Dynamics and regulation of ABC transporters elucidated by EPR

Abhandlung zur Erlangung des Titels

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“I hate to advocate drugs, alcohol, violence, or insanity to anyone but they’ve always worked for me”

Hunter S. Thompson
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Abstract

ATP-binding cassette transporters (ABC transporters) harness energy from hydrolyzing ATP to transport many different substrates across cell membranes. ABC transporters occur in all three kingdoms of life and are commonly divided into four families: the type I and type II importers, which appear in prokaryotes only, the exporters, which exist ubiquitously, and the energy coupling factor (ECF) type transporters. ABC importers of type I and II transport lots of different substrates into the cell, with a corresponding substrate binding protein (SBP) being involved. Exporters usually are less substrate specific than importers and transport products from metabolism or lethal substances outside of the cytosol. Their function makes them very important for cell viability in general and for multidrug resistance properties in particular.

All ABC transporters consist of two transmembrane domains (TMDs) which form the translocation pathway and two nucleotide binding domains (NBDs) which hydrolyze ATP and thus deliver the energy for switching the transporter from an inward to an outward facing conformation or vice versa. Although several high resolution X-ray structures of quite a number of transporters are known, essential characteristics of the transport mechanism, the substrate translocation pathway, conformational changes during the nucleotide cycle, regulatory mechanisms etc. are still not completely understood. In this thesis I provide three case studies performed on one ABC importer (MalFGK$_2$) and two exporters (MsbA and TM287/288) with electron paramagnetic resonance spectroscopy (EPR) in combination with site-directed spin labeling (SDSL) of specific, engineered cysteines in the transporters.

The probably best studied importer is the type I maltose importer from *E. coli* MalFGK$_2$ in conjunction with its corresponding substrate binding protein MalE. Crystal structures of the pretranslocation, ATP- and vanadate trapped state exist and have largely contributed to the general understanding of the transport mechanism. However, the response of the transporter to the presence of liganded or unliganded MalE as well as the stimulation of ATPase activity even if unliganded MalE is present are still hardly understood. In this thesis I present results which show that the transporter binds MalE in the apo- and ADP-state independently on the presence of substrate. Different periplasmic conformations of the transport complex are adopted depending on whether maltose is present or not. In the ATP-state, the NBDs are driven into a fully closed conformation no matter if MalE/maltose or MalE alone are present, triggering the hydrolysis of ATP, thus generating a futile cycle in the latter case. All together, the results could widen the knowledge about the conformational plasticity of the type I ABC transporters.

In case of the homodimeric, lipid A and lipopolysaccharides transporter MsbA from *E. coli* which is homologous to the multidrug exporter LmrA of *L. lactis* and mammalian multidrug resistance transporters such as P-glycoprotein, three structures showing the transporter in the apo, closed apo and AMP-PNP [*Adenosine 5'-(β,γ-imido)triphosphate*] state are known. The apo and closed apo structures exist at a rather low resolution, namely only showing the $C_{\alpha}$-atoms. The outward facing, AMP-PNP-bound structure is similar to the one of Sav1866. Interestingly, in this thesis I show that a 1:1 stoichiometric binding of an in *vitro* selected designed ankyrin repeat protein (DARPin) to MsbA was observed. This revealed an unforeseen asymmetric nature of the homodimeric transporter.
MsbA. In fact, by means of EPR, I show that the binding epitope of the DARPin could be mapped to the TMDs, lying outside of the twofold symmetry axis. Furthermore, no differences concerning the pronounced switch from inward to outward facing conformation in presence of the DARPin were observed. This and additional biochemical experiments showed that the transporter’s ability to translocate substrate is not impaired by binding of the DARPin. Moreover, a stimulation of the ATPase activity is observed if DARPins are present, suggesting that the catalytic cycle is accelerated if MsbA is in an asymmetric state.

Crystal structures of the heterodimeric ABC exporter TM287/288 from *T. maritima* showed an asymmetry in the NBDs in the apo and AMP-PNP-bound state. The current model for the transport mechanism of TM287/288 emanates from ATP being bound and hydrolyzed to the consensus site in each transport cycle and ATP being exchanged from time to time at the degenerate site. However, in the presence of AMP-PNP, only one molecule of this nucleotide analog was crystallized bound to the degenerate NBD site, and, in contrast to many other exporters, the two NBDs were found to be in close contact, with the overall structure of the transporter trapped in an inward-facing state. Interestingly, also in the crystal structure obtained without nucleotides, the NBDs were found to be in close contact, in contrast to apo states of other transporters like MsbA or ABCB10. By comparing the apo and the AMP-PNP-bound conformations by EPR experiments performed in frozen solution, the peculiar behaviour of this heterodimeric exporter in the presence and absence of AMP-PNP was confirmed.
Zusammenfassung


1 Introduction

1.1 EPR techniques for studying macromolecules

Electron paramagnetic resonance (EPR) can be called the big little sister of nuclear magnetic resonance (NMR), the other and more widely used magnetic resonance technique. “Big” refers to the fact that the magnetic moment resulting from an electron spin is approximately 660 times bigger than the one resulting from a proton nuclear spin. Exactly speaking, for an electron bound to an orbit in the semi-classical description the ratio between the magnetic moments is inversely proportional to the ratio of the corresponding masses:

\[
\frac{\gamma_e}{\gamma_P} \sim \frac{m_P}{m_e}
\]

where \(\gamma_e\), \(\gamma_P\) denotes the gyromagnetic ratios of the electron, proton and \(m_e\), \(m_P\) the respective masses. Based on this fact, EPR would have a by two orders of magnitude higher sensitivity compared to NMR, as reflected in the numbers for \(\frac{\gamma_e}{2\pi} = 28\,000\,\text{MHz} T^{-1}\) and \(\frac{\gamma_P}{2\pi} \approx 43\,\text{MHz} T^{-1}\). In reality, however, the advantage in sensitivity of a typical EPR spectrometer at Q band (33–35 GHz) compared to a comparable 800 MHz NMR spectrometer is decreased by one order of magnitude due to the lower magnetic field (1.2T compared to 18.8T) and slightly further decreased by sensitivity advantages on the electronic level.

EPR is the little sister due to the fact that most samples don’t contain any free electron spin and are thus EPR-silent, the scope for applications is far off the routine level of NMR. Even if one or more electron spins are present (e.g. metal-containing samples, natural radical species, spin labels), information which can be gained is only coarse-grained. However, EPR can be applied to biomolecules of any size, with no limitation concerning their environment. In favorable cases, one protein sample suitable for NMR can lead to a complete structure determination with atomic resolution. On the other hand, the biggest advantage for NMR, the omnipresence of nuclear spins, can turn into opposite for large molecules such as most membrane proteins are. Due to faster relaxation, these systems are not suitable for standard NMR techniques for structure determination (COSY, TOCSY, NOESY). Furthermore, the presence of protons in buffer, detergents or lipids makes these problems even worse. The other big technique for structure determination with atomic resolution, X-ray crystallography, is always dependent on the availability of good crystals. If they can be achieved, it is up to date the most powerful method for elucidating protein structures.

1.1.1 Site-directed spin labeling

Most proteins naturally do not contain a paramagnetic center, thus being EPR silent. Site-directed spin labeling (SDSL) is one possibility to introduce a spin center into proteins. It had been first demonstrated on bacteriorhodopsin mutants[1] and until now has been applied to numerous proteins[28]. The technique is based on a reaction of a cysteine’s thiol group with a thiosulfonate-group attached to a 1-Oxo-2,2,5,5-tetramethyl-Δ3-pyrroline
ring bearing a free electron at the NO-group, called MTSSL. Containing a disulphide bridge itself, the label is highly specific to the thiol of a cysteine. The disulphide bridge is re-formed by the cysteine side chain in exchange to the sulphonate, which acts as a leaving group. Although the NO radical is very stable under biochemical conditions, the weak point is that both the disulfide bridge as well as the nitroxide group may easily be cleaved or reduced to hydroxylamine, respectively, under reducing conditions. This leads either to unbound or to EPR silent spin-labels, both cases are usually not useful for addressing structural questions.

The major advantage, because of which SDSL has found such widespread use, is the easiness of labeling, which works in most cases following a standard protocol. Since the label is significantly smaller than a fluorescent dye, much more sites are accessible for the label. Furthermore, since the size of the spin-labeled side chain is of the same order of that of the natural amino acids’ side chains, the probability of distorting the backbone conformation of the respective protein is much smaller than for labels being bigger by almost one order of magnitude.

Figure 1.1: **Skeletal formula of MTSSL side chain.** The conformational space of the side chain is represented by the angles $\chi_1 - \chi_5$, each representing a relevant rotatable bond. Fig. taken from[9].

The MTSSL side chain is highly flexible, thus it can adopt many possible conformations which reduces the probability of distorting the protein’s backbone significantly. On the other hand, this large conformational space can lead in some cases to a very broad spatial distribution of the spin center when MTSSL is attached to a protein. As a possible consequence, distance distributions obtained by measuring the distance between two spin labels in a protein sample may become very broad and thus can create problems in interpretation[10-12]. Besides the most widely used MTSSL radical, further commercially available spin labels exist, such as the iodoacetamido proxyl (IA-proxyl)[13] or maleimide-labels[14]. Being more stable than MTSSL, they are, on the other hand, less selective.

### 1.1.2 The cw-EPR experiment

From the point of view of hardware requirements, the simplest commercially available spectrometers are built for the continuous wave (cw) EPR experiment. If a sample with $S \neq 0$ is placed into a homogenous magnetic field, the spin states split according to $m_S = -S, -S + 1 \ldots S - 1, S$ due to the electron Zeeman interaction. The energy difference between these states is defined by the electron Zeeman interaction, which is described as

$$\Delta E = g_\mu_B B_0 |\Delta m_S|,$$

where $\Delta E$ symbolizes the energy difference, $g$ the $g$-factor of the spin center (which is discussed below), $\mu_B$ Bohr’s magneton and $\Delta m_S$ the difference of the magnetic quantum
numbers associated with the respective transition. This rather simple equation is valid for systems fulfilling the high-field approximation, which means that the electron Zeeman interaction is dominating. As a consequence, all states of nuclear and electron spins are quantized along the external magnetic field vector \( \vec{B}_0 \) with its magnitude \( B_0 \). For spin \( \frac{1}{2} \) systems, two energy levels are obtained with \( \Delta m_S = \pm \frac{1}{2} \), leading to the further simplified term \( \Delta E = g \mu_B B_0 \). From this equation it follows that the splitting of the two states is linearly dependent on the strength of the external magnetic field. If one irradiates the sample with an electromagnetic radiation of a wave-length fulfilling the resonance condition

\[
\Delta E = g \mu_B B_0 = h \nu,
\]  

a transition between the two spin states occurs which can be probed in a spectrometer. Because the energy difference and thus the resonant frequency are linearly dependent on the magnetic field strength, giving the resonant frequency for characterizing a system is not enough. Thus, the g-factor is introduced as a dimensionless gyromagnetic ratio which is field independent and therefore is much more suitable for sample characterization. Equivalently, the gyromagnetic ratio \( \gamma_e \) which is defined as

\[
\gamma_e = \frac{g e}{2m_e} = \frac{g \mu_B}{h},
\]  

could be used, but since it is rather inconvenient to use, it is common to use the g-factor in EPR. Starting from this two-level system, one can understand the basic setup of a cw EPR spectrometer. The parts which are needed comprise a source for radiation (microwave frequency), a magnet (electro or superconducting magnet) and a detector. If one draws an analogy to widely used UV/vis spectrometers, the setup would be build as a transmissive one, measuring the absorbance of the sample. In optics this is possible because only ground states are populated which leads to a well measurable absorbance of the sample. Unfortunately, in EPR (and of course NMR) the levels are populated nearly equally with only a small fraction of spins difference. Consequently, a setup like this is not sensitive enough. This has led to a reflective setup design sketched in fig. 1.2. Briefly, the sample is placed into a cavity within a magnetic field. The cavity is designed such that nearly no microwave intensity which is coupled into it and reaches the sample is reflected. If a sample fulfills the resonance condition of eq. 1.3, a small amount of the intensity is reflected and leaves the cavity towards the detector. Since the cavity works only for a narrow frequency range, the microwave frequency is kept constant and the magnetic field is swept.

The sensitivity can be further increased by modulating the magnetic field around its main value by up to a few Gauss (\( 1 \text{G} = 1 \cdot 10^{-4} \text{T} \)). This results in a modulation of the wanted signal which can be selectively detected. Since microwave detectors are sensitive on a very broad frequency range, they collect a lot of noise among the desired signal. If the signal is modulated with a certain frequency, a narrow band filter can be applied to the detector signal, thus reducing the noise a lot.

1.1.3 cw-EPR on nitroxyl labeled biomolecules

With a rather simple cw experiment it is possible to obtain information from different fields which will be introduced below. From the shape of a room temperature (RT) spectrum one can extract the reorientational motion of the spin label. Water exposed sites or loop regions display a high degree of mobility resulting in a low hyperfine splitting and small
linewidth. The problem is, that one has to deal with several correlation times which are described as follows\cite{16}:

- the rotational correlation time of the entire protein
- the effective correlation time of the rotational motion of the spin label linker
- the effective correlation time of the segmental motion of the protein backbone

It is impossible to obtain all three correlation times with one experiment since the characteristic timescales range from $\mu$s to ps. To overcome this, EPR spectra at different frequencies have to be measured to separate the different motional regimes. This approach is called multifrequency EPR and was shown on spin-labeled T4 lysozyme as a model system\cite{17,18}.

Similar to the mobility, one can get information about the water accessibility of a spin labeled site. For this, a paramagnetic quencher agent (like NiEDDA) is added to the solution. Then, a cw power saturation experiment is performed, where the signal intensity is plotted against the applied microwave power in absence and presence of the quenching agent. From this, an accessibility parameter $\Pi$ can be gained\cite{19-21}.

A polar environment of the spin label shifts the hyperfine value for $A_{zz}$ to higher and $g_{xx}$ to lower values. Whereas $A_{zz}$ can be well extracted from X band spectra, where the hyperfine interaction dominates, it is necessary to go to W band for obtaining $g_{xx}$ due to the larger electron Zeeman interaction at higher frequencies. Nevertheless, in principle both values can be extracted from only W band spectra. If one performs this experiment for several sites of a spin labeled protein, one can plot the measured $A_{zz}$ vs. the $g_{xx}$ values and derive a structural model\cite{21}.

Figure 1.2: Sketch of a basic cw EPR setup. Taken from\cite{15}.
1.1.4 Measuring distances with EPR: dipolar interaction

For measuring distances between two electron spins, the two mainly applied techniques are both based on the dipole-dipole interaction of two electron spins. The “full” static spin Hamiltonian can be written as

\[ H = H_{EZ} + H_{HF} + H_{NZ} + H_{NQ} + H_{ZFS} + H_{EX} + H_{DD}. \]  \hspace{1cm} (1.5)

Here, \( H_{EZ} \) denotes the electron Zeeman interaction, describing the interaction of an electron spin with an external magnetic field. If a nucleus with non-zero nuclear spin quantum number is present, the term \( H_{HF} \) describes the interaction between electron and nuclear spins. In this case, the nuclear Zeeman interaction term \( H_{NZ} \) needs to be considered if it is of similar order of magnitude as \( H_{HF} \) or if nuclear frequencies are measured. For nuclear spin quantum numbers \( I \geq \frac{1}{2} \), an interaction between the electric field gradient and the nuclear quadrupole moment is present, described by \( H_{NQ} \). It occurs in the absence of electron spins and influences EPR spectra only to second order. Finally, the last three terms only exist if more than one electron spin is present. In the case these spins are very close to each other in space, equivalent to strongly overlapping orbitals, the coupling of the electron spins described by \( H_{ZFS} \) is in the same order of magnitude as the electron Zeeman interaction, thus a description in terms of a group spin needs to be applied. Then, the coupling of the electron spins leads to a splitting into Kramer doublets even in absence of an external \( B_0 \) field (thus the name zero field splitting). In case the spins are further apart, it is no longer convenient to treat the electron spins as a group spin, thus they are treated as separate spins. As a consequence, the electron electron interaction is described by the two terms exchange and dipolar coupling. The first one decays to a good approximation exponentially and in case of an isolating medium and a non-conjugated linker between the spin centers it decays much faster with increasing distance than the dipolar coupling.

The dipolar coupling can be understood from the classical model of two interacting magnetic dipoles given from electrodynamics. If one makes use of the correspondence principle, which means introducing operators instead of vectors, one obtains

\[ H_{DD} = \frac{\mu_0}{4\pi\hbar r_{1,2}^3} g_1 g_2 \mu_B^2 (\vec{S}_1 \cdot \vec{S}_2) - \frac{3(\vec{S}_1 \cdot \vec{r}_{1,2})(\vec{S}_2 \cdot \vec{r}_{1,2})}{r_{1,2}^5}. \]  \hspace{1cm} (1.6)

Here, \( \mu_0 \) describes the magnetic constant, \( \vec{r}_{1,2} \) and \( r_{1,2} \) represent the interspin vector and its magnitude, which is equivalent to the distance between the spin centers 1 and 2, \( g_1 \) and \( g_2 \) are the g-factors of spin centers 1, 2, \( \mu_B \) Bohr’s magneton and \( \vec{S}_{1,2} = (\vec{S}_{1,2,x}, \vec{S}_{1,2,y}, \vec{S}_{1,2,z}) \) are the spin operators of spin 1, 2. A more often used form of this expression is obtained if one transforms it to spherical coordinates \( \vec{r}_{1,2} = r_{1,2}(r, \phi, \theta) \) and makes use of the ladder operators \( \hat{S}^z = \hat{S}_z \pm i\hat{S}_y \). Doing that, equation 1.6 can be written in form of the so called “dipolar alphabet”:

\[ H_{DD} = \frac{\mu_0}{4\pi\hbar r^3} g_1 g_2 \mu_B^2 (\hat{A} + \hat{B} + \hat{C} + \hat{D} + \hat{E} + \hat{F}) \]  \hspace{1cm} (1.7)

with
\[
\begin{align*}
\hat{A} &= \hat{S}_{1,z} \hat{S}_{2,z} (1 - 3 \cos^2 \theta) \quad (1.8) \\
\hat{B} &= -\frac{1}{4} (\hat{S}_{1}^+ \hat{S}_{2}^- + \hat{S}_{1}^- \hat{S}_{2}^+) (1 - 3 \cos^2 \theta) \quad (1.9) \\
\hat{C} &= -\frac{3}{2} (\hat{S}_{1,z} \hat{S}_{2}^z + \hat{S}_{1} \hat{S}_{2,z}) (\sin \theta \cos \theta e^{-i\phi}) \quad (1.10) \\
\hat{D} &= -\frac{3}{2} (\hat{S}_{1,z} \hat{S}_{2}^z + \hat{S}_{1} \hat{S}_{2,z}) (\sin \theta \cos \theta e^{i\phi}) \quad (1.11) \\
\hat{E} &= -\frac{3}{4} \hat{S}_1^+ \hat{S}_2^z (\sin^2 \theta e^{-2i\phi}) \quad (1.12) \\
\hat{F} &= -\frac{3}{4} \hat{S}_1^- \hat{S}_2^z (\sin^2 \theta e^{2i\phi}) \quad (1.13)
\end{align*}
\]

Looking at the terms with respect to a four-level diagram which describes a coupled system of two spin \( \frac{1}{2} \) species, one can come to the following interpretation: term \( \hat{A} \) doesn’t mix states since it commutes with the electron Zeeman Hamiltonian. It therefore changes the energy levels of the states in the four-level diagram, but doesn’t mix them. Term \( \hat{B} \) consists of combinations of the raising and lowering operator, thus it describes a zero quantum transition (also called flip-flop-transition). The following terms \( \hat{C} \) and \( \hat{D} \) describe a single quantum transition since they consist of either a lowering or a raising operator and a \( z \)-operator. Finally, the terms \( \hat{E} \) and \( \hat{F} \) correspond to double quantum transitions, because they contain both solely a combination of raising (\( \hat{E} \)) or lowering (\( \hat{F} \)) operators, respectively. In case the coupling between the two spins is big compared to their respective electron-Zeeman interactions, potentially all terms of the dipolar alphabet have to be taken into account since double and zero quantum transitions are possible (“strong coupling”). In contrast, if the coupling between the two spins is small compared to their respective electron-Zeeman interactions and the spin centers fulfill the point-dipole-approximation, the interaction is called “weak coupling” and can be described with only secular terms of \( \mathcal{H}_{DD} \). If the coupling is comparable to or larger than the difference of the two electron-Zeeman interactions, the \( \hat{B} \)-term must be considered. The case where only the \( \hat{A} \)-term is relevant typically applies for two spin centers separated by 1.5\,nm or more. The fact that exchange coupling can be neglected under these circumstances and as the dipolar Hamiltonian can be described by only secular terms (equivalent to the term \( \hat{A} \)), one obtains the rather simple description

\[
\mathcal{H}_{DD} = \omega_{DD} (1 - 3 \cos^2 \theta) \hat{S}_{1,z} \hat{S}_{2,z} \quad (1.14)
\]

with

\[
\omega_{DD} = \frac{\mu_0}{4 \pi h r^3} g_1 g_2 \mu_B^2.
\]

Here, \( \theta \) describes the angle between the interspin vector and the external magnetic field. For the mostly used spin pair consisting of two nitroxides, the splitting of the levels due to the dipolar interaction becomes\[^{22}\]:

\[
\omega_{DD} = \frac{52.18}{r_{1,2}^3} \cdot \text{MHz} \cdot \text{nm}^{-3}, \quad (1.16)
\]

assuming \( g_1 = g_2 = 2.005 \).
If two neighbouring spins are within 1.5 nm of distance, a broadening of a cw EPR spectrum can be observed due to the dipole-dipole interaction. From this broadening, in principle one can derive an interspin distance and width of the distribution. There are two limiting cases under which these measurements can be performed. The rigid lattice case, where no motion of the spin labeled side chain is present and the fast tumbling case, where the rotational correlation time is low enough for isotropic averaging of the dipolar interaction. In the first case, the difference between a spectrum composed by the two singly labeled spectra and the measured dipolar broadened spectrum allows for determination of a broadening function resembling the dipolar interaction. To achieve the rigid lattice limit, the spectra have to be recorded in solid state powder samples or in frozen solution forming a powder-like glass.

