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Investigating transport proteins by solid state NMR

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Abstract Transporters form an interesting and complex class of membrane proteins. Many of them are potential drug targets due to their role in translocation of ions, small molecules and peptides across the membrane or due to their role in multidrug resistance. Hence elucidating their structure and mechanism is of great importance and may lead to a host of new drugs and methods to alter or inhibit their function. Solid state NMR is an emerging technique for investigating transport proteins. Along with other biochemical and biophysical techniques solid state NMR can provide data on drug binding, protein dynamics and structure at the interface between structural biology and functional analysis. Here, we review solid state NMR applications to primary active and secondary transporters involved in translocation of small molecules. We discuss current experimental limitations and give an overall perspective on how the technique may be used to address some pertinent questions relevant to transporters.

Keywords Drug resistance · EmrE · FucP · GalP · GusB · LacS · NupC · LmrA · Membrane · Multidrug · NMR · Protein · Transporter

Introduction

Transporters are important membrane proteins Membrane proteins and specifically transporters provide the entry and exit sites for proteins and solutes through biological lipid membranes. Recently sequenced bacterial genomes have

revealed that 3–10% (3 % in *Mycobacterium tuberculosis* and 10% in *Salmonella typhimurium*) of open reading frames are predicted to encode membrane transport proteins (Cole et al. 1998; McClelland et al. 2001). These transporters are vital for cell nutrition, environmental sensing, ATP synthesis, protein/toxin secretion as well as influx and efflux of solutes. Furthermore, some of these transport proteins exhibit surprisingly broad substrate specificity which allows them to play important roles in multidrug resistance, cell volume regulation and peptide selection for translocation across membranes.

In order to fulfil their function, transport proteins must negotiate a cycle which includes steps associated with substrate recognition, binding, translocation and release. This transport cycle may be coupled to ATP hydrolysis in the case of ATP binding cassette (ABC) proteins or ion translocation in the case of secondary transporters.

So far, 10 structures of transport proteins have been determined (see Table 1) (Striebek 2005). This information is clearly of great value, although this small number of structures does not yet contain a representative from all transporter families. Furthermore, we must remember that each structure corresponds to a snapshot of the protein during its transport cycle. Therefore, additional approaches are needed to elucidate the transport kinetics, substrate-protein interactions or conformational changes during substrate translocation.

Solid state NMR for the study of membrane proteins Solid state NMR (ssNMR) has been widely used in membrane biophysics, to study membrane bound peptides and increasingly for investigations of larger integral membrane proteins. Areas of application cover protein structure and dynamics, lipid-protein, substrate-protein, and substrate-membrane interactions (Fig. 1). Static ssNMR applied to macroscopically ordered membrane samples has been used to elucidate secondary structure and topological organisation of membrane bound peptides (Ketchum et al. 1997). However, for larger membrane proteins, that fall within the scope of this review, static ssNMR techniques are of limited use. Instead, magic angle sample spinning (MAS)

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Table 1 Overview of publicly available transport protein structures

Protein	Function	Family	Organism	Resolution	PDB
GlpT	Sugar uptake	Major facilitator superfamily (MFS)	<i>E. coli</i>	3.30 Å	1PW4
LacY	Lactose uptake	Major facilitator superfamily (MFS)	<i>E. coli</i>	3.50 Å	1PV6, 1PV7
AcrB	Multidrug efflux	Resistance-nodulation-cell division (RND)	<i>E. coli</i>	3.50 Å	1IWG, 1OY6, 1OY8, 1OYD, 1OYE
EmrE	Multidrug efflux	Small multidrug resistance (SMR)	<i>E. coli</i>	3.70 Å	1S7B, 2F2M
BtuCD	Vitamin B ₁₂ uptake	ATP-binding cassette (ABC)	<i>E. coli</i>	3.20 Å	1L7V
MsbA (closed)	Lipid and drug export	ATP-binding cassette (ABC)	<i>E. coli</i>	4.50 Å	1JSQ
MsbA (open)	Lipid and drug export	ATP-binding cassette (ABC)	<i>Vibrio cholera</i>	3.80 Å	1PF4
SecYEB	Protein translocation	General secretory pathway (Sec)	<i>Methanococcus jannaschii</i>	3.50 Å	1RHZ, 1RH5
AmtB	Ammonium transport	Ammonium transporter (Amt)	<i>E. coli</i>	1.40 Å	1U77, 1U7G, 1U7C, 1XQE, 1XQF
GltPH	Glutamate transport	Dicarboxylate / amino acid:cation symporter (DAACS)	<i>Pyrococcus horikoshii</i>	3.50 Å	1XFH

Data were compiled from a web-database maintained at the MPI for Biophysics, Frankfurt, Germany (Striebek 2005)

seems more promising as it imposes fewer restrictions with respect to sample preparations: The proteins can be studied in a number of states (discussed in detail below). But most importantly, anisotropic NMR interactions such as chemical shift or dipolar couplings are averaged out by fast sample rotation (in practice 5–20 kHz) about the magic angle (54.7° with respect to the magnetic field), leading to the type of well resolved spectra shown in Fig. 2.

