Weak Substrate Binding to Transport Proteins Studied by NMR

Paul J. R. Spooner,* W. John O'Reilly,# Steven W. Homans,§ Nicholas G. Rutherford,# Peter J. F. Henderson,# and Anthony Watts*

*Biomembrane Structure Unit, Department of Biochemistry, University of Oxford, Oxford OX1 3QU; #Department of Biochemistry and Molecular Biology, University of Leeds, Leeds LS 2 9JT; §Department of Chemistry, University of St. Andrews, St. Andrews, Scotland, United Kingdom

ABSTRACT The weak binding of sugar substrates fails to induce any quantifiable physical changes in the L-fucose-H^+ symport protein, FucP, from *Escherichia coli*, and this protein lacks any strongly binding ligands for competitive binding assays. Access to substrate binding behavior is however possible using NMR methods which rely on substrate immobilization for detection. Cross-polarization from proton to carbon spins could detect the portion of ^{13}C-labeled substrate associated with 0.2 μmol of the functional transport system overexpressed in the native membranes. The detected substrate was shown to be in the FucP binding site because its signal was diminished by the unlabeled substrates L-fucose and L-galactose but was unaffected by a three- to fivefold molar excess of the non-transportable stereoisomer D-fucose. FucP appeared to bind both anomers of its substrates equally well. An NMR method, designed to measure the rate of substrate exchange, could show that substrate exchanged slowly with the carrier center ($>10^{-1}$ s), although its dynamics are not necessarily coupled strongly to this site within the protein. Relaxation measurements support this view that fluctuations in the interaction with substrate would be confined to the binding site in this transport system.

INTRODUCTION

Many membrane transport proteins exhibit low affinities for their substrates (K_d values in the millimolar range). This presents a particular challenge to describing substrate binding characteristics and to the use of ligand binding for exploring the functional and structural characteristics of this family of proteins. A good case in point is FucP, the L-fucose-H^+ symport protein from *Escherichia coli* (Bradley et al., 1987; Muiry et al., 1993). This protein has been cloned to achieve high levels of overexpression in the native organism (nominally ~20% of total inner membrane protein), and its sequence of 438 amino acids (M_r = 47,773) is predicted to fold into 12 transmembrane helices (Gunn et al., 1994, 1995). Fusions of β-lactamase with segments of the FucP sequence support this 12-helix topology (Gunn et al., 1995), a common prediction for membrane transport proteins (Henderson, 1993). FucP therefore represents one of the few model systems for 12-helix membrane transport proteins currently available in the quantities required to allow a wide range of structural and physical studies.

The scope of biophysical studies on FucP has been very limited. The sole source of information on substrate specificity for FucP has been from monitoring its transport activity, which identified D-arabinose, L-galactose, and L-fucose as transportable substrates, but not their respective optical stereoisomers (Bradley et al., 1987; Muiry et al., 1993). It is not, however, susceptible to recognized inhibitors of sugar transport activity, such as the fungal antibiotics cytochalasin B and forskolin. This absence of any strongly associated ligands has precluded any evaluation of substrate binding from competitive binding assays. Substrate binding to FucP also fails to induce any fluorescence changes (A. R. Walmsley and P. J. F. Henderson, unpublished data) which have been previously used to quantify binding with other symporters (Walmsley et al., 1994). This weak yet rather conservative ligand binding behavior makes FucP a particularly interesting transport protein, which requires novel methods for analysis.

Previously, we began to explore the scope of solid state NMR for elucidating structural and functional characteristics of membrane transport proteins by combining magic-angle spinning (MAS) NMR with site-specific labeling to observe features of substrate binding to the galactose-H^+ symport protein GalP (Spooner et al., 1994). The low binding affinities of ligands to this family of transporters means that a significant portion of substrate remains unbound even in compact pellets of the membranes vesicles containing these proteins. NMR methods are therefore required to be selective for that fraction restricted by its binding with the protein and this can be achieved by exploiting magnetization transfer from proton to heteronuclear spins by the cross-polarization (CP) method. Detection of only bound ligand by this means requires that the membranes are maintained in their native (non-frozen) state. This application of conventional solid state NMR methodology to the relatively fluid and dynamically complex membrane systems might, at first sight, appear unpromising. However, this approach was effective in the detection of substrate selectively bound to GalP protein in native membranes (Spooner et al., 1994) and is applied here to examine the hitherto intractable characteristics of substrate binding to FucP in its native membranes. The "fluid" membranes allow for observation of the functional state of proteins and so can provide access

Received for publication 29 April 1998 and in final form 25 August 1998.

Address reprint requests to Dr. Paul J. R. Spooner, Biomembrane Structure Unit, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK. Tel.: 1865-275270; Fax: 1865-275259; E-mail: spooner@bioch.ox.ac.uk.

0006-3495/98/12/2794/07 $2.00

to dynamic features of substrate translocation and ensures that the observations have physiological relevance.

Here we show that the NMR methods can discriminate selective binding of both D-arabinose and L-galactose to the FucP protein in native membranes with very good sensitivity. The methods yield information on the dynamic behavior of bound substrate as well as for the overall translocation process and describes an extremely weak association of substrate in the binding center.

