipolar coupling during magic angle spinning (MAS) which permit efficient creation of double-quantum (DQ) coherence has only recently been effectively overcome with the introduction of the HORROR (HOmonucleaR ROtarary Resonance) [15] or C7 (7-fold symmetric phase Cycles) [16] techniques. With efficiencies typically approaching 50% of the available magnetization being recovered through the DQ filter, the use of these methods for studies on complex macromolecular systems has become a practical proposition, as illustrated by the determination of torsional angles within the retinal side chain of rhodopsin [17] following double-quantum selection using the C7 method. The work reported here is extended to assess the effectiveness of the C7 method, which is relatively insensitive to chemical shift differences between coupled spins [16], for the detection of selective binding of a sugar ($^{13}C_2$-labelled glucose) and the more strongly binding inhibitor, $^{13}C_2$-labelled forskolin, to GalP overexpressed in native membranes. The results show that this method is applicable to membranes converted into the solid state by freezing and that the physical characteristics of the particular ligand can then play a role in confining observations to the binding centre of the protein.

## 2. Material and methods

All $^{13}$C-labelled compounds were purchased from Cambridge Isotope Laboratories (Andover, MA) and the non-labelled inhibitors forskolin and cytochalasin B were obtained from Sigma (Poole, Dorset).

### 2.1. Synthesis of [7-OCO$^{13}$CH$_3$] forskolin (Fig. 1A)

Forskolin (102 mg, 0.25 mmol) was converted into its desacetyl derivative as previously reported [18]. The reaction mixture was evaporated in vacuo and

the residue was purified by isocratic reverse phase HPLC (Polymer Laboratories C-18, PLRP-S100, 10 μm, 300×25 mm) using acetonitrile/water 7:3 (v/v) as eluting solvent to yield purified desacetylforskolin (72 mg, 0.2 mmol, yield: 80%). $^{1}$H NMR (250 MHz) and $^{13}$C NMR (63 MHz) spectra differ from those of forskolin by the absence of a 3H singlet $^{1}$H resonance at 2.2 ppm, by shift of the H-7 resonance from 5.48 to 4.23 ppm, and by the absence of $^{13}$C resonances at 170 and 21 ppm due to the loss of the acetyl group (TLC 4:1 $CH_2Cl_2$/EtOAc, $R_f$: 0.29; mass spectrum: ES (−ve ion), *m/z* 367.1 (M-1, 100%)).

The desacetylforskolin prepared as above (33 mg, 0.09 mmol) was acetylated with [2,2′-$^{13}C_2$]acetic anhydride (0.25 g, 2.4 mmol) [19]. The reaction mixture was evaporated in vacuo and the crude product was purified by reverse phase HPLC as described above to yield [7-OCO$^{13}$CH$_3$]forskolin as a white solid (7.5 mg, 0.018 mmol, yield: 20%). The solution NMR spectra were identical to those of forskolin except that the $^{1}$H resonance at 2.2 ppm appeared as a 3H doublet ($J_{CH}$ = 130 Hz); also an enriched singlet $^{13}$C resonance at 21.6 ppm and a natural abundance doublet at 170 ppm ($J_{CC}$ = 60 Hz) was observed. These observations confirm that the acetylation was successful and that the enriched $^{13}$C nucleus was incorporated into forskolin (TLC 4:1 $CH_2Cl_2$/EtOAc, $R_f$: 0.68; mass spectrum: EI, *m/z* 411 (M+, 15%), 393 (M-OH, 50%)).

### 2.2. Synthesis of [7-O$^{13}$CO$^{13}$CH$_3$] forskolin (Fig. 1B)

Desacetylforskolin (72 mg, 0.2 mmol) was prepared and then acetylated as described above with [1,1′2,2′-$^{13}C_4$]acetic anhydride (100 mg, 0.943 mmol) to give [7-O$^{13}$CO$^{13}$CH$_3$]forskolin as a white solid (34 mg, 0.083 mmol, yield: 42%). The structure and isotopic labelling of the product were confirmed by $^{1}$H NMR, $^{13}$C NMR ($^{13}$C enriched signals: 21.2 and 169.6 ppm, each a doublet, $J_{CC}$ = 60 Hz) and mass spectrometry (ES, *m/z* 413; M+ +1, 100%) and its purity checked by TLC.