Fig. 1 Solid state NMR can be used for investigating the interaction between substrate and protein (a), for studying changes in protein conformation and dynamics upon substrate binding (b), for protein-lipid interactions (c) or for observing the binding of small molecules to lipid membranes (d)

The number of membrane proteins available for biophysical studies has been dramatically increased by the advent of microbial expression systems (e.g. *Escherichia coli*, yeast and baculovirus) optimised for membrane proteins, the availability of detergent screens and advanced purification technologies, in addition to the sequencing of a number of prokaryotic and eukaryotic genomes. For a review, see (Wang et al. 2003). An overview of sample preparation and biophysical investigations possible at each step is given in Fig. 3. Solid state NMR is increasingly applied to isotope labelled membrane proteins in order to derive data about protein structure and dynamics. The possibility of a complete de novo structure determination purely based on MAS NMR recoupling techniques was first demonstrated for small insoluble peptides (Jaroniec et al. 2004; Rienstra et al. 2002) and has been extended to soluble proteins studied in the solid state (Castellani et al. 2002; Seidel et al. 2005; Zech et al. 2005). However, similar studies have not yet been successfully applied to membrane proteins largely due to limitations in spectral resolution. However, a number of studies have reported promising MAS NMR spectra of membrane proteins prepared as 2D crystals (Hiller et al. 2005), 3D crystals (Lorch et al. 2005a) and also in proteoliposomes (Andronesi et al. 2005). The latter is clearly the preparation of choice as it is closest to the protein's native environment. Lipid reconstituted samples also offer the option of investigating the dynamics of the protein as it binds ligands (Patching et al. 2004a), negotiates its reaction cycle (Mason et al. 2005) or responds to changes in the lipid environment (Yamaguchi et al. 2004). However, the currently very limited knowledge base does not yet allow us to derive general rules about how to prepare membrane proteins for solid state NMR and so screens have to be performed for each individual protein.

Fig. 2 Basic principles of solid state NMR. Large macromolecular complexes such as membrane proteins do not rotate fast enough to average dipolar couplings or anisotropic chemical shifts to zero, which results in a complex NMR interaction network. Isotropic tumbling can be emulated by rotating the sample rapidly (1–20 kHz) about the magic angle (MAS NMR) (a). The presence of all dipolar couplings and anisotropic chemical shifts causes broad featureless spectra as shown here for 1–2-¹³C glycine, which improves by

proton decoupling and cross polarisation, and magic angle sample spinning at increasing rotation rates (from top to bottom) (b). MAS NMR combined with suitable pulse sequences for polarisation transfer, decoupling and recoupling of NMR interactions can then be used for example for resonance assignment and structural studies. Shown here is a typical double-quantum–single-quantum correlation spectrum of crystalline U-¹³C–glutamic acid (c)

The potential role of solid state NMR in the process of drug discovery has been highlighted in a recent review (Watts 2005). The structures of ligands and drugs can be determined at their site of action by solid state NMR. This aids in defining the ligand binding site. In addition, drug partitioning, drug-lipid interactions and drug polymorphism can be assessed by solid state NMR. It has also recently been shown to be a method capable of determining the backbone structure of a peptide ligand

(neuropeptide) bound to its native G protein-coupled receptor (GPCR) (Luca et al. 2003).

Solid state NMR studies on transporters So far, only a limited number of solid state NMR studies on transporters have been reported (Appleyard et al. 2000; Glaubitz et al. 2000; Mason et al. 2004; Patching et al. 2004a,b, 2005; Spooner et al. 1993, 1994a,b, 1998, 1999). Applications cover in principle three areas: interactions of substrates with the membrane, detection of substrates bound to transporters, and methodological studies describing how to prepare isotope labelled transporters.

The first solid state NMR applications to transporters were presented by the laboratories of Watts and Henderson who demonstrated the detection of substrate bound to the sugar transporter GalP (Appleyard et al. 2000; Spooner et al. 1993). This has triggered a number of further studies aimed at obtaining structural information concerning the position of the binding site (Spooner et al. 1998), as well as the ligand/protein association constant (Patching et al. 2004a). Recently, it has also been shown that isotope labelled transporters can be prepared at a quantity and purity suitable for ssNMR (Mason et al. 2004).

In the following, we will give an overview about published work on transporters and discuss perspectives

Fig. 3 Schematic diagram depicting transport protein preparation and the biophysical techniques that can be utilised at each preparation stage

for further studies in the light of biochemical challenges. This overview is not intended to serve as a technical introduction into solid state NMR. For this, the reader is referred to introductory texts such as Laws et al. (2002) or textbooks.

Sample preparation

Availability of transport proteins

ssNMR imposes strict constraints on sample preparation with respect to purity and homogeneity. The greatest hurdles to overcome, however, are those of quantity and concentration of the isotope labelled transport protein and/or labelled ligand bound to the protein. Usually, the amount of nuclear spins in the sample has to be in the order of μmol for a decent signal-to-noise ratio. Considering that the active sample volume used for MAS NMR is typically in the order of 20–90 μl , the protein concentration must be in the order of 3–20 mM. This means that 2–10 mg of a small 12 kDa protein, like the small multidrug transporter EmrE, needs to be inserted into such a small sample container. When studying proteoliposomes, the concentration problem is compounded by the presence of lipids which take up most of the rotor volume. Whilst functional assays can be performed on proteoliposomes with small protein/lipid mol ratio (1:1,000), ssNMR requires ratios of up to 1:100, but four-fifths of the sample volume is still taken up by lipids. Higher protein/lipid mol ratios are often prevented by the risk of protein aggregation.

Generally, the protein must be overexpressed in a host system. This allows a range of uniform and amino acid selective isotope labelling schemes to be applied, an essential step if the protein is to be studied directly. Therefore, a suitable recombinant expression system must be chosen. Overexpression of sufficient amounts of transport proteins for solid state NMR has been successful in *E. coli*, *Lactococcus lactis* and a cell free expression system.