MATERIALS AND METHODS

Membrane preparation and substrates

E. coli strain AR120(pFG7) (Gunn et al., 1994), containing the *fucP* gene was grown in an MBR Bioreactor with 25 l of medium containing yeast extract and bacto-tryptone (both 10 g/l) with 5 g/l NaCl, 20 mM glycerol, and 50 mg/l carbenicillin. When the growth medium reached an OD (at 680 nm) of 1.0, expression of FucP was induced by the addition of nalidixic acid (40 mg/l) and the induced cells were harvested some hours after by centrifugation (Gunn et al., 1994). For maximizing levels of FucP expression, post-induction growth was optimized for the highest activity of L-fucose uptake by the cells. Harvested cells were disrupted by explosive decompression in a French press and the inner membrane vesicles were isolated by sucrose density gradient ultracentrifugation as previously used for membranes derived from other genetically engineered organisms (Spooner et al., 1994). Inner membrane vesicles were washed and resuspended in 20 mM Tris-HCl buffer at pH 7.5 and stored at −80°C after rapid freezing.

Total protein in the inner membrane preparations was determined spectrophotometrically (Schaffner and Weissmann, 1973) and the proportion of FucP was estimated from densitometry measurements on the protein resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized from staining with Coomassie Brilliant Blue R250 (Henderson and Macpherson, 1985). As indicated in Fig. 1, the overexpressed FucP protein is located in the gels at a position corresponding to an apparent molecular size of 32 kDa. This band was previously ascribed to FucP, based on the knowledge that other sugar-H^+ transport proteins migrate in SDS-PAGE at 65–75% of their true molecular weights (Gunn et al., 1994). By optimizing the growth for the highest specific transport activity, expression of FucP was increased to ~40% of total protein in these preparations. Thawed inner membranes with overexpressed FucP were sedimented for the NMR analysis by ultracentrifugation at $10^5 \times g$ for 1 h. The sedimented membrane vesicles still typically contained greater than 80% by weight of water, although this was reduced during the early stages of the analysis (see below). Once thawed, membranes were maintained at between 0 and 4°C throughout all subsequent sample preparation and analysis.

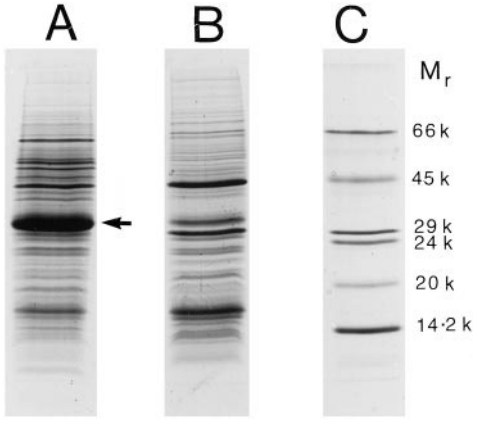

FIGURE 1 SDS-PAGE of membrane samples from the *E. coli* strain expressing FucP (nalidixic acid induced strain of AR120(pFG7); see under Materials and Methods). Each membrane fraction (30 μg) was solubilized in sample buffer at 37°C, 30 min before electrophoresis on a 15% SDS-PAGE. Proteins were visualized by staining the gel with Coomassie Brilliant Blue R-250. The FucP protein is indicated by an *arrow. Lane A*, inner membrane fraction; *lane B*, outer membrane fraction; *lane C*, molecular weight markers (SDS-7 Dalton Mark V11-L, Sigma), bovine albumin (M_r = 66,000), egg albumin (45,000), bovine carbonic anhydrase (29,000), bovine pancreas trypsinogen (24,000), soybean trypsin inhibitor (20,000), and bovine milk α-lactalbumin (14,200).

D-[1-^{13}C]Arabinose was purchased from Cambridge Isotope Laboratories (Andover, MA) and L-galactose, uniformly labeled with ^{13}C, (L-[$^{13}C_6$] galactose), was a gift from the Martek Corp. (Columbia MD). All non-labeled sugars were obtained from Sigma (St. Louis, MO). Solutions of sugars were prepared in 20 mM Tris-HCl buffer at pH 7.5 for adding to inner membrane pellets in 10-μl aliquots (for transported isomers) or 5-μl aliquots (for non-transported isomers). Sugars were equilibrated with membrane pellets for at least 30 min before measurement (see below).

NMR methods

^{13}C NMR spectra were recorded at 100.63 MHz (400.13 MHz for protons) using a Bruker MSL spectrometer with double-bearing MAS probeheads for 7 mm sample rotors. MAS rates were 3.0 kHz, during which sample temperature was controlled with the bearing gas maintained at a temperature of 2°C. The CP experiments used a proton field strength of 50 kHz for contact, but this was attenuated by 50% for decoupling in membrane samples during acquisition from both CP and single-pulse experiments. The field strength applied to the ^{13}C spins was adjusted to satisfy the Hartmann-Hahn condition for CP and then used in all pulsed experiments. All membrane pellets were first examined without substrate to record control spectra from natural abundance nuclei. Before adding the sugars, excess aqueous phase which had separated from the membrane mass was drained off to improve sample homogeneity and to promote binding with sugar substrates. However, these membrane vesicle preparations always remained expanded and well hydrated during their analysis and at no stage resembled collapsed or dried membranes. Solutions of the sugars were mixed directly into the membrane mass within the MAS sample rotors and allowed to equilibrate at 4°C. Subsequent analysis of substrate binding by NMR indicated that substrate can equilibrate rapidly (minutes) with the membranes containing active protein and gentle stirring was used only to ensure sample homogeneity before spinning. Proton spin-lattice relaxation in the laboratory and rotating frames used CP for indirect detection on resolved ^{13}C spins as described (Schaefer et al., 1977; Sullivan and Maciel, 1982).