In the fast tumbling limit, techniques based on $T_2$ relaxation effects can be applied. Basis for this is the Redfield relaxation theory. This approach was used in studies with T4 lysozyme[23]. Concerning physiological temperatures, none of the two limits is fulfilled since the rotational correlation time of a soluble protein of higher mass or a membrane bound protein is slow, thus no sufficient averaging occurs. It was shown, that the rigid lattice model can be applied even at RT, if the rotational correlation time is slow enough which can be supported by an increased viscosity of the buffer solution[24].

1.1.5 Measuring distances with EPR: DEER

To extract distances between spin labels in the weak coupling regime, the four-pulse DEER (double electron-electron resonance) sequence[25] is applied at cryogenic temperatures (powder-like samples) to obtain the dipolar interaction from an intensity modulated spin echo. The DEER sequence (see fig.1.3) first creates a classical Hahn Echo. Then, on a second frequency, typically 65–100 MHz apart in X or Q band, a $180^\circ$ pump pulse is applied to flip the coupled spins.

By this, the additional magnetic field at the observer spin, resulting from the pumped spin, changes its direction, thereby changing the resonance frequency of the observer spin by the dipolar interaction. With the magnitude of the dipolar interaction being inversely proportional to $r_{1,2}^3$, the change of the resonance frequency of the observer spin is related to the distance between them. Then, a refocusing $180^\circ$ pulse is applied on the observer spin. Depending when the pump pulse is applied, the intensity of the echo in the time domain is modulated with the frequencies of the dipolar interaction. Since the dipolar frequency is dependent on the angle $\theta$ between the external magnetic field and the interspin vector, one would obtain always a distribution of frequencies in a powder sample, even if there is only one distance present. The orientation dependence is given by the term $(1 - 3 \cos^2 \theta)$ of equation 1.14. The probability density function for a given angle $\theta$ is given by $\sin \theta d\theta$. If this weighting is applied to the angular dependent frequency function one obtains a so called Pulse pattern. For each transition two identical patterns with opposite sign are obtained since the transition can occur in both ways.

The signal function $V(t)$ of the DEER experiment can be written as a product of the background function $B(t)$ and the form factor $F(t)$:

$$V(t) = F(t) \cdot B(t) \quad (1.17)$$

Extracting distances from the obtained DEER signal therefore first requires removal of the background resulting from intermolecular spin-spin distances. The probability of finding spins of another molecule within the distance $r dr$ leads to an exponential decay
Figure 1.3: Four pulse DEER sequence and the steps from raw DEER trace V(t) to the extracted distance distribution P(r) via background-corrected secondary data, the so called form factor F(t).

of the echo signal in the time domain for the ideal case of homogenously distributed spin centers. Deviations from this ideal case, e.g. due to the excluded volume of particles, spins on membrane surfaces etc., can be fitted with a stretched exponential, which leads to the formula \( B(t) = \exp(-kt^D) \) for the background. It can be seen easily, that for \( D = 3 \) (a homogenous 3D background) the formula converts into a normal exponential decay, which is stretched for violations of the ideal 3D case.

For fitting the dipolar evolution time (also called form factor), a solution to the moderately ill-posed problem is needed. The ill-posedness results from the dependence of the angular frequency not only from distance, but additionally from the angle \( \theta \) between the interspin vector and the external magnetic field. An in reality rather broad distribution can be fitted as multiple more narrow distributions as well without being able to discriminate these cases at the level of the form factor. This can be solved by applying a Tikhonov regularization. It consists of two parameters, one being the mean square deviation of the fit of the form factor, the other representing the roughness of the distance distribution. These two factors are weighted against each other with a Tikhonov parameter \( \alpha \). Plotting the log of MSD \( (\rho(\alpha) = \| S(t) - D(t) \|^2) \) against the log of the second derivative representing the roughness \( (\eta(\alpha) = \| \frac{d^2}{dr^2} P(r) \|^2) \) for different weighting factors \( \alpha \) ideally results in an L-curve, where the optimal weighting between good fit and smoothness of the distribution lies in the kink of the “L”. The Tikhonov analysis is implemented in the freely available software DeerAnalysis\(^{[26]} \), which was used throughout in this thesis.
1.1.6 Optimal conditions for the DEER experiment

![Q band ESE-spectrum](image)

Figure 1.4: Real field sweep ESE-spectrum with simulated pulse shapes. Red is the pump pulse, blue the pulse on the observer spins.

To obtain a well analyzable DEER curve, it is fundamental that the two frequencies differ enough to avoid significant overlapping. If this is not the case, artifacts are obtained which cannot be easily deconvoluted and are not considered in the freely available standard program DEER Analysis working under MATLAB. In X band, the frequency of the pump pulse is set 65 MHz below the observer frequency for DEER applied on nitroxide spin pairs. Performing the DEER experiment at higher frequencies such as Q band is advantageous concerning the signal to noise ratio and thus duration of measurement. In Q band, the field sweep of nitroxides looks different from X band (see 1.1.2) and thus the pump frequency is set 100 MHz above the observer frequency (see fig. 1.4). The higher separation of the two frequencies results from the pulse width which is approximately 85 MHz (width at half height) for a rectangular 12 ns pulse. The optimal concentration for proteins lies around 50 µM\textsuperscript{[27]}. Going to much higher concentrations is usually not successful since the background function decay is steeper which leads to a higher relative signal loss. Additionally, it decreases the accuracy of the background fitting. In the end, achievable concentrations for protein samples may be clearly below this value and still can be measured.

Up to date, most of the DEER experiments have been performed at X band (typically at 9 – 10 GHz). Recently, it was shown that sensitivity increases by a factor of 17 – 25 when going from X to Q band with a home-built resonator able to contain oversized sample tubes (up to 3.1 mm) and a home-built high power setup\textsuperscript{[28]}. Since the signal-to-noise ratio increases with the square root of the number of scans, this means a gain of measurement time by a factor of up to 625 for achieving the same signal to noise ratio when going from X to Q band. The reason why DEER hadn’t been performed in Q band more often before was the lack of a commercially available high-power Q band spectrometer.

When talking about sensitivity, temperature is always a matter for EPR experiments. The signal intensity decays exponentially with the transverse relaxation constant of the observer spins $T_{2,\text{obs}}$. Thus, going to low temperatures may look advantageous on a first glance, but going too low doesn’t help anymore since there is a low temperature limit of $T_{2,\text{obs}}$ which lies between 40 and 60 K for proteins\textsuperscript{[10]}. Below this limit, spin diffusion is the driving force of relaxation which can only be decreased by deuterating the protein and/or buffer.
If a protein is reconstituted into lipid bilayers such as liposomes, a steep background decay can be observed. The reason is, that the space for the protein is restricted to only the membrane plane, thus the sample has a lot of excluded volume and the proteins are crowded much more than in detergent solution. This problem is hardly avoidable, but reconstitution parameters might be optimized regarding the protein to lipid ratio. If possible, reconstitution into nanodiscs may be another way of avoiding this problem but this is not as straightforward as the procedure for liposomes.

1.1.7 Limitations of the DEER experiment

The DEER experiment works very well and reliably, if certain restrictions are taken into account\textsuperscript{10}. The overall requirement is, that the sample contains two (or more) electron spins in suitable distance, meaning roughly between 1 and 10 nm. Limitations on the long side come from the fact that the dipolar frequency becomes very small at long distances and ideally one should measure one full oscillation or even more for a reliable background correction. The length of the dipolar trace is inherently limited by transverse relaxation and instantaneous diffusion leading to signal decay. For complex systems like membrane proteins, the achievable limit often decreases to 5–6 nm. On the short side, the limit is defined by the excitation bandwidth of the pump pulse. If the splitting due to the dipolar interaction exceeds the excitation bandwidth of the pump pulse, there is no possibility that it can flip the spins and therefore no effect in the refocused echo is observed anymore. The typical width of a pump pulse achievable by a 10 ns pulse is 100 MHz, corresponding to the width of the Pulse pattern for an internuclear distance of ca. 1 nm. Additional limitations at distances < 1.0 nm come from the breakdown of the point-dipole approximation due to increasing exchange coupling of the electrons, which leads to a non-secular dipolar Hamiltonian which is not considered in the standard analysis of the DEER traces.

1.1.8 Comparison between EPR and NMR on proteins

As mentioned before, most macromolecules are, in contrast to NMR, EPR silent. NMR experiments can lead to a huge amount of information, which can lead to a full determination of the structure. Distance restraints or constraints, respectively, can be gathered by nuclear Overhauser effect (NOE) based experiments. If two protons are within typically 1.8 to 6 Å, a cross peak between these protons can be observed in the nuclear Overhauser effect spectroscopy (NOESY) experiment. The intensity of this peak is proportional to $r^{-6}$, with $r$ being the distance between the two nuclear spins. For analyzing the NOE peaks, it is fundamental with macromolecules such as peptides or proteins to have assigned the respective proton peaks and the complete backbone correctly. Achieving this requires usually several multidimensional NMR experiments, such as correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY) or heteronuclear experiments like heteronuclear single-quantum correlation (HSQC) or heteronuclear multiple-bond coherence (HMBC). Simple 1D experiments are not able to resolve the peaks since too many peaks cluster at typical chemical shift regions. In case, the 2D experiments are still not able to resolve the peaks, 3D or even higher dimensional experiments need to be applied (HNCA, HNCACO, and many more), which requires $^{15}$N and $^{13}$C labeling of the amino acids. This is a very costly approach. If the proton peaks are fully and correctly assigned, the distance re- and constraints can be extracted from NOE experiments. Furthermore, angle restraints can be extracted from the dependence of J-coupling constants on the dihedral angle between the involved nuclear spins.

If enough restraints are given, finally the structure can be solved by using available
programs for structure calculations based on NMR restraints. They use the experimental restraints and an energy function to gain the structure of minimal energy. Since one has only a certain number of restraints, one gets an ensemble of the minimal energy structures, which converged in different calculations, as an output. Usually, the structure ensemble shows parts of low and high flexibility of different protein domains and thus can give a broader view on conformational dynamics of the respective protein than can be achieved with structures from X-ray crystallography.

It is even possible to gain orientation restraints between different protein domains by applying residual dipolar coupling (RDC) experiments. Here, the protein is embedded into a matrix which leads to a partially preferred orientation of the macromolecules. By this, the dipolar through-space couplings between nuclear spins are not fully averaged out like in samples where the molecules can freely tumble in solution. RDC restraints are obtained like NOE restraints by through-space interaction, but show a $r^{-3}$ dependence instead of a $r^{-6}$ dependence. Since a dependence of the coupling frequency from the angle between the interspin vector and the external magnetic field exists, information of the relative orientation of different parts of the molecule can be gained. Although it was shown that on model systems a full structure model can be obtained by solely using RDCs[29], in practice they are combined with restraints from NOE experiments.

Nonetheless, there is a upper limit for determining structures only by NMR techniques. It comes from the fact, that signal decay due to relaxation processes increases the bigger a molecules gets. If too much of the magnetization is decayed during the evolution time of any experiment, no signal can be detected anymore. Currently, this limit lies at around 25 to 30kDa for soluble proteins. Many relevant and especially membrane proteins are thus too big to be studied by NMR exclusively. The relaxation problem can be partially reduced by deuteration[30] of the protein and its environment or by applying the TROSY experiment[31].

It is generally not possible with EPR to solve complete protein structures, since the abundance of electron spins is not comparable to the omnipresent nuclear spins. Hence, only coarse grained information can be gathered. Nevertheless, this can give valuable insight into conformational changes of proteins since conclusions about domain movements can directly be drawn with suitable long range distance constraints. Since the higher gyromagnetic ratio of the electron spin allows distance measurements in the range from 1.5 to 8nm, it is possible to observe directly and with one suitable spin pair conformational changes of a protein. This method is even more successful, if a structure of the protein is known beforehand from NMR or X-ray crystallography.

1.1.9 Comparison of EPR and X-ray crystallography

X-ray crystallography is up to date the most powerful and most widely used technique for structure determination of macromolecules. Currently (as of January 2016) the protein data base (PDB) lists over 102000 entries for structures obtained by X-ray crystallography[1]. In comparison, around 11000 entries are listed for NMR. These numbers show the dominance of X-ray crystallography on the field. There is no size limitation, it all depends on finding conditions to successfully crystallize the molecule. For membrane proteins it is often very difficult and time consuming to find the right conditions. Lipids, detergents or other additives needed to stabilize the protein often prevent successful crystallization. Additionally, the technique itself has certain drawbacks. Structural artifacts due to crystal packing can occur and lead to misinterpretations. Even in crystals, dynamic

\[ \text{http://www.rcsb.org/pdb/statistics/holdings.do} \]
regions within a macromolecule can exist and prevent structural assignment. And, last
but not least, a crystal always reflects the state of minimal energy under the corresponding
conditions. Usually, proteins have a natural environment different from a crystal, either
being in solution or incorporated into membrane bilayers. It is, e. g., impossible to reveal a
structural equilibrium between two possible conformations under the same conditions for
crystallization. Whereas with NMR (working in solution at ambient temperature) or with
EPR (working in solution or frozen solution) such equilibria can in principle be observed.
Thus, a good approach is to combine diffraction techniques with atomic resolution and
techniques able to work in more natural environments for getting an as much as possible
complete picture of conformational states of a biomacromolecule.

1.1.10 From sidechain to backbone: MMM

The ability to measure long range distance distributions of a spin labeled side chain leads
to a need for a possibility to connect side chain conformations with conformations of the
protein backbone. One approach is to represent the conformational space of the spin-
labeled side chain by an ensemble of discrete rotamer states\(^8\). This rotamer library is
gained from molecular dynamics (MD) simulations of the free spin label. The histogram
of dihedral angles \(\chi_1 - \chi_5\) (see fig.1.1) of MTSSL during this simulation shows that they
are distributed around a set of characteristic values for each rotatable bond. Nonetheless, a
library generated from these sets of angles led to rotamers with internal atomic clashes and
after their removal the remaining rotamers didn’t represent the MD results well. The reason
may lay in a systematic deviation of the single rotamers from the respective canonical
angles. Thus, the rotamers from the MD trajectory were clustered correspondingly to
the canonical ensembles and finally the dihedral angles were averaged over the whole sub
ensemble. Finally, the frames of the MD trajectory were re-clustered with respect to the
newly averaged dihedral angles and new averages were computed from these new sub-
ensembles. The rotamer from the MD simulation with the smallest MSD to the averaged
angles was taken as the representative structure for this combination of angles and taken
into the rotamer library. The normalization for the rotamer library was done by weighting
the single rotamers with the number of assigned frames from the MD trajectory. The MD
simulation was performed at 298K and 175K which reflect the motional regimes in which
most EPR experiments on proteins are performed. 175K represents the glass transition
temperature of water-glycerol matrices, where equilibration of the conformational ensemble
takes place during freezing of the sample. Thus, this temperature is taken to represent
DEER experiments usually performed at 50K.

The rotamer library is used in the software package MMM\(^2\) to simulate DEER experiments by in silico labeling of known structures. Here, the rotamer library is scanned for a
fixed position in the sequence and an energy term for each rotamer is computed. It consists
of a Boltzmann term for the internal energy of the rotamer library and an external energy
which reflects the interaction between the spin label and the protein environment. This is
computed by pairwise Lennard-Jones potentials for each rotamer with a cutoff distance to
avoid too heavy computations.

With the help of this software tool, one can identify favorable sites for spin labeling
by avoiding too restricted sites where the partition function of possible rotamers is small.
Especially for studies of protein dynamics resembled in different conformational states or
for interaction studies of biological molecules in general this software package provides a
very valuable tool for EPR experiments. Potentially lots of hard work can be saved if it

\(^2\)http://www.epr.ethz.ch/software/mmm-older-versions.html
1.2 ABC transporters

The family of ABC transporters is ubiquitous in all three domains of life. So far, four different types of transporters are known (see fig.1.6). They are involved in many physiological phenomena such as transport of e.g. metabolites, nutrients or co-factors through the cell membrane [32]. In eukaryotes, only exporters are known up to date, being involved in several human diseases such as for example cystic fibrosis or in other biological processes like immuno protection or multi-drug resistance (TAP1/2 or Pgp, respectively). Contrarily, in prokaryotes all subtypes can be found and ABC transporters are generally essential for cell viability in bacteria. Their general function is to use energy from ATP hydrolysis for the transport of substrates through cell membranes. The transporter usually consists of two nucleotide binding domains (NBDs), which are sequentially and structurally highly conserved among the whole family, and two transmembrane domains which differ widely in sequence and structure. This explains the diversity and therefore possible specificity for substrates. The two classes of importers, type I and type II, consist of four individual polypeptides forming the two NBDs and two TMDs. Additionally, they use a fifth subunit called substrate binding protein (SBP), which delivers the substrate to the TMDs (see cyan in fig.1.6). Exporters may consist of two homo- or heterodimers, each consisting of one TMD and one NBD which form a so called half transporter of one single polypeptide chain. Characteristic subsequences for the NBDs are the Walker A (GxxGxGKS/T, x being any residue) and Walker B motif (φϕϕD, ϕ being a hydrophobic residue), which indicate presence of an ATP binding site and the ABC signature motif (LSGGQ) which is unique for these systems. The transmembrane domain of each subunit typically consists of 6–8 helices which anchor the transporter in the membrane and additionally form the

is known beforehand which sites are predictably well suited for labeling and the resulting distance is likely in a measurable distance range.

Figure 1.5: Superimposition of dihedral angles distributions obtained from a MD trajectory (blue). By averaging these distributions to a canonical ensemble (green), the final rotamer library is obtained as described in 1.1.10. Figure taken from [9].
translocation pathway.

Figure 1.6: The four types of ABC transporters which are known up to date with respective examples. Depicted in light and dark grey are the two TMD subunits, in red and dark red the NBD dimer and in cyan the bound substrate binding protein. ATP Hydrolysis takes place in the NBDs which transfer the conformational change to the TMD via coupling helices (black).