Overexpression and labelling in *E. coli* For bacterial transporters, the classical *E. coli* expression is the system of choice because of its low cost, the bacteria's rapid doubling time, well understood genetics and the availability of a range of expression vectors and bacterial strains. Overexpression of bacterial transporters has been repeatedly demonstrated (Auer et al. 2001; Curnow et al. 2004; Masi et al. 2003; Xie et al. 2004; Yerushalmi et al. 1995). A more detailed summary of expression levels of various secondary membrane transporters is given in a review by Wang et al. (2003).

E. coli is also the most used expression system for preparing isotope labelled proteins for NMR spectroscopy. Complete labelling can be achieved by using minimal media which contains all necessary nutrients and an isotope enriched carbon and/or nitrogen source. These well established standard procedures have been used, for example, to prepare the *E. coli* transporter EmrE for

solution state NMR (Schwaiger et al. 1998). Unfortunately, α -helical membrane proteins often have a low spectral dispersion (Krueger-Koplin et al. 2004), and therefore uniformly labelled proteins yield very crowded NMR spectra with many overlapping peaks. To circumvent this problem, selective labelling of single amino acid types can be achieved with a defined medium (synthetic rich) containing all amino acids (Muchmore et al. 1989). Selective labelling can be aided, and metabolic scrambling of NMR active nuclei avoided, with the use of auxotrophic *E. coli* strains. However, auxotrophs are not available for all amino acids and usually support only low levels of protein overexpression. An alternative to auxotrophs makes use of the T7 promoter and the action of the antibiotic rifampicin (Arkin et al. 1996; Lee et al. 1995a). Rifampicin selectively binds to the *E. coli* RNA polymerase and blocks its transcription initiation, whilst the T7 RNA polymerase is not affected. This can be exploited by growing the *E. coli* in unlabelled media to the target cell density, pelleting the cells and inducing in fresh isotope labelled media (Almeida et al. 2001). Shortly after induction, rifampicin is added and thus it is assured that protein contaminants are not isotope labelled and *E. coli* cell metabolism is reduced.

***L. lactis* overexpression and labelling** Overexpression and labelling for NMR has been demonstrated for the ABC multidrug transporter LmrA (Mason et al. 2004). Amplified expression in *L. lactis* offers a number of advantages. The cells grow rapidly to a high cell density and overexpressed membrane proteins are found exclusively within the cytoplasmic membrane. Proteins can then be solubilised directly from the membrane with mild detergents and purification is simplified by the small proteome size (Kunji et al. 2003).

Cell Free Expression and labelling Cell free expression systems have been produced from many eukaryotic and prokaryotic cells including *E. coli*, rabbit reticulocyte and wheat germ (Klammt et al. 2004; Madin et al. 2000; Pelham and Jackson 1976). Crude cell extracts supply most of the necessary enzymes, ribosomes and cofactors for expression. Currently, most published applications utilise *E. coli* based coupled transcription/translation expression systems. Early cell free expression systems were performed in batch mode, but the yields were very low due to extract deactivation and exhaustion of substrates (Jewett and Swartz 2004). Development of continuous-flow reactors allowed dramatically prolonged production times of up to 40 h but the yield only increased marginally due to loss of translation components (Spirin et al. 1988). More successful were approaches using semi-continuous cell free expression which yield up to 6 mg/ml for soluble proteins (Kigawa et al. 1999). Using *E. coli* based coupled transcription/translation systems, integral membrane proteins have been expressed and yields of 1 mg/ml for GPCRs (Ishihara et al. 2005), 1.3 mg/ml for MscL (Berrier et al. 2004) and 3 mg/ml for EmrE (Rotem et al. 2001) have been achieved. These proteins can be

expressed either as precipitate or directly inserted into a hydrophobic environment when in the presence of detergents and/or lipids (Fig. 4). However, yields in the presence of detergents are reduced in a detergent dependent fashion correlating with critical micellar concentration (CMC) (Berrier et al. 2004). Bacteriorhodopsin, for example, has been expressed as aggregate in quantities sufficient for biophysical studies such as Fourier Transform Infrared Spectroscopy (FTIR) and could be shown to refold correctly into its native and active conformation using sodium dodecyl sulfate (SDS) and halobacterial lipids (Sonar et al. 1993). Meanwhile, small multidrug resistance (SMR) proteins, GPCRs and ion channels have been expressed successfully in the presence of detergents and lipids. Furthermore, they could be shown to be active and were reconstituted successfully into artificial membranes (Berrier et al. 2004; Ishihara et al. 2005; Klammt et al. 2004). In conclusion, *in vitro* expression is a viable alternative to *in vivo* expression under some circumstances. The high yield of protein per mass of labelled amino acid and low detergent requirements make cell free expression of some membrane proteins economically viable despite its inherent high reagent costs and its manifold of optimisation parameters (Guignard et al. 2002; Jewett and Swartz 2004). Special consideration has to be given to prove the correct fold of the expressed protein. Direct manipulation of reaction conditions enables the use of cofactors and unnatural amino acids (Betton 2003). And, of course, *in vitro* production of toxic proteins is also possible.

