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Review

NMR and EPR studies of membrane transporters

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Abstract

In order to fulfill their function, membrane transport proteins have to cycle through a number of conformational and/or energetic states. Thus, understanding the role of conformational dynamics seems to be the key for elucidation of the functional mechanism of these proteins. However, membrane proteins in general are often difficult to express heterologously and in sufficient amounts for structural studies. It is especially challenging to trap a stable energy minimum, e.g., for crystallographic analysis. Furthermore, crystallization is often only possible by subjecting the protein to conditions that do not resemble its native environment and crystals can only be snapshots of selected conformational states. Nuclear magnetic resonance (NMR) and electron paramagnetic resonance (EPR) spectroscopy are complementary methods that offer unique possibilities for studying membrane proteins in their natural membrane environment and for investigating functional conformational changes, lipid interactions, substrate-lipid and substrate-protein interactions, oligomerization states and overall dynamics of membrane transporters. Here, we review recent progress in the field including studies from primary and secondary active transporters.

Keywords: electron paramagnetic resonance (EPR); membrane transporters; nuclear magnetic resonance (NMR).

Introduction: membrane proteins

Membrane proteins represent approximately one-third of all proteins (Wallin and von Heijne, 1998; von Heijne, 2007), but approximately two-thirds of all current drug targets are membrane proteins (Drews, 2000; Hopkins and Groom, 2002; von Heijne, 2007). Membrane transporters are a highly heterogeneous class of membrane proteins which fulfill a plethora of different tasks within cells ranging from the transport of small solutes to the extrusion of drugs. The energy for these tasks is derived from either ATP hydrolysis in primary active transporters [ATP-binding cassette (ABC)-transporters] or from ion translocation in the case of secondary active transporters. In *Escherichia coli* for example, approximately 40% of all membrane proteins are transporters. Of these transporters, approximately 33% are importers and 7% are involved in efflux (von Heijne, 2007). Approximately 5% of the *E. coli* genome encodes ABC transporters (Linton and Higgins, 1998). The interaction of membrane proteins with lipids and substrates, as well as the interaction of their substrates with lipids, are highly relevant for membrane transporter function. Here, we review some examples in which nuclear magnetic resonance (NMR) and electron paramagnetic resonance (EPR) have been successfully applied to primary and secondary active membrane transporters to yield unique functional insight complementary to other biochemical and structural data (Table 1).

Nuclear magnetic resonance

NMR is a biophysical ensemble method that relies on detecting NMR-active nuclei, such as ^{1}H, ^{2}H, ^{13}C, ^{15}N, ^{31}P or ^{19}F. However, NMR is rather insensitive, so that a large amount of sample is usually needed (typically in a μM to mM concentration range). By using clever labeling schemes, many different types of NMR experiments yielding a variety of structural and dynamic information in atomic resolution are possible. Importantly, NMR is a non-invasive method that allows obtaining information on structure, protein dynamics, substrate binding, and interaction between different proteins, ligands or lipids in the case of membrane proteins (reviewed, e.g., by Marassi and Opella, 1998; Arora and Tamm, 2001; Thompson, 2002; McDermott, 2004; Basting et al., 2006; Hong, 2006; McDermott and Polenova, 2007).

In solution NMR, the dipolar spin interactions are averaged through the rapid movement of molecules in solution which results in narrow line-widths of the NMR signals. However, the dependency of solution NMR on fast molecular tumbling imposes a molecular weight (MW) limit for this method that is quickly exceeded for membrane proteins in detergent micelles. Progress in solution NMR techniques, such as transverse relaxation-optimized spectroscopy (TROSY) (Pervushin et al., 1997), made it possible to investigate very large proteins and even membrane proteins with multidimensional NMR (Arora et al., 2001; Fernandez et al., 2001, 2004; Hwang et al., 2002; Fernandez and Wider, 2003). For example, the GroEL-GroES complex (900 kDa) has been investigated in TROSY-based NMR experiments (Fiaux et al., 2002), and recently the NMR backbone assignment and secondary structure of sensory rhodopsin, a seven transmembrane helical G protein coupled receptor (GPCR) analog, was determined in a detergent micelle (Gautier et al., 2008). However, the natural environment for a

Table 1 Examples of NMR and EPR applications to membrane transporters discussed in this review.

Application	NMR	EPR
Folding/secondary structure	EmrE[1, 2, 3]	BtuB[10]
	MRP1[4]	MsbA[11]
	ABCB6-NBD2[5]	
	MJ1267[6, 7]	
	MalF-P2[8]	
	CFTR[9]	
Oligomerization		EmrE[12]
Topology		EmrE[13]
Structural asymmetry	EmrE[14]	BtuCD[15, 16]
Domain-domain interactions	CFTR[9]	MBP-$MalFGK_2$[18, 19]
	MRP1[17]	
Domain rearrangements/ protein dynamics	ABCB6-NBD2[5]	MsbA[21, 22]
	MJ1267[6, 7]	BtuCD[14, 15]
	LmrA[20]	$MalGFK_2$[23]
Real time kinetics	LmrA[24]	P-glycoprotein[25]
Substrate/inhibitor binding	EmrE[26]	MsbA[22]
	GalP[27, 28, 29]	BtuB[34, 35]
	FucP[30]	
	LacS[31]	
	NupC[32]	
	GusB[32]	
	ZitB[33]	
Nucleotide interaction	MRP1[4, 17]	MsbA[11, 36, 37]
	MJ1267[7]	BtuCD[15, 16]
	ABCB6-NBD2[5]	$MalFGK_2$[18, 19]
	LmrA[20]	
Posttranslational modification	CFTR[9]	

Reference key: [1]Arkin et al. (1996); [2]Schwaiger et al. (1998); [3]Agarwal et al. (2007); [4]Ramaen et al. (2003); [5]Kurashima-Ito et al. (2006); [6]Wang et al. (2002); [7]Wang et al. (2004); [8]Jacso et al. (2009); [9]Baker et al. (2007); [10]Fanucci et al. (2002); [11]Buchaklian and Klug (2005); [12]Koteiche et al. (2003); [13]McHaourab et al. (2008); [14]Lehner et al. (2008); [15]Hvorup et al. (2007); [16]Goetz et al. (2009); [17]Ramaen et al. (2005); [18]Orelle et al. (2008a); [19]Grote et al. (2008); [20]Siarheyeva et al. (2007); [21]Dong et al. (2005); [22]Borbat et al. (2007); [23]Austermuhle et al. (2004); [24]Hellmich et al. (2008); [25]Omote and Al-Shawi (2002); [26]Glaubitz et al. (2000); [27]Appleyard et al. (2000); [28]Patching et al. (2008); [29]Spooner et al. (1994); [30]Spooner et al. (1998); [31]Spooner et al. (1999); [32]Patching et al. (2004b); [33]Rahman et al. (2008); [34]Fanucci et al. (2003a,b,c); [35]Merianos et al. (2000); [36]Buchaklian and Klug (2006); [37]Westfahl et al. (2008).

membrane protein must be the lipid bilayer. Solid-state NMR (ssNMR) is an established method for hypothesis-driven biophysics research on membrane proteins and an emerging tool for structural biology. Thus, ssNMR is very valuable for the investigation of membrane proteins in lipid bilayers, a much closer mimic of their native environment than the detergent micelle. In theory, ssNMR is not subject to any weight limit. However, in large molecules the number of signals becomes so large that individual signals cannot be resolved anymore due to spectral overlap. This can be overcome by selective labeling schemes and/or 3D/4D NMR spectroscopy (Tugarinov et al., 2006; Agarwal and Reif, 2008; Schedlbauer et al., 2008). ssNMR is also a powerful tool to investigate membrane protein interaction partners and the incorporation of substrates, such as hydrophobic drugs, into the membrane (Watts, 1999; Xu et al., 2002). The term ‘solid’ refers to the tumbling rate of the protein in bilayers, which is slow on the NMR experimental time scale. Magic angle spinning (MAS) of the NMR sample around an angle of 54.7° with regard to the external magnetic field (where the orientation dependence of the chemical shift and the dipolar coupling disappears) is often used to average out anisotropic interactions (Figure 1) (Andrew et al., 1958; Lowe, 1959). This enables the measurement of well-resolved spectra of reconstituted, two-dimensional (2D) or 3D crystalline proteins. The loss of structural information in these spectra due to MAS can be overcome by specially adapted pulse sequences (Guillon and Schaefer, 1989; Griffin, 1998; Jaroniec et al., 2001; Thompson, 2002, and references therein).

Electron paramagnetic resonance

Electron paramagnetic resonance (EPR) is especially amenable to membrane proteins because its application is neither limited by the protein size nor by the optical properties of the sample. The method requires that the protein contains a paramagnetic center, such as metal ions, amino acid radicals or cofactor radicals. Continuous wave EPR (cw-EPR) yields information concerning the dynamics of a protein and allows distance measurements in the range of approximately 8–25 Å for doubly labeled peptides and proteins based on dipolar splitting and line-broadening in the spectra due to dipole-dipole interactions (Figure 2) (Rabenstein and Shin, 1995; Steinhoff et al., 1997; Steinhoff, 2004). Additionally, the mobility of the spin label can be directly deduced from the line-shape of the cw-EPR spectra (Figures 2 and 3). The lower distance limit of this method derives from an increasing influence of exchange interactions of the overlapping π-orbitals of the coupled radicals. The upper distance limit stems from the fact that homo- and heterogeneous contributions obscure the dipolar couplings of interest between the spins influencing the line-broadening on cw-spectra (Steinhoff, 2004).