1.2.1 ATP hydrolysis and conformational changes in ABC importers: the case study of the maltose importer

To allow active transport through the cell membrane, energy from ATP hydrolysis has to be harnessed and transduced into conformational changes, which allow the substrate to pass. Generally, ABC transporters are subdivided functionally into the NBDs which hydrolyze ATP and thus deliver the energy for the transport cycle and into the TMDs, which bind and translocate the substrate. Both kinds of transporters, importers and exporters, have a resting state which is usually outward facing for importers, and inward facing for exporters. The common notion is, that upon binding of ATP to the NBDs and, in case of importers, the SBP to the TMDs, the NBDs close and switch the transporter from the open, resting state into an inward facing state (importers) or outward facing state (exporters), respectively. Upon closing of the NBDs and in the presence of Mg\(^{2+}\) ions, ATP is being hydrolyzed leading to an intermediate state of the transporter consisting of bound ADP and bound inorganic phosphate (P\(_i\)). This state can be trapped by adding orthovanadate in the presence of ATP/Mg\(^{2+}\). Usually, P\(_i\) is released after ATP hydrolysis which leaves the transporter in the so called posthydrolytic state with ADP and Mg\(^{2+}\) bound to the NBDs. If vanadate is present, it occurs that a vanadate molecule binds upon release of P\(_i\) which traps the intermediate state since the vanadium interacts with five oxygens. Thus, orthovanadate acts as an analog to P\(_i\). One can drive the transporter in certain states of the nucleotide cycle by simply adding the required reagents, like e.g., adding ATP/Mg\(^{2+}\) in the presence of SBP leads to the inward facing state in case of importers and to the outward facing state in case of exporters. Interestingly, for exporters ATP and Mg\(^{2+}\) both need to be present to populate the outward facing state, whereas for the MalFGK\(_2\) importer ATP and SBP alone are sufficient to obtain the inward facing state.
The first hints for the mechanism of hydrolysis were proposed when the isolated NBD dimers MalK from the maltose transporter of *E. coli* were crystallized with and without ATP and ADP[33,34]. Upon binding of ATP, a tweezer-like motion leading to closure of the dimer could be observed. This general behavior was later supported by the crystal structures of the full maltose importer MalFGK2 which was crystallized in the ATP-, apo- and vanadate trapped states in presence of substrate. [35–37]. From these structures it was concluded that the closing motion of the NBDs is transduced via the Q-loop and the coupling helices of the TMDs. The TMDs consequently undergo a transition from an outward facing to an inward facing state for importers or vice versa in case of exporters.

![Diagram of nucleotide cycle](image)

**Figure 1.7:** *Simplified model of the nucleotide cycle of MalFGK*$_2$, adapted from [8]. The substrate binding protein binds maltose with relatively high affinity (green apo, orange closed) and with quite low affinity to the transporter in the apo state (light green, open NBDs). Upon bindin of ATP the transporter switches to the inward facing state (red, closed NBDs), the SBP opens (green) and releases maltose to the binding cavity within the transporter. ATP is being hydrolyzed, SBP and maltose are released (blue NBDs).

### 1.2.2 The role of the substrate binding protein

As mentioned before, importers usually have a substrate (or solute) binding protein (SBP). In case of Gram-negative bacteria, SBPs are found usually in the periplasm, in rare cases however they are fused to the transporter[18] as it is usually the case in Gram-positive bacteria. The SBP binds the solute with high affinity and presents it to the transporter upon binding to its extracellular part. Most of the known SBPs have two domains consisting of a central β-sheet flanked by α-helices which are connected by a hinge region. SBPs are thus postulated to be able to adopt four conformations: open-unliganded, open-liganded, closed-unliganded and closed-liganded. Up to date, the common notion is that they are mostly in the open-unliganded form in the absence of substrate, but a small fraction may be in the closed-unliganded form[39,40], which is thermodynamically stabilized in presence of bound substrate. Substrate binding on the conformational level is normally accompanied by a rigid body rotation of the two domains around the hinge region. The extent of rotation however is differing a lot[41] among the various species, from non-existent in TroA[42], a few degrees for BtuF[43] to as much as 60° in LivJ[44]. Despite all the valu-
able snapshots obtained by many crystal structures of different SBPs in various states, the key mechanism of how substrate translocation takes place, and how substrate availability is communicated to the transporter, is still poorly understood. EPR investigations performed on the MalFGK2-E complex to pinpoint the role of substrate in the formation of the complex and on the nucleotide cycle are presented in chapter 2.

1.2.3 The ABC exporters MsbA and TM287/288

The first ABC exporter whose structure was solved is Sav1866 from *S. aureus*. It was captured in an ADP bound, outward facing state. Soon later, it was crystallized in the physiologically more relevant AMP-PNP [adenosine 5'-\((\beta,\gamma\text{-imido})\text{triphosphate}\)] bound state, which didn’t show significant conformational differences with respect to the previous structure. The structure of the homologous exporter MsbA from *E. coli* was determined before, but the structures had the wrong hand due to a data processing error. The correctly treated diffraction data led to a structure very similar to the Sav1866 in the outward facing AMP-PNP bound state. Interestingly, two different inward facing apo conformations were found, the so called closed and open apo structures. Though, as a drawback, the resolution of these structures is pretty low (5.3 Å resolution for open apo, 5.5 Å for closed apo), thus only Cα models are given in the PDB files. The open apo structure shows NBDs being apart as much as 5nm and was supported by aqueous accessibility measurements with EPR, suggesting a large change in accessibility upon nucleotide binding. In this thesis I present further results confirming the large conformational space sampled by MsbA and hints for an asymmetric behavior of the homodimeric MsbA.

In contrast to many bacterial ABC exporters which are homodimers, the transporter TM287/288 from *T. maritima* is a heterodimer like most of the eukaryotic ABC exporters. Nearly half of the mammalian heterodimeric ABC transporters contain two non-homodimeric NBDs. There, one NBD differs in the ABC motif, the Walker B motif and the switch histidine from the consensus sequence and is thus called the degenerate site. The second NBD is identical to the consensus sequence and therefore referred to as the consensus site. The structure of TM287/288, which has a sequence identity of 36% to the well-studied ABC exporter LmrCD from *L. lactis* transporting Hoechst33342 and daunomycin, was determined with a resolution of 2.9Å. The TM287/288 shows the typical architecture for mammalian exporters which was first described for Sav1866. Two TMD helices of one subunit are entangled with four helices of the other subunit and vice versa. The coupling helices are found at the kink of the two TMD helices which cross over and interact with the second subunit, thus each NBD acts on the TMD of the opposite subunit. The two subunits share only 33% of sequence identity, but show very similar conformations (rmsd of 2.29Å). Notably, the transporter was crystallized in presence of the nucleotide analogue AMP-PNP but the structure shows that only one molecule of AMP-PNP is bound to the transporter’s degenerate site. The consensus site doesn’t show any hints for a second AMP-PNP molecule. Furthermore, the two NBDs are not fully closed as it would be expected under these conditions, but are ≈9 Å apart (distance from the nucleotide to the signature motif of TM288). Based on this crystal structure, a mechanism of transport was proposed where the transporter rests with one ATP bound to the degenerate site, upon binding of substrate and a second ATP switches to the outward facing state, where the substrate is released. Then, either one ATP molecule at the consensus site is hydrolyzed, leading to the previous conformation of the resting state with one ATP bound to the degenerate site. Much less frequently, ATP is hydrolyzed both at the consensus and degenerate site, which is in line with previous findings for LmrCD. Understanding the mechanics of conformational changes dur-
ing the nucleotide cycle and addressing the questions concerning the asymmetric behavior of mammalian ABC exporters will help to gain insight how transporters like CFTR and TAP1/2, which play fundamental roles in cell physiology, work. Here, I will present results obtained by applying SDSL and DEER to investigate the conformational changes in different states of the nucleotide cycle to support findings obtained from crystal structures.

Experiments performed on the heterodimeric ABC exporter BmrCD from Bacillus subtilis in the presence and absence of nucleotides and substrate, respectively, revealed that in the apo state the NBDs are largely displaced. Additionally, a distinct conformation of the NBDs in the AMP-PNP (and ADP) bound state was found, with the TMDs in the inward facing state\textsuperscript{[53]}. Interestingly, a fully closed NBD conformation, which is also transduced to the TMDs (leading to the outward facing state), could be populated in the vanadate trapped state. Further experiments on the homodimer MsbA in contrast didn’t show an asymmetric behavior of the NBDs, showing two distinct conformations (outward and inward facing) with no difference whether AMP-PNP or ADP-Vi is present.

Additionally, a structural model of the heterodimeric ABC transporter TmrAB from the gram negative bacterium T. thermophilus which is homologue to various multidrug transporters with one degenerate nucleotide binding site was obtained by applying electron cryomicroscopy (cryo-EM) in combination with fragment antigen binding (FAB) and homology modeling together with molecular dynamics flexible fitting\textsuperscript{[54]}. A sequence identity of 31.3\% from TmrB to TM287 and of 37.8\% from TmrA to TM288 was found. The single particle cryo-EM was performed in absence of nucleotides and led to a resolution of 8.2 Å. The structure shows an inward facing transporter with a cavity which is open to the cytosol and (to a lower extent) to the inner leaflet of the surrounding cell membrane. The structure is not as widely open as the MsbA apo conformations, but very comparable to the structure of TM287/288 with one AMP-PNP bound to the NBDs. Together, these two publications confirm and widen the findings I present in this thesis concerning MsbA and TM288/287 which had been published before.
2 Conformational plasticity of the type I maltose ABC importer

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2.1 Introduction

ATP-binding cassette (ABC) systems are found in all kingdoms of life, forming one of the largest protein superfamilies. ABC transporters comprise two transmembrane domains (TMDs) that form the translocation pathway and two nucleotide-binding domains (NBDs) that bind and hydrolyze ATP. Based on biochemical and structural evidence, all ABC transporters are thought to function by an 'alternate-access' mode with the translocation path shuttling between an inward-facing and outward-facing conformation in response to substrate and ATP binding, the latter causing the NBD dimer to close.

Canonical ABC importers are sub-divided into type I and type II based on structural and biochemical evidence and are dependent on extracellular (or periplasmic) solute binding proteins (SBP), which play a crucial role in initial steps of the transport cycle. SBPs generally consist of two symmetrical lobes which rotate towards each other upon substrate binding.

The type I maltose transporter of E. coli/Salmonella is probably the best-understood ABC transporter to date. It is composed of the periplasmic maltose binding protein, MalE, the membrane-integral subunits, MalF and MalG, and the nucleotide-binding subunits, MalK2. The available crystal structures in the pre-translocation, ATP-, and vanadate-trapped states have largely contributed to the understanding of the details of the inward-to-outward-facing mechanism. The post-hydrolytic state has not yet been crystallized, but data exist proposing this state to have a distinct structure from the other three known crystal snapshots. MalE interacts with the transporter throughout the nucleotide cycle and the X-ray structures revealed the switch of the binding protein from the liganded (closed) to the substrate-free (open) conformation concomitantly with ATP binding to the NBDs. Despite all these structural insights, the response of the transporter to substrate availability is poorly understood. Furthermore, the mechanism behind the stimulation of the ATPase activity of the transporter by unliganded MalE is still elusive.

Our results show that the apo- and ADP-states of the transporter bind both open and closed MalE but the complex adopts different periplasmic configurations. The ATP-state of the transporter can bind either closed MalE, inducing its opening and release of substrate to MalF or directly unliganded MalE - thus a futile cycle is generated. In both cases the
NBDs are triggered into a fully closed dimer primed for ATP hydrolysis. Furthermore, we demonstrate that dissociation of the binding protein is not a prerequisite for substrate translocation. These novel features highlight the conformational plasticity of a type I ABC importer.

2.2 Results

2.2.1 Experimental system and techniques used

We performed chemical cross-linking and EPR spectroscopy in detergent-solubilized and reconstituted transporters to detect MalE-MalG and MalK-MalK distances between cysteines or spin-labeled cysteines shown in Fig. 2.1. The cysteine mutations and subsequent labeling did not affect ATPase activity of the variants (Tab. 2.1). Site-directed chemical cross-linking was performed using flexible homobifunctional thioisulfonate linkers with defined spacer lengths in the extended conformation of 5.2Å (EBS), 10.4Å (HBS), and 24.7Å (PBS)

Mean distances and distance distributions between singly spin-labeled MalE and singly labeled MalFGK$_2$ and between doubly spin-labeled NBDs were obtained by double electron-electron resonance (DEER) at cryogenic temperatures, which provides a "frozen" snapshot of the equilibrium population of the complex. As an example of the complementarity and reliability of the two methods, Fig. 2.1B shows the agreement between experimental distance constraints obtained in the apo-state of the complex by DEER or cross-linking and the simulations performed on the corresponding crystal structure (3PV0).

2.2.2 Maltose- and nucleotide-mediated interaction between MalE and MalG

Cross-linking between P78C in MalG and two cysteines placed in the N-lobe of MalE was performed to follow complex formation under different conditions. In the presence of MalE(G13C) a strong cross-link to MalG(P78C) was found with all linkers (marked EG in Fig. 2.2A), regardless of the presence of maltose and/or nucleotides. This agrees with the available crystal structures[35,36] which show no significant change in the C$_\alpha$−C$_\alpha$ distance between both residues in different nucleotide states (Fig. 2.2) and confirms that both open and closed MalE bind to the transporter. The results were confirmed in proteoliposomes, thus strongly indicating that the properties of the detergent-solubilized maltose transport complex reflect those in lipid environment (Fig. 2.7A). The cross-link between MalG(P78C) and another Cys in the N-lobe of MalE (position 36) was likewise found to be independent of maltose (Fig. 2.8C). Reliability and specificity of this approach were validated using a Cys at position 246 in MalE located 3.3nm apart from MalG(P78C) (see Fig. 2.1A), which showed no cross-linking under any condition as expected. Moreover, reducing the incubation time with the cross linker to 5 seconds, lowering the concentration of the cross-linker to 1µM and of MalE to 1/8 relative to MalFGK$_2$ still resulted in a cross-linked product. In contrast, the formation of MalE dimers by simple collision was found to be clearly dependent on MalE concentration (Fig. 2.9).

DEER measurements were performed to extract accurate interspin distances between MalG(78R1) (with R1 being the spin-labeled side chain) and spin-labeled sites in the N-lobe of MalE. A broad distance distribution centered at 4nm was measured between MalG(78R1) and position 80 in MalE in the apo-state (Fig. 2.2B) independent of maltose. In the presence of 3mM ATP (with additional 0.075mM EDTA to abolish hydrolysis) the distance decreased to 3.8nm, again independent of maltose (Fig. 2.2C). In the ADP-state
Figure 2.1: **DEER and cross-linking distance restraints vs MalFGK2-E structure.**

(A) Apo-state crystal structure (PDB 3PV0). The Cα atoms of the residues replaced by cysteines in this study are shown in ball representation. Some pairs were chosen for both cross-linking and DEER measurements (solid lines), others only for cross-linking (dotted lines). (B) Experimental DEER (straight black line) and cross-linking (violet bar) distances obtained with the pairs G78-E36 and G78-E352 (the letters denote the subunit of the site) compared to the simulated nitro xide-nitro xide (NO-NO, dotted line) and sulfur-sulfur (Sγ−Sγ, black bar) distances simulated with MMM using spin-labeled rotamers on the structure 3PV0. The insets show the corresponding bands in the gel.

(3 mM ADP, 5 mM MgCl₂), in presence and absence of maltose, two distinct distance distributions were measured, centered at 3.3 or 4 nm, respectively (Fig. 2.2D), demonstrating the role of the substrate for the periplasmic configuration of the N-lobe (see also Figs. 2.10, 2.11). The data confirmed that the ADP-state of the complex differs from both the apo- and ATP- snapshots (Fig. 2.12). The interspin distance between MalG(78R1) and another spin label at position 36 in MalE was found to be below 2 nm (borderline region of DEER sensitivity), thus only the apo-state was measured and the distance was found to be unaffected by maltose, corroborating the cross-linking findings (Fig. 2.10A). It is worth noting that the low modulation depth (0.1–0.2) observed in all DEER traces is to be attributed to the similar low affinity of MalE for the transporter in the three states investigated ($K_d \approx 10^{-4}$ M). The availability of a home-made high-power Q-band spectrometer allowed reliable detection of the DEER traces with optimal signal-to-noise and dipolar evolution time.$^{[28]}$

In response to maltose, the relative displacement between residues in the C-lobe of MalE (positions 211, 352) and MalG was remarkably different from those in the N-lobe. From the crystal structures, the $C_\alpha - C_\alpha$ distance between MalG-P78 and MalE-S352 changes from
Figure 2.2: Cross-linking and DEER data between MalFG(P78C)K₂ and sites in the N-lobe of MalE. (A) MalFG(P78C)K₂ in DDM (n-Dodecyl \( \beta \)-D-Maltopyranoside) micelles (2.5 \( \mu \)M) was incubated with MalE(G13C) (5 \( \mu \)M) and homobifunctional thiosulfonate cross-linkers in the presence of different cofactors as indicated. The cross-linked products were subsequently analyzed by SDS-PAGE. The cross-linkers EBS, HBS and PBS have approximate spacer lengths of 5.2 Å, 10.4 Å and 24.7 Å (see Supplementary materials and methods for more details). (B) Deer analysis on MalFGK₂ transporters solubilized in DDM carrying the spin label at position 78 in MalG incubated with MalE spin-labeled at the N-lobe (positions 80) in different nucleotide states in the presence and absence of maltose, as indicated. Upper panels, normalized DEER Form factors \( F(t) \); bottom panels, distance distributions obtained with Tikhonov regularization using the software DeerAnalysis2010[26]. The mutants are denoted by the name of the relative subunit (e.g. G or E) and the corresponding residue number.

3.4 to 4.2 nm going from the pre-translocation to the ATP-bound state (Tab. 2.2). A cross-linked product was observed with PBS in the apo-state which was significantly enhanced (1.6 fold, 4 independent measurements) upon binding of maltose (Fig. 2.3A). We suggest a weak interaction between the C-lobe of unliganded MalE and the transporter, which is strengthened by maltose presence. Upon addition of ATP or in the vanadate-trapped state no cross-linked product was obtained, independent of maltose, suggesting a shift of the C-lobe away from position 78 in MalG, consistent with the crystal data[35]. The weak interaction of the C-lobe of MalE was restored by ATP hydrolysis and remained unchanged in the presence of ADP (Fig. 2.3A). These results were reproduced in proteoliposomes (Fig. 2.7B) and were corroborated with other pairs in MalG and in the C-lobe of MalE (Fig.
Figure 2.3: Cross-linking and DEER data between MalFG(P78C)K2 and sites in the C-lobe of MalE. (A) MalFG(P78C)K2 in DDM micelles (2.5 µM) was incubated with MalE(S352C) (5 µM) and homobifunctional thiosulfonate cross-linkers in the presence of different cofactors as indicated. (B) Deer analysis on MalFGK2 transporters solubilized in DDM carrying the spin label at position 78 in MalG incubated with MalE spin-labeled at the C-lobe (positions 352) in different nucleotide states in the presence and absence of maltose, as indicated. Upper panels, normalized DEER Form factors \(F(t)\); bottom panels, distance distributions obtained with Tikhonov regularization using the software DeerAnalysis2010. The mutants are denoted by the name of the relative subunit (e.g. G or E) and the corresponding residue number.

The distance distributions obtained by DEER between spin labels attached at position 352 in the C-lobe of MalE and position 78 in MalG (Figs. 2.3B-D, 2.10, 2.11) substantiate the cross-linking information. In fact, the C-lobe interaction in the pre-translocation state shows remarkable substrate dependence as revealed by the change from a broad (3–6 nm) to a narrower distance distribution (centered at 2.8 nm) upon maltose binding to MalE (Fig. 2.3B). Interestingly, in the ATP-state a unique 4 nm mean distance was measured independent on maltose (Fig. 2.3C). In contrast to the N-lobe, in the ADP-state the C-lobe adopts the same relative position with respect to MalG independent of substrate (Fig. 2.3D). The maltose-dependent interactions in the pre-translocation state were further validated using a second position in the C-lobe (Fig. 2.9) and the results obtained between MalG(P78R1) and MalE(S352R1) were confirmed in proteoliposomes (Fig. 2.12).

Notably, all experimental interspin distances between MalE and the reporter position 78 in MalG in the apo-state (with closed MalE) and in the ATP-state (with both MalE
variants) agree well with those simulated using the program MMM\cite{9} on the corresponding crystal structures (Fig. 2.10, 2.11, 2.12).

In summary, the data show that apo- and ADP-states of the transporter are not selective for open or closed MalE and that the complex has different periplasmic configurations in the absence or presence of substrate. In contrast, the ATP-state of the transporter can bind either closed MalE, switching it into the open conformation, or directly unliganded open MalE.