A pulse sequence designed to measure the rate of substrate exchange with the carrier center in the transporter proteins is represented schematically in Fig. 2 and referred to here as dephasing-delayed cross-polarization (DDCP). Proton magnetization is prepared by a non-selective $\pi/2$ pulse and then allowed to dephase over a single rotation period ($1/\omega_r$; ω_r = rotation speed in Hz), before storing residual magnetization back along the longitudinal axis in the laboratory frame ($+z$). The π pulse centered at $\omega_r/2$ refocuses isotropic chemical shifts over the dephasing delay and incorporates a 180° phase shift to compensate for errors in setting the pulse width. At normal speeds, the single rotation period is sufficient to dephase all magnetization for the protons associated with the membranes but is too short to degrade the magnetization of substrate that remains free in solution, which is stored. The mixing time, τ_m, allows any exchange with free substrate, retaining full magnetization, which can then be detected by the conventional CP sequence. Since magnetization is also recovered by longitudinal relaxation processes, the method will selectively detect exchange that occurs faster than the rate of spin-lattice relaxation in the laboratory frame ($1/T_{1Z}$).

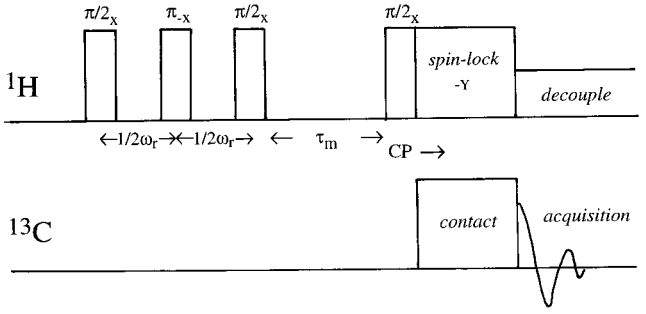

FIGURE 2 DDCP pulse sequence designed to monitor the rate of substrate exchange with the protein. The creation and then dephasing of proton magnetization in the membranes occurs over a single rotor period, $1/\omega_r$, where ω_r is the sample rotation speed and is followed by a variable mixing time τ_m to allow substrate to exchange.

RESULTS

NMR observation of FucP membranes and bound substrates

Fig. 3 *A* shows a CP MAS spectrum recorded from the membranes with overexpressed FucP. Although the membranes are maintained in their "fluid" state, the spectrum shows strong contributions from the protein component, appearing as broad envelopes of intensity from groups with wide chemical shift dispersion. This is especially apparent in the spectral region 50–70 ppm, which mainly contains spectral intensity from the α-carbons of the proteins. The innate complexity of the natural abundance ^{13}C spectra from membranes makes it difficult to identify resonances in Fig. 3 *A* that arise exclusively from the induced FucP protein. However, some partly resolved resonances in the alkyl region between 25 and 30 ppm are not observable in CP MAS spectra from non-induced ("wild-type") membranes and can be attributed to alkyl side chain carbons of the FucP protein.

On adding L-[$^{13}C_6$]galactose, signals arising from a motion-restricted portion of this substrate are clearly identifiable in the spectral region, 90–100 ppm (Fig. 3 *B*). This corresponds to signal from ^{13}C in the C-1 position of the sugar which falls in a spectral region essentially free of natural-abundance ^{13}C contribution from the membranes (Fig. 3 *A*). Expanding this region (Fig. 3 *C*) clearly displays signals from both anomers at this position and, in spite of a large artificial line broadening (30 Hz) used in signal processing, it is even possible to resolve the doublet multiplicity in each, due to the J-coupling in this uniformly labeled substrate (~50 Hz coupling with C-2). D-[1-^{13}C]Arabinose is also detected with good sensitivity for both anomers (expanded spectrum, Fig. 3 *D*) and these resonances appear narrower with the absence of J-coupling effects in this single-labeled material. The dominant signal from C-1 in bound substrates appears at the higher frequency which corresponds to the anomer having the C-1 hydroxyl in *trans* configuration with respect to the C-2 hydroxyl in both cases. This assignment is trivial, as the C-1 anomers show no significant change in their chemical shift compared with their NMR observation in free solution, which have typical anomeric shifts (Levy et al., 1980). The spectral intensities detected from these anomers with the FucP membranes also agrees closely with the ratio of ~2:1 observed in equilibrated aqueous solutions of both substrates by direct ^{13}C observation. The similarity in spectral characteristics for