Pulsed EPR techniques, such as pulsed electron-electron double resonance (PELDOR) (Milov et al., 1984) or double electron-electron resonance (DEER) (Larsen and Singel, 1993), allow to determine the distance between two electron spins based on the distance dependence of their dipole-dipole coupling in the range of approximately 15–80 Å (Figures 3 and 4) (Martin et al., 1998; Pannier et al., 2000; Prisner et al., 2001; Jeschke et al., 2004; Steinhoff, 2004; Schiemann and Prisner, 2007). The lower range distance limitation comes from the fact that for very short distances short microwave pulses are needed to excite the dipolar spectrum, which are restricted by hardware limitations. The upper distance limit is given by the spin-spin electron relaxation time which limits the detection window (Martin et al., 1998; Pannier et al., 2000).

However, many membrane proteins do not naturally contain a paramagnetic center. Thus, site-directed spin labeling (SDSL) made EPR applicable for all proteins, not just those that contain natural radicals or metal centers (Hubbell and Altenbach, 1994; Hubbell et al., 1996, 2000; Schiemann and Prisner, 2007; Klug and Feix, 2008). SDSL is a straightforward method that can be applied

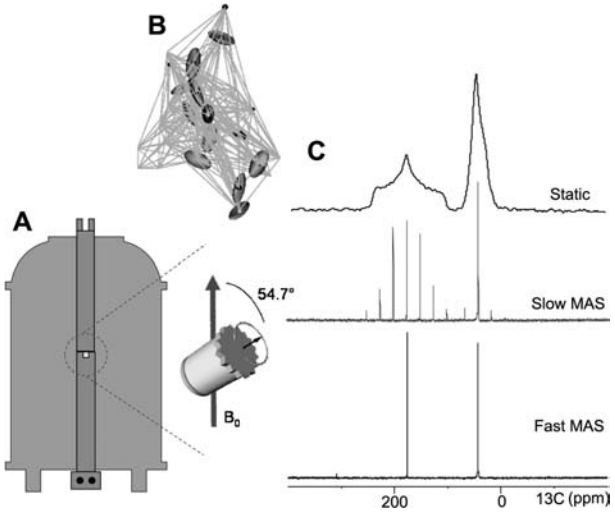

Figure 1 Solid-state NMR.
(A) Magic angle spinning (MAS) around 54.7° removes (B) anisotropic interactions from the spectra. Static ssNMR spectra yield very broad lines (C, top). Slow MAS causes the spectra to dissociate into spinning sidebands around the isotropic resonance, with each sideband appearing at an integer of the spinning speed (C, middle). Fast MAS allows the detection of the isotropic lines at high resolution (C, bottom). All spectra were measured on ^{13}C glycine. Figure modified from Basting et al. (2006).

also to samples for NMR. Cysteine residues are introduced in positions of interest through site-directed mutagenesis and are subsequently chemically labeled with either a nitroxide spin label for EPR, or an NMR active spin label, such as ^{19}F (Huestis and Raftery, 1978; Berliner et al., 1982; Hubbell et al., 1996; Klein-Seetharaman et al., 1999; Hellmich et al., 2009). Of course, it needs to be carefully verified that the cysteine mutation and labeling do not influence the protein function. Bearing this in mind, the local environment of the spin label, secondary structure and sites of tertiary interaction can be probed and protein dynamics, the solvent accessibility of the labeling site and the distance between two spin labels can easily be investigated (Hubbell and Altenbach, 1994; Klug and Feix, 2008).

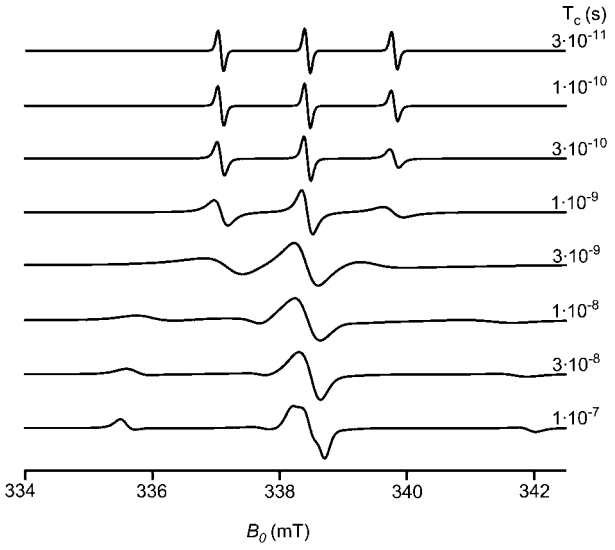

Figure 2 Continuous wave EPR spectra for different correlation times τ_c of a nitroxide spin label simulated with chili, EasySpin (Stoll and Schweiger, 2006).
For short correlation times (fast movement), the lines are narrow. With decreasing movement (long correlation times), the linewidth increases and line-shape changes to yield an anisotropic spectrum.

The intermolecular distances that can be probed by dipolar NMR are limited by the size of the magnetic moments (gyromagnetic ratios) of the nuclear spins in question and vary in dependence of the nuclei used, but are usually in the range <10 Å. Thus, solution and solid-state NMR as well as cw-EPR and pulsed EPR nicely complement one another (Figure 3).

Additionally, EPR spin labels are quenchers in NMR measurements inducing line-broadening or the disappearance of resonances in the proximity of the spin label in an NMR spectrum (as has been demonstrated, e.g., on LacS; Spooner et al., 1999). This allows for the simplification of NMR spectra and the extraction of additional structural information, such as the identification of amino acids in close proximity to the spin label (Dyson and Wright, 2004).

In the following sections, relevant methodology will be explained in more detail on specific examples of secondary active transporters, namely on small multidrug resistance (SMR) proteins and a member of the cation diffusion facilitator (CDF) family. Because they present a functionally very diverse and ubiquitous family of membrane transporters from bacteria to man (Higgins, 1992), the main focus in this review is placed on ABC transporters.

NMR and EPR studies on secondary active transporters

EmrE – a small multidrug resistance protein from *E. coli*

EmrE is a SMR protein from *E. coli* that has been thoroughly investigated with both NMR and EPR. Like all SMR proteins, EmrE contains four putative transmem-

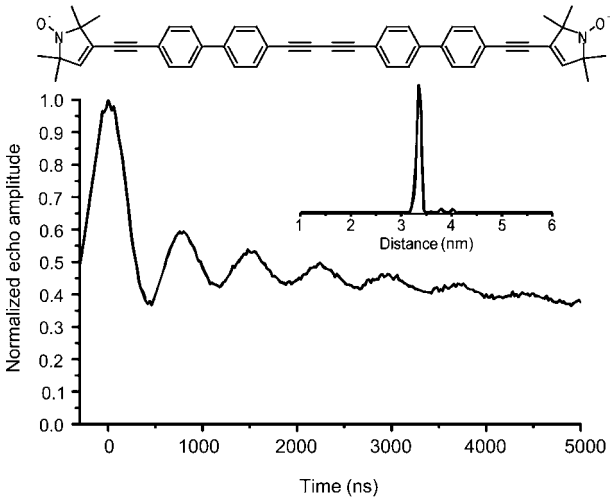

Figure 3 PELDOR trace and resulting distance distribution of a model biradical (modified from Bode et al., 2007).