2.2.3 Effects of maltose and MalE on the conformation of the NBDs

The observed maltose effects on the interactions between MalE and MalFGK$_2$ prompted us to investigate whether maltose has an effect also on the NBDs’ motions. Two cysteine mutations (V17C, E128C) were introduced in each MalK subunit close to positions already used in a previous study\cite{5}. The 17-128' (the prime denotes the second MalK monomer) and the 128-17' distances are distinctly shorter than all other intra- and inter-MalK distances and should report closure and reopening of the NBDs, according to the crystal structures. The apo-state of the MalFGK$_2$ alone (Fig. 2.4A, turquoise trace) showed a distance peak centered at 3.5 nm which was associated with the 17-128' and 17'-128 distances. Binding
of open (Fig. 2.4A grey) or closed (Fig. 2.4A black) MalE only slightly affected this
distance (Fig. 2.4A grey), indicating that the binding of MalE to the periplasmic side of
the transporter does not alter the relative position of the open NBDs. Binding of ATP
alone to MalFGK₂ did not close the NBDs (Fig. 2.4B, orange trace), in agreement with
previous EPR studies[5]. Only after MalE binding (independent of maltose) the full closure
of the NBDs was observed, resulting in a mean distance centered at about 2.5 nm (Fig.
2.4B, red and pink traces).

Interestingly, we found that binding of ADP-Mg to MalFGK₂ induced a semi-open con-
formation of the NBDs with a mean interspin distance of about 3 nm (Fig. 2.4C, brown
trace), which was unaffected by subsequent binding of open or closed MalE (Fig. 2.4C,
blue and cyan traces). The latter findings highlight that this NBDs’ conformation is purely
nucleotide-driven.

In contrast, the ATP-state of the NBDs is driven by both MalE and ATP and the complex
adopts a unique conformation independent of maltose. The experimental distances show
good agreement with those simulated on the available crystal structures (Fig. 2.13).

Cross-linking data performed on the MalE-MalFGK(V117C, E128C)₂ complex (Fig.
2.14) confirmed the MalE/nucleotides-driven NBD motions independent of maltose.

![Cross-linking data](image)

Figure 2.5: ATPase and transport activities of cross-linked MalE-F GK₂ supercomplexes. (A) ATPase activity of MalFG(P78C), EBS- or PBS-
linked MalFG(P78C)-MalE(G13C) (named EBS-G78-E13, PBS-G78-E13) in
the presence of increasing concentration of freely diffusible MalE(G13C) and
of MalFG(T46C) and PBS-linked MalFG(T46C)-MalE(S352C) (named PBS-
G46-E13) in the presence of increasing concentration of freely diffusible
MalE(S352C). (B) [¹⁴C] maltose transport of reconstituted MalFG(P78C) and
MalFG(T46C) with added MalE(G13C) and MalE(S352C), respectively at
molar ratios 10:1 (grey) or 1:1 (dark grey) and of the supercomplexes EBS-G78-
E13, PBS-G78_E13 and PBS-G46-E352. The asterisks in the EBS-linked super-
complex denotes the observed basal maltose incorporation, with no time-
dependent increase. Each value is the mean of three separate determinations
with S.D. shown as error bars.

2.2.4 The N-lobe of MalE requires conformational freedom for substrate
import

How important are the different periplasmic configurations of MalE for substrate import?
And is the dissociation of MalE during the nucleotide cycle required for substrate trans-
port? To address these questions, we created supercomplex variants containing PBS-
(≈ 25 Å)-linked MalFG(P78C)K₂-MalE(G13C) and MalFG(T46C)K₂-MalE(S352C), rep-
resenting linkage of the N- and C-lobes of MalE to the transporter, respectively. The supercomplexes were passed through a molecular sizing column to remove excess MalE and subsequently analyzed for ATPase activity and maltose transport. Both variants displayed ATPase activities comparable to those in the presence of freely diffusible MalE at a 1:1 molar ratio under the same conditions (Fig. 2.5A). However, and unlike the control, addition of excess soluble MalE to the cross-linked complex did not increase activity, suggesting a lack in space to accommodate more than one copy of MalE in the vicinity of the transport complex. The two supercomplex variants were also shown to retain the ability to transport maltose with similar efficiency as the complex in the presence of a 1:1 molar ratio of MalE (Fig. 2.5B). Thus, we conclude that dissociation is not a necessary requirement for transport. Strikingly, we found that the activity of a supercomplex was affected by the spacer length of the cross-linker. When linked by EBS (≈ 5 Å), the complex containing MalG(P78C)-MalE(G13C) exhibited only basal ATPase activity (Fig. 2.5A) and showed no substrate transport (Fig. 2.5B), suggesting that the N-lobe’s conformational freedom in the periplasmic region of the complex is required to accomplish the switch from the outward- to the inward-facing state leading to substrate import.

2.3 Discussion

Site-directed chemical cross-linking and DEER spectroscopy were applied to investigate the substrate-dependency of the MalE-transporter interactions in different nucleotide states and the stimulatory effect of liganded and substrate-free MalE on the ATPase activity of the transporter.

In an intact E. coli cell and under inducing conditions of the maltose regulon, a 30–50-fold excess of MalE was observed relative to its membrane partners. However, only 20% of the normal MalE amount was required for full transporter activity[71]. Together, these findings indicate that the high concentration of MalE in the periplasm is not mechanistically essential for transport. Our results corroborate this notion as cross-linked products and DEER traces were observed at only two-fold excess of MalE over transporter which allowed 70% of ATPase activity compared to a 10-fold excess. Moreover, we found that dissociation of MalE is not a necessary step to accomplish the full nucleotide cycle, as supercomplexes formed by cross-linking MalE to MalG with long linkers (PBS) showed normal ATPase activity and substrate transport. However, the conformational freedom of MalE in the periplasmic region of the complex is necessary for transport, in fact blocking the N-lobe movements with a short linker (EBS) abolished ATPase activity and transport. This finding is reminiscent of type I ABC importers with one or two solute binding domains fused to one TMD[38]. The analysis of such a transporter, the OpuA complex of L. lactis revealed that two SBPs cooperatively interact but one is sufficient for transport[72].

Both in the presence and absence of substrate, three distinct conformations of the periplasmic region of the complex could be identified in the apo-, ATP- and ADP-states. The almost constant low modulation depth in the DEER traces suggests that the affinity of MalE for the transporter stays in the 10⁻⁴ M range in the three states investigated and it is independent of maltose, in contrast to the nanomolar affinity between open MalE and the AMP-PNP state of MalFGK2 reported recently[73].

Notably, we found that the structure of the MalE-transporter complex in the periplasmic region differs if MalE is added in the unliganded or maltose-loaded form to the apo- or ADP-state of the transporter. The interspin distances simulated on the X-ray structure of the complex in the pre-translocation state[39] are in good agreement only with the experimental distances in the presence of maltose (Figs. 2.10, 2.11, 2.12). In line with
the finding that the ADP-structure of the complex differs from that in the apo-state, we found a different behavior of the N- and C-lobes of MalE upon maltose addition: in the ADP-state the interaction with the C-lobe of MalE is unaffected but the N-lobe adopts a slightly different conformation depending on substrate.

Schematic models of the different conformations monitored by EPR and cross-linking are depicted in Fig. 2.6 superimposed to the available structural data. In summary, the apo-state of the transporter, with open NBDs, binds the N-lobe of unliganded (open) MalE while the C-lobe is more disordered. The presence of maltose leads to a tighter interaction between the C-lobe and the transporter, leaving the NBDs unaffected. Binding of ATP alone does not affect the ABC cassettes, but the presence of either open or closed MalE induces the full closure of the MalK dimer and the complex resembles the ATP crystal structure (Fig. 2.11B, 2.13B-C). Closed NBDs are primed to hydrolyze ATP, which explains the observed stimulation of ATPase by open MalE. We thus exclude that the observed stimulation by open MalE is due to the small fraction of closed MalE in equilibrium. The different conformations adopted during the remaining steps of the cycle in the periplasmic region of the complex are suggested to decrease the efficiency of the hydrolysis (Fig. 2.6B).

Hydrolysis of ATP switches the NBDs to a semi-open conformation, reintroducing the maltose-dependent interactions in the periplasmic region of the complex. The semi-open NBDs’ conformation is found here to be purely nucleotide-driven, namely ADP-Mg is
sufficient to induce the partial closure of the ABC cassettes in the absence of MalE (Fig. 2.6A). This is remarkably different from the negligible distance changes induced by ATP (or AMP-PNP-Mg) between positions 17 and 128 introduced as reporter side chains in MalK, in agreement with previous EPR studies. It is worth mentioning that a partial closure was observed by EPR and cross-linking upon ATP binding in the absence of MalE using the interfacial positions 83 and 85 in the Q-loop, while no distance changes were observed between spin labels in the regulatory domain or attached at position 117 located at the outer surface in the vicinity of position 128. Possibly, in the absence of MalE, binding of ATP to MalK slightly modifies only the Q-loop regions, leaving the overall ATP cassette dimer in an open state.

Under physiological conditions, it is assumed that the resting state of the transporter has ATP bound and the MalK dimer is in an open conformation (Fig. 2.6A). Unliganded (open) or liganded (closed) MalE would then contact the transporter with both lobes inducing the full closure of the NBDs. When substrate is available, the up-regulation of malE expression assures that the closed (liganded) form, thus a productive cycle, will prevail. Following ATP hydrolysis, maltose is translocated to the cytoplasm and, upon dissociation of phosphate, the transporter might exchange the unliganded MalE with a closed liganded copy in the presence of maltose or with an open unliganded MalE if maltose is becoming unavailable in the environment (Fig. 2.6C).

These data, obtained by two independent experimental tools, working either at ambient temperature (cross-linking) or in a frozen environment (DEER) highlight the conformational plasticity of a type I ABC importer and expand the knowledge on binding protein-transporter interactions which lead to coupling conformational changes to substrate translocation. One intriguing point is that this interaction is mediated by the extended extracellular MalF-P2 loop, confined to maltose transporters from enteric bacteria and a few other prokaryotes. Thus, assuming a basically common mode of SBP-transporter interactions and transmembrane signaling, in the vast majority of canonical importers the periplasmic contact must be maintained differently. How this is achieved remains to be elucidated.

2.4 Materials and methods

More details are provided in SI.

2.4.1 Protein expression, purification and spin labeling

Expression and purification of MalFGK2 and of MalE were performed according to methods described elsewhere. Spin labeling of the transporter mutants was performed according to ref. ATPase activity of all proteins was tested in detergent solution and in liposomes before and after spin labeling. Two selected cysteine mutants in MalG as well as the three supercomplex variants were tested for substrate transport in liposomes using [14C] maltose.

2.4.2 Cross-linking experiments

Cross-linking experiments using homobifunctional thiosulfonate linkers were performed as described earlier.
2.4.3 DEER experiments

Dipolar time evolution data were acquired using the four-pulse DEER experiment\(^{[25]}\). DEER traces were measured either in a conventional X-band spectrometer (Bruker ELEXSYS-II E580), or in a home-made high power Q-band spectrometer equipped with a home-made oversized resonator\(^{[28]}\).

2.5 Supporting information

2.5.1 Construction of cysteine variants

Cysteine residues replacing MalG-T46, MalE-T36, MalE-S352 and MalK-V17C/E128C were introduced by site-directed mutagenesis into the respective plasmid-borne alleles using the Stratagene’s Quik change mutagenesis kit according to the manufacturer’s instructions. Constructions of MalG-P78C, MalE-G13C, MalE-S211C, and MalE-T80C were described elsewhere\(^{[66,76]}\).

2.5.2 Purification of MalFGK\(_2\) and MalE variants and spin labeling

Histidine-tagged MalFGK\(_2\) variants were overproduced and purified essentially as described earlier\(^{[77]}\), except for omitting MgCl\(_2\) from all buffers. Briefly, cells were disrupted by one passage through a French Press at 18,000 psi. The membrane fraction was isolated by centrifugation (200,000 × g for 1 h at 4°C) and resuspended at a total protein concentration of 5 mg/mL in 50 mM Tris-HCl (pH 7.5), 20% glycerol and 1.1% n-Dodecyl β-D-Maltoside (DDM, buffer 1). After incubation for 1 h on ice, extracted proteins were separated from the remaining membrane fraction by ultracentrifugation and subsequently subjected to Ni-NTA chromatography. Pooled complex protein-containing fractions were desalted by passage through a PD10 column, equilibrated with 50 mM Tris-HCl (pH 7.5), 10% glycerol and 0.2% DDM (buffer 2). His6-MalE (wild type and variants) was purified from the cytosolic fraction of \(E.\ coli\) strain JM109 harbouring plasmid pCB06 or derivatives according to ref.\(^{[76]}\). To remove tightly bound maltose, purified MalE variants were subjected to a denaturation/renaturation procedure using 6M guanidine hydrochloride as described in ref.\(^{[79]}\). Cysteine mutants of MalE (20 μM protein concentration) were spin labeled with a 10 fold molar excess of MTSSL [(1-Oxyl-2,2,5,5-tetramethyl-∆3-pyrroline-3-methyl) methanethiosulfonate label Toronto Research Chemicals] and incubated 1 h at 4°C under gentle shaking. Excess of MTSSL was removed with a PD10 column and the labeled protein was concentrated to approximately 200 μM. Spin labeling efficiency was about 80–90%. The cysteine mutants of the transporter were spin labeled with the same procedure but using a 2–4-fold molar excess of MTSSL with respect to the cysteines.

2.5.3 Labeling with 2-(4’-maleimidylanilino)naphthalene-6-sulfonic acid

MIANS [2-(4’-maleimidylanilino)naphthalene-6-sulfonic acid] modification of MalFGK(V17C/E128C) was performed after cross-linking of 2.5 μM of complex in the presence of 5 μM wild-type MalE, 4 mM ATP and 1 μM 1,6-hexandiyl-bimethane-thiosulfonate (HBS) as described in ref.\(^{[78]}\), except that buffer 2 was used and no dithiothreitol (DTT) was added to terminate the reaction.
2.5.4 Preparation of proteoliposomes

For ATPase activity measurements, MalFGK₂ variants were reconstituted into liposomes at a lipid to protein ratio of 50:1 (E. coli total lipid extract, Avanti Polar Lipids). Reconstitution was performed by incubating the mixture with Bio-Beads (100 mg; Bio-Rad) for 18 h at 4°C. Proteoliposomes were isolated by centrifugation at 200,000 × g for 1 h at 4°C and the equivalent of 100 µg complex (determined by quantitation of MalK after SDS-PAGE) was resuspended in 600 µl buffer 3 containing 50 mM Tris (pH 7.5). For site-specific cross-linking, MalFGK₂ variants were reconstituted at a 10:1 lipid-to-protein ratio in the absence or presence of 10 mM ADP or 10 mM ATP. MalFGK₂(V17C/E128C)₂ was reconstituted in the presence of a two-fold molar excess of wild-type MalE. After incubation with Bio-Beads (300 mg) for 1 h at 4°C the equivalent of 100 µg of complex was resuspended in 120 µl of buffer 3.

For double electron-electron resonance (DEER) measurements, transporters were reconstituted at a 10:1 lipid to protein ratio either in the absence of nucleotides or in the presence of 5 mM AMP-PNP. The total assay comprised 1 ml total volume, 700 µg transporter and 800 µg MalE. For suitable sample concentrations, after centrifugation at 180,000 × g the whole amount was resuspended in buffer 3. 10 mM MgCl₂ was stepwise added to the reconstituted samples before DEER measurements.

2.5.5 Cross-linking experiments

Cross-linking experiments using homobifunctional thiosulfonate linkers were performed as described earlier[78]. In brief, the thiosulfonate cross-linkers 1,2-ethanediyl-bismethane-thiosulfonate (EBS), HBS and 3,6,9,12,15-pentaoxaheptadecane-1,17-diyldibismethanethiosulfonate (PBS) with approximate spacer lengths of 5.2 Å, 10.4 Å and 24.7 Å, respectively[70], were purchased from Toronto Chemicals. After incubation of the indicated protein samples (2.5 µM MalFGK₂ variants, 5 µM MalE variants) in buffer 2 for 10 min at 37°C, reactions were started by adding the respective cross-linker (final concentration 1 mM). After 20 min at room temperature, reactions were terminated by adding 5 mM N-ethylmaleimide. Additives used were: ADP (4 mM), ATP (4 mM), maltose (10 µM), MgCl₂ (10 mM) and vanadate (0.5 mM).

For ATPase measurements of cross-linked MalE-FGK₂ complexes, cross-linking reactions were carried out with the respective protein samples in the presence of 10 µM maltose and 5 mM of PBS or EBS as indicated for 20 min at room temperature. Subsequently, cross-linked complexes were isolated by size-exclusion chromatography (Superdex 200, GE Healthcare) in buffer 2.

Cross-linking of complex variants reconstituted in the absence or presence of ADP or ATP were performed essentially as described above, except of using 5 µM MalFGK₂ variants and 10 µM MalE variants, unliganded or supplemented with 20 µM maltose, in 50 mM Tris (pH 7.5). Vanadate-trapped complexes were prepared as described in ref.[78].

2.5.6 ATPase assay

ATPase measurements were carried out with reconstituted complex variants at a final protein concentration of 0.5 µM in 50 mM Tris (pH 7.5), 5 mM MgCl₂ buffer. The respective purified MalE variant was added at a constant concentration of 5 µM in the absence or presence of 20 µM maltose. The reaction was initiated by addition of 2 mM ATP and ATP hydrolysis was measured by assaying the release of inorganic phosphate at 37°C using ammonium molybdate as described in ref. [80].
Activity measurements of MalFGK\textsubscript{2} variants and cross-linked complexes in detergent solution were performed accordingly except for using 10 mM MgCl\textsubscript{2}. Purified MalE variants were added to a final concentration of 5 \(\mu\)M. Incorporated into proteoliposomes, all complex variants displayed basal ATPase activities that were slightly increased by open unliganded MalE and further stimulated in the presence of maltose-loaded MalE. In detergent solution, stimulation of the intrinsic enzymatic activity by MalE was also observed but less pronounced, consistent with earlier reports\textsuperscript{[78,81,82]} (see Tab. 2.1 and 2.2). All MalG and MalK cysteine mutations were found to be active before and after spin labeling (see Tab. 2.1 and 2.2). Spin-labeled MalE variants stimulated the activity of the wild type complex between 67 and 102\% compared to the unlabeled protein, with exception of MalE(G13R1), which was not further considered for EPR experiments (Tab. 2.1).

### 2.5.7 Transport assay of [\textsuperscript{14}C] maltose

Transport of [\textsuperscript{14}C] maltose was measured using proteoliposomes formed by fast dilution of 100 \(\mu\)g of the indicated complex variants mixed with 250 \(\mu\)l of E. coli total lipids sonicated in 50 mM Tris-HCl (pH 7.5), 25 \(\mu\)l 10\% (wt/vol) n-Octyl \(\beta\)-D-Glucopyranoside in 30 ml 50 mM Tris-HCl (pH 7.5) containing 7.5 mM ATP. After ultracentrifugation at 200,000 g for 30 min, proteoliposomes were resuspended in 400 \(\mu\)l 50 mM Tris-HCl (pH 7.5). For transport assays, 60 \(\mu\)l of proteoliposomes (final complex concentration of 0.5 \(\mu\)M) were mixed with MalE(G13C) or MalE(S352C) variants (final concentrations of 5 \(\mu\)M and 0.5 \(\mu\)M, respectively) and 10 \(\mu\)M [\textsuperscript{14}C] maltose (600 \(\mu\)Ci/\(\mu\)mol; American Radiolabeled Chemicals) in 50 mM Tris-HCl (pH 7.5) buffer containing 1 mM MgCl\textsubscript{2}. After 20, 40, 60, and 80 s, 25 \(\mu\)l aliquots were diluted in 1 ml 50 mM Tris-HCl (pH 7.5) containing 1 mM maltose followed by rapid filtration through a 0.22 \(\mu\)m nitrocellulose Millipore filter. Subsequently, the filters were washed once with 5 ml of 50 mM Tris-HCl (pH 7.5) and retained radioactivity was determined by liquid scintillation counting. For the cross-linked supercomplexes, no MalE was added. For background correction, proteoliposomes formed without adding of ATP were used.

### 2.5.8 DEER experiments and simulations

DEER traces were recorded on detergent-solubilized transporters in three different conditions: absence of nucleotides (apo-state), 3 mM ATP and 0.075 mM EDTA (ATP-state), 3 mM ADP and 5 mM MgCl\textsubscript{2} (ADP-state). The proteoliposome samples contained AMP-PNP instead of ATP/EDTA.