Solubilisation, purification, choice of detergents

Highly amplified expression levels were achieved for some transporters (GalP, NucP, LacS, FucP) allowing substrate binding studies by ssNMR of these proteins in their natural membranes (Patching et al. 2004a; Spooner et al. 1999,

Fig. 4 Schematic diagram of a semi-continuous *E. coli* based cell free expression system with coupled transcription and translation (see text for details) (Klammt et al. 2004)

1998, 1994a). Nevertheless, in most cases proteins have to be solubilised from membranes, purified and reconstituted into phospholipids. For one transporter, *E. coli* EmrE, organic solvent based protein extraction has been described (Yerushalmi et al. 1995), but in general, carefully selected detergents have to be used for protein solubilisation. Ideally, the detergent should fulfil several critical requirements: it must be capable of solubilising the protein, the protein must be stable, and finally it must be compatible with a reconstitution procedure to yield active protein. Rarely does one detergent fulfil all requirements, and so the choice of detergent is often a compromise.

Solubilisation is followed by one or more chromatographic purification steps, e.g. affinity, size exclusion, ionic or hydrophobic chromatography. Unfortunately, there is an inevitable trade-off between quantity, stability, activity and very pure protein preparations. The success of protein purification is routinely monitored using SDS PAGE and western blot analysis. Protein homogeneity can also be evaluated using analytical size exclusion chromatography and mass spectrometry. A very good review surveying sample preparation and optimization was written by Wang et al. (2003).

High protein stability in detergent solution is necessary for all applications. For example, detergents used during protein crystallisation are screened for their ability to maintain the integrity of the protein for long periods of time often judged by size exclusion chromatography. Long term stability is also a prerequisite for solution state NMR, where the best detergent is usually judged by the quality of the protein NMR spectrum. Using this strategy, a thorough screen of 25 different detergents was performed on a *Staphylococcus aureus* Smr (Krueger-Koplin et al. 2004) where a number of promising candidates were found, such as, for example, 1-palmitoyl-2-hydroxy-sn-glycero-3-[phospho-RAC-(1-glycerol)] (LPPG). Despite the fact that the protein detergent complexes appeared to be larger than 100 kDa, the rotational correlation time corresponded to that of a 15–20 kDa protein tumbling isotropically in solution. This has been interpreted as tumbling of the protein inside the LPPG detergent micelle. Unfortunately, the best detergents for high quality solution state NMR spectra do not necessarily correspond to the detergents found to be most suitable for maintaining a functional folded protein or long term stability. For example, it has been demonstrated using radioactive ligand binding assays that detergent solubilised EmrE, (an Smr homologue) binds the substrate tetraphenylphosphonium (TPP⁺) only in dodecyl maltoside (DDM) amongst a dozen tested detergents (Muth and Schuldiner 2000). The requirements for ssNMR are similar to those above since the protein should be stable and functional in detergent before the protein is reconstituted into liposomes.

Reconstitution into lipid vesicles

Reconstitution of membrane proteins is commonly achieved by one of three methods: mechanical means

such as freeze-thawing and sonication, dissolving protein and lipid in organic solvent with subsequent evaporation of the solvent, or detergent mediated procedures. Although reconstitution by codissolving of lipids and protein in organic solvents has been shown for EmrE (Yerushalmi et al. 1995) and was used to prepare samples for solid state NMR (Glaubitz et al. 2000), transport proteins have been mainly reconstituted using detergent based methods which also facilitate a smooth transition to 2D crystallization. Stability and activity of membrane proteins during and after the reconstitution process are mainly governed by the detergent choice and homogeneous incorporation. In general, detergent solubilised membrane proteins are mixed with lipids followed by a decrease of detergent concentration which causes incorporation of the protein into liposomes. Detergent removal can be achieved using dialysis (Gorzelle et al. 1999), gel chromatography (Kiefer et al. 1996), dilution (Curnow et al. 2004) or hydrophobic absorption (Paternostre et al. 1988). The method of choice depends mainly on detergent properties such as CMC and hydrophobicity, but also on the desired speed of reconstitution and completeness of detergent removal. Detergents with a low CMC can only be removed efficiently by hydrophobic adsorption, which is also the best method for almost complete detergent removal (Allen et al. 1980; Holloway 1973), while dilution of detergents allows fastest reconstitution. Rapid hydrophobic absorption of detergents by polystyrene beads seems to be the most promising approach for SMR transport proteins (Curnow et al. 2004; Sikora and Turner 2005; Winstone et al. 2002), but has also been used for the ABC multidrug transporter LmrA. This efflux pump has been reconstituted for solid state NMR by mixing solubilised protein with preformed, detergent destabilised vesicles followed by detergent removal through polystyrene beads (Mason et al. 2004). Phospholipid adsorption onto the hydrophobic beads can be minimised by bead presaturation with lipids, and protein adsorption has been shown to be negligible (Rigaud et al. 1997). An excellent review on biobead based reconstitution was written by Rigaud et al. (1998).

Successful reconstitution can be monitored using continuous or discontinuous density gradient centrifugation to separate empty liposomes and proteoliposomes with

different protein:lipid ratios (Chi-Rosso and Toole 1987). Further characterisation of proteoliposomes can be achieved by freeze fracture electron microscopy (Fig. 5) to assess sample homogeneity and protein aggregation (Viitanen et al. 1986). Successful reconstitution also requires a functional test to verify the protein's correct fold within the liposome, for which transport, ligand binding and ATPase assays in the case of ABC transporters can be used. However, assays on proteoliposomes are not without their limitations and drawbacks. False negative results will arise from drug binding sites being trapped on the inside of liposomes or within multilamellar complexes and thus being inaccessible to the substrate.

Crystallisation

From the biochemical point of view, solid state NMR studies on proteoliposomes are clearly the approach of choice as this allows preparation of functional membrane proteins. But it has been shown for two cases, the β -barrel OmpG and the α -helical diacylglycerol kinase (DGK), that 2D (Hiller et al. 2005) or 3D crystals (Lorch et al. 2005a) of membrane proteins can also be used for solid state NMR. The advantage of crystals stems mainly from the higher protein concentration that can be achieved and from very well resolved ssNMR spectra which can be obtained under favourable circumstances.