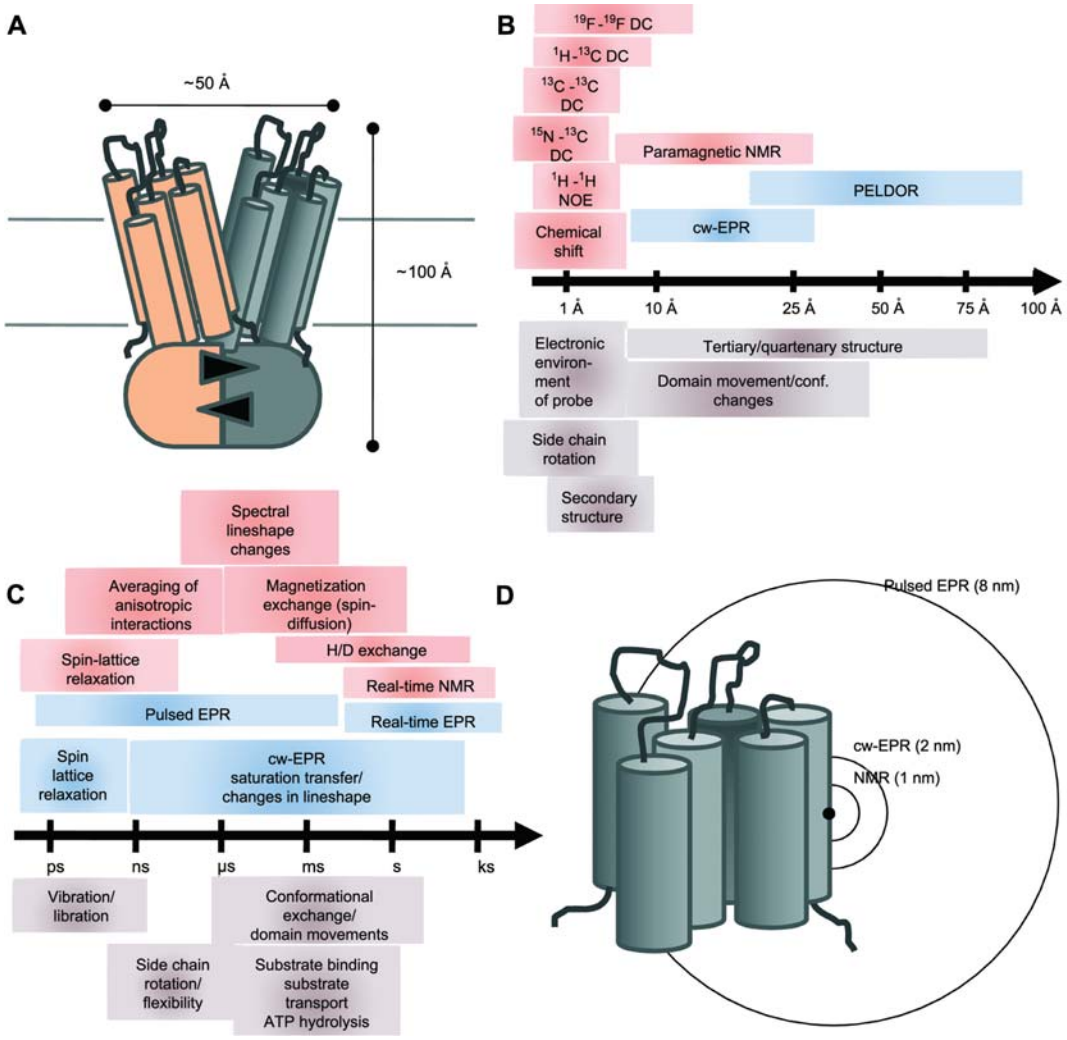

Figure 4 NMR and EPR as complementary methods for the investigation of membrane proteins.
(A, B) Distances that are covered by tertiary and quaternary structure of transporters and in transporter dynamics. (B) EPR and NMR experiments that yield information around these distances. (C) Timescale of biological processes and dynamics and NMR and EPR experiments that can be applied to investigate these dynamic processes. (D) Overview of distances that can be covered by NMR and EPR methods. The respective spin label (radical or paramagnetic center for EPR, NMR-active nucleus for NMR is depicted as a black dot). For detailed reviews see, e.g., Hubbell et al. (2000); Krushelnitsky and Reichert (2005); Boehr et al. (2006); Henzler-Wildman and Kern (2007).

brane helices (Arkin et al., 1996). This has been supported by hydrophobicity analysis (Schuldiner et al., 1997) and also by NMR studies on solubilized EmrE (Schwaiger et al., 1998). The members of the SMR family are rather small with a molecular weight of 10–12 kDa and are found in bacteria and archaea (Grinius et al., 1992; Ninio and Schuldiner, 2003). EmrE is a proton/substrate-antiporter that recognizes cationic aromatic drugs (Yerushalmi et al., 1995). The relevant residue for substrate binding is glutamate-14 embedded in the membrane in helix 1 and is highly conserved in this transporter family (Yerushalmi and Schuldiner, 2000a).

Evaluation of protein fold and sample integrity – a solid-state NMR study on EmrE

A MAS NMR study on EmrE in the 2D crystalline form investigated the role of E14 for the protein fold (Agarwal et al., 2007). Sample integrity was probed with a 2D ^{13}C-^{13}C correlation experiment that allows visualizing carbons that sense each other through dipolar couplings. Different amino acid types and secondary structures in proteins give rise to unique peak distributions in these spectra, thus yielding a 'fingerprint' of the protein at hand. Amino acids that are present in helices will, in general, show a different chemical shift from those in β-sheets, allowing the determination of the structural state of the protein (Spera and Bax, 1991). For proteins with pronounced α-helical regions, this is an elegant way to determine long-time stability of the sample. For very rare amino acids within the protein sequence of a homodimer, such an experiment also has the benefit to reveal the symmetry of the system. For identical residues within an identical chemical environment, the chemical shifts should be the same. If the resonances do not superimpose, this is a good indication that the two nuclei are not

chemically equivalent (e.g., different protonation state, conformation or environment).

Agarwal et al. (2007) also used an EmrE E14C mutant in addition to wildtype EmrE in their NMR study. Interestingly, the spectral quality of the uniformly ^{13}C-labeled E14C mutant was significantly decreased in comparison to the wildtype, indicating a structurally heterogeneous sample, but an investigation of the secondary structure showed that the protein is still dominantly α-helical. This might indicate that E14 is important for the integrity of the tertiary structure of EmrE (Agarwal et al., 2007). Such information, with atomic resolution, obtainable either by X-ray crystallography or ssNMR, is essential to understand the actual protein condition after mutations have been introduced. This indicates how important the sample condition is and demonstrates the unique advantage of ssNMR over other methods in the investigation of membrane proteins due to the possibility to carry out measurements in the native lipid environment which will allow insights into protein function and dynamics that are inaccessible with other methods.

Drug binding to EmrE – a cross polarization solid-state NMR study

The possibility of measuring bound substrate (tetraphenylphosphonium, TPP^+) on the reconstituted EmrE protein has been shown by Glaubitz et al. with cross polarization MAS NMR (CP MAS NMR; Glaubitz et al., 2000). CP MAS is a very valuable method for such experiments since it allows the selective detection of immobilized species among an ensemble, such as a bound substrate while the unbound fraction remains invisible. The feasibility of this method was also proved by the detection of bound substrates by CP MAS NMR in sugar transporters GalP, FucP and LacS and the nucleoside-H^+ symporter NupC, as well as the glucuronid-H^+ symporter GusB (Spooner et al., 1994, 1998, 1999; Appleyard et al., 2000; Patching et al., 2004a,b, 2008; Basting et al., 2006). CP is based on the transfer of magnetization via dipolar couplings from highly abundant nuclei (such as ^1H) to those that are intended to be observed (such as ^{13}C, ^{15}N, ^{31}P) but are inherently less sensitive (Pines et al., 1973; Urbina and Waugh, 1974; Frye et al., 1985). The transfer mechanism works best in rigid samples, either frozen or immobilized by other means. CP thus constitutes a method to either enhance signal or to present a dynamic filter for detection of mobility. The fact that the bound substrate could be detected with CP NMR also shows that the lifetime of the bound species is in the range of ms and therefore quite long lived (Glaubitz et al., 2000) as would have been expected for a substrate with nM affinity (Muth and Schuldiner, 2000).

Parallel or antiparallel topology? EPR studies on EmrE

Interestingly, the oligomeric state of EmrE has been described as any oligomeric species from dimer to tetramer (Yerushalmi et al., 1996; Muth and Schuldiner, 2000; Tate et al., 2001; Koteiche et al., 2003; Butler et al., 2004; Elbaz et al., 2004). Despite the large amount of controversial data, it now seems clear that the functional oligomeric state of EmrE is a homodimer (Soskine et al., 2002; Ubarretxena-Belandia et al., 2003; Butler et al., 2004; Schuldiner, 2009). This was also observed in the cryo-EM projection structure of EmrE (Tate et al., 2001) and the X-ray structure (Chen et al., 2007). The dimer has a shared interface for H^+ and drug binding where transport is possibly achieved by alternating the accessibility of the two sites (Yerushalmi and Schuldiner, 2000b). When EmrE is forced into an antiparallel topology (through coexpression of two engineered mutants that have a changed insertion bias yielding either a $N_{in}C_{in}$ or a $N_{out}C_{out}$ polypeptide) it confers resistance to cells against ethidium bromide and the two glutamates show functional inequivalence (Rapp et al., 2007). The X-ray and electron microscopy data also suggest an antiparallel dimer, the 'dual topology model' (Tate et al., 2001; Ubarretxena-Belandia et al., 2003; Fleishman et al., 2006; Chen et al., 2007; Korkhov and Tate, 2009). On the other hand, crosslinking data, experiments on genetically fused dimers and negative dominance studies yield arguments for a parallel topology (Soskine et al., 2002, 2006; Ninio et al., 2004; Steiner-Mordoch et al., 2008; Schuldiner, 2009). Studying the topology of EmrE is of importance not only to understand the functionality of SMR proteins but also to investigate the underlying evolution of the protein.