### 2.3. Membrane preparation

*E. coli* strain JM1100 containing the plasmid pPER$_3$(His$_6$) for overexpression of the GalP(His)$_6$ protein (unpublished work), was grown in liquid medium as described [8]. *Inside-out* vesicles were prepared from the genetically engineered organisms by explosive decompression in a French press and the inner membrane fraction was isolated by sucrose density ultracentrifugation [8]. Membrane vesicles were washed three times by suspension in 20 mM Tris-HCl buffer, pH 7.5, followed by ultracentrifugation before final suspension in the same buffer for storage at −80°C after rapid freezing. Total protein concentration in the vesicle suspension was determined by the method of Schaffner and Weissmann [20], of which GalP was estimated to comprise between 50 and 55% according to densitometry measurements on the proteins resolved by SDS-PAGE and stained with Coomassie blue. Membranes without GalP were prepared from *E. coli* strain JM1100 (pBR322) for use in control experiments.

### 2.4. NMR analysis

Preparation of all samples and their subsequent use in the NMR experiments was conducted at temperatures of 4°C or less. Quantities of membrane suspension in 20 mM Tris-HCl buffer, pH 7.5, estimated to contain 300 nmol of GalP protein, were equilibrated with substrate or inhibitor for 30 min at 4°C. $^{13}$C-Labelled D-glucose and D-galactose were added as an aqueous solution to the membrane suspension and the inhibitors, forskolin and cytochalasin B, were added gradually as ethanolic solutions to the membrane suspension with vortex mixing. Volumes of the membrane suspension were adjusted prior to adding inhibitor so that the final ethanol concentration was less than 2 vol%. Following equilibration for 30 min, membranes were sedimented for NMR analysis by ultracentrifugation (100 000×*g*; 30 min).

$^{13}$C NMR spectra were recorded at 100.6 MHz (400 MHz for protons) with a Bruker MSL spectrometer with double-bearing MAS probe heads for 7 mm sample rotors or a Chemagnetics (Varian) CMX Infinity Spectrometer using a Chemagnetics Apex MAS probe for 6 mm diameter sample spinners. Sample spinning speed ($\omega_r$) was maintained at 3.0 or 4.0 kHz unless otherwise stated and the sample temperature in the probe was maintained at 2°C

or below with dried air supplied through a simple heat exchange coil that was immersed in an acetone-dry ice bath.

The C7 pulse sequence used for double-quantum excitation and observation of carbon spins was essentially the same as originally described for this method [16] and is illustrated in Fig. 2. Carbon magnetization is first created by CP from protons, using a ramp on the carbon contact pulse to reduce sensitivity to Hartmann-Hahn mismatch between the two nuclei. Following a 90° pulse on carbons, double-quantum coherence is created during the C7 sequence of pulses in which the nutation frequency ($\omega_1^S$) for the carbon spins satisfies the condition $\omega_1^S = 7\omega_r$ and C7 elements have their phase progressively incremented by $2\pi/7$. In the version used here, each element comprises of 90, 360 and 270° pulses whose phases alternate by $\pi$, which is described as the POST-C7 version of this sequence [21]. For normal solid samples (−35°C) the proton field strength was 70 kHz for both cross-polarization (CP) and decoupling during acquisition, but was increased to 100 kHz for decoupling during the C7 sequence of pulses. These field strengths were reduced for fluid membranes (approx. 2°C) to 50 kHz for both CP and decoupling during the C7 sequence and a decoupling field of 25 kHz was used to avoid sample heating during signal acquisition. Contact times during CP were 0.5 ms for solid or frozen samples and 1 ms for non-frozen fluid membranes. The exchange time for creation of DQ coherences, $\tau_{exc}$, was found to be optimal at 0.5 ms throughout these measurements. Dephasing-delayed CP (DDCP) was implemented for estimating the rates of inhibitor exchange as previously described [7].

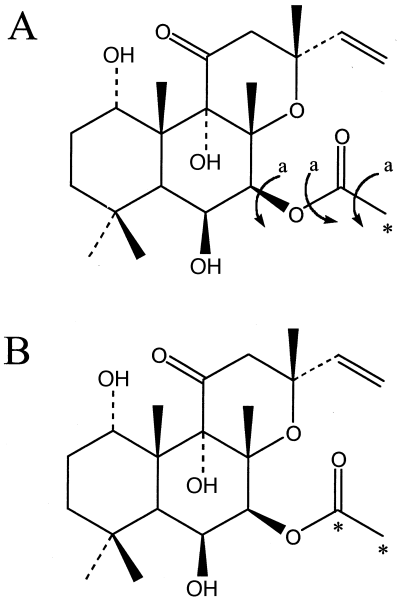


Fig. 1. Structure of forskolin with the sites of isotopic labelling in [7-OCO$^{13}$CH$_3$]forskolin (A) and [7-O$^{13}$CO$^{13}$CH$_3$]forskolin (B) indicated with asterisks. Possible bond rotations within the labelled acetyl group are shown (a).