These well resolved spectra have been attributed to high 'short-range' order in crystalline samples compared to proteoliposomes. Therefore, spectral improvements are most likely due to restricted protein dynamics in crystals, as protein dynamics give rise to line-broadening due to conformational exchange. The drawbacks of crystals are the extensive screens needed for crystallisation conditions, despite the fact that well diffracting crystals are not a requirement.

2D crystallisation is essentially a reconstitution process optimised to induce local order and crystallinity at very high protein:lipid mol ratios of 1:15–30. During 2D crystallisation slow, controlled, removal of detergent is desired and usually a narrow range of near physiological

Fig. 5 Reconstitution of membrane proteins. Protein incorporation into liposomes is achieved by replacing detergents with lipids which can be done by a number of methods (see text). Successful incorporation is monitored by freeze fracture electron microscopy as shown here for the ABC multidrug transport LmrA (16 k magnification) (Mason et al. 2004). The image was kindly provided by Dr. W. Haase, MPI Biophysik

crystallisation conditions is screened. This is a major advantage compared to 3D crystallisation.

Solid state NMR on transporters

Sugar transporters

Sugar transporter GalP

The first solid state NMR studies on a transporter were carried out on GalP, a member of the major facilitator superfamily (MFS). GalP is a galactose- H^+ symport protein located in the inner membrane of *E. coli*. It is closely related to sugar transport systems in higher organisms, including Glut1 in humans (Baldwin and Henderson 1989). GalP has a molecular weight of 50 kDa, is predicted to consist of 6 transmembrane helices and is thought to function as a homodimer. The protein can be overexpressed in *E. coli* where it is targeted to the inner membrane. These GalP-containing membranes can be extracted and worked on directly, removing the need for any further purification steps and allowing the protein to be studied in its native membrane. Such samples have been used for ssNMR based ligand binding studies.

The approach presented by Spooner et al. (1994a) relies on the effect of molecular dynamics on cross polarisation (CP) (Schaefer et al. 1977). Cross polarisation is usually used to transfer polarisation via dipolar couplings from protons to less sensitive nuclei such as ^{13}C in order to enhance their signal intensity. Spooner et al. (1994a) have used CP as a 'dynamic filter' since dipolar couplings are reduced with molecular motions causing decay in signal intensity. A GalP substrate such as D-[1- ^{13}C]glucose will therefore be visible under CP conditions when bound to GalP in a fluid membrane, while no cross polarisation will take place for its free state. Typical ^{13}C -CPMAS spectra of GalP containing membranes with and without D-[1- ^{13}C]glucose are shown in Fig. 6. Two resonances are detected because glucose is present in two anomeric forms. The chemical shifts of glucose are very similar to those detected in solution, but the peak intensities (Fig. 6d) reveal a greater spectral contribution from the β -form of glucose indicating its preferred binding to GalP. It was also possible to show that ^{13}C -labelled D-glucose and D-galactose were displaced from GalP with singly labelled [7- ^{13}C]forskolin, a known inhibitor of sugar transport in GalP (Appleyard et al. 2000). No alternative binding site for forskolin was found.

The timescales of the molecular dynamics of bound D-[1- ^{13}C]glucose have been determined from NMR relaxation measurements (Spooner et al. 1994a). The proton spin-lattice relaxation in the laboratory frame $T_{1\rho}$ is influenced by high frequency motions (10^{-9} s) and was found to be much faster for bound substrate compared to its crystalline state. Therefore, the substrate in its binding site undergoes fast motions on the nanosecond timescale. The relaxation $T_{1\rho}$ in the rotating frame of the spin-locking field used for CP is sensitive to low frequency motions in the

Fig. 6 ^{13}C -CPMAS spectra from *E. coli* membranes containing GalP (a) and with bound D-[1- ^{13}C]glucose (b). Expanded regions show an increase of signal upon addition of 0.25 μ mol (c) and 1 μ mol of D-[1- ^{13}C]glucose (d). Adapted, with the authors' permission, from Spooner et al. (1994a)

microsecond to millisecond range and was found to be very similar for bound substrate, protein and lipid resonances. This has been interpreted by the authors as an indication that slow collective motions influence all membrane components equally.

The presented relaxation and CP data show that the lifetime of the transporter-substrate complex corresponds to the ms timescale of the NMR experiment, i.e. slow exchange takes place. These findings are consistent with a previously suggested 'mobile-barrier' mechanism, in which the substrate-transporter complex is alternatively exposed to both sides of the membrane by conformational fluctuations.

The general application of cross-polarisation as a dynamic filter to detect substrates bound to transporters is limited by the presence of large background signals arising from the ^{13}C natural abundance in lipids and proteins. However, the use of multiply ^{13}C -labelled substrates with strongly dipolar coupled nuclei in close proximity offers a solution: the application of double-quantum filtering NMR experiments will only select those coupled nuclei while suppressing the ^{13}C natural abundance background (Lee et al. 1995b). This approach has been attempted on doubly labelled glucose and forskolin. Unfortunately, it was found that in fluid membranes at 2°C no substrate detection was possible, but freezing samples at

−35°C allowed an efficient double-quantum filtering and hence substrate detection, most likely due to restrictions of molecular motion. At this temperature, both bound and non-bound substrates start to become immobilised which makes dynamic filtering difficult (Appleyard et al. 2000).