Koteiche et al. (2003) have investigated the question of oligomerization using cw-EPR spectroscopy at room temperature with site-directed spin labeling and single-cysteine mutational screening in a cysteine-free background. They found that the transmembrane helices 1 (TM1) (containing the catalytically active glutamate) from two different monomers come into close proximity in the reconstituted protein. However, no higher oligomers were detected. The same study also gave an insight into the binding pocket of EmrE by investigating the accessibility of helix 1 to NiEDDA [nickel(II) ethylenediaminediacetate], a paramagnetic relaxation reagent that is exclusively found in the aqueous phase. TM1 is partially buried in the membrane and partially accessible to the environment forming a V-shaped (N-terminus exposed to lipids, C-terminus tightly packed between other helices) solvent-accessible pore with high side-chain mobility for the binding of diverse substrates. This EPR study on spin-labeled EmrE considered a parallel topology of the EmrE dimer, but their data do not necessarily contradict an antiparallel topology, since the exposure of single residues to lipids and solvents and the proximity of the two glutamate residues is not necessarily changed in the dual topology model (Fleishman et al., 2006; Korkhov and Tate, 2009).

The topology of EmrE has also been investigated by SDSL and multipulse EPR (McHaourab et al., 2008). This EPR approach employed DEER to measure distances between spin labels. Spin labels were introduced along the symmetry axis of the dimer as predicted by X-ray crystallography and cryo EM on either side of the helices. The authors reached the conclusion that the dimer topology has to be parallel because the distances between spin labels in the two monomers should be rather small in a parallel topology and distinctly larger in an antiparallel topology as predicted by the dual topology model.

However, their distance measurements are based on detergent-solubilized EmrE and it thus remains an open question whether enhanced protein flexibility in these non-native conditions could have biased the observed distance distribution.

Functional asymmetry of the conserved glutamates – a solid-state NMR study on EmrE

So far, solution NMR studies on SMR proteins in detergents or organic solvents were mostly carried out on monomeric protein and preparations with dimers could not identify any asymmetry in the glutamates (Schwaiger et al., 1998; Krueger-Koplin et al., 2004; Poget et al., 2006, 2007). The possible structural and functional asymmetry of the glutamates in reconstituted EmrE has been investigated with ssNMR on EmrE (Lehner et al., 2008). In a glutamate-free background, E14 of EmrE was ^{13}C-labeled in a cell free expression approach, because glutamate could scramble when labeled in bacteria. Cell free expression is a new promising approach to prepare isotope labeled membrane protein samples for NMR spectroscopy, especially when scrambling should be avoided or if the membrane protein of interest is toxic for the bacterial expression host (Klammt et al., 2004; Koglin et al., 2006). Because of the high natural abundance of the ^{13}C isotope (approximately 1%) and the high amount of lipids used in reconstituted samples, double-quantum filter experiments had to be applied which allow only the observation of ^{13}C nuclei whose direct neighbor is also a ^{13}C nucleus (Lee et al., 1995). This suppresses unwanted signals from the ^{13}C natural abundance background. The two E14 in the EmrE dimer exhibited NMR signals with two distinct chemical shifts of similar intensities in the protein reconstituted into native *E. coli* lipids. The largest chemical shift separations were found for the backbone resonances which increased upon ethidium bromide binding. Ethidium bromide is a known substrate of EmrE (Ninio and Schuldiner, 2003; Lehner et al., 2008). The ^{13}C chemical shifts of the glutamate backbone resonances Cα and C′ are mainly influenced by the local secondary structure, while the carboxyl resonance Cδ is a sensitive reporter of the side chain protonation state (Betz et al., 2004). The observed chemical shift differences indicate structurally non-equivalent E14s in the EmrE dimer. The chemical shift changes of Cδ upon substrate binding can be explained by substrate induced shielding but also hint at a change in the protonation status or newly formed long-range electrostatic contacts. The observed line narrowing of the backbone resonances show a reduced conformational flexibility upon substrate binding. It seems that in spite of the detected asymmetry within the homodimer, both functional residues are involved in drug interaction. This agrees with previous biochemical experiments which showed that after a conservative exchange of one glutamate to a glutamine the protein only recognizes monovalent substrates and has a significantly slower transport rate (Steiner-Mordoch et al., 2008). The asymmetry of the functional residues is compatible with both a parallel and an antiparallel conformation of the homodimer, thereby not resolving this part of the controversial issue.

The biophysical data available to date has not been able to resolve the question of topology. The NMR and EPR studies were, however, able to allow us to gain an insight into the influence of single residues on protein integrity, the effect of substrate binding on different time scales, the environment of the binding pocket and the functional asymmetry of the glutamates upon drug binding that would not have been achievable with other methods.

Zn^{2+} extrusion by ZitB – a metal ion exporter from *E. coli*

ZitB is a metal ion exporter from *E. coli* from the CDF family involved in metal ion homeostasis. It contains six putative membrane helices and histidine-rich cytosolic motifs. Its overexpression leads to zinc resistance (Grass et al., 2001; Lee et al., 2002). ZitB is a Zn^{2+}/H^{+} antiporter that extrudes zinc ions out of the cell (Chao and Fu, 2004). It can also recognize and transport other ions, e.g., the cell toxin cadmium (Chao and Fu, 2004). In a recent study, ^{113}Cd CP-ssNMR has been used to probe the ion binding to ZitB in native membranes (Rahman et al., 2008). Zn was replaced by Cd, because no natural Zn spin-half NMR suitable isotope exists. $^{113}Cd^{2+}$ can be used as an NMR-active replacement of either Zn^{2+} or Cu^{2+}, because of its comparable sterical properties and similar functionality (Kidambi et al., 2003). With CP NMR, it was possible to detect the fraction of ^{113}Cd that was bound to ZitB. Upon addition of excess of Zn^{2+}, the original substrate, the Cd^{2+} resonance was diminished as Zn replaces Cd and Cd is more mobile in solution than bound to the protein. The addition of Cu^{2+} and Ni^{2+} had the same effect on the ^{113}Cd spectrum, indicating that these ions may represent further substrates for ZitB.

To focus on the ligand instead of the protein itself has a high potential for ligand screening and visualization for membrane transporters of all types, not just metal transporters. It allows to measure substrate interactions without size limitations and even when only little protein is available, the ligand as the isotope labeled interaction partner in the measurement is usually more abundantly available. For example, the conformation of isotope labeled neurotensin bound to its GPCR NTS-1 was solved by ssNMR (Luca et al., 2003). And recently, the conformation of the isotope labeled peptide hormone bradykinin bound to the unlabeled human GPCR bradykinin-receptor-2, which was only available in quantities of approximately 1 mg, was solved (Lopez et al., 2008).

NMR and EPR studies on primary active transporters

ABC transporters

ABC transporters participate in a large number of cell processes, including nutrient import (Davidson et al., 1992), protein secretion (Koronakis and Hughes, 1993), drug efflux (Gottesman and Pastan, 1993; van Veen et al., 2000), ion transport (Jetten et al., 1989), and peptide transport in adaptive immunity (Schölz and Tampé, 2005). They share a common architecture of two nucle-

otide binding domains (NBDs) and usually two times six transmembrane helices forming two transmembrane domains (TMDs) (Holland and Blight, 1999). However, there are some important exceptions to this common theme of 12 helices in the TMDs, such as BtuCD (Locher et al., 2002; Bass et al., 2003) or MalFG (Ehrmann et al., 1998) and some members of the ABCC family, such as MRP1 (Hipfner et al., 1997). Often, the NBDs and TMDs are translated on a single polypeptide, but there are all other types of domain organization possible. In half-transporters, for example, a single NBD is fused with one TMD and the functional transporter forms through dimerization (Linton and Higgins, 1998; Holland and Blight, 1999). While the TMDs are sequentially diverse, the NBDs, which harbor the conserved sequential motifs that identify an ABC transporter – Walker A and Walker B motif (Walker et al., 1982) and the C-loop (ABC signature motif) – share significant sequence identity and structural conservation (Higgins et al., 1986). Other conserved motifs include the D-, Q- and H-loops. The ATP molecule interacts with the Walker A motif from one NBD and the C-loop of the other, thereby acting as the 'molecular glue' that keeps the NBD sandwich dimer together. The Walker B domain coordinates the divalent magnesium cation in the binding site via hydrogen bonds. The D-loop interacts with the H-loop of the opposing NBD and 'senses' the conformational state of this second NBD. The Q-loop interacts with the TMDs and enables conformational crosstalk between NBD and TMD. A conserved aromatic residue upstream of the Walker A domain (A-loop) stacks to the base of ATP and is essential for nucleotide binding (Ambudkar et al., 2006; Kim et al., 2006). (For recent reviews on NBD architecture and interactions see, e.g., Moody and Thomas, 2005; Hanekop et al., 2006; Davidson and Maloney, 2007; Sauna and Ambudkar, 2007; Seeger and van Veen, 2008.) In principle, an ABC transporter couples two cycles: upon ATP binding, the NBDs dimerize, hydrolyze ATP, subsequently release P_i and ADP and finally dissociate. During this cycle, conformational changes are relayed to the TMDs which utilize the energy from ATP binding or hydrolysis to translocate the respective substrate (Senior et al., 1995; Smith et al., 2002; Linton and Higgins, 2007; McDevitt et al., 2008; Orelle et al., 2008b).