DEER measurements were performed at Q-band frequencies (34–35 GHz) on a home-made spectrometer equipped with a TWT amplifier (150 W) and a home-made rectangular resonator enabling the insertion of sample tubes with 3 mm outer diameter.\textsuperscript{[28]} Dipolar time evolution data were acquired using the four-pulse DEER experiment. All DEER measurements were performed at 50 K. All pulses were set to 12 ns and deuterium nuclear modulations were averaged by increasing the first interpulse delay by 16 ns for 8 steps. The electron double resonance frequency was set at the maximum of the echo-detected field swept spectrum, 80 MHz higher than the observed frequency. The background of the DEER primary data (\(V(t)\)) was fitted and the resulting secondary data (\(F(t)\)) were converted by a model-free Tikhonov regularization to distance distributions with the software DeerAnalysis2011\textsuperscript{[26]}. Few DEER measurements were also performed at X-band with the conventional scheme. The observer frequency was 65 MHz higher than the pump frequency, all observer pulses were set to 32 ns and the pump pulse to 12 ns. Hydrogen and deuterium modulation artifacts were averaged (8 times increment of 56 ns). All samples
were prepared independently from different batches and the DEER traces were found to be reproducible. Samples for DEER measurements contained 10% (vol/vol) d8-glycerol and were shock-frozen in liquid nitrogen before being inserted in the precooled cavity at 50K.

The simulation of the possible spin label rotamers attached at positions in MalFGK2-E was performed using the Matlab program package MMM\(^1\) based on a rotamer library approach\[^9\].

The low affinity of MalE to MalFGK\(_2\) results in low modulation depths of the DEER traces, which dramatically decreases the S/N ratio. With the usual concentrations for DEER (\(\approx 150\) \(\mu\)M transporter and \(\approx 300\) \(\mu\)M MalE), the maximal modulation depth assuming a \(K_d\) of about \(10^{-4}\)M and 100% labeling efficiency is estimated to be 0.2. Measuring in Q-band is advantageous to maximize the S/N ratio and to avoid residual artifacts due to modulation of the signal with proton and deuterium frequencies.

It is worth noting that MalK\(_2\) is a symmetric homodimer, thus spin labeling of the double cysteine mutants (V17C-E128C) in the nucleotide binding domains leads to a four-spin system with inherent peculiarities. The 17-128\(^{\prime}\) (the prime denotes the second MalK monomer) and the 128-17\(^{\prime}\) pairs are characterized by short distances which should report the closure and reopening of the NBDs and are clearly separable from the longer distances between the other intra- and inter-MalK pairs. Moreover, due to the separation between the different dipolar frequencies, ghost peaks\[^8\] resulting from four spin artifacts were found to be negligible.

### 2.5.9 Supplemental figures

![Figure 2.7: Site-specific cross-linking of MalFGK\(_2\) to N- and C-lobe cysteine variants of MalE. Homobifunctional thiosulfonate cross-linkers with defined spacer lengths (EBS, 5.2 Å; HBS, 10.4 Å; PBS, 24.7 Å) and cofactors were added to different combinations of MalE-transporter cysteine mutants as described in the figure in liposomes [(A) and (B)]. The results of the SDS-PAGE are presented.](http://www.epr.ethz.ch/software/index)

\[^1\]freely available at http://www.epr.ethz.ch/software/index
2.5.10 Table 2.1

Table 2.1: ATPase activities of maltose transport complex variants

<table>
<thead>
<tr>
<th>MalE variant</th>
<th>ATPase activity [µmol P$_i$ min$^{-1}$ mg$^{-1}$]</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MalF$^+$G$^+$K$^2_2$</td>
<td>MalF$^+$G(T46C)K$^2_2$</td>
<td>MalF$^+$G(P78C)K$^2_2$ [MalF$^+$G(P78R1)K$^2_2$]</td>
</tr>
<tr>
<td><strong>No addition</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In liposomes</td>
<td>0.10 ± 0.02</td>
<td>0.11 ± 0.01</td>
<td>0.07 ± 0.01[0.05 ± 0.01]</td>
</tr>
<tr>
<td>In detergent solution</td>
<td>0.03 ± 0.03</td>
<td>0.28 ± 0.02</td>
<td>0.23 ± 0.04[0.25 ± 0.07]</td>
</tr>
<tr>
<td>+ MalE wt</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In liposomes</td>
<td>0.26 ± 0.02</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>- maltose</td>
<td>1.60 ± 0.04</td>
<td>1.38 ± 0.10</td>
<td>1.57 ± 0.13[0.90 ± 0.10]</td>
</tr>
<tr>
<td>In detergent solution</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- maltose</td>
<td>0.52 ± 0.03</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>+ maltose</td>
<td>1.20 ± 0.06</td>
<td>1.00 ± 0.05</td>
<td>0.98 ± 0.07[0.61 ± 0.05]</td>
</tr>
<tr>
<td>+ MalE(G13C)/maltose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In liposomes</td>
<td>1.71 ± 0.04</td>
<td>ND</td>
<td>1.40 ± 0.13</td>
</tr>
<tr>
<td>In detergent solution</td>
<td>1.26 ± 0.11</td>
<td>ND</td>
<td>0.82 ± 0.08</td>
</tr>
<tr>
<td>+ MalE(T36C)/maltose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In liposomes</td>
<td>1.34 ± 0.05</td>
<td>ND</td>
<td>1.08 ± 0.09</td>
</tr>
<tr>
<td>In detergent solution</td>
<td>1.09 ± 0.08</td>
<td>ND</td>
<td>0.76 ± 0.03</td>
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<tr>
<td>+ MalE(T80C)/maltose</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>In liposomes</td>
<td>1.55 ± 0.06</td>
<td>ND</td>
<td>1.72 ± 0.04</td>
</tr>
<tr>
<td>In detergent solution</td>
<td>1.14 ± 0.04</td>
<td>ND</td>
<td>1.15 ± 0.09</td>
</tr>
<tr>
<td>+ MalE(211C)/maltose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In liposomes</td>
<td>1.55 ± 0.06</td>
<td>1.46 ± 0.04</td>
<td>1.34 ± 0.12</td>
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<tr>
<td>In detergent solution</td>
<td>1.22 ± 0.16</td>
<td>0.96 ± 0.06</td>
<td>1.06 ± 0.11</td>
</tr>
<tr>
<td>+ MalE(S352C)/maltose</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>In liposomes</td>
<td>1.66 ± 0.09</td>
<td>1.30 ± 0.25</td>
<td>1.44 ± 0.11</td>
</tr>
<tr>
<td>In detergent solution</td>
<td>1.25 ± 0.10</td>
<td>0.99 ± 0.14</td>
<td>1.13 ± 0.05</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>MalE variant</th>
<th>ATPase activity [µmol P$_i$ min$^{-1}$ mg$^{-1}$]</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MalF$^+$G$^+$K(V17C/E128C)$_2$</td>
<td>MalF$^+$G$^+$K(C17R1/C128R1)$_2$</td>
</tr>
<tr>
<td><strong>No addition</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In liposomes</td>
<td>0.11 ± 0.01</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td>In detergent solution</td>
<td>0.37 ± 0.04</td>
<td>0.31 ± 0.02</td>
</tr>
<tr>
<td>+ MalE wt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>In liposomes</td>
<td>0.19 ± 0.01 (+ DTT)</td>
<td>0.14 ± 0.02</td>
</tr>
<tr>
<td>- maltose</td>
<td>1.59 ± 0.03 (+ DTT)</td>
<td>1.76 ± 0.06</td>
</tr>
<tr>
<td>In detergent solution</td>
<td>0.46 ± 0.03 (+ DTT)</td>
<td>0.41 ± 0.05</td>
</tr>
<tr>
<td>+ maltose</td>
<td>1.10 ± 0.04 (+ DTT)</td>
<td>1.07 ± 0.04</td>
</tr>
</tbody>
</table>

ATPase activities of purified complex variants in liposomes or detergent solution (0.5 µM) were measured in the absence or presence of de- and re-natured MalE variants (5 µM).
and maltose (10 µM). Proteoliposomes were prepared as described above. Data generally represents means of at least two independent experiments. ND, not determined; * denotes cys-less variant, R1 denotes the spin-labeled side chain. For the NBD’s cys-mutants, DTT was added to prevent internal cross-linking of the unlabeled ATPase subunits.

2.5.11 Table 2.2

Table 2.2: Summary of inter-residue distances (in Å) determined by site-specific cross-linking in detergent micelles compared to Cα – Cα distances in the available X-ray structures.

<table>
<thead>
<tr>
<th>MalG</th>
<th>MalE</th>
<th>Cα – Cα-distance</th>
<th>X-link</th>
</tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td>apo</td>
</tr>
<tr>
<td>C78</td>
<td>C13</td>
<td>(3PV0) apo</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PNP</td>
<td>18</td>
</tr>
<tr>
<td>C46</td>
<td>C46</td>
<td>C211</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Underlined distances denote increase in intensity of cross-linked product; see text for further details. MalE-C246 was used as a negative control (see also Fig. 2.1) to demonstrate the reliability of the cross-link approach. In line with the crystal structures, no cross-link product was obtained.

Author contribution

E.S. and E.B. designed the research and provided funding; S.B. and E.B. performed EPR experiments, S.B. performed spin labeling and reconstitution; A.L. and S.W. performed protein expression and purification, ATPase experiments and cross linking; S.B. and E.B. analyzed EPR data; A.L. and E.S. analyzed ATPase and cross linking data; S.B., A.L. and E.B. prepared figures; and S.B., A.L., E.S., and E.B. wrote the paper.
Figure 2.8: Site-specific cross-linking of MalFGK₂ to N- and C-lobe cysteine variants of MalE. Homobifunctional thiosulfonate cross-linkers with defined spacer lengths (EBS, 5.2 Å; HBS, 10.4 Å; PBS, 24.7 Å) and cofactors were added to different combinations of MalE-transporter cysteine mutants as described in the figure in detergent [(C)-(F)]. The results of the SDS-PAGE are presented.
Figure 2.9: SDS-PAGE analyses of cross-linked products obtained under varying experimental conditions in n-Dodecyl β-D-Maltoside micelles. (A) Time-dependent cross-linking of MalFG(T46C)K₂ (2.5 μM) with MalE(S211C) (5 μM) in the presence of maltose (10 μM) and low concentration of the homobifunctional thiosulfonate cross-linker HBS (1 μM). (B) MalFG(T46C)K₂ (2.5 μM) was incubated for 5 s with n-fold molar excess of MalE(S211C) in the presence of maltose (10 μM) and HBS (1 μM). (C) MalFG(P78)K₂ (2.5 μM) was incubated with MalE(V246C) (5 μM) and linkers with defined spacer lengths (EBS, 5.2 Å; HBS, 10.4 Å; PBS, 24.7 Å; 1 mM) in the presence of cofactors as indicated. The arrow marks the position where the MalE-MalG cross-linked band (EG band) would appear. No cross-linked EG band is visible under the conditions tested. Please note that the faint band always present around this molecular weight is a contaminant of the MalE (V246C) preparation (first three lanes).
Figure 2.10: **Experimental interspin distances between MalE and MalG compared with simulations.** (Left) DEER primary data $V(t)$ and background fits obtained with DeerAnalysis2011. (Middle) Normalized DEER form factors $F(t)$ and corresponding fits. (Right) Distance distributions obtained with Tikhonov regularization parameters 100 or 1,000. For clarity, the mutants are named according to the subunit at which the spin label is attached and the amino acid position (e.g., G78, E36). (A) Apo-state, in the presence (black) and absence (gray) of maltose. The dotted lines show the corresponding simulated distance distribution performed with MMM based on the crystal structure of the apo-state [Protein Data Bank (PDB) 3PV0, black] and of the AMP–PNP-state (PDB 3PUY, red).
Figure 2.11: **Experimental interspin distances between MalE and MalG compared with simulations.** (Left) DEER primary data $V(t)$ and background fits obtained with DeerAnalysis2011. (Middle) Normalized DEER form factors $F(t)$ and corresponding fits. (Right) Distance distributions obtained with Tikhonov regularization parameters 100 or 1,000. For clarity, the mutants are named according to the subunit at which the spin label is attached and the amino acid position (e.g., G78, E36). (B) ATP-EDTA-state, in the presence (red) and absence (pink) of maltose. (C) ADP-state, in the presence (blue) and absence (cyan) of maltose. The dotted lines show the corresponding simulated distance distribution performed with MMM based on the crystal structure of the apo-state [Protein Data Bank (PDB) 3PV0, black] and of the AMP-PNP-state (PDB 3PUY, red).
Figure 2.12: **Maltose effect on reconstituted samples.** DEER analysis of the mutant pair MalFG(P78R1)K2-MalE(S352R1) reconstituted into liposomes. (Upper) Apo state in presence (black) and absence (gray) of 10 mM maltose. (Lower) AMP-PNP (10 mM) was added during reconstitution in the presence (red) and absence (pink) of 10 mM maltose. (Left) Normalized DEER raw data $V(t)$ and background fits obtained with DeerAnalysis2011. (Middle) Normalized DEER form factors $F(t)$ and fits obtained with DeerAnalysis2011. (Right) Distance distributions obtained with Tikhonov regularization parameter 1,000. The results of the corresponding simulated distance distribution (dotted black, dotted red) performed with MMM based on the crystal structures of the apo- (3PV0) and AMP-PNP- (3PUY) state are superimposed to the experimental distance distributions.
Figure 2.13: MalE and maltose effects on MalK₂ interspin distances in different nucleotide states (different comparisons with respect to Fig. 2.4). (Upper) Normalized DEER primary data $V(t)$ and background fits obtained with DeerAnalysis2011 on MalFGK₂ transporters solubilized in DDM spin labeled at positions 17 and 128 in MalK. (Middle) Normalized DEER form factors $F(t)$ and fits obtained with DeerAnalysis2011. (Bottom) Corresponding distance distributions obtained with Tikhonov regularization. The short distance peak ($< 4.5 \text{nm}$) represents the 17-128’ and 128-17’ contributions. (A) MalFGK₂ transporter alone in the apo-state (turquoise), AMP-PNP-Mg²⁺ (10 mM, orange trace), and ADP-Mg²⁺ (10 mM, brown trace). (B) Analogous DEER analysis performed after incubation of MalFGK₂ with wt-MalE and 1 mM maltose without nucleotides (black), in the presence of AMP-PNP-Mg²⁺ (10 mM, red trace) or ADP-Mg²⁺ (10 mM, blue trace). (C) Analogous DEER analysis performed after incubation with wt-MalE in the absence of maltose without nucleotides (gray), with AMP-PNP-Mg²⁺ (pink) and ADP-Mg²⁺ (cyan). The distance distributions obtained from simulations with MMM are superimposed as dotted lines in the color of the corresponding state. For clarity only the 128-17’ and 17-128’ distance contributions are shown. The interspin distances of the transporter alone (A) are based on PDB 3FH6 (turquoise) and PDB 2AWO (brown), the latter representing only the isolated NBDs in the ADP-state. In case of MalFGK₂-E-complexes in the presence of maltose (B), the simulations are based on PDB 3PV0 (black) and 3PUY (red).
Figure 2.14: **Site-specific cross-linking of MalFGK(V17C/E128C)_2.** (A) Purified complex protein was incubated in the absence or presence of wild-type MalE with crosslinkers and cofactors as indicated, and subsequently analyzed by SDS-PAGE. (B) The cross-linked complex obtained in the presence of wild-type MalE, ATP, and HBS (Coomassie-stained SDS gel, lane 1) was further analyzed by immunoblotting using an antiserum raised against MalK (lane 2), and after additional incubation with MIANS (lane 3) as described in Materials and Methods. The MIANS-labeled complex was visualized under UV light following SDS-PAGE. The data reveal that after cross-linking, the upper of the two MalK dimers has still free SH-groups, whereas the lower band cannot be labeled with MIANS. Consequently, we conclude that the lower MalK dimer represents the doubly cross-linked product, whereas the upper band originated from incomplete cross-linking.
3 Asymmetry in the homodimeric ABC transporter MsbA recognized by a DARPin

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3.1 Introduction

ATP-binding cassette (ABC) transporters are primary active transporters, which act as exporters, importers, receptors, and channels. These transmembrane proteins mediate a plethora of physiological phenomena, including transport of metabolic products, nutrients, proteins, peptides, lipids, polysaccharides, ions, and drugs. In humans, malfunction of ABC transporters leads to several genetic disorders including cystic fibrosis, neurological diseases, retinal degeneration, cholesterol, and bile transport defects, anemia, and insufficient drug response.

MsbA is a homodimeric ABC exporter involved in the transport of lipid A and lipopolysaccharides to the outer leaflet of the inner membrane of several Gram-negative bacteria, and the msbA gene is essential in *Escherichia coli*. MsbA is homologous to the multidrug transporter LmrA of Gram-positive *Lactococcus lactis* and mammalian multidrug resistance transporters such as P-glycoprotein and shows overlapping substrate specificity with them. At a molecular level, ABC transporters consist of four domains, two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs). In ABC exporters, the TMDs are composed of at least six transmembrane helices, which form the substrate transport pathway and provide specificity. The NBDs are also known as ATP-binding cassettes and drive the transport cycle by binding and hydrolyzing ATP. The motional energy of the NBDs are coupled to conformational changes in the TMDs which alternate between an inward- and an outward-facing state and thereby form a pathway to mediate unidirectional transport across the membrane.

Current molecular understanding of ABC exporters is based on crystal structures of Sav1866 solved at 3Å in its outward-facing state, P-glycoprotein solved at 3.8Å in its inward-facing state, and the structures of three homologues of MsbA solved in three different conformations at resolutions ranging from 3.7Å to 5.5Å. Based on these crystal structures, large conformational changes are expected to occur in ABC exporters upon nucleotide binding and hydrolysis, which could be confirmed by cross-linking experiments and EPR measurements.

The mechanistic details of how the binding and hydrolysis of ATP at the NBDs is coupled to the transport process at the TMDs are still a matter of controversy. In many...
heterodimeric ABC transporters, one of the two ATPase sites deviates from the consensus sequence and is therefore called the degenerate site[49]. Such heterodimeric ABC exporters mainly hydrolyze ATP at the consensus site and thus operate in an asymmetric fashion. In homodimeric ABC exporters such as BmrA as well as in P-glycoprotein which is a fused heterodimer but exhibits two consensus ATPase sites, biochemical experiments demonstrated that only one ATP molecule is trapped by vanadate in the post-hydrolytic state per ABC transporter dimer[85–86]. It was concluded that ABC exporters hydrolyze the two ATP molecules sequentially instead of simultaneously, possibly involving a mechanism of alternating catalytic sites[87]. This mechanism implies that the NBD homodimer must switch to an asymmetric conformation before ATP is hydrolyzed at one of the two catalytic sites. In support of this notion, asymmetric homodimers have been observed in crystal structures of the ATP-bound NBDs of the ABC transporter HlyB[88]. On the other hand, current crystal structures of outward-facing MsbA and Sav1866 feature two bound AMP-PNP [Adenosine 5'- (β,γ-imido)triphosphate] at the NBDs (thus representing a pre-hydrolytic state) and display only minor structural differences between the two chains, that are related by 2-fold non-crystallographic symmetry[46,48]. Molecular dynamics simulation using the symmetric Sav1866 and MsbA structures as starting points revealed that the NBDs readily switch to an asymmetric state in which only one molecule of ATP enters the hydrolysis reaction at a time[89,100]. Further work addressing the question of asymmetry in ABC exporters is clearly needed to resolve the apparent discrepancies between asymmetries found based on biochemical experiments and the symmetric crystal structures of the homodimers Sav1866 and MsbA.

Designed Ankyrin Repeat Proteins (DARPins) represent a well-established randomized scaffold used as an alternative to antibody fragments (Fabs and scFvs) and have been selected against a variety of target proteins[101–103]. A growing number of DARPin co-crystal structures demonstrate the successful application of such designed binding proteins as crystallization aids[102]. Besides possible improvements of crystal diffraction, DARPinDNA have the potential to trap the targeted protein in alternate conformations. DARPinDNA selected against the multidrug transporter AcrB for example trap this protein in an asymmetric state with two DARPinDNA bound to the homotrimer[103,104]. In the absence of DARPinDNA, AcrB was initially crystallized in its symmetric and later in its asymmetric state[105,106].

