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NMR observation of substrate in the binding site of an active sugar-H^+ symport protein in native membranes

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ABSTRACT **NMR methods have been adopted to observe directly the characteristics of substrate binding to the galactose-H^+ symport protein GalP, in its native environment, the inner membranes of *Escherichia coli*. Sedimented inner-membrane vesicles containing the GalP protein, overexpressed to levels above 50% of total protein, were analyzed by ^{13}C magic-angle spinning NMR, when in their normal "fluid" state and with incorporated D-[1-^{13}C]glucose. Using conditions of cross-polarization intended to discriminate bound substrate alone, it was possible to detect as little as 250 nmol of substrate added to the membranes containing about 0.5 μmol (≈26 mg) of GalP protein. Such high measuring sensitivity was possible from the fluid membranes by virtue of their motional contributions to rapid relaxation recovery of the observed nuclei and due to a high-resolution response that approached the static field inhomogeneity in these experiments. This good spectral resolution showed that the native state of the membranes presents a substrate binding environment with high structural homogeneity. Inhibitors of the GalP protein, cytochalasin B and forskolin, which are specific, and D-galactose, but not L-galactose, prevent or suppress detection of the ^{13}C-labeled glucose substrate, confirming that the observed signal was due to specific interactions with the GalP protein. This specific substrate binding exhibits a preference for the β-anomer of D-glucose and substrate translocation is determined to be slow, on the 10^{-2} s time scale. The work describes a straightforward NMR approach, which achieves high sensitivity, selectivity, and resolution for nuclei associated with complex membrane proteins and which may be combined with other NMR methodologies to yield additional structural information on the binding site for the current transport system without isolating it from its native membrane environment.**

X-ray crystallography has so far only succeeded in providing three-dimensional structures for two classes of membrane proteins: the photosynthetic reaction centers (1, 2) and the porins (3, 4), both from bacterial sources. Opportunities for using NMR, the major noncrystallographic approach to resolving three-dimensional structures, to analyze complete integral membrane proteins in their native or membrane-like environment are presently confined to the solid state methods. Such methods promise to provide a rich source of information for the complex macromolecules in these motionally anisotropic environments (5, 6). Magic-angle spinning (MAS) is frequently employed to generate the spectral resolution required in these methods, to distinguish resonances from individually labeled sites, which may then be used to yield very limited but rather precise information on spatial geometry in specific segments of the macromolecule (7, 8).

The major factor restricting a more general application of these solid state NMR methods is their requirement for large quantities of proteins (generally tens of milligrams) for which some means is available to introduce nonperturbing labels at appropriate sites within these macromolecules. The system studied in the current work, GalP, the galactose-H^+ symport protein from *Escherichia coli*, represents an ideal candidate in this regard since its expression in native membranes has been amplified to very high levels using a plasmid containing the *galP* gene expressed under the control of its own promoter (P. E. Roberts & P.J.F.H., unpublished data), which should allow practically unlimited manipulation of the amino acid sequence of the protein. Furthermore, this system represents an important class of membrane proteins (9), which has yet to be addressed by any of the more direct methods of structural analyses.

GalP is closely related to many sugar transport systems occurring in the higher organisms, including man (9). This structural homology was first recognized between sugar transporters in *E. coli* and human tissue (10) and remarkably has since been extended to define a superfamily of >50 diverse facilitator and porter systems (11). GalP has a molecular mass of 50,950 and from its sequence can be predicted to adopt the 6+6 helix topology (12), in common with a wide variety of transport systems (11). Substrate translocation is believed to occur via an oscillating conformation of the transporter, a concept originally referred to as the "mobile barrier" mechanism (13, 14). Therefore, apart from providing structural information, analytical methods should ideally be capable of evaluating the motional characteristics of the macromolecule in response to complexation with substrate, so as to arrive ultimately at some functional mechanism integrating structural and dynamic information. Solid state NMR is well suited to such a broad application, although these methods have invariably been applied to biological systems that were converted to a rigid or frozen state so as to allow efficient application of cross-polarization (CP) for enhancing measuring sensitivity (15) or to suppress motional interference at individually labeled sites. In previous studies, however, we have advocated that maintaining the native "fluid" state of membrane systems for MAS studies can reveal important dynamic features of the constituent molecules, as demonstrated for model membrane systems (16) and protein–lipid complexes (17). Thus, retaining the fluid state of the membrane, although not generally favorable for applying solid state NMR methodology, can provide opportunities to investigate biological macromolecules in their functioning state. The current work seeks to establish the feasibility of such studies on fluid membrane systems, whether, in the first instance, measuring sensitivity is adequate to detect specific substrate binding to the protein. Substrate binding not only reports on measuring sensitivity but also gives information on