Investigation of unfolded protein domain and phosphorylation by NMR – CFTR

Out of the 48 human ABC transporters, 17 have been linked to genetic diseases (Dean and Annilo, 2005) and the most common one is the lung disease cystic fibrosis caused by reduced or absent chloride transport by the cystic fibrosis transmembrane conductance regulator (CFTR, ABCC7) (Moody and Thomas, 2005). CFTR is a member of the ABC superfamily and a chloride channel (Jetten et al., 1989; Riordan et al., 1989). Point mutations (most commonly ΔF508 in NBD1), nonsense mutations or frame shifts in CFTR cause cystic fibrosis (Welsh and Smith, 1993). CFTR shares the basic ABC transporter topology of two times six transmembrane helices and two nucleotide binding domains, but it also contains a unique hydrophilic regulatory (R) domain which connects the two halves of the channel. This R domain contains multiple serine residues that are known to be phosphorylated by protein kinase A (PKA) and protein kinase C (Kennelly and Krebs, 1991; Gadsby and Nairn, 1999; Ostedgaard et al., 2001; Gadsby et al., 2006). This phosphorylation, together with ATP binding and hydrolysis at the NBDs, regulate CFTR channel function and the phosphorylation of the R domain enhances the affinity for ATP at the NBDs (Gadsby and Nairn, 1999; Ostedgaard et al., 2001). Initial NMR studies were carried out on NBD1 of CFTR and on peptides from the F508 region (Massiah et al., 1999; Duffieux et al., 2000). Using fragments or isolated domains of proteins for NMR studies is especially helpful if the protein is not stable over prolonged periods of time or if it exceeds the size limits for solution NMR. Massiah et al. (1999) could show that the region around F508 forms a labile α-helix which is destabilized by the deletion of F508. This might explain, in part, why the pathological CFTR ΔF508 mutant is retained in the endoplasmatic reticulum (ER) and is subsequently degraded (Cheng et al., 1990; Jensen et al., 1995; Ward et al., 1995), although the protein was found to be functional in the ER (Pasyk and Foskett, 1995) and is targeted to the plasma membrane in cell cultures at room temperature rather than blood temperature (Gadsby et al., 2006).

To date, structural information of CFTR includes the crystal structure of mouse and human wildtype NBD1, ΔF508 NBD1 and a number of single site NBD1 mutants at position F508 (Lewis et al., 2004, 2005; Thibodeau et al., 2005). The crystal structure by Lewis et al. shows a distinctly changed surface topography of ΔF508 NBD1 in comparison to the wildtype NBD (Lewis et al., 2005). Comparison of the NBD with crystal structures of other full-length ABC transporters places the F508 region in a position to interact with the transmembrane domain (Bass et al., 2003; Mendoza and Thomas, 2007). The mutation may therefore alter the interaction of the NBD with its transmembrane domain significantly. The R domain is intrinsically 'unstructured' with a small percentage of residues favoring the α-helical state (probably through a continuously interchanging ensemble of heterogeneous structures) in solution with the phosphorylation status being of no detectable influence (Ostedgaard et al., 2000). The R domain has been resistant towards crystallization approaches so far but was successfully investigated by NMR (Baker et al., 2007). The unstructured nature of the R domain might explain why no single phosphorylation site is essential for stimulation of channel activity and why not all phosphorylation sites have the same effect (Gadsby and Nairn, 1999; Ostedgaard et al., 2000). It has been speculated that the phosphorylated R domain must somehow interact with other regions in CFTR in order to regulate channel activity (Gadsby and Nairn, 1999; Ostedgaard et al., 2000, 2001). Baker and colleagues investigated the mobility restrictions of the R domain in the presence and absence of CFTR NBD1 in their NMR study and identified sites through which both domains interact. The structural implications of R domain phosphorylation have been probed: the NMR data support the model of an unstructured protein with rapid interconversion of heterogeneous conformations in both the phosphorylated and unphosphorylated state. The method could also identify those

residues that display a propensity for a transient α-helical structure within the unordered domain. However, the unphosphorylated, unstructured R domain decreases in α-helical population upon phosphorylation without an overall structural ordering taking place. Upon phosphorylation, the overall mobility of the R domain increases and it loses contact sites with NBD1. This finding supports the hypothesis that the R domain inhibits the dimerization of the NBDs until it is phosphorylated (Cheung et al., 2008). Upon treatment with PKA, every previously reported serine phosphorylation site was verified by NMR and a previously unreported threonine residue was discovered as an additional PKA phosphorylation site. In this study, phosphorylation sites were identified through the ^{1}H chemical shifts of the phosphorylated amino acids, but this is of course also possible through ^{13}C, ^{15}N or directly through ^{31}P chemical shifts (Chen et al., 1993; Gemmecker et al., 1997; Seifert et al., 2002).

Within the last two decades, it has been increasingly recognized that many proteins involved in many of the most fundamental regulatory processes in cells are indeed intrinsically unstructured (Wright and Dyson, 1999). Natively unfolded proteins also play key roles in a number of neurodegenerative diseases (Dobson, 2001). The inaccessibility of these proteins to the 'usual' structural methods calls for techniques, such as NMR, to investigate protein interactions, posttranslational modifications and their effect on dynamics and function (Dyson and Wright, 2004; Dedmon et al., 2005; Boehr et al., 2006; Mukrasch et al., 2007).

Investigating NBD-NBD and NBD-nucleotide interactions – MRP1 and MJ1267

Multidrug resistance protein 1 (MRP1, ABCC1) confers resistance to chemotherapeutic agents in human cancer cells and transports anionic conjugate compounds (Cole et al., 1992; Cole and Deeley, 1993, 1998; Leslie et al., 2001). It is also a close relative to CFTR (19% identity) (Cole et al., 1992; Gadsby et al., 2006). In addition to the usual ABC transporter topology, MRP1 contains an extra N-transmembrane domain of five helices in proximity to the N-terminus and an additional cytosolic N-terminal extension (Hipfner et al., 1997; Cole and Deeley, 2006). The NBDs, henceforth denominated as NBD1 (N-terminally bound) and NBD2 (C-terminally bound), are not identical: although they both contain the conserved ABC motifs Walker A and Walker B domain, they display some significant sequence differences. Most importantly, the ABC signature motif LSGGQ (the C-loop) is replaced by LSVGQ in NBD2 and the catalytic glutamate is replaced by an aspartate in NBD1. The Q-loop is preceded by a 13 amino acid deletion in NBD1 in comparison to the NBD2 sequence (Cole et al., 1992; Hipfner et al., 1999; Gao et al., 2000). The two NBDs of MRP1 are also functionally non-equivalent (with NBD1 having a higher binding affinity for ATP and NBD2 a greater hydrolysis capacity), but both are essential for ATP hydrolysis and the inactivation of one abolishes transport activity altogether (Gao et al., 2000; Nagata et al., 2000; Hou et al., 2003; Payen et al., 2003; Cole and Deeley, 2006). There are very few crystals of eukaryotic NBDs known, all of which are monomeric (Gaudet and Wiley, 2001; Lewis et al., 2004, 2005; Thibodeau et al., 2005). Accordingly, the crystal structure from MRP1 NBD1 is also a monomer (Ramaen et al., 2006). This means that other methods for the investigation of catalytic residues and the NBD interface pose attractive alternatives to X-ray crystallography. Isolated NBD1 (Asn^{642}-Ser^{871}) of MRP1 was investigated with NMR and shown to be folded correctly (Ramaen et al., 2003). Upon addition of Mg^{2+}, no spectral changes were observed, but addition of ATP led to an increase of the line-width of Trp^{653} indicating that this residue is involved in ATP binding. The NBD remained monomeric throughout the measurements and thus no ATP hydrolysis occurred. Additionally, peaks corresponding to glycine residues reacted to the addition to ATP and Mg^{2+} supporting the notion that these peaks are those of the glycine residues of the Walker A motif. This observation presents a nice internal control of the correct fold and binding properties of NBD1 under NMR conditions. The observed importance of Trp^{653} for ATP binding in NMR measurements concurs with data from crystal structures and biochemical data of other NBDs, where aromatic stacking with the adenine was shown (Gaudet and Wiley, 2001; Karpowich et al., 2001; Kim et al., 2006). This was later verified by the crystal structure of MRP1 NBD1 (Ramaen et al., 2006). In a later NMR study, the interaction of MRP1 NBD1 with NBD2 (Ser^{1286}-Val^{1531}) and its dependence on ATP was investigated by NMR to probe whether stable dimers form (Ramaen et al., 2005). Isolated NBD1 has a low intrinsic ATPase activity and it has been proposed that NBD1 is the site for ATP binding, whereas its interaction with NBD2 promotes ATP hydrolysis (Payen et al., 2003; Yang et al., 2003). Interestingly, neither with native PAGE nor gel filtration was it possible to detect dimer formation of the two MRP1 NBDs promoted by the addition of Mg^{2+} or ATP (Ramaen et al., 2005). Again, NMR is the method of choice to investigate weak or transient interactions that are not detectable otherwise. Changes in dynamics or other perturbations caused by interaction with ligands or proteins will be directly observable in the fingerprint spectra of the protein of interest. A chemical shift perturbation experiment monitors changes in the chemical shift of a labeled protein when the unlabeled interaction partner is titrated into the sample. For very stable complexes, two sets of peaks will be observed – one for the bound and one for the free species with the latter slowly diminishing during the titration. For low affinity or transient interactions the interface changes constantly (fast exchange) and the observed single peak population will be the weighted average of the bound and free species (Zuiderweg, 2002). Upon addition of MRP1 NBD2 to NBD1, NBD1 does not change its conformation but spectral changes indicate that both partners interact transiently in an ATP dependent manner (but not in the presence of Mg^{2+} and absence of ATP). Some residues show a particularly strong effect upon NBD2 addition, such as Gly^{771} of the C-loop in NBD1 indicating that this motif is located at the NBD interface and is directly involved in ATP dependent dimerization (Ramaen et al., 2005), a fact that is well known from the dimeric NBD crystal structures of bacterial ABC NBDs (Smith et al., 2002; Zaitseva et al., 2005).