## 3. Results and discussion

### 3.1. Binding of $^{13}$C-labelled forskolin

The CP MAS technique is capable of detecting ligand that is constrained by binding within the membrane samples and was shown to result in exclusive observation of sugars that are selectively bound to sugar symport proteins, including GalP, that are overexpressed in their native membranes [6,7]. When using these simple ligands, it was easy to select the position of $^{13}$C enrichment so that it appeared in spectral regions relatively free from the natural abundance contribution from the membrane sample. The labelling options for the inhibitor, forskolin, were more restricted and the only practical site of labelling was in the acetyl group on carbon-7 (Fig. 1A), a resonance which falls into a crowded alkyl region of the spectrum recorded from the GalP membranes alone (Fig. 3A and expanded region Fig. 3C). However, when the singly labelled forskolin (Fig. 1A) was titrated into the membranes a signal from the ligand was detected in this region with increasing intensity following additions of between 200 and 400 nmol of ligand as shown in Fig. 3B and expanded spectral regions Fig. 3F and G. The resonance from the bound ligand appears at around 21.1 ppm, which is shifted by 0.5 ppm to lower frequency compared with its chemical shift observed in $CDCl_3$ solution (not shown). Spectra from inner membranes without GalP protein showed no ligand signal at this position when equilibrated with up to 500 nmol of labelled forskolin as shown in Fig. 3E, demonstrating that the forskolin binding observed was selective for GalP. The lack of any signal without selective binding to the protein is also important for showing that inhibitor which is not selectively bound, remains suf-

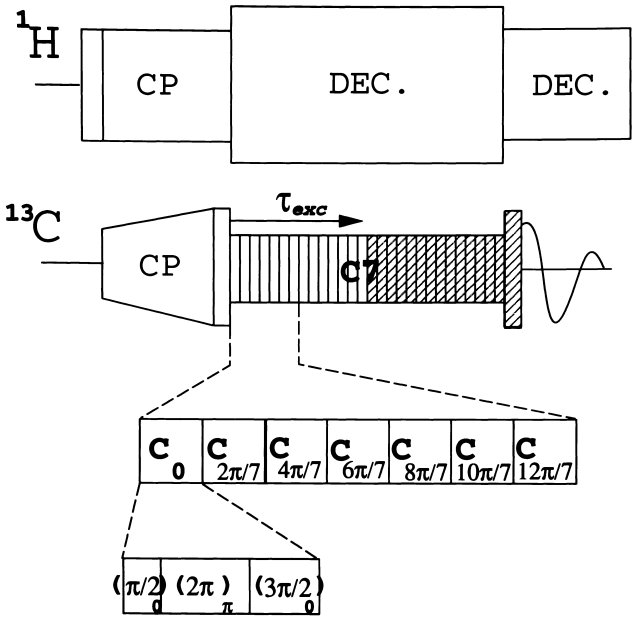


Fig. 2. The C7 pulse sequence used for recording DQ-filtered MAS $^{13}C$ NMR spectra. CP from proton to carbon spins is followed by an integral number of C7 sequences for the exchange time $\tau_{exc}$, selected to be optimal for converting transverse magnetization into the DQ state. The C7 elements have phases incremented by $2\pi/7$ and each comprises of three pulses whose phases are alternated by 180° in this POST-C7 version of the sequence. The shaded region follows the inverse process for re-conversion to transverse magnetization and employs a conventional phase scheme for selecting only magnetization passing through the DQ state. Proton decoupling (DEC.) is applied at levels described in Section 2.4.

ficiently mobile to avoid detection. Studies also reported here indicate that sufficient mobility is retained even in membranes at reduced temperature (−35°C).