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Abbreviations: MAS, magic-angle spinning; CP, cross-polarization; T_{1z}, spin-lattice relaxation time in the laboratory frame; $T_{1\rho}$, spin-lattice relaxation time in the rotating frame.

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the structural and dynamic characteristics of complex formation and translocation.

MATERIALS AND METHODS

Membrane Preparations and Substrates. *E. coli* strain JM1100 (pPER3) was grown in liquid medium with minimal salts (18) supplemented with glucose (27 mM), L-histidine (80 μg/ml), and thymine (20 μg/ml) and containing tetracycline (15 μg/ml). Inner-membrane vesicles were prepared from the genetically engineered organisms by explosive decompression in a French press and isolated by sucrose density ultracentrifugation. Membrane vesicles were normally resuspended in 20 mM Tris·HCl buffer at pH 7.5 and sedimented once using ultracentrifugation before final resuspension in the same buffer and stored at −80°C following rapid freezing. Total protein concentration in the vesicle suspension was determined by the method of Schaffner and Weissmann (19), and GalP was estimated to comprise between 50% and 55% of the total membrane protein from densitometry measurements on the proteins resolved by SDS/polyacrylamide gel electrophoresis and stained with Coomassie blue. NMR analyses were conducted on membrane pellets prepared by ultracentrifugation at $10^5 \times g$ for 1 hr and were maintained throughout these and all subsequent manipulations at temperatures between 0°C and 4°C.

D-[1-^{13}C]Glucose was supplied by MSD Isotopes in its anhydrous crystalline form. All other substrates and inhibitors of sugar transport used in this work were obtained from Sigma. Sugar substrates were added directly to the membrane pellets as small volumes (<15 μl) of solution in the buffer and allowed to equilibrate at 4°C for 30 min following brief mixing, prior to NMR measurement.

NMR Methods. MAS NMR was carried out at 100.63 MHz for the ^{13}C nuclei (400.13 MHz for protons) using a Bruker (Billerica, MA) MSL spectrometer and Bruker double-bearing MAS probeheads for 7-mm sample rotors. A spinning speed of 3.0 kHz was used for all samples and their temperature was controlled by the probe bearing air, maintained at a temperature of 2°C. A proton field strength of around 50 kHz was applied for CP and for decoupling protons in samples of the solid substrate but was attenuated by 60% for decoupling in membrane samples. The field strength applied to the ^{13}C spins was adjusted to satisfy the Hartmann–Hahn condition for CP from protons in the samples and yielded a $\pi/2$ pulse width of 5–7 μs. These power levels and pulse widths were also used for making observations by direct irradiation of ^{13}C spins. Proton spin-lattice relaxations in the laboratory and rotating frames were measured by employing CP to accomplish detection on the resolved ^{13}C resonances as described (20, 21). These methods will generally yield relaxation rates for protons that are directly bonded to the detected carbon sites (20, 21).

RESULTS AND DISCUSSION

Solid Substrate. A MAS spectrum obtained from crystalline D-[1-^{13}C]glucose by direct irradiation of the ^{13}C nuclei (without CP) is shown in Fig. 1*A* to consist of two major components, corresponding to the two anomeric configurations existing for the labeled 1-position. The downfield component (95.6 ppm) is assigned to the β-form, following previous observations by solution state NMR (22), and has the configuration shown in Fig. 1*B*. The small resonance appearing downfield of the β component is assumed to represent an alternative form of the solid substrate, possibly a hydrate. The differing spectral intensities measured for the two anomeric forms under these conditions are due to differences in their spin-lattice relaxation rates. This is also evident from the time constants for proton relaxation in the laboratory