The problem of strong ^{13}C natural abundance background has also been addressed by preparing ^{13}C -depleted membranes containing GalP. *E. coli*, expressing GalP, were grown on ^{13}C -depleted glucose (Patching et al. 2004b). The interfering ^{13}C background signals were significantly reduced to $^{13}\text{C} \leq 0.07\%$ (compared to natural ^{13}C abundance of 1.1%). Based on this very low background, the simultaneous detection of both sugar substrate and inhibitor by cross polarisation was possible.

Fucose transporter FucP

The L-fucose- H^+ symport protein FucP from *E. coli* has a monomeric size of 47 kDa and is predicted to form 12 transmembrane segments (TMS). High expression levels, FucP constitutes 20% total inner membrane protein, enabled ssNMR studies directly on native membranes (Spooner et al. 1998).

FucP is known to transport D-arabinose, L-galactose and L-fucose, but not their respective stereoisomers (Muiry et al. 1993). It binds its ligands weakly making competitive binding assays impractical. However, detection of substrate binding by solid state NMR has been done following the approach discussed for GalP. It was possible to show by cross polarisation that both substrate anomers of D-[1- ^{13}C] arabinose and L-[$^{13}\text{C}6$]galactose are bound equally well.

To measure the substrate exchange time, the authors went a step further by suggesting a dephased delayed cross polarisation (DDCP) experiment: All magnetisation of protons associated with the membrane is dephased, i.e. removed, and only magnetisation of protons free in solution, such as in unbound substrate, is retained. During a variable mixing time, this free substrate can bind to FucP which is detected by cross polarisation. The substrate exchange time has been estimated by analysing the cross polarisation signal intensity as a function of these mixing times. It was found that a very slow exchange between free and bound substrates takes place ($>10^{-1}$ sec).

Lactose Transporter LacS

The 69 kDa lactose transport protein LacS from *Streptococcus thermophilus* belongs to the glycoside-pentoside-hexuronide (GPH):cation symporter family. It is predicted to fold into 12 TMS and appears to form a functional dimer with one sugar translocation channel per monomer (Veenhoff et al. 2001). Members of the GPH family are characterised by sequence conservation in helices II, IV and in the interhelical loop between helices 10 and 11 (Poolman et al. 1996). Overexpression in *Streptococcus thermophilus* results in 25% of the total protein content in native membranes being LacS. These membranes have

been used for MAS NMR substrate binding studies on the fully active mutant LacS (K373C) (Spooner et al. 1999).

Cross polarisation experiments on D-[1- ^{13}C]galactose revealed that both galactose anomers are bound by LacS, though there is no significant chemical shift change compared to galactose in solution. The cross polarisation signal follows classical saturation binding behaviour with respect to the substrate concentration. Consequently, the authors were able to determine a K_d of 4 mM for D-[1- ^{13}C] galactose directly from solid state NMR data.

After attaching a nitroxide maleimide spin label to C373, located in the interhelix loop 10–11, the ^{13}C CPMAS signals of bound D-[1- ^{13}C]galactose disappeared almost completely. Based on this paramagnetic signal quenching, the authors estimated the sugar binding site to be within 15 Å of the nitroxide spin label at residue C373 and concluded that the interhelix loop 10–11 is in close proximity to the substrate binding site.

Nucleoside transporter NupC and glucuronide transporter GusB

Further improvements on the quantitative characterisation of substrate binding by MAS NMR were presented by Henderson, Middleton and colleagues on the bacterial transporters GusB and NupC (Patching et al. 2004a). GusB is a 50 kDa glucuronide- H^+ symporter from *E. coli*. It transports glucuronides, which are often conjugated to hormones, xenobiotics and drugs excreted from the human body (Liang et al. 2005). The 43 kDa nucleoside transporter NupC from *E. coli* is a member of the concentrative nucleoside transporter (CNT) family. It shares structural motifs with the three human members of this family (hCNT1-3) and thus can serve as a experimental model for the clinically relevant mammalian proteins (Patching et al. 2005). For both proteins, a 20–50-fold amplification of their expression levels has been shown (Patching et al. 2004a). Hence, the concentration of the protein in the native membrane is high enough for substrate binding studies to be carried out. It had been demonstrated for LacS, that a K_d can be obtained from signal intensities of bound substrate, filtered by cross polarisation, as a function of substrate concentration (Spooner et al. 1999). This normally requires substrate titration to the sample into the MAS rotor or the preparation of many different samples. Patching et al. (2004a) suggest an alternative. They show that binding constants and dissociation rate constants k_{off} can be determined directly from a single sample. This was achieved by following the cross polarisation signal intensity as a function of the time period during which cross polarisation takes place (contact time). The method is based on a statistical analysis of substrate binding kinetics during ^1H - ^{13}C cross polarisation. If substrate binds, cross polarisation is enabled and magnetisation builds up during the residence time of the substrate in the binding site, after which the substrate returns to its free state. The longer the contact time, the higher is the probability that a binding event will occur

during that period. It is then possible to simulate the effect of many random binding events with different substrate-transporter lifetimes, substrate binding constants and varying contact times on the CP signal intensity. As a result, experimental CP build-up curves can be analysed to obtain values for K_d and k_{off} . The authors were able to demonstrate and verify this approach for the binding of $[1-^{13}\text{C}]\beta\text{-glucuronide}$ for which K_d and k_{off} values of 4.17 mM and 698 s^{-1} have been obtained (Fig. 7). It was even possible to determine binding constants for unlabelled ligands by carrying out competition binding experiments.