The signal observed with 400 nmol of labelled forskolin in GalP membranes was reduced by sequential addition of non-labelled cytochalasin B (data not shown), another inhibitor of GalP that binds with similar affinity as forskolin [6]. The direct observations alone provide good evidence that the methods detect inhibitor that is selectively bound to the GalP protein alone. The reasonable sensitivities observed from short (1 ms) Hartmann-Hahn contact indicate that possible motions involving bond rotations with and within the labelled acetyl group (see arrows 'a' in Fig. 1A) do not interfere with efficient CP within ligand that is bound to GalP.

In further experiments, the rate at which forskolin exchanges with its site of specific binding on the protein was determined to be slow on the NMR time scale ($> 10^{-2}$ s), since no exchange could be detected from observations using the DDCP method (data not shown), in common with the binding of D-glucose to this protein [6].

### 3.2. Binding of $^{13}C$-labelled sugars and their displacement by [$^{13}C$]forskolin

The CPMAS spectrum recorded from GalP membranes equilibrated with 20 mM (60 μmol) D-[1-$^{13}C$]galactose (Fig. 4A), shows well resolved resonances from both α and β anomers of the bound sugar in a region of the spectrum (expanded in Fig. 4B) relatively free from natural abundance $^{13}C$ contributions (90–100 ppm). This is similar to the spectrum from bound D-[1-$^{13}C$]glucose in GalP membranes [6]. The spectra of bound galactose anomers show no significant difference in chemical shift (96.7 and 92.5 ppm) compared with their observation in aqueous solution, in common with the weak binding observed for other sugar substrates to this and other membrane transport proteins [6,7,22]. As increasing amounts of labelled forskolin were added, the intensity of the galactose resonances decreased, ultimately close to background levels (expanded spectral regions in Fig. 4C–E) as the intensity of the signal from bound forskolin increased (Fig. 4F–I). A similar progressive displacement by forskolin was observed for the binding of D-[1-$^{13}C$]glucose (10 mM; 30 μmol) to the GalP membranes (data not shown).

The observations here indicate that when the inhibitor forskolin binds to GalP, sugar ligand is displaced and is not bound to any alternate site within the protein. It has previously been concluded [11] that the inhibitor binds only to the cytoplasmic surface of the protein that is predominantly exposed to the outer surface of the *inside-out* membrane vesicles prepared for this work. Furthermore, being non-transportable, added forskolin cannot interfere with sugar at the protein surface exposed to the inner surface of the membrane vesicles (periplasmic membrane surface). According to the observations here, when forskolin is bound to the cytoplasmic surface of the protein, the sugars cannot bind from either surface of GalP. If two (or more) physically distinct sites or conformations for sugar binding exist, then it