The only other NMR studies on ABC transporter NBDs to our knowledge so far include only the C-terminal NBD

of ABCB6 (NBD2), a human mitochondrial ABC transporter involved in ion homeostasis, and MJ1267, the ABC of a branched amino acid transporter (homologous to *E. coli* LivG ABC) from *Methanococcus janaschii* (Wang et al., 2002, 2004; Kurashima-Ito et al., 2006). In the case of MJ1267, they nicely complement the known crystal structures (Karpowich et al., 2001) and for ABCB6 NBD2 they present the first structural and dynamic information.

The branched amino acid transporters share a unique sequence motif, the LivG insert in the NBD which is separated by over 30 Å from the nucleotide-binding site. The correct fold of MJ1267 was verified with NMR and the changes in domain flexibility were probed upon Mg^{2+} ADP binding (Wang et al., 2004). In the apo state, the LivG insert was found to be the most dynamic part within MJ1267. The binding of the nucleotide had especially strong effects on resonances belonging to the Walker A and Walker B motifs, the H-loop and the LivG insert, indicating major conformational changes in these areas. Because the LivG insert is far away from the nucleotide binding core, there seems to be an allosteric dependency of the flexibility of LivG upon nucleotide binding. The LivG insert might interact with the TMDs and communicate the occupancy of the NBD. The Walker A domain shows some structural ordering upon nucleotide binding, whereas the C-loop remains flexible in the apo- as well as the nucleotide-bound form. On the μs-ms timescale, the aromatic residue responsible for adenosine stacking (F17) and the D-loop become rigid upon nucleotide binding. The same effect was observed in B-factor changes of the crystal structure (Karpowich et al., 2001). These dynamic changes support the hypothesis that nucleotides bind within the NBDs of ABC transporters through a conformational capture mechanism where ligand binding selects the most favorable interactions from a preexisting conformational ensemble (James and Tawfik, 2003). These data nicely demonstrate how NMR can provide dynamic data to complement crystal structures.

Similarly, the NMR study on NBD2 of ABCB6 also demonstrated a rather flexible domain in the apo state with reduced dynamics for the residues involved in nucleotide binding upon ADP addition (Kurashima-Ito et al., 2006).

The influence of nucleotide binding on the dynamics of the NBDs in a full-length transporter – LmrA

LmrA is a homodimeric half-transporter from *Lactococcus lactis*. It is a functional homolog of human P-glycoprotein (van Veen et al., 1996). Just like its famous relative, it extrudes a plethora of hydrophobic compounds and therefore conveys multidrug resistance (mdr) to its host organism (van Veen et al., 2000). This transporter has been successfully isotope labeled and reconstituted for NMR (Mason et al., 2004). Under MAS conditions, reconstituted LmrA remains functional as was shown by a ssNMR ATPase assay that detected ^{31}P in order to follow ATP hydrolysis in real time (Hellmich et al., 2008). This is the first time that real-time ssNMR has been carried out on a membrane protein. Deuterium ssNMR has been used to qualitatively probe the overall molecular dynamics of full-length LmrA and to investigate the influence of nucleotide binding to NBD mobility (Siarheyeva et al., 2007). This is the only study to date that directly measured the influence of nucleotides on NBD mobility on the full-length transporter. LmrA contains 48 alanines which are widely dispersed throughout the system (12 in TMDs, 17 in loops and intracellular domains and 19 in the NBD). The alanine side chains were deuterated (L-[β-$^2H_3$]alanine, ala-d_3) which allows monitoring backbone movements due to the direct attachment of the C^2H_3 group to the α-carbon throughout the protein. With ^2H-NMR correlation times from 10^{-10} to 10^{-4} s are covered thus including rotational diffusion of membrane proteins, internal motions and side chain movements (Prosser and Davis, 1994). Ala-d_3-LmrA features two components in its spectrum: a narrow isotropic peak and a broad Pake pattern, typical for deuterium quadrupole couplings in the absence of motional averaging. Going to lower temperatures, the isotropic component gradually disappears from the spectrum, indicating that this initially fast moving component is now "frozen" into many non-interchanging conformations. When similar measurements are repeated with the isolated TMD lacking the NBDs, the isotropic component of the spectrum is not visible even at high temperatures, thus confirming that it really corresponds to the NBDs (and not the loops) which tumble faster than 10^{-5} s under the experimental conditions, whereas the isolated TMDs appear to be immobile on the experimental timescale. In order to investigate the NBD mobility in the pre- and the post-hydrolysis state, Ala-d_3-LmrA was incubated with either AMP-PNP, a non-hydrolyzable ATP analog, or ATP and vanadate, yielding an ADP, V_i trapped NBD in the post-hydrolysis state (Urbatsch et al., 1995; Ecker et al., 2004). At low temperatures, the pre- and post-hydrolysis state preparations show spectra similar to the ground state Ala-d_3-LmrA. At higher temperatures (where the isotropic peak is pronounced for ground state Ala-d_3-LmrA), the prehydrolysis state shows a highly reduced mobility for the NBDs, whereas the post-hydrolysis state shows a mobility that is reminiscent of the ground state. The data therefore indicate that the NBDs as whole domains are highly flexible. Upon ATP binding, their motion is significantly restricted whereas the post-hydrolysis state is motionally similar to the ground state.

Drug-extrusion and drug-membrane interactions – P-glycoprotein

Some cancer cell lines show 'multidrug resistance', a resistance against a multitude of compounds that do not share structural or functional similarities (Endicott and Ling, 1989; Gottesman and Pastan, 1993). These cells express a number of efflux pumps that recognize the detrimental compound and detoxify the cell (Gottesman and Pastan, 1993; Ambudkar et al., 1999). One of these is the ABC transporter P-glycoprotein (Pgp, MDR1, ABCB1), the first ABC transporter to be connected to a mdr phenotype (Juliano and Ling, 1976) through ATP-dependent efflux of a variety of hydrophobic but structurally diverse drugs (Senior et al., 1995). Some possible similarities of Pgp substrates have been elucidated through an *in silico*

comparison of a library of known substrates (Seelig, 1998), but the precise transport mechanisms remain unclear. Models as to how Pgp removes drugs from the lipid bilayer include the 'hydrophobic vacuum cleaner' model and the flippase model (Raviv et al., 1990; Higgins and Gottesman, 1992; Gottesman and Pastan, 1993; Higgins, 1994). To understand the mechanisms of drug extrusion, the transporter mechanism as well as the location of possible substrates within the membrane must be determined. Interestingly, the K_m values of the respective drugs correlate with their hydrophobicity indicating that drug-lipid interactions comprise an initial step in drug recognition by Pgp (Seelig and Landwojtowicz, 2000). It was shown with MAS NMR that drugs that are recognized by P-glycoprotein can be found in the membrane within a restricted area underneath the lipid headgroups (Siarheyeva et al., 2006). The same position of drugs within membranes was predicted in molecular dynamics simulations. Based on these simulations it was proposed that charged drugs experience a deficit of potential hydrogen bonds within the bilayer and that Pgp provides alternative bonds thus picking up the respective drug at the water/lipid interface (Omote and Al-Shawi, 2006). To monitor drug transport by Pgp, a real-time transport assay with spin-labeled verapamil (SLV) by EPR was developed which has the advantage that drug location can be monitored directly through the mobility of the probe (Omote and Al-Shawi, 2002; Al-Shawi and Omote, 2005). SLV was found to have an even higher affinity than unlabeled verapamil making it a very specific substrate. Upon mixing with liposomes, a large amount of SLV was found to have reduced mobility (thus had entered the membrane) and could be easily distinguished from the unbound, mobile population. However, SLV was measured to be 120-fold more hydrophilic than verapamil without the fixed positive charge. Upon addition of MgATP to reconstituted Pgp, a time-dependent decrease of the high-mobility population could be monitored which corresponds to a decrease of the SLV population in the aqueous phase. This was interpreted as the transport of SLV from the outer membrane leaflet into the lumen (supporting the hydrophobic vacuum cleaner model) and the subsequent fast diffusion of aqueous SLV into the outer membrane, rendering transport and not substrate availability the rate-limiting step. In this transport assay, Pgp was able to establish a nearly 13-fold (in some cases up to 25-fold) substrate gradient across the membrane. It was proposed that this maximum value was due to inhibition of 'drug unloading' in the lumen due to the high intralumenal SLV concentration, because substrate leakage induced changes of lipid fluidity could be ruled out.