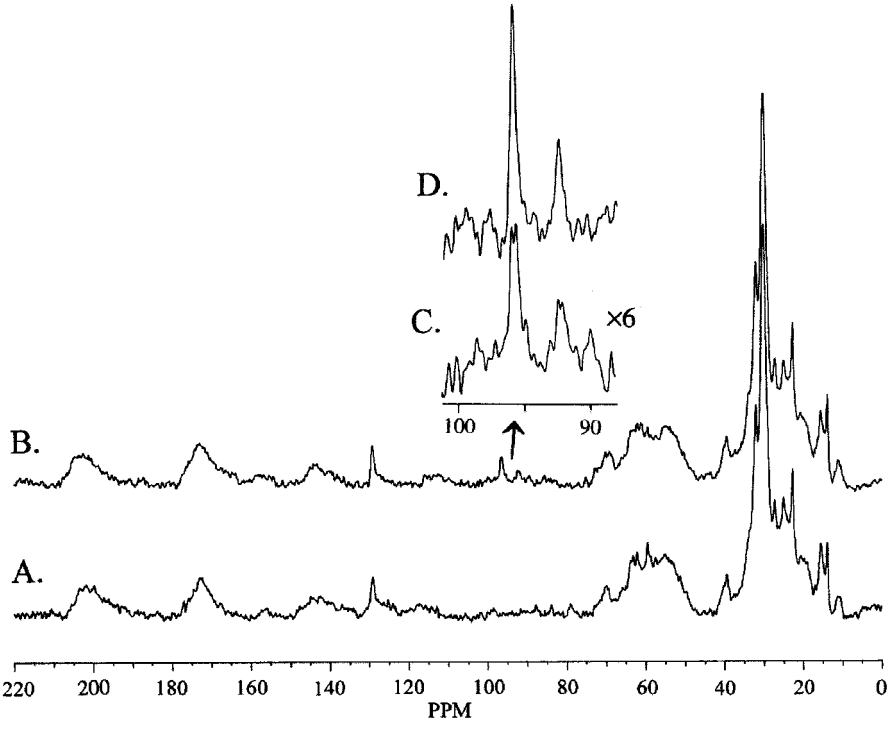

FIGURE 3 CP MAS ^{13}C spectra from FucP membranes (with ~0.2 μmol of FucP). Spectra recorded before (*A*) or after (*B*) incorporation of 1 μmol of L-[$^{13}C_6$]galactose (with 5 μmol of D-fucose). (*C*) Expanded region showing spectral intensity (×6 expansion) appearing from C-1 in bound L-[$^{13}C_6$]galactose or (*D*) D-[1-^{13}C]arabinose bound from adding 1 μmol of this labeled substrate with 3 μmol D-fucose to untreated membranes. All spectra were acquired at 2°C from 8000 accumulations, using a recycle time of 1 s and then processed using 30 Hz of line-broadening for exponential filtering.

free and bound substrate makes it especially important to show that the substrate detected with FucP membranes is selective only for the transport system and that its observation by NMR is not affected by exchange processes.

Specificity of substrate binding for FucP

All substrate binding was measured in the presence of three- to fivefold concentrations of unlabeled D-fucose, which is not a substrate for FucP. This non-transportable sugar was initially introduced to discourage any nonspecific binding of labeled substrate which might have been indistinguishable from the substrate binding selectively to FucP. However, D-fucose appeared to have little or no effect on the measurements and so its presence serves only to emphasize the selectivity for the FucP protein in the binding measured by NMR. This selectivity is further demonstrated by competitive binding with unlabeled transportable sugars as described below.

As shown from the expanded regions of ^{13}C CP MAS spectra in Fig. 4, the strong signal from D-[1-^{13}C]arabinose bound in the presence of excess D-fucose is depleted by sequential additions of the unlabeled substrate L-fucose (*spectra A–C*). L-Fucose, the native substrate, does not appear to be an especially potent competitor for binding to the protein and may bind at lower affinity than D-arabinose. Unlabeled L-galactose also inhibits the D-arabinose binding, as shown by the series of spectra in Fig. 4, *D–F*, but appears to be an even less effective competitive substrate than L-fucose. Further evidence that L-galactose is the weakest binding substrate of those studied is given in the spectra *G* and *H*, which show that L-[$^{13}C_6$]galactose binding in the presence of D-fucose appears completely blocked, with no detectable NMR signal, after adding an equimolar amount of L-fucose.

Substrate exchange and the dynamics of substrate bound to FucP

The membranes in their "fluid" state display a complex array of motional processes and spin-lattice relaxation is used to examine the prevalence of motions occurring within two quite separate time regimes. Relaxation in the laboratory frame is sensitive to motions with rates in the vicinity of the NMR observation (Larmor) frequency ($\omega_0 = 0.4$ GHz for protons), where we expect a minimum in the time constant, T_{1Z}, for this process. Relaxation in the rotating frame, with time constant $T_{1\rho}$, is mediated by fluctuations with rates comparable to the applied proton field strength (10^4-10^5 Hz). Following CP, relaxation is observed for protons in the vicinity of the detected ^{13}C nuclei, which takes advantage of the greater spectral resolution of this heteronucleus. Nonetheless, only for the enriched substrate is there the opportunity for measurements on individual sites or environments within these systems, due to the low sensitivity and complexity of other signals arising from the natural abundance ^{13}C contribution. Instead, relaxation information are gathered for the identifiable types of chemical groups in the membranes which, despite differing in their environments, do offer a general description of relaxation