A slightly different situation was observed for the binding of $[1'-^{13}\text{C}]\text{uridine}$ to NupC: using cross polarisation, a signal from bound ligand was identified in native *E. coli* membrane with and without induced expression of NupC. The chemical shift did not differ significantly in either case, but signal intensity was higher in the presence of protein, indicating that nonspecific binding to the membrane or other membrane proteins takes place. Any quantitative analysis would have to take this signal contribution into account by either difference spectroscopy or suitable filtering techniques. The method used by Patching et al. (2004a) relies on cross polarisation with polarisation inversion (CPPI) normally applied as a spectral editing technique to simplify NMR spectra (Wu and Zilm 1993). After cross polarisation, magnetisation is transferred back from ^{13}C to ^1H for a short period of time, t_p . Depending on the length of this time period and the motional characteristic of the observed molecular group, the carbon signal will disappear. If specifically and nonspecifically bound substrates differ in rates and amplitudes of their anisotropic motion, signal from nonspecifically bound populations can be eliminated by appropriate choice of t_p . The experiment was first calibrated on native membranes containing $[1'-^{13}\text{C}]\text{uridine}$,

but without NupC, and then applied to membranes with NupC. Using this filtering trick in conjunction with the method described above for the LacS measurements, a value for K_d of 2.6 mM was obtained.

Multidrug Efflux Pumps

Antibiotic resistance of pathogenic bacteria is a major worldwide problem. Bacteria rapidly acquire resistance to new antibiotics and their widespread use over a number of years means they no longer provide effective control against many infectious diseases. Several resistance mechanisms, namely, drug inactivation, target alteration, prevention of drug influx and active drug extrusion are recognised and have been found to act synergistically. One of these mechanisms, active drug extrusion, can be assigned to membrane-bound primary or secondary efflux pumps. The primary efflux pumps, otherwise known as ABC transporters, utilise energy-derived from ATP hydrolysis for active transport of substrates. Among the secondary transporters, four families, SMR (small multidrug resistance), multidrug and toxic compound extrusion (MATE), resistance nodulation cell division (RND) and major facilitator superfamily (MFS), have currently been described (Putman et al. 2000).

SMR protein *EmrE*

Proteins of the SMR family are small multidrug efflux pumps of 10–12 kDa with a predicted four helix trans-membrane topology and have been found in archaea and bacteria (De Rossi et al. 1998; Grinius et al. 1992; Ninio and Schuldiner 2003). In one case, SMR proteins have

Fig. 7 The cross polarisation build-up of $[1-^{13}\text{C}]\beta\text{-glucuronide}$ bound to GusB can be analysed using a statistical approach in terms of K_D and k_{off} . Adapted, with the authors' permission, from Patching et al. (2004a)

been detected in up to 31% of clinical isolates of methicillin resistant *S. aureus* (Noguchi et al. 2005).

SMR proteins contain approximately 110 amino acids and are characterised by five amino acid sequence motifs, highlighted in the topology diagram (Fig. 8a) of EmrE, the most studied SMR protein.

It has been suggested that EmrE transports a diverse array of aromatic and positively charged substrates in a proton/drug antiport fashion (Paulsen et al. 1996). Direct structural information is only available for EmrE. Solution state NMR of EmrE in the highly artificial environment of a chloroform:methanol:water mixture achieved a secondary structure determination, but no tertiary structure could be proposed (Schwaiger et al. 1998). Low resolution tertiary structures both with and without bound substrate were achieved more recently by electron microscopy of 2D crystals (Tate et al. 2001; Ubarretxena-Belandia et al. 2003). The electron density reveals that three helices in each monomer are crossing the membrane and are nearly perpendicular to the membrane plane whereas the remaining fourth helix is highly tilted (Tate et al. 2001; Ubarretxena-Belandia et al. 2003). A 3.8 Å x-ray crystallographic structure of EmrE suggests a highly unusual dimer of conformational heterodimers as the functional unit. Monomers of the conformational heterodimer are roughly inverted with respect to each other. Helices 1–3 form a six helix bundle and helix 4 of one monomer is positioned nearly parallel to the membrane surface, while the remaining helix 4 of the second monomer protrudes from the membrane (Ma and Chang 2004). A new 3.7 Å x-ray structure of EmrE in complex with tetraphenylphosphonium (TPP⁺) confirms the opposite direction of both subunits, but agrees with the transmembrane arrangement of all helices detected by electron microscopy (Pornillos et al. 2005).

Despite these data, the oligomeric state of SMR proteins remains highly controversial. Biochemical and structural data supporting dimers, trimers or tetramers have been reported and are summarised in Table 2.

Solid state NMR studies of EmrE followed a similar approach to those described for GalP. ³¹P-cross-polarisation dynamics was used to differentiate between bound and unbound TPP⁺, which has been shown to bind to EmrE (Muth and Schuldiner 2000). In contrast to GalP, expression levels of EmrE are not sufficient to perform binding studies in the native membrane. Therefore, experiments have to be performed on purified EmrE in proteoliposomes. TPP⁺ is known to diffuse into the membrane (Ahmed and Krishnamoorthy 1990), but once there remains very mobile. Consequently, the TPP⁺ signal is not enhanced by cross polarisation in either DMPC (Glaubitx et al. 2000) or *E. coli* liposomes. A signal from TPP⁺ can, however, be seen when the sample is polarised directly (i.e. without the motion filters imparted by cross polarisation). When EmrE is included in the liposomes two signals appear (Fig. 8c). Based on the chemical shifts, the cross polarisation dynamics and signal enhancement upon adding more substrate, the resonances have been interpreted as arising from nonspecific and specific bindings sites. The ssNMR