EPR studies on MsbA

The lipid A transporter MsbA is present in the inner membrane of Gram-negative bacteria (Karow and Georgopoulos, 1993). MsbA is essential and its loss is lethal for *E. coli* as the bacterium then accumulates lipid A in the inner membrane (Karow and Georgopoulos, 1993; Polissi and Georgopoulos, 1996; Zhou et al., 1998). It has also been shown to confer multidrug resistance (Reuter et al., 2003; Woebking et al., 2005). The membrane transporter from *E. coli* has been extensively investigated with SDSL EPR (Buchaklian et al., 2004; Buchaklian and Klug, 2005, 2006; Dong et al., 2005; Westfahl et al., 2008) and DEER (Borbat et al., 2007). The depth of individual amino acids within the membrane and the solvent accessibility in the resting state were probed by cw-EPR (Buchaklian et al., 2004; Dong et al., 2005).

cw-EPR on the NBDs of MsbA

The conserved ABC transporter motifs (Walker A and Walker B domain, C-loop, H-loop, Q-loop) were investigated through the introduction of a large library of single cysteine mutations in the full-length transporter (Buchaklian and Klug, 2005, 2006; Westfahl et al., 2008). A study by Buchaklian and Klug investigated the effect of ATP binding to the Walker A motif (Buchaklian and Klug, 2005). Its α-helical character in a full-length ABC transporter was investigated by EPR and is in accordance with the then known crystal structures of the NBDs of other ABC transporters (Hung et al., 1998; Diederichs et al., 2000; Yuan et al., 2001). The resting state as well as the pre- and post-hydrolysis states were investigated. Those residues that had been previously implicated as being in direct contact with ATP showed significant decrease in spin label mobility upon ATP binding, but the secondary active motifs remain intact (Buchaklian and Klug, 2005). As fits the mechanism of a sandwich dimer formation upon ATP binding and hydrolysis (Smith et al., 2002), the accessibility of the spin labeled Walker A domain was significantly reduced in the transition state (V_i, ADP trapped state) as opposed to the resting state. Interestingly, MgAMP-PNP, which is often used as a non-hydrolysable analog of ATP in functional studies, did not induce the same structural changes as ATP. In a related cw-EPR study, Buchaklian and Klug (2006) investigated the effect of the ATP hydrolysis cycle on the C-loop and the H-loop. Again, these residues were blocked from solvent access and showed tighter packing when ATP was bound. A recent comprehensive study of the MsbA NBDs showed that the H-loop, C-loop and Q-loop all display significant structural rearrangements upon ATP hydrolysis but not in the preceding binding step (Westfahl et al., 2008). The transition from the ATP bound pre-hydrolysis state to the post-hydrolysis state was shown to induce the most distinct structural changes within the NBD and might therefore be the moment in the hydrolysis cycle where important structural information is relayed to the TMDs. Only the Walker A motif seems to react structurally to the addition of ATP (Buchaklian and Klug, 2005). Indeed, this could indicate that the Walker A motifs interact with the ATP molecules in the aqueous environment and that NBD dimerization does not occur directly upon ATP binding (in the absence of Mg^{2+}) but only when Mg^{2+} is present and hydrolysis begins. In the multidrug ABC transporter BmrA from *Bacillus subtilis*, Mg^{2+} has been found to either greatly improve ATP affinity for the NBDs or to enhance NBD closure (Orelle et al., 2003). On the other hand, ATP binding has been shown to be Mg^{2+} dependent in P-glycoprotein (Hrycyna et al., 1999) and the presence of the maltose binding protein and ATP (without Mg^{2+}) are sufficient to lead to dimer formation of the NBDs of the maltose transporter $MIFGK_2$ (Orelle et al., 2008a). Mg^{2+} may therefore have different effects on

ATP binding and NBD dimerization in various ABC transporters.

Domain movements in an ABC transporter – EPR studies on MsbA

Two crystal conformations of the apo state MsbA from *E. coli* exist. One shows a large tilt between the two membrane bundles of the MsbA homodimer giving rise to a large cavity (Ward et al., 2007). However, it seems difficult to reconcile the large distance between the fully separated NBDs in the MsbA crystal structure (Ward et al., 2007) with an ATP dependent dimerization model of the NBDs. The structures of other ABC transporters, e.g., BtuCD or HI1470/1, show a nucleotide free state where the NBDs are still in close contact (Locher et al., 2002; Pinkett et al., 2007). The second structure of MsbA without nucleotide more closely resembles these structures where the NBDs remain in contact at their base despite the absence of nucleotides (Ward et al., 2007) and better suits a tweezer-like motion such as has been described, e.g., for the NBDs of the maltose ABC transporter during their hydrolysis cycle (Chen et al., 2003). However, the presence of a large cavity in MsbA was confirmed by EPR studies (Buchaklian et al., 2004; Dong et al., 2005), so it remains to be clarified whether the opening of such a large cavity is part of the hydrolysis cycle of MsbA. Borbat et al. (2007), using pulsed EPR on singly labeled MsbA (resulting in two spin labels in corresponding environments in the homodimer), found that the transporter populates a number of conformations in the apo state, whereas in the more rigid post-hydrolysis state well-defined distances between spin labels are observed. Alternating access from cytoplasmic or periplasmic side is due to extensive rigid body movement. Borbat et al. have presented the only study to date which included a MsbA substrate, lipopolysaccharide (LPS), but the recently described spin-labeled lipid A – which was shown to remain a substrate of MsbA – will probably remedy this in the near future (Bretscher et al., 2008). Addition of LPS to spin-labeled MsbA increased the average distance of spin labels on the periplasmic side thus supporting an outward facing conformation (Borbat et al., 2007). The study by Dong et al. also revealed a mechanism of extensive repacking and rigid body movements upon transition from one state of the ATP hydrolysis cycle to the next. These data from both studies are compatible with the elegant ATP switch model that was proposed for the mechanism of action of ABC transporters regardless of their physiological function by Higgins and Linton based on experimental data on a number of transporters (Higgins and Linton, 2004; Linton and Higgins, 2007).

The NBDs in the crystal structure by Ward et al. show a distance of approximately 50 Å. In the EPR study by Borbat et al., a large distance distribution centering approximately 50–60 Å is observed for the apo state MsbA. When the protein is trapped in the transition state (ADP, V_i), the NBDs come into closer proximity and distance changes of 30 Å for a spin label at the base of the NBDs are observed (Borbat et al., 2007). This study was carried out with only one EPR label on the NBDs, and NBDs, even in full-length transporters, have been observed to be quite flexible in the apo state (Siarheyeva et al., 2007). It will be interesting to see whether EPR studies with spin labels at other positions of the NBD (and also in other ABC transporters) can confirm this type of distance distribution.

These combined studies on MsbA allowed, however, for the first time to appreciate global conformational changes and flexibility under physiological conditions in such a large membrane transporter.

ABC importers – BtuCD and MalFGK$_2$

EPR on the outer membrane protein BtuB

Since the substrates transported into the cytosol of Gram-negative bacteria need to pass across two membranes, every importer in the inner membrane needs an additional outer membrane transporter to translocate substrates into the periplasm, where they are recognized by their respective substrate binding protein and are passed on to the specific transporter of the inner membrane (Quiocho and Ledvina, 1996). In the case of vitamin B_{12} import, both transport proteins, BtuB, a TonB-dependent β-barrel receptor in the outer membrane as well as the ABC transporter BtuCD in the inner membrane have been investigated by EPR (Merianos et al., 2000; Coggshall et al., 2001; Fanucci et al., 2002, 2003a,bc; Xu et al., 2006; Hvorup et al., 2007; Goetz et al., 2009). TonB-dependent receptors are coupled to the inner membrane proteins TonB, ExbB and ExbD which allows them to utilize the protonmotive force (pmf) of the inner membrane to translocate substrates across the outer membrane through conformational changes energized by the pmf (Bradbeer, 1993; Ferguson and Deisenhofer, 2002; Koebnik, 2005). They all share a highly conserved region of eight amino acids, the Ton box that reaches into the periplasm to contact TonB, when a substrate is bound to the extracellular side (Gudmundsdottir et al., 1989; Merianos et al., 2000). The β-barrel character of BtuB and the position of the labeled residues within the membrane have been investigated by SDSL EPR, and interestingly a 'mobility gradient' of increased label mobility through the pore from the extracellular to the periplasmic site was observed by Fanucci et al. (2002). The Ton box itself and the influence of vitamin B_{12} as well as of a *ton*-protein targeting toxic protein, an E-colicin, were investigated by Kadner, Cafiso and co-workers (Merianos et al., 2000; Coggshall et al., 2001; Fanucci et al., 2003a,b,c; Xu et al., 2006). In the absence of the substrate, the Ton box rests within the β-barrel. Upon substrate addition, this domain becomes highly mobile, is extended and reaches 20 to 30 Å into the periplasm (as measured by DEER) in order to contact TonB. This means that the extracellular presence of substrate is required for contact with TonB and the subsequent energy relay that is necessary to transport the substrate across the outer membrane. The colicin investigated by Fanucci et al. competes with vitamin B_{12} for the binding site on BtuB (Di Masi et al., 1973; Cao and Klebba, 2002). The colicin was found to independently stabilize the docked form of the Ton box (where it rests within the β-barrel of BtuB) and continued to do so even if sub-

strates favoring the extended, undocked conformation were present thus allowing some conclusions for a possible mechanism of action for this toxin (Fanucci et al., 2003a).