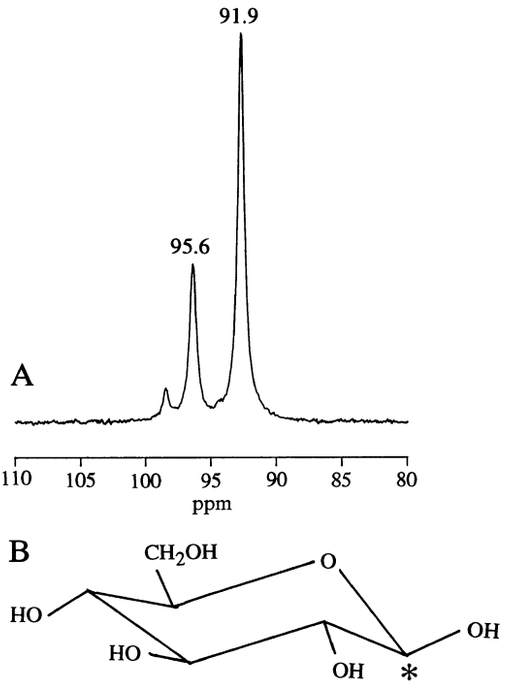

FIG. 1. (*A*) Proton-decoupled ^{13}C MAS spectrum from the crystalline D-[1-^{13}C]glucose showing resonances from the labeled position in the α (91.9 ppm) and β (95.6 ppm) configuration. The spectrum was recorded from 64 acquisitions using a recycle delay of 3 s. (*B*) Configuration at the 1-position (asterisk) in the β-anomeric state. The α-form has the alternative orientation of the 1-hydroxyl, toward the same side of the ring as the adjacent 2-hydroxyl.

frame, T_{1z}, detected at position 1 in the crystalline material, entered in Table 1. Such lengthy time constants testify to the high stability of the pyranoside ring, particularly in the β-configuration, which restricts motions of higher frequencies ($\approx 10^{-9}$ s) that influence T_{1z}. Motions are also restricted in the low-frequency realm ($\approx 10^{-5}$ s) that determine $T_{1\rho}$ for relaxation in the rotating frame of the spin-locking field used for CP.

These rates of relaxation recorded for solid substrate are too slow to allow effective detection of substrate, even with CP, at the low levels expected from binding to GalP in the current study (see below), perhaps rendering any observation in the solid state impracticable. Opportunities for analysis in the solid state are also precluded by the nature of sugar binding with the GalP protein, described below.

Substrate Binding. The dissociation constants given in Table 2 reveal only weak binding for the sugar substrates, D-galactose and D-glucose, to the membrane vesicles (ref. 23; A. R. Walmsley and P.J.F.H., unpublished data). Since the

Table 1. Proton spin-lattice relaxation times in the laboratory (T_{1z}) and rotating ($T_{1\rho}$) frames, detected at the labeled 1-carbon for both anomers in crystalline D-glucose and for the β-anomer bound to GalP membranes

Relaxation time	Crystalline D-glucose		Membrane					
	α	β	Bound glucose (β)	CH_2	Cα	C=C	Cφ	C=O
T_{1z}, s	108	255	0.6	0.2	0.6	0.5	0.2	0.5
$T_{1\rho}$, ms	150	150	6	4	6	5	5	4

Mean values of net proton relaxation detected at various carbon sites within the membrane molecules are as follows: CH_2, methylenes; Cα, α-carbons in the polypeptide backbones; C=C, lipid chain unsaturation; Cφ, aromatic protein side chains; C=O, carbonyls.

Table 2. Dissociation constants (K_d), determined for equilibrium binding of substrates and inhibitors used in the current study, to GalP in inner-membrane vesicles

Substrate	K_d, mM
D-Galactose	9
D-Glucose	0.5
Cytochalasin B	0.002–0.006
Forskolin	0.002

Data were obtained from competitive binding assays with cytochalasin B or forskolin (23–25).