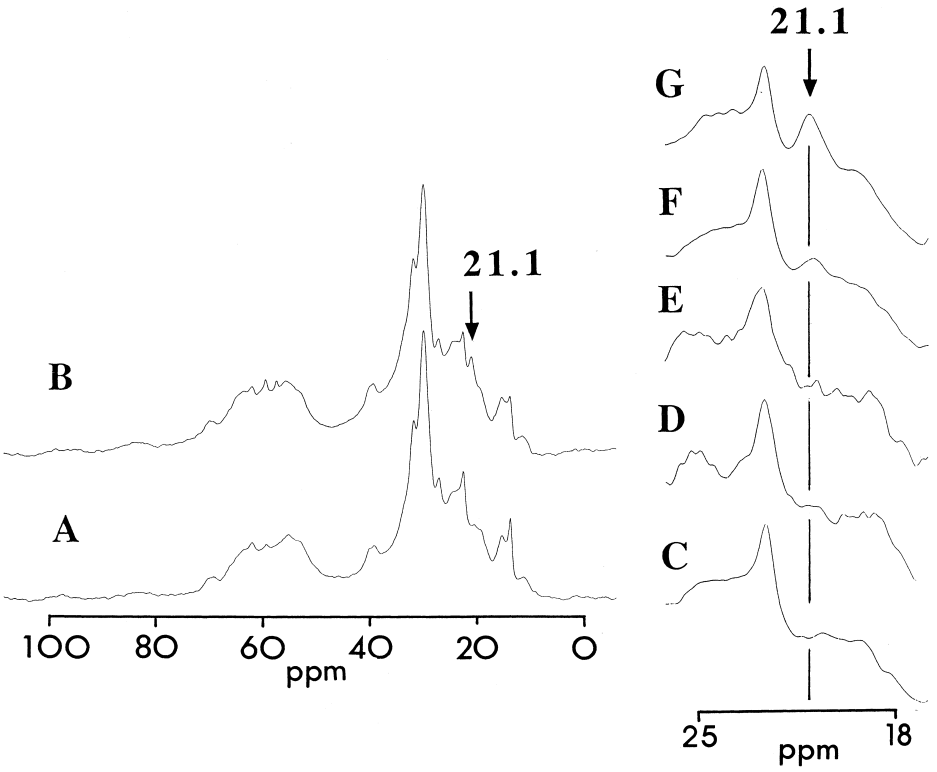


Fig. 3. Detection of singly labelled forskolin bound to GalP. $^{13}C$ CP MAS NMR spectra from GalP (300 nmol) in inner membranes (A) and after addition of 400 nmol of [7-OCO$^{13}CH_3$]forskolin (B). Expanded regions show resonances detected from bound [7-OCO$^{13}CH_3$]forskolin after adding 200 nmol (F) and 400 nmol (G) of the inhibitor. C and G are the relevant expanded regions from spectra A and B, respectively. Expanded regions are shown from spectra recorded from membranes containing no GalP without (D) and with (E) 500 nmol of [7-OCO$^{13}CH_3$]forskolin.

may be concluded that neither of these sites or conformations is accessible in the presence of the inhibitor forskolin.

The existence of a large background signal from natural abundance $^{13}C$ in the membranes prevented any quantitative comparison between forskolin binding and sugar displacement using the singly labelled materials. In addition, such interference would impede more detailed studies of ligand interactions with its immediate environment within the protein. Ways of further improving selectivity in the observation of bound ligand by using DQ-filtered NMR methods are described in the remainder of this work.

### 3.3. DQ-filtered MAS NMR of doubly labelled glucose

Preliminary efforts were made to observe D-[1,2-$^{13}C_2$]glucose selectively bound to GalP membranes in their fluid state using the C7 technique as a DQ filter for elimination of $^{13}C$ background signal that arises from the isolated natural abundance spins. Extended application of the C7 pulse sequence (Fig. 2) failed to generate an adequate double-quantum filtered signal from the spin pair in the substrate bound to the membranes at 2°C (data not shown). Failure to create a DQ-filtered signal in this case could have been due to an inability to recover the dipolar interaction between the carbon spin pair due to motional averaging experienced by the bound sugar in 'fluid' membrane systems. It was previously deduced from relaxation analyses [6] that this sugar remains associated with the binding site in GalP on the NMR time scale ($>10^{-1}$ s) and so does not exchange rapidly between bound and unbound forms. Similarly, the DQ measurements should be unaffected by any complete dissociation of sugar from the binding site in the protein. It was also inferred from the previous observations [6,7] that the overall reorientation of the protein in the membranes is too slow to interfere

with detection of bound sugar. What remains of the motional contributions are the fast local motions within the protein which are too restricted to attenuate seriously the proton-carbon couplings, and are beneficial in facilitating relaxation recovery in the