Here, we describe the selection of DARPinDNA against detergent-purified MsbA using ribosome display. We identified a DARPin which binds to the homodimeric transporter in a 1:1 stoichiometric ratio. The DARPin DNA binding epitope was mapped to the TMDs and was found to be located away from the 2-fold symmetry axis of MsbA, suggesting that the DARPin recognizes asymmetries in MsbA. DEER measurements revealed that addition of the DARPin to MsbA does not influence the large ATP-induced conformational changes during transporter cycling. Nevertheless, DARPinDNA binding enhances the basal ATPase activity of the transporter without impairing the substrate-induced stimulation suggesting that the catalytic cycle is accelerated when asymmetric MsbA conformations are populated.

3.2 Experimental procedures

3.2.1 Purification, biotinylation, and spin labeling of MsbA and DARPinDNA

A detailed description of the cloning, expression, and purification of MsbA is given in the supplemental Experimental Procedures. MsbA was expressed in E. coli C43 (DE3) cells from a pBAD vector and purified using β-UDM (n-Undecyl β-D-Maltopyranoside) as
detergent. DARPin expression and purification has been described previously\textsuperscript{[107]}\textsuperscript{[107]}. Enzymatic biotinylation was facilitated by fusing an Avi-tag sequence to the one of MsbA. Single cysteine mutants at positions A25C, R103C, N191C, S206C, and S246C of MsbA were introduced by site-directed mutagenesis taking Cys-less msbA (C88S/C315S) as template. Likewise, single cysteine mutants were also introduced into the N- and C-terminal caps of DARPin\_55 (which is Cys-less by design) at the positions S12C, K16C, E29C, D151C, and D160C. For methanethiosulfonate cross-linking and spin labeling, single cysteine mutants of MsbA and DARPin were purified by supplementing 5mM dithiothreitol (DTT), which was removed (PD-10 column, GE Healthcare) prior to labeling with 10 times molar excess of MTSSL ((1-oxyl-2,2,5,5-tetramethyl-\(\Delta_3\)-pyrroline-3-methyl) methanethiosulfonate; Toronto Research Chemicals). Excess spin label was removed by size exclusion chromatography. For the EPR measurements, DARPin-MsbA complexes were made by spin labeling the freshly purified proteins individually, followed by gel-filtration of the protein complex.

3.2.2 Selection and screening of DARPin specific to MsbA

The N3C DARPin library\textsuperscript{[101]} was used for binder selection by ribosome display as described\textsuperscript{[103,108]}\textsuperscript{[107]}. Four rounds of selections were carried out following the solution panning strategy\textsuperscript{[107]}\textsuperscript{[107]}. 0.05% of \(\beta\)-UDM were used to maintain MsbA in active form. Single clones of the enriched pool of DARPins from the 4th selection round were expressed from the vector pQE30myc\textsuperscript{[107]} in E. coli XL-1 Blue yielding DARPins carrying an N-terminal RGS-His6 tag (MRGSHHHHHH) and a C-terminal Myc5 tag (5 times amino acid sequence MEQKLISEEDLNE). Single DARPin clones were analyzed for binding to bMsbAA viaC by crude cell extract ELISAs as described previously\textsuperscript{[107]}\textsuperscript{[107]}.

3.2.3 SEC of isolated DARPins and the MsbA-DARPin complexes

ELISA-positive MsbA-specific DARPins were subcloned into pQE30 (Qiagen) devoid of the C-terminal Myc5 tag for further biochemical analysis. A molar excess (2 to 3 times) of freshly gel-filtrated monomeric DARPins were mixed with MsbA and incubated in 20mM Tris-HCl (pH 7.5), 150mM NaCl, and 0.05% \(\beta\)-UDM for at least 30min at 4°C for complex formation. The protein complex was separated on a Superdex 200 10/300 GL column (GE Healthcare) (Fig. 3.10). The fractions corresponding to the MsbA-DARPin complex were analyzed by on-chip protein analysis according to the manufacturer’s protocol (Protein 80 Kit, Agilent Technologies) (Fig. 3.14).

3.2.4 Cysteine cross-linking of MsbA and DARPin\_55 mutants

For cross-linking studies, two thiol-specific homobifunctional cross-linkers namely M5M (1,5-pentanediyl bismethanethiosulfonate, Toronto Research Chemicals) and M11M (3,6,9-trioxaundecane-1,11-diyl-bismethanethiosulfonate, Toronto Research Chemicals) with spacer arms of 9.1 Å and 16.9 Å were used\textsuperscript{[70]}\textsuperscript{[70]}. The MsbA and DARPin\_55 mutant proteins were mixed at ratios of 1:3 and incubated with 0.5mM of cross-linker for 15min at 4°C (if not stated otherwise). The reactions were stopped by addition of SDS sample buffer and separated by non-reducing SDS-PAGE. The ability of DARPin\_55\_29C to cross-link to MsbA\_191C was also tested using inside-out membrane vesicles (ISOVs) containing overexpressed MsbA\_191C (with detergent purified MsbA\_191C, this cross-link is readily formed). To this end, MsbA\_191C containing ISOVs (6mg in 3ml) were mixed with DARPin\_55\_29C (35mg in 7ml) and M11M (1mM) was added. After incubation for 30min, N-ethylmaleimide (NEM, 10mM) was added to react with the remaining free cys-
tein for another 30 min. Subsequently, MsbA was purified according to the standard protocol and subjected to non-reducing SDS-PAGE (Fig. 3.14).

### 3.2.5 ATPase assay

The ATPase activity of purified protein was measured from the release of $P_i$ from ATP in a colorimetric malachite green assay as described\[^{[109]}\]. Briefly, purified protein (200 nM) in 50 mM K-HEPES buffer pH 7.4 containing 0.05% $\beta$-UDM, 2.5 mM MgCl$_2$ and 2 mM ATP was incubated at 30°C in a total reaction volume of 50 $\mu$l for 10–15 min. MsbA was inactivated by heating at 80°C for 2 min and free $P_i$ was measured using malachite green solution. The A600 of the samples was measured in a Microplate reader Infinite M100 (TECAN) and compared with the A600 of standards containing 0.5–4 nmol $P_i$. Readings were corrected for $P_i$ contamination originating from the ATP and buffer. DARPin$_{55}$-stimulated ATPase activity of MsbA was performed by adding DARPin$_{55}$ at concentrations as indicated to MsbA (200 nM) 15 min prior to the assay start. All cysteine mutants used in this study showed ATPase activity comparable to the wild-type. Spin labeling did not affect the activity of MsbA (Table 3.5.2).

### 3.2.6 Reconstitution of MsbA into liposomes

Detergent-purified MsbA was reconstituted into lipid membrane vesicles made of polar *E. coli* lipids and egg phosphatidylycholine mixed at a ratio of 3:1\[^{[110]}\]. For EPR and DARPin binding experiments, the reconstitution buffer was 20 mM Tris-HCl pH 7.4, 150 mM NaCl, for ATPase activity measurements it was 50 mM K-HEPES pH 7.0. MgCl$_2$ was added only where indicated shortly before performing ATPase activity or EPR experiments to avoid aggregation of the liposomes. The amount of reconstituted MsbA was determined by the Bio-Rad DC Protein Assay according to manufacturer’s protocol and compared BSA protein concentration standards. Binding of DARPin$_{55}$ to the reconstituted MsbA was tested by incubating the DARPin$_{55}$ with reconstituted MsbA (3:1 ratios) or empty liposomes for 15 min at 4°C. Unbound DARPin$_{55}$ was removed by three buffer washes after centrifugation at 20,000 $\times$ g for 20 min at 4°C. The pelleted liposomes were dissolved in SDS buffer and were separated by SDS-PAGE (15% acrylamide gel) followed by SYPRO ruby staining (Fig. 3.12B).

### 3.2.7 Distance measurements by pulse EPR experiments

Dipolar time evolution data were acquired using the four-pulse DEER (Double Electron Electron Resonance) experiment\[^{[25]}\]. DEER traces were measured in a home-made high power Q band spectrometer equipped with a home-made oversized resonator\[^{[111]}\]. All pulse lengths were set to 12 ns and deuterium modulation artifacts were averaged (8 times d1 time increment of 16 ns). The pump frequency was placed at the maximum intensity of the nitroxide spectrum, and the observer frequency 80 MHz lower. All samples were prepared independently from different batches and the DEER traces were found to be reproducible. Samples contained 10% (vol/vol) d8-glycerol and were shock-frozen in liquid nitrogen before measurement. Data analysis was performed with the software DeerAnalysis 2011\[^{[26]}\]. The simulation of the possible spin label rotamers attached at selected positions in MsbA was performed using the Matlab program package MMM based on a rotamer library approach\[^{[9]}\].
3.2.8 8-N$_3$-[\(\alpha$-\textsuperscript{32}P]ATP photolabeling

MsbA191C (1.5 µM) and DARPin\_55\_29C (6 µM) were cross-linked with M11M (1.5 mM) in 20 mM Tris, pH 7.5, 150 mM NaCl, 0.05% β-UDM containing 3 mM MgCl\_2. 8-N$_3$-[\(\alpha$-\textsuperscript{32}P]ATP (1 µM, 0.6 Ci mmol) was added to 10 µl protein sample (quadruplicates), and the reaction mixtures were incubated at 25°C for 5 min followed by UV irradiation (100 millijoules, UV cross-linker, Hoefer) on ice. Labeled cross-linked MsbA-DARPin complex was analyzed by SDS-PAGE and photolabeling was quantified using a phosphor imager (Molecular Dynamics, Storm 840). To analyze the amount of cross-linking, four aliquots were supplemented with cold 8-N$_3$-ATP followed by UV radiation, SDS-PAGE and staining with SYPRO Ruby (Invitrogen). The fluorescent signal was read with the LAS-3000 imaging system, and the bands were quantified using the Aida software (Raytest).

3.3 Results

3.3.1 \textit{In vitro} selection and identification of DARPins specific for MsbA

DARPin selection against biotinylated avi-tagged MsbA (bMsbAAviC) was performed using ribosome display with a DARPin library containing three randomized modules\cite{101,112} (Fig. 3.7). From 2000 DARPin clones analyzed for binding to bMsbAAviC by an initial ELISA (Fig. 3.8), 42 DARPins were further analyzed using a specificity ELISA in which besides bMsbAAviC the ABC transporter LmrCD and the secondary-active multidrug transporter AcrB (both prepared as biotinylated Avi-tagged proteins) were used (Fig. 3.9). This assay revealed 37 MsbA-specific DARPins that were further analyzed by size-exclusion chromatography. 27 of these DARPins displayed various degrees of oligomerization (soluble aggregates) and 6 of them showed monomer/dimer equilibrium and were therefore excluded. The remaining 4 MsbA-specific DARPins were monomeric but only one DARPin (named DARPin\_55) formed a stable complex with MsbA that could be isolated by size exclusion chromatography (Fig. 3.10). The affinity of DARPin\_55 for MsbA was estimated by competition ELISA\cite{101}, in which the binding of bMsbAAviC to immobilized DARPin\_55\_myc5 was competed by pre-incubation with increasing amounts of free DARPin\_55. The dissociation constant $K_d$ derived from these experiments was 80 nM (Fig. 3.11). Hence, from 2000 potential DARPin binders analyzed, we could identify one specific high-affinity binder (Fig. 3.1A).

3.3.2 The ATPase activity of MsbA is stimulated upon complex formation with DARPin\_55

The effect of DARPin binding to MsbA was studied with respect to the ATPase activity of the transporter. The basal ATPase activity of detergent-purified MsbA (200 nM) was stimulated up to 2.1-fold upon complex formation with DARPin\_55 (serial dilution from 50 nM to 1.6 µM) in a dose-dependent manner reaching its maximum at a concentration of 400 nM DARPin\_55 (Fig. 3.1C). Addition of the control DARPin E3\_5\cite{112,113} did not alter the ATPase activity of MsbA and DARPin\_55 alone did not hydrolyze ATP. By determining the Michaelis-Menten kinetics of ATP hydrolysis it was found that DARPin\_55 binding increases the maximal velocity $V_{max}$ (increase from 162 ± 9 to 255 ± 8 nmolP min$^{-1}$mg(MsbA)$^{-1}$) while the apparent $K_m$ of the ATPase activity remains unchanged (102 ± 22 and 117 ± 14 µM ATP for MsbA and the MsbA/DARPin\_55 complex, respectively) (Fig. 3.1D). Lipid A and daunomycin are known substrates of MsbA and were found to stimulate the ATPase activity of the detergent-purified transporter about 2-fold in
Figure 3.1: Characterization of DARPin_55: Specificity, epitope mapping and modulation of ATPase activity of MsbA. (A), ELISA to determine binding specificity of DARPin_55. DARPin_55 specifically binds to its target protein bMsbA\textsubscript{AviC} but not to the control proteins bLmrCD\textsubscript{AviC} and bAcrB\textsubscript{AviC}. DARPin_110819 specifically recognizing bAcrB\textsubscript{AviC} was used as a control. (B), ELISA assay to delineate DARPin_55 binding to subdomains of MsbA. DARPin_55 binds to the transmembrane domain (bTMD\textsubscript{AviC}) but not to the nucleotide binding domain (bNBD\textsubscript{AviC}) of MsbA. bAcrB\textsubscript{AviC} is used as a negative control. (C), modulation of basal ATPase activity of detergent-solubilized MsbA in complex with DARPin_55. DARPin_55 was added to MsbA (200nM) in a 2-fold serial dilution series ranging from 50nM to 1.6 \textmu M leading to a stimulation of the ATPase activity of MsbA by up to 2.1-fold. Addition of the unselected DARPin E3_5 (1 \textmu M) to MsbA does not increase its ATPase activity. DARPin_55 (1 \textmu M) alone does not hydrolyze ATP. (D), ATPase activity of MsbA was determined in the presence of DARPin_55 (1 \textmu M) or E3_5 (1 \textmu M) as well as in absence of DARPins at varying ATP concentrations. Addition of DARPin_55 results in a higher maximal rate of ATP hydrolysis (V\textsubscript{max}), while the apparent affinity for ATP (K\textsubscript{M}) is not affected. (E) and (F), stimulations of the ATPase activity of MsbA by substrates and DARPin_55 are additive. Lipid A was added to detergent-purified MsbA at concentrations of 0, 0.05, 0.1, and 0.2 mg mL\textsuperscript{-1} (E), daunomycin was added at concentrations of 0, 50, 100, and 200 \textmu M (F) and ATPase was measured in the absence and presence of DARPin_55 (1 \textmu M). Error bars represent standard deviations.

the absence\textsuperscript{[114]} and 3–4 fold in complex with DARPin_55. This implies that drug-induced and DARPin_55-mediated stimulations of the basal ATPase activity of MsbA are additive (Fig. 3.1, E and F). Therefore, DARPin binding does not appear to interfere with MsbA’s capability to bind its cargo substrates. MsbA and the MsbA\textsubscript{E506Q}\textsuperscript{[115]} mutant (whose ATPase activity is abolished) were reconstituted in proteoliposomes\textsuperscript{[110]}.

The addition of
lipid A and daunomycin stimulate the ATPase activity of reconstituted MsbA about 2-fold as described previously (Fig. 3.12)\textsuperscript{[114]}. In contrast to detergent-purified MsbA the basal ATPase activity of reconstituted MsbA was not stimulated by the addition of DARPin_55, because DARPin_55 does not bind to membrane-embedded MsbA as discussed below (Fig. 3.13).

### 3.3.3 Binding of DARPin does not impair MsbA ability to undergo conformational changes

To study the possible structural effects induced by DARPin binding to the transporter, single cysteine mutants of MsbA were labeled with MTSSL and interspin distances between the two labeled sites were measured by DEER in the apo- and AMP-PNP-states. The DEER analysis on two representative spin-labeled sites (position 191 in the TMDs and position 539 in the NBDs) is presented in Fig. 3.2. The 191-191 distance in the apo-state centered at 5.8 nm was found to be in line with the apo crystal structure\textsuperscript{[48]}, showing a C\textsubscript{α} - C\textsubscript{α} distance of 4.7 nm. The distance decreased to 4.1 nm upon addition of AMP-PNP and MgCl\textsubscript{2} (Fig. 3.2A), which fully agrees with the interspin distance simulated with the program MMM\textsuperscript{[9]} based on the AMP-PNP structure of MsbA. Upon complex formation with DARPin_55, MsbA showed the same distance distributions, indicating that the detergent-solubilized MsbA retained the ability to accomplish the conformational changes induced by nucleotide binding (Fig. 3.2A).

The AMP-PNP-state exhibited a sharp 1.8 nm distance between the two 539 labels located in the NBDs, both in the absence and presence of DARPin_55 (Fig. 3.2B). The obtained distance is again in perfect agreement with the crystallographic data, strongly indicating that DARPin binding does not lead to major structural changes in MsbA. The apo-state of the 539 mutant of MsbA showed a peak at about 6 nm in line with the inverted V-shaped apo crystal structure, which is characterized by a C\textsubscript{α} - C\textsubscript{α} distance of 6.4 nm. A second broad peak between 2 and 4 nm was present which might represent intermediate conformations adopted during NBD closure or disengagement. However, these intermediate states were only observed in this particular mutant and they were strongly suppressed after reconstitution into liposomes (Fig. 3.12). Interestingly, adding DARPin_55 to the apo-state of the 539 mutant of MsbA diminished the short distances but did not affect the 6 nm peak suggesting a stabilization of the open-apo structure by DARPin_55 in analogy to the reconstitution into liposomes (Fig. 3.2B, 3.12). Taking into consideration all tested mutants, the inverted V-shaped crystal structure and the outward-facing AMP-PNP bound structure\textsuperscript{[48]} appear to describe the conformational changes of detergent-solubilized MsbA appropriately.

### 3.3.4 DARPin_55 binds to the transmembrane domain of detergent-purified MsbA

To delineate the binding epitope of DARPin_55 on full-length MsbA we used purified sub-domains of MsbA, namely the TMD and the NBD. For this purpose, the domains were purified as Avi-tagged biotinylated proteins (bTMD\textsubscript{AviC} and bNBD\textsubscript{AviC}) and binding to DARPin_55 was assessed by the same ELISA setup as used to identify MsbA-specific DARPins described above (Fig. 3.1B, 3.8). This ELISA experiment revealed that DARPin_55 binds to the transmembrane domain (bTMD\textsubscript{AviC}) but not to the nucleotide binding domain (bNBD\textsubscript{AviC}) of MsbA. Binding of DARPin_55 to bTMD\textsubscript{AviC} resulted in a comparatively smaller ELISA signal than binding to full length bMsbAAviC. This could be due to partially unstructured regions on the TMDs in the absence of the NBDs. Binding
Figure 3.2: *Intra-MsbA distance measurements by DEER reveal no major conformational changes of the transporter upon binding of wild-type DARPin.* (A), left, normalized DEER form factors $F(t)$ and fits obtained with DeerAnalysis2010 on detergent-purified MsbA_191R1 alone and in complex with wild-type DARPin_55. Traces were detected in the apo-state and in the AMP-PNP-state (5 mM AMP-PNP and 5 mM MgCl$_2$) in the absence and in the presence of DARPin_55, as indicated. Right, distance distributions obtained with Tikhonov regularization parameters 100 or 1000. The intra-MsbA distances simulated based on the AMP-PNP x-ray structure (PDB 3B60) are superimposed (red dotted). (B), DEER analysis on MsbA_539R1 alone and in complex with DARPin_55 analogous to (A).

of DARPin_55 was further tested using membrane vesicles containing overexpressed MsbA and MsbA reconstituted in proteoliposomes (Fig. 3.13). In contrast to detergent-purified MsbA, DARPin_55 did not bind to the membrane-embedded transporter indicating that the binding epitope on MsbA cannot be accessed by DARPin_55 because it is (at least partially) masked by the lipid bilayer.
3.3.5 Cysteine cross-linking of DARPin and MsbA mutants

To further specify the binding epitope of DARPin_55 on MsbA, single cysteines were introduced into MsbA and DARPin_55 and proximities between these cysteines were detected using the thiol-specific homobifunctional cross-linkers M5M and M11M. A total of nine single cysteine mutants were made at different positions in the cytoplasmic part of the TMD of MsbA and in DARPin_55 (four in MsbA at positions 103, 191, 206, and 246 and five in DARPin_55 at positions 12, 16, 29, 151, and 160). Using ELISA, each mutated DARPin was confirmed to bind to every single cysteine MsbA mutant (data not shown). All possible combinations of MsbA and DARPin_55 cysteine mutants were then cross-linked as described in the experimental procedures. The single cysteine mutants of MsbA alone did not form any cross-linked products in the presence of M11M and M5M (Fig. 3.3A) nor could any of the DARPin mutants be cross-linked to Cys-less MsbA (Fig. 3.14). Cysteines introduced at the N terminus of DARPin_55 (at positions 12, 16, and 29) were efficiently cross-linked to MsbA_191C and to a lesser extent to MsbA_206C and MsbA_246C with both cross-linkers (Fig. 3.14). MsbA_103C formed only faint cross-linking products with all DARPin mutants tested. Likewise, the cysteines at the C terminus of DARPin_55 (at positions 151 and 160) were only poorly cross-linked with the four MsbA single cysteine mutants with either cross-linker. These faint cross-linking products seemed promiscuous and were therefore considered as background. In summary, the N terminus of DARPin_55 is in close proximity to the MsbA_191C position whereas the orientation of the C-terminal part of DARPin_55 could not be resolved by only this analysis.