pellets of membrane vesicles prepared for NMR analysis remain quite expanded, having an aqueous content in excess of 80% by weight, then a significant proportion of sugar substrate will remain unbound in these preparations. In contrast to conventional useage, CP is applied in the current study to distinguish the fraction of substrate immobilized by binding with the membranes. To allow this, preparations must remain in the fluid state (i.e., above 0°C) so that, due to motional averaging of dipolar interactions within free solution substrate, CP should then be incapable of detecting the nonassociated fraction. However, it can by no means be assumed, *a priori*, that CP will be effective for substrate bound to proteins within the native fluid state of the membranes. Neither can it be predicted that the relaxation behavior of bound substrate in this state will be particularly favorable for its detection by NMR. For these reasons, the maintenance of membranes in their native fluid state, although largely dictated here by the unfavorable binding characteristics of substrate and necessary for studying the native functional state, might normally be expected to impede or even defeat the application of conventional solid state NMR methods such as CP MAS. Nonetheless, the analysis reported below was conducted using operating conditions quite typical for observations on rigid systems.

NMR Characteristics for the Fluid State Membranes. The CP MAS NMR spectrum recorded from membrane vesicles containing 0.5 μmol (26 mg) of GalP protein is shown in Fig. 2*A*. The spectral region between 90 and 100 ppm, expected to accommodate resonances from the substrate, is shown to be essentially free of contributions from natural abundance ^{13}C nuclei in the membranes. As D-[1-^{13}C]glucose is titrated into the membrane pellet, the CP signal from this substrate emerges in the anticipated spectral region as shown in Fig. 2*B*. A few hours of accumulation time was found to be sufficient to detect as little as 250 nmol of added substrate, the lowest amount incorporated for analysis, as shown by the expanded region C in Fig. 2. The resonances detected from substrate increase progressively in intensity as more of this labeled material is incorporated into the sample, up to the maximum amount added of 1 μmol (2 equivalents with respect to GalP protein), as shown in the expanded spectral region D in Fig. 2. The spectrum of substrate recorded at these levels exhibits a good lineshape and resolution between resonances for the two anomeric forms, which in this instance is dominated by the downfield contribution from the β-form. Chemical shifts of the detected resonances are slightly downfield from those recorded from the solid substrate (compare with Fig. 1*A*) and indistinguishable from those recorded in aqueous solution (not shown). No substrate binding could be detected in this way to membrane vesicles from the organisms not expressing GalP: *E. coli* strain JM1100 (pBR322), but having an approximately equivalent total amount of membrane protein exposed to 1 μmol of labeled glucose. The detected signal was therefore associated with expression of the GalP protein.

The high sensitivity for detection of bound substrate demonstrated in these CP MAS NMR measurements can be partly attributed to the favorable relaxation characteristics of substrate combined with the fluid membrane samples. Spin-lattice relaxation in the laboratory frame was rapid for the protons in bound D-glucose and similar to that estimated for various classes of sites identified within the constituent membrane molecules, as shown from the relaxation data (T_{1z}) from membrane samples included in Table 1. The much faster spin-lattice relaxation for the substrate bound to membrane preparations compared with its crystalline state is due to it acquiring a high density of fast (approximately nanosecond) motions from its site(s) of binding on the membranes. The uniformity and efficiency of rotating-frame relaxation indicated by the $T_{1\rho}$ values for all sites within the membrane (Table 1) are in agreement with suggestions made by us (26) and others (27) that this process is dominated by "slow" collective molecular motions that are transmitted to all components within natural membranes and protein–lipid systems. Despite bound substrate being influenced by high densities of motions within both fast and slow regimes, the overall motions in this environment remained sufficiently restricted to allow rapid polarization transfer via the proton–carbon dipolar interactions. Apart from not compromising the CP process, the overall fluctuations in the binding envi-