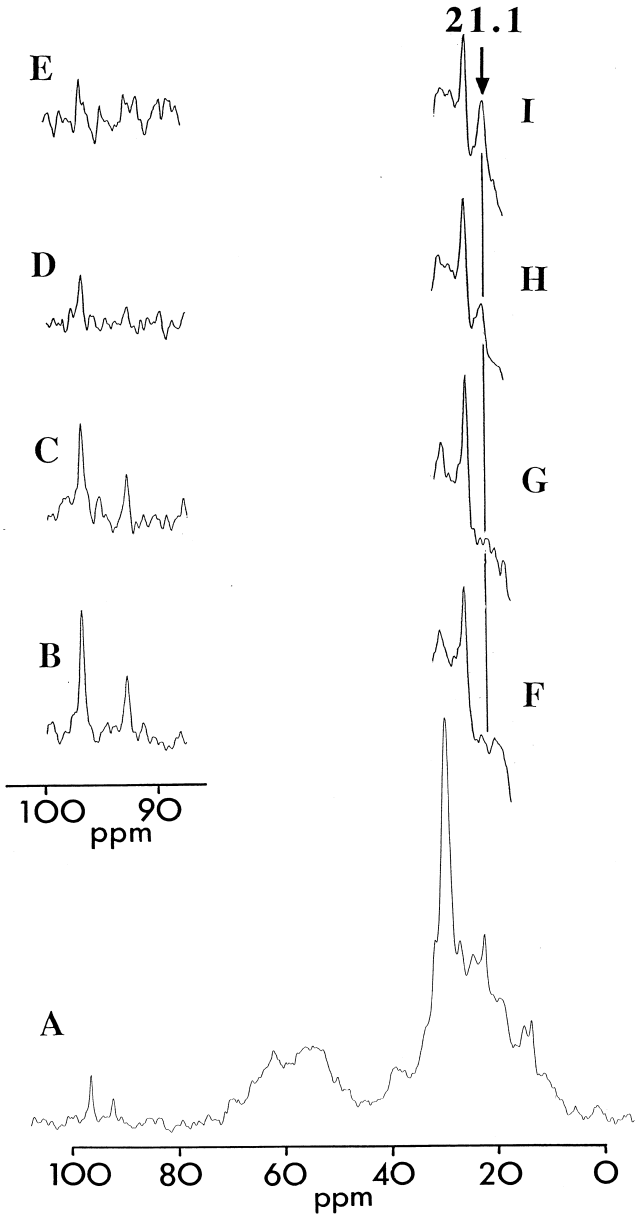


Fig. 4. Binding of D-[1-$^{13}$C]galactose to GalP and its displacement by [7-OCO$^{13}$CH$_3$]forskolin. CP MAS spectra from membranes with GalP (300 nmol) and 20 mM D-[1-$^{13}$C]galactose (A) with expanded spectral regions accommodating the signal from bound sugar (B–E) or bound forskolin (F–I) and following addition of 0 nmol (B,F), 100 nmol (C and G), 300 nmol (D,H) and 500 nmol (E,I) of [7-OCO$^{13}$CH$_3$]forskolin.

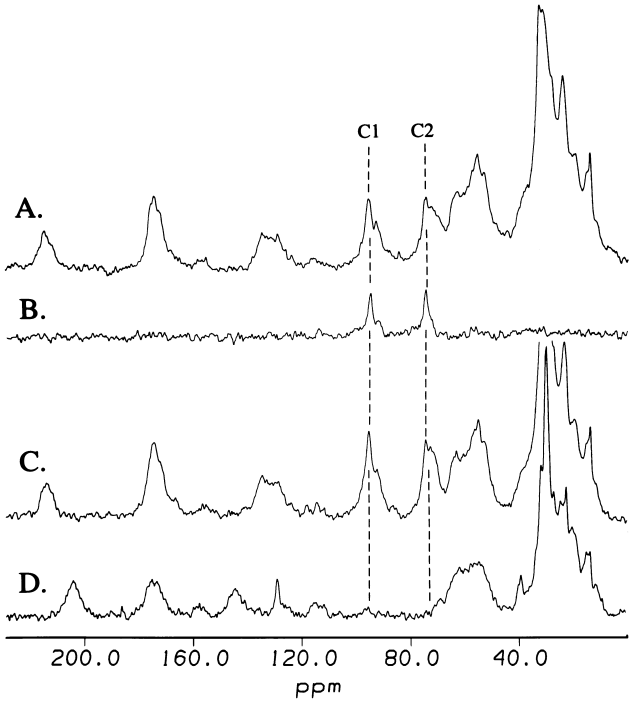


Fig. 5. Measurements recorded at −35°C for (A) the membranes containing 300 nmol of GalP with 10 mM D-[1,2-$^{13}$C$_2$]glucose by CP MAS, showing strong signal from both positions (C1/C2) labelled within the sugar. (B) Selective detection of substrate in a DQ-filtered spectrum. (C) Substrate detected in a CP MAS spectrum from membranes with 300 nmol of GalP, pretreated with 1 μmol unlabelled forskolin before addition of 10 mM D-[1,2-$^{13}$C]glucose and (D) the CP MAS spectrum of this sample recorded in a non-frozen state at 2°C.

NMR measurement [6,7]. Although the *homonuclear* dipolar couplings between carbon spins are smaller and therefore more susceptible to motional averaging than the proton-carbon couplings, it does not seem likely that these would be completely removed by the restricted local motion experienced by sugar bound to the protein. A more likely explanation for failure to create DQ signal is that because the 'fluid' membranes exhibit such a broad Hartmann-Hahn frequency response, CP persists during the C7 elements of the sequence (Fig. 2) and so interferes with the requisite precession of the carbon spins for efficient creation and reconversion of the double-quantum states.