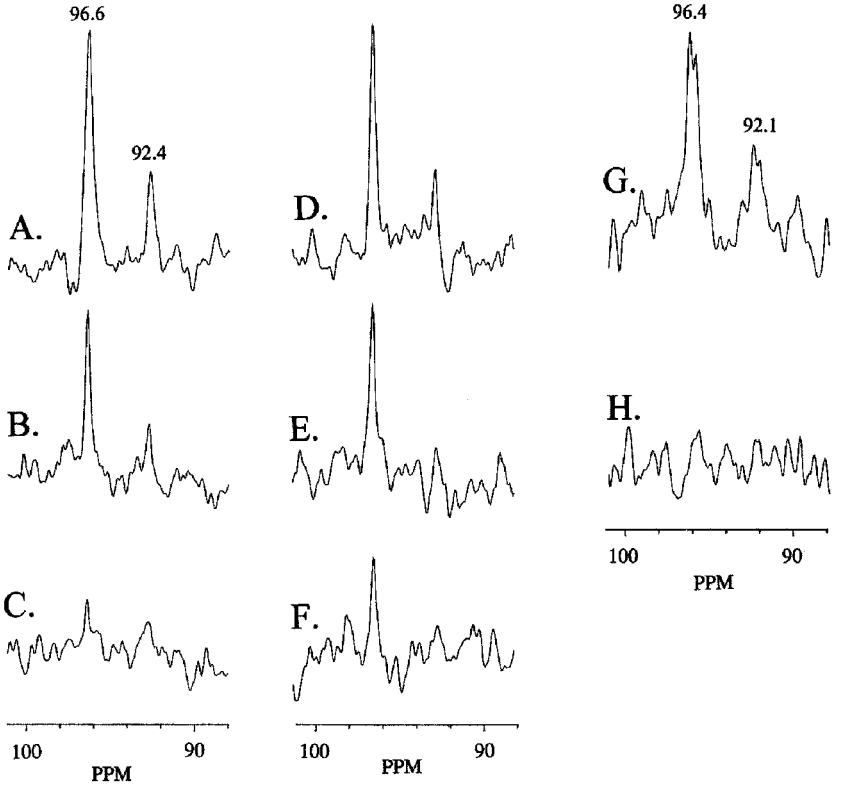

FIGURE 4 Effects of competitive binding on the CP MAS spectrum of labeled substrates bound to FucP membranes. (*A*) Expanded spectra from C-1 in bound substrate after adding 1 μmol of D-[1-^{13}C] arabinose with 3 μmol of unlabeled D-fucose to 0.2 μmol FucP and then (*B*) after additions of 1 μmol and (*C*) 2 μmol of L-fucose. (*D*) Spectra from bound substrate as for *A* but in the presence of 5 μmol of unlabeled D-fucose and then (*E*) after adding 1 μmol or (*F*) 2 μmol of L-galactose. (*G*) Expanded spectra from C-1 in L-[$^{13}C_6$]galactose bound from adding 1 μmol of this substrate to FucP membranes in the presence of 5 fold D-fucose and then (*H*) after adding 1 μmol of L-fucose. Chemical shift values in *G* correspond to midpoint of splittings. All spectra acquired and processed as for full spectra in Fig. 3, except for series *A–C* which were each recorded from 16,000 accumulations.

TABLE 1 Proton spin-lattice relaxation times in the laboratory (T_{1Z}) and rotating ($T_{1\rho}$) frames

	Membranes						
	CH_2	Cα	C═C	C═O	Bound arabinose	Crystalline arabinose	Solution arabinose
T_{1Z} (s)	0.7	0.7	0.4	0.8	0.9	213	2.1
$T_{1\rho}$ (ms)	4	5	6	5	8	378	104

Detected at the labeled 1-carbon in D-[1-^{13}C]arabinose and mean values measured at the major resolvable classes of natural abundance ^{13}C species in the FucP membranes as follows: CH_2, methylenes; Cα, α-carbons in the polypeptide backbones; C═C, lipid chain unsaturation; C═O, carbonyls. Solution measurements from 100 mM arabinose in D_2O.

processes occurring within the membranes and provides a useful reference against which the motion behavior of substrate can be compared. The rapid relaxation rates for bound arabinose, recorded in Table 1, show that it experiences a high density of motions in both regimes, in common with other groups associated with the membranes (Table 1). All relaxation monitored in the fluid membranes appeared to follow simple kinetics and is not thought to be significantly driven by nonspecific exchange processes (i.e., spin-diffusion). The extent to which the motions promote efficient spin-lattice relaxation in bound substrate is clear by comparing these data with the very slow relaxation for substrate in its rigid crystalline lattice (Table 1). The relaxation rates for bound arabinose fall at the lower end of the both ranges recorded for groups within the membranes but are much faster than for arabinose in solution (Table 1), whose motions are mostly more rapid than ω_0 (i.e., in the region of extreme narrowing). According to these relaxation data, the substrate detected by CP clearly reflects quite well the dynamic environment of the membranes and is quite distinct from that for substrate in free solution.