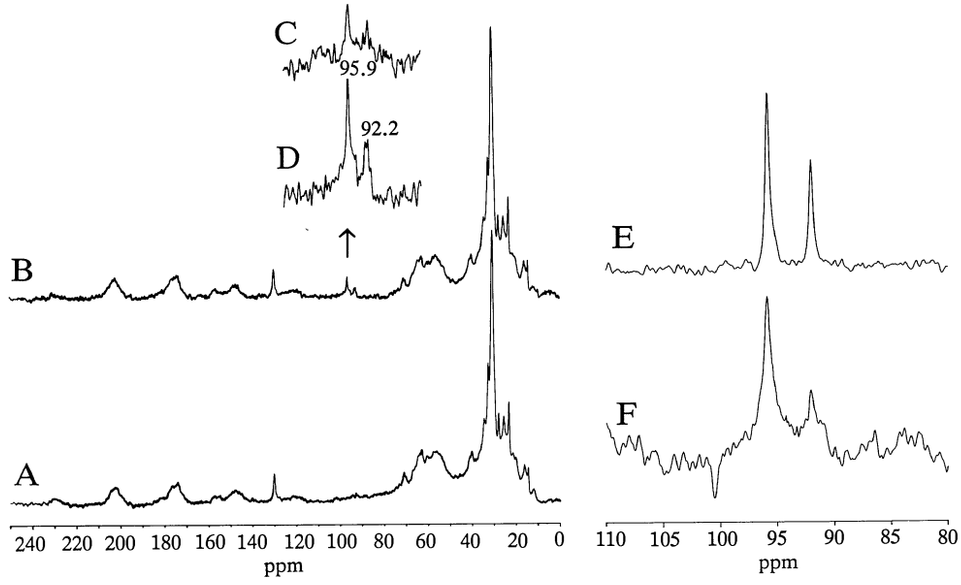

FIG. 2. Proton-decoupled ^{13}C CP MAS spectra from inner membranes containing 0.5 μmol (≈26 mg) of GalP (*A*) and with D-[1-^{13}C]glucose incorporated (*B*). Expanded regions show resonances detected from bound substrate following additions of 0.25 (*C*) and 1.0 (*D*) μmol of D-[1-^{13}C]glucose to the membranes. Spectra were recorded using a 1-ms contact time for CP and a 3-s recycle delay and were averaged from 8000 acquisitions. Other expanded regions show resonances detected following the addition of 1 μmol of substrate from a solution containing the β- and α-anomers at a ratio of 1.8:1, by direct single-pulse excitation of carbon spins (no CP) from 2400 acquisitions and a 6-s recycle time (*E*) or from the conventional CP sequence as used previously, but with a 2-s recycle delay (*F*).

ronment of the substrate also appear not to fall within a motional regime that can inopportunely defeat the MAS averaging. Since rotating-frame relaxation in this system can be considered a property of the membrane environment and disregarded for solution substrate, the dynamics of this process is therefore highly sensitive to the state of substrate association. Consequently, the fact that the detectable substrate faithfully reproduces the collective relaxation characteristics of the membrane constituents confirms not only that the method discriminates only bound substrate but also that this fraction appears to remain bound on the millisecond time scale of this observation. Another feature that made an important contribution to the sensitivity of the measurements is the narrow linewidths obtainable from membranes in the fluid state. Linewidths observed for the bound substrate could be as low as 15 Hz, close to the field inhomogeneity permitted in these experiments. This contrasts strikingly with linewidths observed from membrane samples analyzed in the solid (frozen) state, which have been reported to be as great as 150 Hz (28). Maintaining the membranes in their native fluid state evidently preserves the structural homogeneity of the binding environment for the substrate.

Substrate Recognition and Translocation. The anomeric mixture of D-glucose mutarotates toward the more stable β-anomer in aqueous solution. The ratio of β- to α-anomer was determined by solution state NMR to be 1.8:1 in a buffer solution equilibrated at 2°C for 48 hr. Direct, nonselective single-pulse observation on this sample when incorporated into the membrane preparation showed that substrate binding did not significantly perturb this anomeric equilibrium for total substrate, as indicated by the resonance intensities shown in Fig. 2*E*. However, the CP spectra from this sample shown in Fig. 2*F*, as well as in all other samples examined, reveal a greater spectral contribution from the β-form, illustrating a preference for binding of this anomer with the protein. In this regard, GalP behaves similarly to the mammalian glucose transporter, which was deduced (29) to recognize the β configuration at the 1-position, based on its binding of glucose analogues with nonmutarotating substituents (methyl ester; fluoro-) in this position. The current observations arrive at a similar conclusion for substrate recognition by the GalP protein, in a more direct, less-perturbing way, although results here do indicate a measurable affinity for the α-form of the substrate.