![Image](image.png)

Figure 3.3: Methanethiosulfonate-mediated cross-linking of one DARPin to homodimeric MsbA. (A–C), MsbA_191C (1 µM) was incubated with increasing amounts of DARPin_55_29C (from 500 nM to 16 µM) in the absence of nucleotides (A), in the presence of MgCl₂ (3.5 mM) and AMP-PNP (3 mM) (B), or ATP-vanadate (2.5 mM, each) (C). Protein complexes were cross-linked using the homobifunctional M11M cross-linker (2 mM) for 15 min at 4 °C and separated by non-reducing SDS-PAGE. (D), ATPase activities of cross-linked MsbA_191C-DARPin_55_29C complexes are equal to those of non-treated MsbA-DARPin complexes and are consistently increased by a factor of two compared with MsbA alone.
3.3.6 One molecule of DARPin_55 binds to the MsbA homodimer

The maximal degree of DARPin cross-linking to MsbA never seemed to exceed 50% of the MsbA chains (supplemental Fig. 3.14). To further investigate this phenomenon, cross-linking between DARPin_55_29C and MsbA_191C was carried out in the absence of nucleotides as well as in the presence of AMP-PNP or ATP-vanadate using increasing amounts of DARPin (Fig. 3.3). Interestingly, cross-linking was saturated at a stoichiometry of approximately one DARPin binding to homodimeric MsbA in its apo-state (Fig. 3.3A). The exact stoichiometry was quantified by SYPRO Ruby staining to 0.73 ± 0.01 DARPins cross-linked to MsbA (Fig. 3.4A). The presence of AMP-PNP or ATP-vanadate appears to diminish the cross-linking reaction (Fig. 3.3, B and C), which might be caused by a longer distance between the thiol groups in this state (see EPR data below) and/or a decreased binding affinity of the DARPin for the nucleotide-bound state(s) of MsbA. Despite multiple attempts, we were not able to perform surface plasmon resonance (SPR) measurements which would have allowed for the determination of binding constants of DARPin_55 recognizing different conformational states of MsbA. The ATPase activity of the DARPin_55_29C/MsbA_191C complex is stimulated by a factor of two compared with MsbA_191C alone and remains unchanged upon cross-linking using M11M (Fig. 3.3D). This indicates that the DARPin_55 remains bound to MsbA during its entire catalytic cycle. Binding stoichiometries were further studied by gel filtration experiments. The MsbA/DARPin_55 complex was separated from excess DARPin_55 by size-exclusion chromatography (Fig. 3.10) followed by a quantitative analysis of DARPin_55 and MsbA using the protein chip technology (Agilent Technologies) (Fig. 3.15). In agreement with the cross-linking results, the ratio between DARPin_55 and MsbA dimer was quantified to 0.8. The binding stoichiometries in the gel filtration assay as well as in the cross-linking experiment were slightly less than one DARPin bound per MsbA dimer. For the gel filtration assay, this is likely due to partial dissociation of DARPin_55 from MsbA during the size-exclusion run whereas in the cross-linking experiment, some of the thiol moieties of the cysteines might have undergone oxidation during the purification and as a consequence would not react with the methanethiosulfonate cross-linker. At this point, two scenarios can explain the binding stoichiometry of one DARPin bound per homodimeric MsbA: (i) Binding of the first DARPin molecule to MsbA sterically prevents binding of the second or (ii) the DARPin recognizes asymmetries in MsbA.

3.3.7 EPR distances between DARPin_55 and MsbA

To rule out possible steric clashes between the two DARPins in the complex, the binding epitope of DARPin_55 on MsbA was mapped by EPR. Various combinations of five spin-labeled single cysteine mutants spanning the MsbA structure (positions 25, 65, 103, 191, 482) and two spin-labeled DARPin_55 (positions 29 or 160) were measured to extract interspin distances by DEER.

Because of the 1:1 stoichiometric ratio between DARPin_55 and MsbA, binding of one spin-labeled DARPin to the homodimeric spin-labeled transporter gives rise to a three-spin complex. This was confirmed by measuring the spin concentration in the MsbA-DARPin complexes using continuous wave EPR (data not shown). To disentangle the intra-MsbA from the MsbA-DARPin distances, we measured the MsbA alone and in the presence of the DARPin. To reduce the known effects of three-spin artifacts in the distance distributions[83], the intra-MsbA and the MsbA-DARPin distances should be as distinct as possible.

Among all combinations tested, only two (MsbA_191R1-DARPin_55_29R1 and MsbA_
Figure 3.4: Preferential nucleotide binding to one chain of asymmetrically stabilized MsbA as determined by 8-N$_3$-[α-32P]ATP photolabeling. (A) and (B), DARPin$_{55}$-29C was cross-linked to MsbA$_{191}$C using M11M, followed by addition of 8-N$_3$-[α-32P]ATP (1 µM) and UV-cross-linking (performed as quadruplicates). The samples were separated by non-reducing SDS-PAGE and analyzed by SYPRO Ruby staining (A) and autoradiography (B). (C), band intensities corresponding to cross-linked DARPin-MsbA complex and free MsbA monomer obtained in (A) and (B) were quantified.

65R1-DARPin$_{55}$-160R1, with R1 denoting the MTSSL spin-labeled side chain) resulted in extractable MsbA-DARPin distances (Fig. 3.5) (all DEER distances are presented in Fig. 3.6). The intra-MsbA$_{191}$R1 distances in the apo- and AMP-PNP- states are shown in Fig. 3.5A. Addition of DARPin$_{55}$-29R1 resulted in the appearance of a short distance peak at 1.5–2 nm confirming the close proximity of the N-terminal region (N-cap) of DARPin$_{55}$ to position 191 in the TMDs already observed by cross-linking (Fig. 3.14). Interestingly, the distance between these two positions becomes slightly longer (∼2 nm) in the presence of AMP-PNP. In agreement with the DEER results obtained with unlabeled DARPin$_{55}$ (Fig. 3.2A), the intra-MsbA$_{191}$R1 distance remains unchanged upon addition of DARPin$_{55}$-29R1 in the apo-state and in the presence of AMP-PNP (Fig. 3.5A). The longer distance observed between the two nitroxide labels in MsbA and DARPin$_{55}$ might explain the reduced degree of M11M mediated cross-linking observed upon addition of nucleotides (Fig. 3.3, B and C).

Whereas the position of the N-cap of DARPin$_{55}$ with respect to the TMD could be readily mapped by cross-linking and EPR, the orientation of the DARPin’s C-cap was challenging to find, also because no cross-link could be established between the 151 and 160 positions of DARPin$_{55}$ and any of the MsbA mutants used (Fig. 3.14). After screening by DEER a number of complexes with DARPin$_{55}$ labeled at the C-cap (position 160), we found a clear distance constraint between the C-cap and the extracellular region of the transporter (position 65). In agreement with the structural changes expected from the X-ray data, addition of AMP-PNP to isolated MsbA resulted in an increase of the 65–65 distance from 4 to 6 nm (Fig. 3.4B). When DARPin$_{55}$-160R1 was analyzed in complex with MsbA$_{65}$R1, we found a distinct MsbA-DARPin$_{55}$ distance centered at 3.9 nm in the AMP-PNP-state, while the overlap with the intra-MsbA distance prevented a clear MsbA-DARPin$_{55}$ distance assignment in the apo-state. In contrast, we could exclude distances < 5 nm between the C-cap of DARPin$_{55}$ and other spin labels in the NBDs (position 482) and in the cytoplasmic-facing region of the TMDs (positions 25, 103, 191) (Fig. 3.6). Based on these observations we could rule out that the C-cap is oriented toward
Figure 3.5: Determination of the binding epitope of DARPin_55 by DEER. (A), left, normalized DEER form factors $F(t)$ and fits obtained with Deer-Analysis2010 on detergent-purified MsbA_191R1 alone and in complex with DARPin_55 spin labeled at position 29 (N-cap). Traces were detected in the apo-state and in the AMP-PNP-state ($5\text{mM AMP-PNP}$ and $5\text{mM MgCl}_2$) in the absence and in the presence of DARPin_55_29R1, as indicated. Right, distance distributions obtained with Tikhonov regularization parameters 100 or 1000. Assigned MsbA-DARPin distances are marked in the distance distribution. (B), DEER analysis on MsbA_65R1 alone and in complex with DARPin_55 spin labeled at position 160 (C-cap) analogous to (A). The vicinity of the intra-MsbA and DARPin-MsbA distances in the apo-state prevented a clear assignment of the DEER constraint.

the NBDs, thus the transmembrane domain of MsbA seems to be covered at least partially by DARPin_55. Importantly, the DARPin binding epitope was found to be far away from the 2-fold symmetry axis of MsbA, thus ruling out the possibility of steric clashes between two DARPins as the cause for the stoichiometric ratio observed.
Figure 3.6: The MsbA-DARPin complex based on cross-linking and DEER data. (A), overview of the cross-linking data (Fig. 3.14) using the X-ray structure of MsbA (PDB 3B60) and a homology model of DARPin_55 as templates. The black straight lines indicate thiol pairs showing strong cross-links, the black dotted lines denote pairs showing weak cross-links. (B), overview of interspin distances between MsbA and DARPin_55. The possible spin label rotamers attached at selected positions in MsbA were simulated using the Matlab program package MMM and are indicated as green clouds. The black straight lines highlight the pairs for which DEER constraints were obtained. All DEER data including the restraints are presented in the table.

3.3.8 The ATPase sites of asymmetrically stabilized MsbA display different nucleotide binding properties

Cross-linking DARPin_55_29C to asymmetric MsbA_191C provides the unprecedented advantage that the conformationally unequal chains of homodimeric MsbA can be separated by SDS-PAGE. Thus, questions as whether the two ATPase sites of asymmetric MsbA display differences with respect to nucleotide binding can be for the first time addressed. The cross-linked DARPin-MsbA complex was incubated with 8-N3-[α-32P]ATP followed by UV cross-linking of the nucleotide to the two chains of MsbA. Subsequently, the proteins were separated by SDS-PAGE and the amount of cross-linking was determined by autoradiography (Fig. 3.4). The ratio between the bands of DARPin_55 cross-linked to MsbA and MsbA alone was quantified to be 0.73 ± 0.01 by SYPRO Ruby staining. Interestingly, the MsbA chain cross-linked to DARPin_55 showed a stronger photolabeling with 8-N3-[α-32P]ATP than the MsbA chain alone (ratio between MsbA-DARPin_55 to MsbA of 1.39 ± 0.07). This indicates that the MsbA monomer to which the DARPin was cross-linked to was photolabeled 1.9 times stronger than the free MsbA chain.

3.4 Discussion

Asymmetries in the NBDs of ABC exporters arising from trapping of nucleotides by vanadate have been discussed since the mid-nineties when Urbatsch and Senior first described the catalytic cycle of P-glycoprotein and later were also found in the homodimeric transporter BmrA [85,86]. Using an in vitro selected DARPin binder, we provide here novel
evidence supporting considerable asymmetries to be found in a well-studied ABC exporter.

Our biochemical analysis shows that DARPin_55 binds to the TMDs of MsbA in a stoichiometry of one DARPin per homodimeric transporter. The binding epitope was mapped to the TMDs of MsbA and therefore, the possibility that binding of the first DARPin sterically hinders binding of a second DARPin to the symmetry-related epitope on MsbA could be excluded. In further support of this notion, DARPin binding does not affect the closure of the NBDs and the transition to the outward-facing state of MsbA upon addition of AMP-PNP. The DARPin therefore recognizes asymmetries in MsbA.

Asymmetric homodimers are not unusual. The epidermal growth factor receptor from *Drosophila* has been recently crystallized in an asymmetric state in complex with two growth factor ligands binding in a low- and a high-affinity mode.[116] Caspase-9 is activated by dimerization and was crystallized as asymmetric homodimer exhibiting one active and one inactive catalytic site.[117] However, despite serious efforts we did not succeed to solve the co-crystal structure of the MsbA-DARPins complex and therefore the molecular details of the asymmetries in MsbA remain elusive.

Epitope mapping revealed that the DARPin binding site includes membrane-spanning portions of the TMD (Fig. 3.6). There are two observations which further support this unexpected finding. First, DARPin_55 only binds to MsbA in its detergent-purified but not in its membrane-bound form (Fig. 3.13). In a detergent-purified MsbA sample, free detergent monomers, protein-free detergent micelles and detergent bound to the membrane protein are in equilibrium.[118] There is constant rapid exchange of detergent molecules between these pools. Lipid bilayers in contrast are more rigid.[119] It is therefore likely that DARPin_55 partly displaces the detergent micelle, but not the relatively stiffer lipid membrane to bind to its epitope. Second, in agreement with the positioning of DARPin_55 with respect to the membrane boundary, the randomized positions in the \( \beta \)-turns of the second and the third DARPin repeat exclusively harbor hydrophobic amino acids (Fig. 3.16). These residues could mediate hydrophobic contacts with the transmembrane helices of MsbA, whereas in the first repeat, there are two glutamates which could interact with the cytoplasmic portion of the TMD. Importantly, DARPin_55 only recognizes MsbA, but not its homologue LmrCD or the multidrug transporter AcrB. Therefore, unspecific hydrophobic interactions of DARPin_55 with membrane proteins in general can be excluded. Interestingly, the presence of DARPin_55 increased the ATPase activity of the \( \beta \)-UDM-solubilized transporter by influencing the \( V_{\text{max}} \) term, independent of the substrate-induced stimulations. This finding is reminiscent of a recent biochemical characterization of the P-glycoprotein-specific antibody MRC16, the addition of which was found to increase the ATPase activity of the transporter in the absence as well as in the presence of the substrate nicardipine.[120]

It has often been argued that *in vitro* selected binding proteins may distort the target protein thereby trapping it in an unnatural and non-physiological state. However, numerous examples of crystal structures solved alone and in complex with a binding partner in very similar conformations speak against this argument (as reviewed in refs.[102,121,122]. One prominent example reminiscent to the study presented here is the homotrimeric multidrug transporter AcrB, that has been crystallized alone and in complex with DARPins in an asymmetric conformation in which each conformer represents a consecutive step of the transport cycle.[103,105,106] Only two DARPins were found to bind to the trimer, and at the third expected DARPin binding site, a steric clash between a conformationally altered subdomain of AcrB with the back side of the DARPin prevented binding.[103,104] The DARPins did not induce the asymmetric state, but rather recognized the intrinsic asymmetry of the targeted protein. It is therefore plausible that the asymmetric conformational
states of MsbA to which DARPin_55 binds belong to the intrinsic ensemble of possible conformations of the transporter.

DEER and cross-linking experiments show that DARPin_55 binds to homodimeric MsbA in the apo-state as well as in the presence of AMP-PNP and ATP-vanadate and therefore the recognized asymmetries in MsbA do not appear to be state-specific. In support of this notion, cross-linked MsbA-DARPin complexes retain their increased ATPase activity suggesting that the DARPin remains bound during the entire catalytic cycle. Importantly, DARPin binding does not trap MsbA in an asymmetric state, which would lead to an inhibition of the ATPase activity, but rather enables the transporter to inter-convert between conformations necessary for function at a higher rate. Our findings stand in partial contrast to a recent study on P-glycoprotein antibodies MRK16 and UIC2, which were shown to bind to the transporter in the apo-state as well as in the presence of AMP-PNP (pre-hydrolytic state), but not if the transporter is trapped with ADP-vanadate (post-hydrolytic state) [120]. In addition to earlier studies dealing with the asymmetric nature of the catalytic cycle at the NBDs [95,96], our results indicate that distinct asymmetries also inhere to the TMDs of MsbA where the DARPin_55 epitope is located. Lipid A, the natural substrate of MsbA, is rather big (molecular weight of around 1.8kDa) and asymmetric. Therefore, it is plausible to assume that only one molecule of lipid A can be accommodated by the substrate binding cavity of MsbA which might require asymmetries in the TMDs. We found that the ATPase activity of detergent-purified MsbA was stimulated by lipid A and daunomycin. Our observations with lipid A agree with a previous study [114], although the stimulation observed by us is not as pronounced as reported, whereas in the same study stimulation of the ATPase by daunomycin was not apparent. These differences might arise from the different detergents used for purification of MsbA (β-UDM in our case and β-DM (n-Decyl β-D-Maltopyranoside) in the mentioned study [114]). Interestingly, the stimulation of MsbA's ATPase activity by lipid A or daunomycin and DARPin_55 are additive (Fig. 3.1, D and E). DARPin binding therefore does not seem to interfere with substrate interactions at the binding cavity. 8-N3-[α-32P]ATP was shown to preferentially photolabel one of the chains of asymmetric homodimeric MsbA, hence supporting the notion that the asymmetries recognized by the DARPin at the TMDs are conformationally linked to the nucleotide binding sites at the NBDs and vice versa. Since photo-cross-linking using 8-N3-[α-32P]ATP is a non-equilibrium binding method, further biochemical analyses are difficult. Nevertheless, in symmetric MsbA both nucleotide binding sites are identical and expected to be labeled by equal amounts of 8-N3-[α-32P]ATP. This is not the case and therefore, the two nucleotide binding sites are different from each other. As previously proposed based on biochemical experiments and molecular dynamics simulations, the asymmetric switching at the NBDs might be a prerequisite for ATP hydrolysis [95,96,98,99]. Our data suggest that DARPin binding to MsbA populates asymmetric states of the transporter from an ensemble of structurally similar conformations which exhibits increased basal ATPase activities and is fully compatible with its function to bind lipid A and drugs.

3.5 Supporting information

3.5.1 Supplementary experimental procedures

Generation of genetic constructs

The E. coli msbA gene was cloned into the pBAD24 [123] for the protein production in E. coli. The 5' end of msbA gene was extended with sequences encoding a His-10 sequence, a C3 protease site (L-E-V-L-F-Q-G-P) and a linker (D-E-A-E-K-L-F-N-Q). The 3' end of msbA
gene was amplified using a reverse primer priming on the msbA gene. The PCR amplified product flanked by NcoI and XbaI restriction sites was ligated into the E. coli expression vector pBAD24 yielding the expression vector pBADMsbA (expressed protein termed MsbAHis). The Avi-tag (G-L-N-D-I-F-E-A-Q-K-I-E-W-H-E) coding sequence was added to the 3' end of msbA, which facilitated site specific biotinylation of the target protein as will be described in another manuscript. The wild type E.coli msbA gene was mutated to cysteine-less MsbA (C88S/C315S) and single cysteine mutants at the positions A25C, R103C, N191C, S206C and S246C of msbA were introduced by site directed mutagenesis. Likewise, single cysteine mutants were also introduced in to the N- and C-terminal caps of DARPIN_55 (which is cysteine-less by design) at the positions S12C, K16C, E29C, D151C and D160C. ATPase-inactive MsbA mutant was generated by mutating the conserved glutamate of the Walker B motif to glutamine (referred to as MsbA_E506Q mutant). The transmembrane domain (amino acid number 1 to 348) and nucleotide binding domain (starting from amino acid number 337 to 582) were cloned to contain an N-terminal His tag and a C-terminal Avi-tag sequence (the expressed protein termed as TMD AviC and NBD AviC respectively) into pBAD24. All constructs were confirmed by DNA sequencing (Microsynth).