Fig. 8 Topology of the SMR transporter EmrE with highly conserved residues (black) and signature sequence motifs (grey) (a). A ³¹P CP MAS spectrum of EmrE in complex with TPP⁺ in DMPC bilayers reveals two resonances (b). The chemical shift of peak 1 is similar to that of free TPP⁺ and is attributed to nonspecific interactions with the protein while resonance 2 is shifted by 4 ppm which is assigned to specifically bound substrate (Glaubitx et al. 2000). While cross polarisation filters and enhances only signals from immobile components such as bound ligands, direct polarisation (by a simple 90° pulse followed by proton decoupling) excites all ³¹P nuclei equally. Here, the spectral contribution from unbound TPP⁺ is much higher compared to the bound fraction

data clearly show that the TPP⁺-EmrE complex is stable on the ms timescale, but further experiments are needed to understand the mechanism of substrate binding and translocation in the light of contradicting biochemical data (see Table 2). Solid state NMR with the help of suitable labelling schemes could help to observe changes in local structure and dynamics upon substrate binding. Of special interest are highly conserved key residues (Fig. 8a),

Table 2 Summary of experimental evidence for the oligomeric states of EmrE (see text for details)

Oligomeric state	Experimental methodology	Reference
Dimer	EPR spectroscopy	(Koteiche et al. 2003)
	EM structure	(Tate et al. 2001) and (Tate et al. 2003)
	Cysteine crosslinking	(Soskine et al. 2002)
	whole cell growth assay	(Jack et al. 2000)
	analytical ultracentrifugation and size exclusion chromatography	(Butler et al. 2004)
Trimer	In vivo and in vitro negative dominance	(Yerushalmi et al. 1996)
	Radioactive ligand binding	(Muth and Schuldiner 2000)
Tetramer	Functional complementation of in vitro produced protein	(Elbaz et al. 2004)
	X-ray crystallography structure	(Ma and Chang 2004)

which could be isotope labelled using the methods discussed above. In combination with double-quantum filtering, only those resonances arising from labelled residues could be selected (Lorch et al. 2005b).

ABC Transporter *LmrA*

The *L. lactis* multidrug efflux pump *LmrA* is a member of the ABC transporter family. It forms a homodimer of two 64 kDa subunits each containing 6 transmembrane helices and one nucleotide binding domain. It has been shown to be a functional homologue of the human P-glycoprotein (van Veen et al. 1998). Substrates of both proteins accumulate within the membrane in the interface region as shown by ¹H-MAS NMR (Siarheyeva et al., submitted).

Overexpression of *LmrA* in *L. lactis* allows sufficient amounts of protein to be obtained for NMR as is the case for all other transporters discussed above. Solid state NMR studies on such complex membrane proteins have been limited due to stability, concentration and reconstitution problems. Recently, it has been shown that it is possible to overcome these restrictions by extensive reconstitution screens and residues selective labelling schemes (Mason et al. 2004). Using this approach, protein dynamic studies have been reported using residue selective ²H-NMR (Lorch et al. 2005b).

Conclusions and perspectives

The number of solid state NMR studies on transporter proteins reported so far is still very small and limited to bacterial systems, but both ABC and secondary systems are covered. Primary and secondary transporters are involved in diverse functions such as multidrug efflux, sugar and nucleoside transport. Some bacterial transporters are

homologues of mammalian transporters. Most notably, in some cases very high expression levels have been reported which made substrate binding studies on native membranes possible. It has been shown that bound substrate can be selectively filtered by cross polarisation allowing binding constants and dissociation rates to be determined. For most cases, except for EmrE, weak substrate binding with K_d values in the mM range was found without significant changes in substrate chemical shifts. The substrate-transporter complex lifetimes were estimated to be in the range of milliseconds. One central difficulty is certainly that isotope labelled substrate is needed and that the use of cross polarisation as a dynamic filter requires the membrane to be in its fluid phase. Furthermore, as in the case of ¹³C or ³¹P labels, natural abundance signals of lipids or other membrane components could obscure the substrate signal. One solution is offered by double-quantum filtering which has the disadvantage that it normally requires frozen samples. This eliminates the dynamic filter effect provided by CP measurements and hence makes it impossible to distinguish between bound and unbound species. Nevertheless, these approaches hold significant potential for drug screening especially in cases where native membrane can be used, but also for purified and reconstituted samples.

The above mentioned examples show, so far, that the main focus has been clearly on drug-protein interaction studies, but solid state NMR on other membrane proteins has yielded unique information about conformations and dynamics. Therefore, first experiments on purified, reconstituted and labelled transporters have begun. This work is in its early stages mainly due to the difficulty of developing suitable methods to overexpress, isotope label and purify sufficient amounts of membrane transporters for NMR and to assess their activity in a reliable fashion. Furthermore, transporters, especially multidrug efflux pumps, may have to be highly flexible to accommodate a number of different substrates during the transport cycle. This may cause further difficulties when it comes to sample preparation, protein stability and NMR spectroscopy. In principle, solid state NMR can be applied at various stages of sample preparation, but membrane proteins are ideally studied in a reconstituted form.

In the authors' opinion, transporters belong to the most exciting membrane proteins as they have to undergo a complex translocation cycle consisting of substrate binding, translocation and substrate release in an energy-dependent fashion. Considering the progress in solid state NMR methodology and advancements in membrane protein sample preparation as reported here, the authors expect solid state NMR to make important contributions to the understanding of the mechanism of energy dependent substrate translocation across membranes in the near future.

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