Fig. 3 *A–C* shows that pretreatment with inhibitors of sugar transport effectively prevents (Fig. 3*B*; forskolin) or, when used below saturation binding levels (Fig. 3*C*; cytocholasin B), suppresses detection of D-glucose that was subsequently incorporated into the membrane preparations. These results, using equivalent or reduced mol ratios of inhibitor to substrate, are consistent with the much stronger association reported for inhibitor binding to GalP protein in Table 2 (23–25), compared to the sugar substrates and provide further confirmation that only selectively bound substrate is detected under the conditions of CP used here. Stereospecificity in the detected sugar binding is illustrated by the spectra in Fig. 3 *D–F*, which show that measurement of bound glucose is unaffected by the addition of a 10-fold greater mol ratio of L-galactose (Fig. 3*E*) but is strongly attenuated by addition of D-galactose (Fig. 3*F*) at these concentrations. The residual binding of D-glucose detected in competition with this molar excess of D-galactose is in accordance with the relative binding affinities measured for these two sugars (Table 2). In these selectivity studies, the degree of substrate binding observed by the NMR methods is characteristic for binding to the GalP symport system alone in the native membranes.

The CP and relaxation results all demonstrate that the specific substrate–carrier center complex exists for a lifetime that is long on the experimental time scale (milliseconds). Attempts were made to measure the overall rate of substrate exchange directly within a "delayed-CP" experiment (P.J.R.S., unpublished method), in which signal from bound substrate is first allowed to decay so that newly exchanged substrate can be monitored as a function of time. Recovery of intensity from bound substrate in these experiments was not significantly faster than expected from transverse relaxation processes and thus exchange of substrate with the carrier center within the protein under these conditions was also deduced to be slow over this extended time scale (10^{-2}–10^{-1} s).

This slow exchange of bound solute might appear surprising considering the weakness of the interaction indicated in the equilibrium binding measurements (Table 2). However both phenomena are reconcilable under the "solvation-substitution" and "mobile-barrier" mechanism proposed for solute translocation in the family of membrane porter proteins (14). In this model, the substrate–carrier center complex is alternately exposed to both sides of the membrane by a conformational fluctuation involving a portion or portions of the polypeptide chain moving in either direction across this site, as schematically represented in Fig. 4. These transport proteins have been classified as "secondary translocators" since complex formation involves exchange of secondary hydration bonds with a network of hydrogen bonds within the carrier center, rather than involving any catalyzed breaking of primary chemical bonds (14, 30). Solvent substitution is