All the undesirable effects mentioned above, that could interfere with effective creation of DQ coherences, can be suppressed by freezing the membrane sample to a solid state. The membranes required

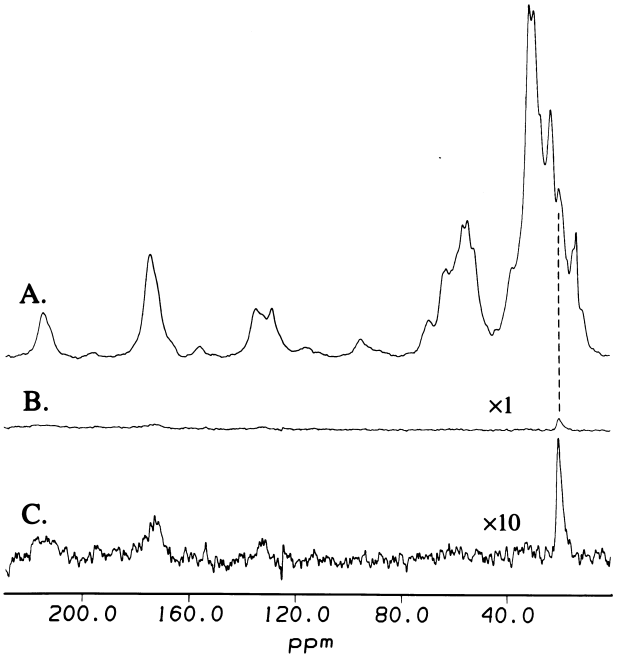


Fig. 6. (A) CP MAS $^{13}C$ NMR spectrum of membranes at −35°C containing 300 nmol of GalP with 500 nmol of the doubly labelled forskolin and (B) a DQ-filtered spectrum of this sample with the same intensity scaling (×1) or (C) displayed with ten-fold (×10) expansion in intensity. All spectra recorded with 21 000 acquisitions.

cooling to −35°C before they approximated to solid samples in terms of their pulse width response to the applied radiofrequency fields. However, this temperature still allowed sufficient local fast motion for rapid relaxation recovery for sugar and inhibitor in the membranes. The CP $^{13}C$ MAS NMR spectrum of the membrane sample with 10 mM D-[1,2-$^{13}C$]glucose at −35°C (Fig. 5A) shows strong signals from both carbon labels in the sugar (C1/C2). The DQ-filtered spectrum recorded under these conditions (Fig. 5B) now only shows signal from the two carbon labels in the sugar. A further membrane sample was pretreated with 1 μmol of unlabelled forskolin, an amount previously found to block detection, and hence binding, of labelled glucose in the fluid GalP membranes [6], but this gave a similar CP spectrum (Fig. 5C) when frozen with the 10 mM D-[1,2-$^{13}C$]glucose to that without any inhibitor binding (Fig. 5A). The transport inhibitor also had little effect on the DQ-filtered spectrum (not shown). Sugar binding in this sample was not, however, detectable by CP MAS with membranes in the fluid state as shown from the absence of any significant high resolution signal at the C1 and C2 spectral positions in Fig. 5D.

The large sugar signal observed in the frozen state and selectively detected with DQ filtration can therefore be deduced to originate mostly from substrate immobilized within ice crystals rather than within the binding site of the protein. On this evidence, selective detection of binding to GalP using the solid state NMR DQ filtration is not possible for the simple sugar substrates that are extensively hydrogen bonded within the aqueous environment and are thus readily immobilized in the ice structure of frozen samples.

### 3.4. DQ filtration of doubly labelled forskolin in frozen membranes

The CP MAS $^{13}C$ NMR spectrum of GalP membranes at −35°C after equilibration with [7-O$^{13}$CO$^{13}$CH$_3$]forskolin in Fig. 6A is superimposed over the DQ-filtered spectrum (Fig. 6B) displayed with the same intensity scaling. The DQ-filtered spectrum appears essentially free from natural abundance