Following the destructive dephasing of the magnetization from membranes with D-[1-^{13}C]arabinose in a DDCP experiment, the recovery of spectral intensity by exchange or relaxation processes (see under Materials and Methods) was measured as a function of the mixing time and these data are displayed in Fig. 5. The recovery of NMR intensity from bound D-arabinose is shown in Fig. 5 to overlay all those for the non-exchangeable groups within the membranes. Otherwise, the overall profile appears to follow quite closely those for the membrane groups over the entire time course of this experiment and so the recovery of substrate magnetization is also expected to be dominated by the longitudinal spin-lattice relaxation processes that are shown in Table 1 to be quite similar. These data provide no indication that the overall recovery profile for bound substrate is accelerated by exchange between substrate bound to the transport system and substrate in free solution and this process is consequently assumed to be slow on this experimental time scale ($>10^{-1}$ s). Any attempt to account for the nominal differences in exchange and relaxation behavior of bound substrate, compared with other membrane groups, will require a discussion of dynamic processes more subtle than complete dissociation from the binding center in the protein, as included below.

DISCUSSION

The NMR methods used here were capable of directly observing the weak binding of sugar substrate to FucP protein in its native membranes with good sensitivity. According to the competitive binding experiments using labeled and non-labeled substrates and non-transportable stereoisomers, the methods detected sugar bound to FucP alone. L-Fucose and D-arabinose did not display any anomeric selectivity in their binding with FucP. This sensitivity

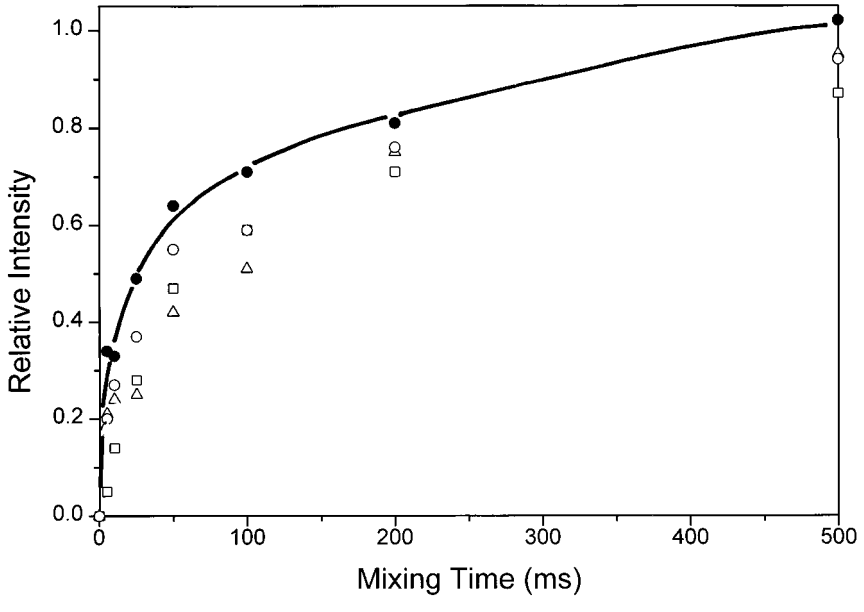

FIGURE 5 NMR spectral intensities as a function of mixing time in the DDCP experiment for (*closed circle*) bound D-[1-^{13}C]arabinose and the following major classes of membrane groups: alkyl CH_2 (*open square*); (*open triangle*) chain unsaturation and (*open circle*) amino acid α-carbons, Cα. Measured intensities are expressed as a ratio of that recorded under normal CP conditions without dephasing.

was due in large part to the narrow line shapes obtainable from these membranes in their "fluid" state. Line widths were close to the static field homogeneity (~15 Hz) routinely achieved for the instrumental magnet-probe combination used in this work. Such narrow line shapes are uncommon in solid state NMR measurements on solid materials, due to inhomogeneous broadening from structural heterogeneity in the solid samples, susceptibility effects and due to the difficulty in eliminating completely the strong homogeneous (Maricq and Waugh, 1979) type of dipolar interactions present in rigid systems (i.e., achieving complete decoupling of protons). These effects appear to be largely circumvented by studying these membranes in their native "fluid" (non-frozen) state. The most distinct properties of the fluid membranes, capable of affecting the NMR response in this way, was their much higher density of local motions compared with rigid systems, as evident in the efficient spin-lattice relaxation recorded for the membranes. These motions presumably help create a more homogeneous local magnetic environment, render homogeneous dipolar interactions more susceptible to conventional decoupling methods and provide for rapid relaxation recovery during the NMR measurement. Given our current appreciation of the molecular dynamics over short time trajectories in biological macromolecules (McCammon and Harvey, 1991), fast local motions can be assumed to offer an efficient source of relaxation recovery for nuclear spins, even without any contribution from overall molecular reorientation. Motions experienced by substrate in the binding site do not, however, seriously compromise its detection by CP which relies on strong dipolar interactions still being available between the protons and heteronuclear spins. Those modes of motion that would seriously interfere with the detection of substrate, such as overall molecular reorientation, therefore appear to be largely excluded.