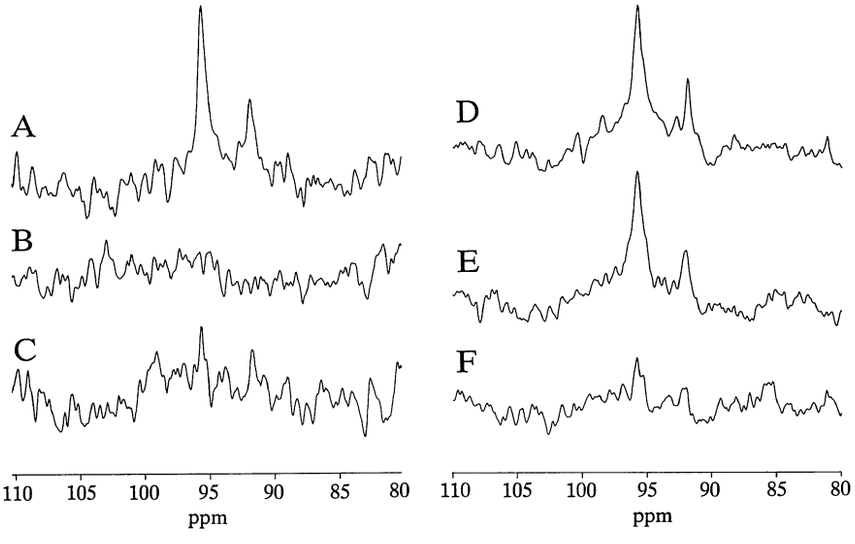

FIG. 3. Expanded regions of the CP MAS spectra indicating the extent of substrate binding (from 1 μmol added) to normal, untreated membranes (*A*) and following pretreatment of membranes with 1 μmol (2 equivalents with respect to GalP) of forskolin (*B*) and 0.55 μmol of cytochalasin B (*C*). At the sample volumes used for pretreatment with inhibitors, this amount of cytochalasin B should provide 90% occupancy of substrate binding sites on GalP; based on a K_d of 5 μM for this inhibitor. Inhibitors were incorporated as ethanolic solutions and both pretreated samples as well as the untreated sample all contained the same final concentration (1% by weight) of this solvent. Detection of bound D-glucose following the addition of 1 μmol to membranes vesicles (*D*) followed by 10 μmol of L-galactose (20 equivalents with respect to GalP) (*E*) and then 10 μmol of D-galactose (glucose-free) (*F*). All conditions for CP MAS measurements were as described for Fig. 2, but using a 1-s recycle delay.

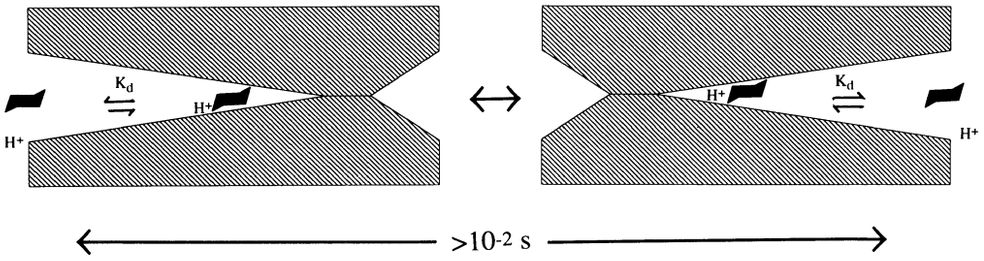

FIG. 4. Schematic representation of the mobile-barrier mechanism of solute translocation with kinetic information appropriate to the conditions of analysis used in this work.

required to occur with an approximately equivalent standard free energy for bonds formed in the carrier center (14). This condition is consistent with the weak binding observed for the sugars, especially for the native substrate, D-galactose (Table 2), and possibly also accounts for the lack of significant chemical shift changes observed on binding the labeled substrate. Thus, we ascribe the lengthy immobilization of substrate observed here directly to the conformational changes induced on binding with the carrier center and the coupled translocation process. Solute binding itself may involve some slow process of deformation or conformational adjustment in the carrier center before complete solvent substitution is accomplished. Some justification for this statement is contained in a separate observation that significant substrate binding can still be detected at elevated pH (8.5) where the proton electrochemical driving force for translocation is depleted (data not shown). The kinetic information incorporated into the schematized representation of the mechanism in Fig. 4 acknowledges that the solute association step may make some contribution to the kinetics of the overall process. Opportunities now exist for elucidating these kinetic characteristics under a larger variety of experimental conditions that are perhaps even more relevant to the biological functioning of the transport system.

In conclusion, the work reveals a straightforward approach that can be combined with site-specific labeling for effective NMR observation of complex membrane proteins in their native state. An essential prerequisite to allow direct observations on whole membrane preparations is that their content of the target protein be strongly augmented above normal native levels by means of genetic amplification. The NMR methods could then be shown to achieve (*i*) good sensitivity by exploiting favorable relaxation rates and from preserving the native structural homogeneity and thus spectral resolution provided by the fluid membranes; (*ii*) discrimination of the bound fraction of weakly binding substrates, leading to information on the structural selectivity for binding with the carrier center; and (*iii*) access to dynamic information on conformational events associated with solute binding and translocation.

All of the above characteristics of high sensitivity and resolution as well as selectivity in the substrate binding combine favorably to encourage extensions of these NMR measurements to pursue more detailed molecular information on the nature of the binding domain. This should not necessarily be restricted to methods developed from the field of solid state NMR. The high-resolution characteristics of the NMR response may enable access to some of the many NMR methods used for structural elucidation of macromolecules in the solution state. Within such "global" NMR approaches involving multiple or uniform heteronuclear enrichment within the protein, it will be necessary to adopt some means of editing or selecting the information required from such complex systems. Toward this end, the current study indicates that it may be possible to exploit the selectivity of substrate binding as an NMR "filter" to confine such observations to sites solely within the substrate binding domain of the protein.

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