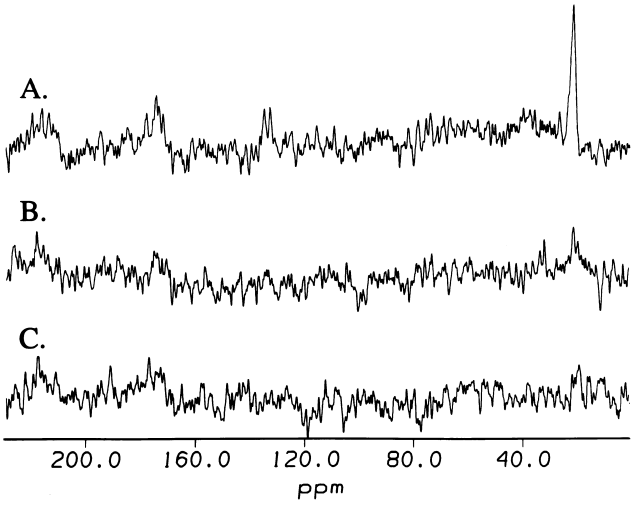


Fig. 7. DQ-filtered CP MAS $^{13}C$ NMR spectra of membranes containing 300 nmol (30 mg) of GalP and 500 nmol of doubly labelled forskolin (A) without and (B) with prebinding of 2 μmol of unlabelled cytochalasin B. (C) DQ-filtered CP $^{13}C$ MAS spectrum of membranes without GalP but with around 60 mg of total membrane protein and 500 nmol of doubly labelled forskolin. All spectra recorded from 24 000 acquisitions at −35°C.

background signal from the membranes leaving a distinct resonance from the forskolin methyl carbon (21.1 ppm) and a broader signal from the carbonyl (approx. 175 ppm) distributed over a number of orders of rotational side bands, spaced at intervals of the rotation frequency (±approx. 40 ppm) as observed more clearly in the intensity expanded spectrum (Fig. 6C). DQ filtration proceeded efficiently, judging by the intensity of the ligand methyl resonance that appears superimposed over the large natural abundance intensity in this region of the normal CP spectrum (Fig. 6A). The ligand resonances in the normal CP spectrum (Fig. 5A) appear broader and are more difficult to discriminate than that observed from the singly labelled forskolin (Fig. 1A) bound to the fluid state membranes (Fig. 3). The further experiments needed to determine the environment of the detected ligand in the frozen membranes were therefore only conducted using the DQ filter.

Fig. 7 shows the DQ-filtered CP MAS $^{13}C$ spectra of the doubly labelled forskolin in GalP membranes at −35°C without (Fig. 7A) and with (Fig. 7B) pretreatment with non-labelled cytochalasin B. The low level of the forskolin methyl intensity (approx. 21 ppm) that remains detectable (Fig. 7B) is roughly consistent with the competition for selective binding expected from using a four-fold higher concentration of the unlabelled cytochalasin B which has a similar affinity for GalP protein [6]. In addition, measurements on doubly labelled forskolin incorporated into membranes, without GalP, showed little or no signal from the methyl label (Fig. 7C). These membranes appear to show more residual intensity in the carbonyl region (approx. 175 ppm) which, as also seen in the competitive binding spectrum (Fig. 7B), may arise from DQ-filtered natural abundance signal from the large population of rigid carbonyls within the membranes.

The measurements with doubly labelled ligand show that in membranes frozen at moderately low temperatures, it is possible to discriminate selective binding to the transport system using DQ filtration, providing the unbound ligand does not form extensive and stable hydrogen bonding with water in the system. Indeed, the more hydrophobic ligands such as forskolin and cytochalasin B, used here, evidently become associated with the membrane environment as concluded from the preceding observations with singly labelled ligand. At −35°C this membrane environment is also regarded as relatively rigid and will restrict motions in the non-specifically bound ligand. However, it should be recognized that this ligand would only be required to reorient during the sample rotation period (0.25 ms) in order to interfere with its detection during the MAS experiment. The ability of a wide range of these *intermediate* rate motions to affect detection during MAS provides greater options for detection of selective binding at reduced temperatures than is generally presumed. However, the amenability of each ligand for selective observation at reduced temperature would need to be assessed on an individual basis, as described in the current work.

This and previous NMR observations of ligand binding to membrane transport systems have relied on the differential immobilization of ligand to discriminate selective interactions within the binding sites of these proteins [6,7,22]. Here, it is shown that DQ filtration not only offers opportunities for selective observation or utilization of magnetization from multiple-labelled ligands but can also introduce an additional, more sensitive level of discrimination in the NMR experiment, based on mobility, due to a reliance on the weaker *homonuclear* dipolar coupling for detection.

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