Apart from severely attenuating the heteronuclear dipolar interactions, large amplitude reorientational motion is not well tolerated in MAS NMR experiments. A wide range of motion rates within the intermediate time scale (10^{-9}-10^{-4} s) can defeat the line narrowing effects of MAS (Haeberlen and Waugh, 1969), compromise the efficiency of proton decoupling (Frydman and Frydman, 1990) or at worse, induce complete dephasing of signal during sample rotation (Maricq and Waugh, 1979). These difficulties were not encountered with the "fluid" membrane systems studied here. Recent observations from electron spin-labels attached to membrane transport proteins, show no significant reorientational motion over this intermediate time scale, in the bacterial membrane expression systems at temperatures between 2 and 5°C (P. J. R. Spooner, W. J. O'Reilly, S. W. Homans, N. G. Rutherford, P. J. F. Henderson, and A. Watts, unpublished observations).

The substrate selectivity demonstrated here, first from binding observed in the presence of excess non-transportable stereoisomer and then from the competitive binding of other transportable substrates, shows that FucP is the target protein of the ligands. Despite this binding selectivity, the bound substrate detected always appeared to be a small fraction of the total substrate recorded by direct ^{13}C observation on these systems (not shown), confirming that the substrates bind only weakly to the transport system. A very weak binding of substrate should not be regarded as unfavorable to its translocation in the overall symport process. On the contrary, a weak binding best describes the process of solvent substitution required to precede translocation via the "moving-barrier" mechanism, as proposed for these "secondary translocators" (Mitchell, 1990). This mechanism requires that secondary hydration bonds with solution substrate can be substituted by a network of hydrogen bonds within the binding center at little or no energy expense, a condition more likely to be satisfied by substrate that appears to associate only weakly with the protein. FucP is concluded to bind all its transportable substrates very weakly, as required for efficient translocation under a "mobile-barrier" mechanism.

Consistent with specificity in the binding, but not implicit for a weak binding process, is the finding that substrate exchanges slowly with the transport system ($>10^{-1}$ s) and that its relaxation characteristics resemble those within the membrane environment. This is interpreted as showing that substrate is restrained within a slow translocation process and, importantly, this allows exclusive observation of substrate within the binding site on the "slow" NMR time scale. The manner in which bound substrate was shown to mimic the membrane environment in these experiments was sufficient to exclude interference from complete substrate exchange or nonspecific binding processes. It is possible, however, that the data reveal that substrate can partake in more subtle dynamic processes occurring within the binding site. For instance, a high local mobility for bound substrate compared with its environment within the protein would resist dephasing of its NMR signal in the DDCP experiment and result in its recovery profile being displaced above those for other membrane groups, as shown in Fig. 5. As little as 10–15% of the signal from associated substrate remaining after the dephasing period, would be enough to account for the differences in its recovery profile shown in Fig. 5, compared with the other groups detected within the membrane. Such a low initial intensity would not, however, be distinguishable from the instrumental noise at these measuring sensitivities.

A degree of motion uncoupling of substrate within the binding site would also be consistent with the moderate differences observed in its relaxation behavior compared with other membrane groups, especially in the rotating-frame relaxation which provides a sensitive measure for association with membranes in their fluid state (Cornell et al., 1983; Spooner et al., 1993). Recent observations on the brush border Na^+-glucose cotransporter provide evidence that the transport of water across membranes is coupled to the movement of solutes by symport proteins and that this plays an important role in the absorption of water by epithelial membranes (Loo et al., 1996). None of the observations reported here are inconsistent with sugars being trans-

ported in a well hydrated binding center in the transport system. The lack of chemical shift perturbation on binding could reveal opportunities for the weakly binding substrate to cycle between solvent substitution and rehydration while remaining within the binding site. These fluctuations in substrate interactions can be of the order anticipated in the above discussion concerning the nominal differences in exchange and relaxation behavior and does not implicate any overall molecular reorientation which appears to be largely excluded.

An important goal for this work would be to exploit the NMR capability demonstrated here for probing the chemical and structural environment of the binding center in several sugar-H^+ symport proteins as well as other membrane transport proteins. With the resolution available here, it may already be possible to estimate torsional angles from J-couplings between multiple labels within ligands that are restrained within the protein. This would facilitate a structural analysis of those ligands that can conform to the binding site geometry. The use of spectral editing via the substrate nuclei has also been used to good effect in some of the most complex proteins observed by high-resolution, solution state NMR (Derrick et al., 1992; Lian et al., 1994). The anisotropic features of membrane systems and their amenability to some solid-state NMR methods as demonstrated here, can open up many more diverse opportunities for detecting and quantifying these interactions by various structural methods (Watts et al., 1995), of which rotational resonance (Levitt et al., 1990) and rotational-echo double resonance (Gullian and Schaefer, 1989) are the best known. The advantages of studying functional systems in their native "fluid" (non-frozen) state are clear for the current observations, although it should be recognized that many of the established solid state NMR methods referred to above are not adapted for such dynamically complex systems, especially if substrate or its interactions can exercise a high degree of motional freedom within the binding site of the membrane transport systems.

This work was supported by Biotechnology and Biological Science Research Council Grant GR/H5512, the Wellcome Trust, The Royal Society, and European Communities Grant BI04 CT96 0129).

REFERENCES

Bradley, S. A., C. R. Tinsley, J. A. R. Muiry, and P. J. F. Henderson. 1987. Protein-linked L-fucose transport in *Escherichia coli. Biochem. J.* 248: 495–500.

Cornell, B. A., R. G. Hiller, J. Raison, F. Separovic, R. Smith, J. C. Vary, and C. Morris. 1983. Biological membranes are rich in low-frequency motions. *Biochim. Biophys. Acta*. 732:473–478.