EPR on the vitamin B_{12} ABC transporter BtuCD

The inner membrane vitamin B_{12} transporter, BtuCD, contains two copies of BtuC that form the transmembrane domains with ten helices each and two NBDs, BtuD (DeVeaux and Kadner, 1985; Locher et al., 2002). Like all other ABC import proteins, such as $MalGFK_2$, it requires a periplasmic substrate binding protein, in this case BtuF, to deliver the substrate to the transporter (Cadieux et al., 2002). A crystal structure of the $BtuC_2D_2$-F complex without the bound substrate and nucleotides was solved recently (Hvorup et al., 2007). It shows BtuF in the open-lobed conformation bound to the periplasmic side of BtuCD. This finding is in agreement with the EPR data from $MalGFK_2$ which also implicated that the open substrate binding protein has a low substrate affinity (Austermuhle et al., 2004; Orelle et al., 2008a). Intriguingly, the transmembrane domain of the BtuF-bound BtuCD homodimer showed structural asymmetry in a subset of four helices (TM3, 4, 5, 5a) yielding an occluded cavity without apparent room for the rather bulky substrate (Hvorup et al., 2007). Such an asymmetry was not observed in the first crystal structure of BtuCD in the absence of BtuF that was also obtained in the nucleotide-free state in an outward facing conformation (Locher et al., 2002). The asymmetry of the helices in BtuCD with and without BtuF was investigated by SDSL EPR through the introduction of single cysteine mutations at the beginning and end of TM5 thereby yielding a set of two spin labels per monomer and four per complete transporter. In the absence of BtuF, the protein shows high mobility on the periplasmic site ('periplasmic gate') which is nearly unaffected by the presence of nucleotides (AMP-PNP or ATP/V_i) (Hvorup et al., 2007; Goetz et al., 2009). Addition of vitamin B_{12}-bound BtuF significantly decreases this mobility, possibly due to protein-protein interactions. Binding of vitamin B_{12}-BtuF also leads to the formation of two distinct spin label populations within the transporter: the spin labels at the respective positions of the two transporter halves are forced into different environments/different sides of their respective helix 5 thus creating the asymmetry that was also observed in the X-ray-structure. When nucleotides were added to the BtuCD-BtuF complex, the labels on the cytoplasmic side of the helices ('cytoplasmic gate') showed high mobility which could correspond to an inward facing conformation in the ATP-bound state. This leads to a functional model where the empty BtuCD waits for vitamin B_{12}-BtuF in the outward facing orientation. Upon binding of BtuF as well as ATP, the transporter opens to the cytoplasmic site and shuttles the substrate across the membrane. Binding of ATP to the BtuCD-BtuF complex is a prerequisite for opening of the cytoplasmic gate. This change occurs through an "asymmetric" intermediate in which both TMDs might not change their conformations simultaneously. ATP hydrolysis and BtuB, P_i and ADP release would then reset the transporter for substrate uptake.

Interaction of $MalGFK_2$ with the substrate binding protein – a solution NMR study

The maltose transporter of *E. coli* consists of two membrane domains, MalF and MalG (with eight and six helices, respectively), as well as two copies of the ATP binding cassettes MalK, and thus belongs to the ABC superfamily (Higgins et al., 1985; Davidson and Nikaido, 1990, 1991; Ehrmann et al., 1998). In contrast to other NBDs, MalK posses an additional C-terminal domain (Diederichs et al., 2000) that apparently increases the affinity of those two subunits for each other (Chen et al., 2003). Since this ABC protein ($MalFGK_2$) is an importer in the inner membrane of Gram-negative bacteria, an outer membrane maltose pore (LamB) and a periplasmic substrate binding protein, maltose binding protein (MBP, MalE), are essential to complete the complex for transport (Kellermann and Szmelcman, 1974). MBP delivers maltose to $MalFGK_2$ for import into the cytoplasm and stimulates ATP hydrolysis at the NBDs (Davidson et al., 1992). Both MalF and MalG are involved in MBP binding (Hor and Shuman, 1993), but the periplasmic loop P2 of MalF (MalF-P2) shows a very distinct fold and seems to be involved in recruiting MBP to the transporter (Daus et al., 2009). A recent NMR study on MalF-P2 expressed as a soluble protein found that it folds independently of the transmembrane part of the transporter (Jacso et al., 2009). The structure of the two-domain protein in solution strongly correlated to that in the crystal structure in complex with MBP (Oldham et al., 2007). In the NMR study, it was found that MBP and MalF-P2 can interact in the absence and presence of bound maltose and the effect on the spectra was the same in both cases indicating a similar binding mode. Upon binding of MBP, resonances of domain 1 of MalF-P2 remained rather unperturbed, whereas domain 2 showed distinct spectral changes due to conformational changes which it had to undergo in order to accommodate the ligand.

Domain movement and interaction with the substrate binding protein – EPR on $MalFGK_2$-MBP

The NBDs, the MBP and the overall conformational changes during the maltose transport cycle have been investigated with SDSL EPR (Hall et al., 1997a,b; Austermuhle et al., 2004; Grote et al., 2008; Orelle et al., 2008a). The MBP can be visualized as a two-lobed protein that binds its ligand in the middle of the two lobes (Spurlino et al., 1991). It had been observed that some molecules bind the MBP and are transported by the $MalFGK_2$ complex, others however that also bind the MBP with high affinity could not be transported across the membrane (Hall et al., 1997a). A previous NMR study with labeled ligands showed that there were different binding modes that distinguished these substrates (Gehring et al., 1991). With a single EPR spin label on each lobe in proximity to the ligand binding site it was shown that the substrates that are transported induce a large conformational change, a 10 Å "closure" of the lobes, upon binding. Non-transported molecules do not invoke closure of MBP, hence no transport is induced (Hall et al., 1997b). Austermuhle et al. (2004) extended the investigation of spin-labeled MBP by observing the

effects of the $MalFGK_2$ complex on MBP binding and conformational changes. Interestingly, MBP can bind to $MalFGK_2$ irrespective of whether bound maltose is present or not (Merino et al., 1995). However, it has a comparably low affinity to the apo state transporter as compared to the ATP-bound (without Mg^{2+} to impede hydrolysis or with non-hydrolyzable ATP analogs) or the transition state (ADP, V_i) $MalFGK_2$. When bound to the apo state, maltose bound MBP remains in the closed formation. In the ATP-bound or transition state $MalFGK_2$, maltose affinity of the MBP is lost and the two lobes reopen towards the transporter transmembrane domain. Of course, in a transport cycle, this step would therefore coincide with the closure of the MalK subunits on the cytoplasmic site. The ADP-bound state was not able to stabilize the MBP-bound state and was spectroscopically similar to the apo state. Based on these results, Austermuhle et al. (2004) suggest a transport model where closure of the NBDs enhances the transporter affinity for MBP which opens its lobes upon binding, loses its affinity for maltose and can thus release the substrate into the transporter lumen which guides it across the inner membrane into the cytoplasm. Release of P_i at the NBDs triggers disintegration of the NBD dimer as well as loss of MBP and resets the transporter to its initial state. A more recent study completed this picture and introduced an even more detailed description of the modus operandi of $MalFGK_2$ (Orelle et al., 2008a). Additional spin-labeling of the MalK domains allowed for investigation of influences of nucleotides and MBP on transporter conformational changes. Neither ATP nor MBP alone were sufficient to trigger NBD closure. This is a significant finding since a number of studies on isolated NBDs as well as full-length transporters showed that ATP binding triggers NBD closure on its own (Moody et al., 2002; Smith et al., 2002; Hollenstein et al., 2007), also in the isolated MalK domains (Chen et al., 2003). In the EPR study, only the combination of both additives could induce concerted structural changes that led to MalK closure. Experiments on other ABC transporters also seem to indicate that substrate binding is the first step in the non-futile ATP-hydrolysis/substrate translocation cycle, reviewed by Linton and Higgins (2007). To test whether ATP hydrolysis would promote reopening of the NBDs, Mg^{2+} was added to the closed $MalFGK_2$ with MBP and ATP present. Strikingly, the transporter did not fully open, but adopted an intermediate conformation between open and closed state that was also observed after the incubation with maltose-MBP and ADP-Mg^{2+}. Upon removal of Mg^{2+} by EDTA, the transporter returned to its open state indicating that Mg^{2+} is an important stabilization agent of this intermediate conformation. This strongly supports the existence of three distinct catalytic states of the transporter during a hydrolysis-transport cycle and may be a hallmark of transport by ABC transporters in general.

Outlook

There has been great progress in the last years, both with the development of new NMR and EPR methods, as well as in sample preparation and availability of many membrane transporters for biophysical studies. This will lead to many novel insights on individual transporters as well as entire transporter classes, further our understanding of the underlying principles of transport across membranes and allow us to appreciate these processes in atomic detail.

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