Derrick, J. P., L.-Y. Lian, G. C. K. Roberts, and W. V. Shaw. 1992. Analysis of the binding of 1.3-diacetyl chloramphenicol to chloramphenicol acetyltransferase by isotope-edited ^{1}H NMR and site-directed mutagenesis. *Biochemistry*. 31:8191–8195.

Frydman, L., and B. Frydman. 1990. A line shape analysis of the effects introduced by motions in the nuclear magnetic resonance spectra of decoupled systems. *J. Chem. Phys*. 92:1620–1626.

Gullian, T., and J. Schaefer. 1989. Detection of weak heteronuclear dipolar couplings by rotational-echo double resonance nuclear magnetic resonance. *In* Advances In Magnetic Resonance, edited by Warren, W. S. Academic Press, New York. 57–83.

Gunn, P. J., C. G. Tate, and P. J. F. Henderson. 1994. Identification of a novel sugar-H^+ symport protein, FucP, for transport of L-fucose into *Escherichia coli. Mol. Microbiol.* 12:799–809.

Gunn, P. J., C. G. Tate, C. E. Samson, and P. J. F. Henderson. 1995. Topological analyses of the L-fucose-H^+ symport protein, FucP, from *Escherichia coli. Mol. Microbiol.* 15:771–783.

Haeberlen, U., and J. S. Waugh. 1969. Spin-lattice relaxation in periodically perturbed systems. *Phys. Rev*. 185:420–429.

Henderson, P. J. F. 1993. The 12-transmembrane helix transporters. *Curr. Opin. Cell Biol*. 5:708–721.

Henderson, P. J. F., and A. J. S. Macpherson. 1985. Assays, genetics, and reconstitution of proton-linked galactose, arabinose, and xylose transport systems of *Escherichia coli. Methods Enzymol.* 125:387–429.

Levitt, M. H., D. P. Raleigh, F. Creuzet, and R. G. Griffin. 1990. Theory and simulations of homonuclear spin pair systems in rotating solids. *J. Chem. Phys*.92:6347–6364.

Levy, G., R. L. Lichter, and G. L. Nelson. 1980. Carbohydrates. *In* Carbon-13 Nuclear Magnetic Resonance Spectroscopy. Wiley. New York. 279–287.

Lian, L. Y., I. L. Barsukov, M. J. Sutcliffe, K. H. Sze, and G. C. K. Roberts. 1994. Protein-ligand interactions: exchange processes and determination of ligand conformation and protein-ligand contacts. *Methods Enzymol*. 239:657–700.

Loo, D. D., T. Zeuthen, G. Chandy, and E. M. Wright. 1996. Cotransport of water by the Na^+/glucose cotransporter. *Proc. Natl. Acad. Sci. USA*. 93:13367–13370.

McCammon, J. A., and S. C. Harvey, 1991. *Dynamics of proteins and nucleic acids*, Cambridge University Press.

Maricq, M. M., and J. S. Waugh. 1979. NMR of rotating solids. *J. Chem. Phys*. 70:3300–3316.

Mitchell, P. 1990. Osmochemistry of solute translocation. *Res. Microbiol*. 141:286–289.

Muiry, J. A. R., T. C. Gunn, T. P. McDonald, S. A. Bradley, C. G. Tate, and P. J. F. Henderson. 1993. Proton-linked L-rhamnose transport, and it comparison with L-fucose transport in *Enterobacteriaceae. Biochem. J.* 290:833–842.

Schaefer, J., E. O. Stejskal, and R. Buchdahl. 1977. Magic-angle ^{13}C NMR analysis of motion in solid glassy polymers. *Macromolecules*. 10: 384–405.

Schaffner, W., and C. Weissmann. 1973. A rapid, sensitive, and specific method for the determination of protein in dilute solution. *Anal. Biochem*. 56:502–517.

Spooner, P. J. R., A. A. Duralski, S. E. Rankin, T. J. T. Pinheiro, and A. Watts. 1993. Dynamics in a protein-lipid complex: nuclear magnetic resonance measurements on the headgroup of cardiolipin when bound to cytochrome *c. Biophys J.* 65:106–112.

Spooner, P. J. R., N. G. Rutherford, A. Watts, and P. J. F. Henderson. 1994. NMR observation of substrate in the binding site of an active sugar-H^+ symport protein in native membranes. *Proc. Natl. Acad. Sci. USA.* 91:3877–3881.

Sullivan, M. J., and G. E. Maciel. 1982. Spin dynamics in the carbon-13 nuclear magnetic resonance spectrometric analysis of coal by cross polarization magic-angle spinning. *Anal. Chem*. 54:1615–1623.

Walmsley, A. R., G. E. M. Martin, and P. J. F. Henderson. 1994. 8-Anilino-1-napthalenesulfonate is a fluorescent probe of conformational changes in the D-galactose-H^+ symport protein of *Escherichia coli. J. Biol. Chem.* 269:17009–17019.

Watts, A., A. S. Ulrich, and D. A. Middleton. 1995. Membrane protein structure: the contribution and potential of novel solid state NMR approaches (review). *Mol. Membrane Biol.* 12:233–246.