Protein expression, purification and spin labeling

MsbA expression was performed in E.coli C43(DE3) cells, grown in 2YT media. Protein production was induced at OD600 of 0.8 with 0.02% L-arabinose (Sigma) for 12-14 hours at 25°C. Harvested cell pellets were resuspended in 20mM Tris-HCl (pH 7.4), 150mM NaCl, 25 μg ml DNaseI and protease inhibitor cocktail (Sigma) and cells were lysed using EmulsiFlex-C3 (Avestin). Unbroken cells and cell debris were removed by centrifugation at 8000 rpm (SS34 rotor) for 10 min at 4°C and membrane vesicles were then harvested by centrifugation at 45,000 rpm (Ti70 rotor) for 60 min at 4°C. MsbA was solubilized from membrane vesicles using 1% β-DDM in 50mM Tris-HCl (pH 7.4), 150mM NaCl (TBS) at 4°C for 90 minutes. The insolubilized membrane fraction was removed by centrifugation at 45,000 rpm (Ti70 rotor) for 60 min at 4°C and cleared lysate was loaded onto a Ni2+-NTA gravity flow column containing 1.5ml resin (Qiagen). The resin was washed with 35mM imidazole pH 7.5, TBS and 0.05% β-UDM (50ml) and MsbA was eluted from the column in the same buffer containing 250mM imidazole pH 7.5 (7ml). Avi-tagged MsbA was enzymatically biotinylated in vitro using purified BirA yielding bMsbAAviC. Every MsbA preparation used in this work was separated from aggregated material by gel filtration (Superdex 200, 10/300, GE Healthcare) in 20mM Tris-HCl (pH 7.5), 150mM NaCl and 0.05% β-UDM and the fractions of the homogenous main peak corresponding to dimeric MsbA were pooled. Purified MsbA was supplemented with 10% glycerol and was snap-frozen in liquid nitrogen and stored at −80°C. Freezing of MsbA did not decrease its ATPase activity even after prolonged storage and did not lead to aggregation of the protein (not shown). Single cysteine mutants of MsbA for methanethiosulfonate cross-linking and spin labeling were purified by supplementing 5mM dithiothreitol (DTT) to the Ni2+-NTA washing and elution buffers. In order to spin label MsbA, DTT was removed on a PD-10 column (GE Healthcare) in TBS containing 0.05% β-UDM buffer according to the manufacturer's protocol. Single cysteine MsbA was spin labeled at 4°C overnight using a 10 times molar excess of MTSSL ((1-Oxyl-2,2,5,5-tetramethyl-∆3-pyrroline-3-methyl) methanethiosulfonate; Toronto Research Chemicals). Excess spin label was removed by size exclusion chromatography. DARPin expression and purification has been described previously. In case of the single cysteine mutants of DARPIN_55, 5mM DTT was added during the Ni2+-NTA purification. DTT was removed by the size-exclusion chro-
matography (Superdex200, 10/300, GE Healthcare) in 20 mM Tris-HCl pH 7.5, 150 mM NaCl. Monomeric DARPIN_55 was snap-frozen in the presence of 10% of glycerol in liquid nitrogen and stored at −80°C. For the EPR measurements, DARPin-MsbA complexes were made by spin labeling the proteins individually, followed by gel-filtration of the protein complex.

### 3.5.2 Table 3.5.2

<table>
<thead>
<tr>
<th>MsbA mutant</th>
<th>ATPase activity [nmol min⁻¹ mg⁻¹ Protein]</th>
</tr>
</thead>
<tbody>
<tr>
<td>MsbA*</td>
<td>136.7 ± 8.3</td>
</tr>
<tr>
<td>MsbA_cysless</td>
<td>130.8 ± 9.2</td>
</tr>
<tr>
<td>MsbA_V65C</td>
<td>230.1 ± 16.9</td>
</tr>
<tr>
<td>MsbA_V65C_R1</td>
<td>234.0 ± 22.2</td>
</tr>
<tr>
<td>MsbA_R103C</td>
<td>121.1 ± 0.7</td>
</tr>
<tr>
<td>MsbA_R103C_R1</td>
<td>115.7 ± 5.9</td>
</tr>
<tr>
<td>MsbA_N191C</td>
<td>111.3 ± 17.8</td>
</tr>
<tr>
<td>MsbA_N191C_R1</td>
<td>126.86 ± 20.6</td>
</tr>
<tr>
<td>MsbA_S482C</td>
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<tr>
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<tr>
<td>MsbA_L539C</td>
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</tr>
<tr>
<td>MsbA_L539C_R1</td>
<td>136.54 ± 9.1</td>
</tr>
</tbody>
</table>

*Wild-type; R1 methanethiosulphonate spin label

**Author contributions**

M.G.G., E.B., and M.S. designed research and provided funding; A.M. performed protein expression, purification, DARPin selection, spin labeling, cross linking, SEC, reconstitution, ATPase assays and photolabeling; S.B. and E.B. performed EPR experiments research; A.M. and M.S. analyzed biochemical data; S.B. and E.B. analyzed EPR data; A.M. and S.B. prepared figures; and A.M., S.B., E.B., M.G.G., and M.S. wrote the paper.
Figure 3.7: **Schematic representation of the binder selection cycle using ribosome display.** An mRNA library is *in vitro* translated and a ternary complex composed of mRNA, nascent polypeptide and ribosome (linkage of genotype to phenotype) is used for the binding selection against the immobilized target protein. Unbound ternary complexes are removed by washing whereas the mRNA encoding for bound DARPins is eluted by dissociating the ribosomal complex using EDTA. The released mRNA is reverse transcribed to DNA and is either used for another round of selection or is analyzed by expressing single clones of the enriched library followed by ELISA (picture adopted from Huber et al.[107]).
Figure 3.8: Schematic drawing of the ELISA set up. First, an anti-myc antibody is immobilized on an ELISA plate coated with Protein A. DARPins are then captured by the anti-myc antibody via their C-terminal Myc5-tag. Biotinylated target protein (LmrCD, AcrB or MsbA in our study) are then allowed to bind to the DARPin of interest and target protein binding is detected using a streptavidin-alkaline phosphatase. Its activity is detected colourimetrically at OD$_{405}$ using p-nitrophenyl phosphate as a substrate.

Figure 3.9: Exclusion of unspecific DARPin binders. 42 DARPin clones were selected from the initial ELISA hits and were further analyzed in this specificity ELISA. Besides bMsbA$_{AvIC}$, ABC transporter bLmrCD$_{AvIC}$ and secondary-active multidrug transporter bAcrB$_{AvIC}$ were used to identify unspecific binders. Out of 42 primary hits, 37 hits were exclusively binding to bMsbA$_{AvIC}$, while 5 clones were promiscuously binding to other target proteins in the ELISA.
Figure 3.10: Size-exclusion chromatography profile of the MsbA-DARPIn_55 complex. MsbA and DARPIn_55 were mixed at a molar ratio of 1:3 and incubated at 4°C for 15 minutes. The MsbA-DARPIn_55 complex and excess DARPIn_55 were separated by size-exclusion chromatography (Superdex 200 10/300 GL column, GE Healthcare). The fractions containing the MsbA-DARPIn_55 complex were quantified by on-chip protein analysis according to the manufacturer’s protocol (Protein 80 Kit, Agilent Technologies; Fig. 3.14). The ratio between DARPIn_55 and MsbA was determined to 0.8 suggesting that one DARPIn binds to homodimeric MsbA.

Figure 3.11: Estimation of DARPin_55 binding affinity to MsbA by a competition ELISA. MsbA (50nM) was pre-incubated with varying concentrations of free DARPIn_55 (10nM to 1000nM) and the mixtures were allowed to bind to immobilized DARPIn_55myc5. At a free DARPIn_55 concentration of 80nM, half-maximal ELISA signal was observed, suggesting that the binding affinity of DARPIn_55 is around 80nM (as described by Binz et al.\[101\]). Addition of the unselected DARPInE3_5 (1μM) did not compete with MsbA binding to immobilized DARPIn_55myc5. Error bars represent standard deviations.
Figure 3.12: Biochemical characterization of MsbA reconstituted into proteoliposomes. (A) and (B) The ATPase activity of reconstituted MsbA was measured in the absence and the presence of DARPin_55 using a concentration range of the MsbA substrates lipid A (A) and daunomycin (B). Error bars represent standard deviations. (C) and (D) DEER analysis of detergent-solubilized and reconstituted MsbA. MsbA spin-labeled at position 539 was used. Left, normalized DEER form factors $F(t)$ and $t_s$ obtained with DeerAnalysis2010. Right, distance distributions obtained with Tikhonov regularization parameters 100 or 1000. (C) Interspin distances in the apo-state in $\beta$-UDM (black), $\beta$-DDM (n-Dodecyl $\beta$-D-Maltopyranoside, green) and reconstituted in proteoliposomes (blue) are shown. This particular mutation was found to destabilize the apo-state conformation in $\beta$-UDM leading to the appearance of a main peak at about 6 nm and of additional short distances. Both $\beta$-DDM and liposomes stabilize the inward-facing conformation. (D) Analogous DEER analysis as in (C) but recorded in the presence of AMP-PNP and MgCl$_2$. 
**Figure 3.13:** **DARPin** _55 does not bind to membrane-embedded MsbA.** (A) Comparison of cross-linking of DARPin_55_29C to MsbA_191C in inside-out membrane vesicles (lane 2), reconstituted in proteoliposomes (lane 3) and in its detergent-solubilized form (lane 4). Cross-linking was not observed for membrane-embedded MsbA_191C. (B) DARPin_55 (3.5 µM) was allowed to bind to reconstituted wild-type MsbA (1 µM) in proteoliposomes and empty liposomes at 4°C for 30 minutes. Unbound DARPin_55 was removed by three washes and the protein was analyzed by SDS-PAGE followed by Ruby staining. DARPin_55 does not bind to reconstituted MsbA confirming the results described in (A).
Figure 3.14: SDS-PAGE analysis of M11M- and M5M-mediated cross-links between single cysteine mutants of MsbA and DARPin_55. Cross-links were performed as described in the materials and methods. The analysis concludes that the N-terminus of DARPin_55 (positions 12, 16 and 29) is in close proximity to positions 191, 206 and 246 of MsbA while the C-terminus of DARPin_55 (positions 151 and 160) is far apart from any of the cysteines introduced into MsbA (see also Fig. 3.6).
**Figure 3.15: Stoichiometry analysis of a DARPin_55-MsbA complex.** (A) Calibration of protein concentration determination by on-chip protein analysis (Agilent Technologies). DARPin_55 at concentrations of 4 µM, 2 µM and 1 µM was analyzed on a protein chip, and the areas of the resulting peaks were plotted against the known protein concentration (determined at A_{280}). The error bars of duplicate measurements were very small and the data could be fitted by linear regression. Note that the curve does not intercept at the origin, which likely reflects the detection threshold of this method. For the quantification of the MsbA-DARPin_55 complex, the protein concentrations were chosen to be in the linear range of detection. (B) Raw data of on-chip analysis of the MsbA-DARPin_55 complex eluted after gel-filtration. Peaks 9 and 10 correspond to DARPin_55 and MsbA, respectively, and the peak area is quantified taking the internal standard peak 11 as reference using the manufacturer's software. Using calibration curves as determined in (A), the stoichiometry between DARPin_55 and MsbA was determined.
Figure 3.16: Surface properties of DARPin_55. (A) A homology model of DARPin_55 was generated based on the coordinates of DARPin E3_5 (PDB entry 1MJ0) using SWISS-MODEL\textsuperscript{122}. The protein surface is colored according to its hydrophobicity (intensity of red color indicates increasing hydrophobicity) using the Eisenberg hydrophobicity scale\textsuperscript{127}. The DARPin scaffold is shown as cartoon and randomized residues are shown as sticks. (B) The sequence of DARPin_55 is depicted and randomized positions of the DARPin library are indicated in the Template_N3C sequence.
4 Structural basis for allosteric cross-talk between the asymmetric nucleotide binding sites of a heterodimeric ABC exporter


4.1 Introduction

ABC exporters are found in every organism. They minimally consist of four domains and exist as homodimers or heterodimers. Two transmembrane domains (TMDs) span the membrane with a total of 12 transmembrane helices and form the substrate permeation pathway by alternating between inward- and outward-oriented states (Fig. 4.5A). A pair of nucleotide binding domains (NBDs) is connected to the TMDs via coupling helices and drive conformational cycling of the transporter by binding and hydrolysis of ATP, a process which is linked to NBD dimerization and dissociation.

In their closed state, the NBDs sandwich two ATP molecules at the dimer interface by composite ATP binding sites involving conserved sequence motifs contributed by both subunits. The A-loop and Walker A motif of one NBD and the ABC signature motif of the opposite NBD are involved in nucleotide binding. The Walker B glutamate and the switch-loop histidine constitute a catalytic dyad required for ATP hydrolysis. In heterodimeric ABC exporters with asymmetric ATP binding sites, these catalytic residues are noncanonical at the degenerate site and ATP is therefore primarily, if not exclusively, hydrolyzed at the consensus site. The Q- and D-loops were associated with interdomain communication, but their functional role remains poorly understood.

Recently, we reported the structure of the heterodimeric ABC exporter TM287/288 from the thermophilic bacterium Thermotoga maritima, which was crystallized in the presence of adenosine 5'-(β,γ-imido)triphosphate (AMP-PNP) and was shown to transport drugs and dyes when expressed in Lactococcus lactis. The transporter adopted an inward-facing state with a nucleotide bound exclusively to the degenerate site. In contrast to the inward-oriented structures of MsbA, ABCB10 and P-glycoprotein in which the NBDs are separated or twisted, we found that the NBDs of TM287/288...
remain in close contact and do not shift in the NBD dimerization plane (Fig. 4.5). The current transport mechanism of TM287/288 envisages the binding of a second nucleotide to the consensus site for the transition to the outward-facing NBD-closed state, which subsequently is hydrolyzed to permit resetting of the transporter.\[51\]

Here, we present the high-resolution structure of the nucleotide-free state of TM287/288. Despite high ATP concentrations in the cell, this state is transiently adopted during transport; at the consensus site, the hydrolysis product ADP is replaced by ATP in each transport cycle, and at the degenerate site, the bound nucleotide is occasionally exchanged. We show that the asymmetric NBDs of TM287/288 remain in contact even in the absence of nucleotides. By comparing the apo state with the AMP-PNP-bound structure, we unravel the structural basis for allosteric coupling between the ATP binding sites.

4.2 Results

4.2.1 In the absence of nucleotides, the NBDs of TM287/288 remain in contact.

To gain insight into the functional and structural role of nucleotide binding at the degenerate site, we solved the crystal structure of TM287/288 in its apo state at 2.53 Å resolution (Table 4.1). Space group and cell edges of the apo state crystals were identical to the ones obtained in the presence of AMP-PNP and, therefore, structural changes discussed here are independent from crystal packing. The overall structures of the apo and the nucleotide-bound state of TM287/288 are highly similar [rmsd of 0.636 Å over residues 1–569 (chain A) and 10–592 (chain B)] and show that the NBDs remain in contact even in the absence of nucleotides (Fig. 4.1 A and B).

To examine conformational changes as a result of nucleotide binding in solution, four spin-label pairs were introduced into TM287/288: one in the extracellular region (150\(^{\text{TM287}}\)/295\(^{\text{TM288}}\)), one in the intracellular region (131\(^{\text{TM288}}\)/248\(^{\text{TM288}}\)) of the TMDs, and two in the asymmetric NBDs (350\(^{\text{TM287}}\)/475\(^{\text{TM288}}\) and 460\(^{\text{TM287}}\)/363\(^{\text{TM288}}\)) (Fig. 4.1B). For the double electron-electron resonance (DEER) measurements, the spin-labeled transporters were incubated at room temperature in the presence or absence of AMP-PNP and MgCl\(_2\) before flash freezing in cold liquid pentane (Fig. 4.1 C–F and Table 4.2).

The experimental DEER distances agree with those simulated with a rotamer library approach\[9\] on the two available crystal structures, considering the 3 to 3.5 Å accuracy achievable with this method\[138\] (Fig. 4.1 C–F and Table 4.2). The DEER measurements with the spin-label pairs 350\(^{\text{TM287}}\)/475\(^{\text{TM288}}\) and 460\(^{\text{TM287}}\)/363\(^{\text{TM288}}\) strongly support the crystal structures in which the NBDs of TM287/288 remain connected in the absence of nucleotides, because the frozen conformational ensembles in the apo and AMP-PNP-bound states are almost indistinguishable.

4.2.2 The conformational equilibrium of TM287/288 is shifted toward the inward-facing state in the presence of AMP-PNP.

In agreement with the crystal structures, but in contrast to what was observed for the homodimeric ABC exporter MsbA\([90,139]\), we found that all intracellular and extracellular interspin distances were only slightly influenced by AMP-PNP addition. We noted a small distance decrease in the intracellular pairs 131\(^{\text{TM288}}\)/248\(^{\text{TM288}}\) and 350\(^{\text{TM287}}\)/475\(^{\text{TM288}}\) upon AMP-PNP binding as well as a slight distance increase in the NBD pair 460\(^{\text{TM287}}\)/363\(^{\text{TM288}}\) (Fig. 4.1 D–F and Table 4.2), indicative of AMP-PNP binding and consequent allosteric effects in the intracellular region of the exporter. There are two reasons that
Figure 4.1: Structural differences between the apo and the nucleotide-bound TM287/288 structures. (A) Side view of the nucleotide-bound TM287/288 structure colored in cyan (TM287) and pink (TM288). AMP-PNP is depicted as colored spheres. Cα positions deviating by more than 1.1 Å between the nucleotide-bound and the apo structure are highlighted in red. Membrane boundaries are indicated in gray. (B) The structural differences are highlighted on the apo structure. TM287 is shown in light gray and TM288 in dark gray. Spin labels used for DEER analysis are shown as orange sticks. (C–F) DEER measurements of four spin-label pairs: in the extracellular region (150\textsuperscript{TM287}/295\textsuperscript{TM288}) (C), in the intracellular region of the TMDs (131\textsuperscript{TM288}/248\textsuperscript{TM288}) (D), in the NBDs at the degenerate site (350\textsuperscript{TM287}/475\textsuperscript{TM288}) (E), and in the NBDs at the consensus site (460\textsuperscript{TM287}/303\textsuperscript{TM288}) (F). Left graphs show background-corrected DEER traces [F(t)/F(0)] in the absence of nucleotides (black) and in the presence of AMP-PNP and MgCl\textsubscript{2} (red). Right graphs show experimental distance distributions (solid lines at the bottom) and simulated distances based on the two corresponding X-ray structures (dotted lines above).

Let us interpret these changes resulting from side-chain rearrangements at the spin-labeled sites and not from the switch to the outward-facing conformation of the exporter. First, the transition to the outward-facing state modeled using Sav1866\textsuperscript{[45]} as a template is expected
to induce a more pronounced interspin distance decrease in all three pairs (> 1 nm) and a concomitant distance increase in the extracellular pair. Second, similar small variations in the mean distances are simulated on the two corresponding structures, corroborating the notion that they can be explained by side-chain rearrangements alone (Fig. 4.1 C-F and Table 4.2).

To address possible influence of the detergent on the conformational cycling of TM287/-288, the exporter spin-labeled at the intracellular pair 131TM288/248TM288 was reconstituted in liposomes. The experimental mean distances in liposomes were similar to those obtained in detergent with deviations in the mean distances of 2.0 and 3.6 Å, in the apo and AMP-PNP-bound state, respectively (Fig. 4.7 A and B and Table 4.2).

The DEER measurements seemingly contradict cross-linking experiments showing that AMP-PNP facilitates NBD closure in TM287/288[51]. However, cross-links irreversibly trap states even if they are only marginally populated. The DEER measurements suggest that in solution, the conformational equilibrium is strongly shifted toward the inward-facing state, because the population representing the outward-facing state was not detectable.

4.2.3 Structural consequences of nucleotide binding at the degenerate site.

The majority of structural differences between the apo and the nucleotide-bound state are located at NBD1 (TM287) (Fig. 4.2 and Movie S11). Residues directly contacting AMP-PNP in the nucleotide-bound structure including the A-loop, the Walker A motif, and the helix following the Walker A respond strongly to nucleotide binding (Fig. 4.2C and Fig. 4.7). The hydrogen bonding network connecting the NBDs at the degenerate site, involving Asn521TM288 of the D-loop, Thr368TM287 of the Walker A motif, and Gln526TM287 of the switch-loop is rearranged. The number of inter-NBD hydrogen bonds is reduced from seven in the AMP-PNP-bound structure to four in the apo state (Fig. 4.3 A and B). Despite the rearrangements, the distance between the NBDs remains unchanged. In the apo state, a hydrogen bond between the ABC signature motif serine (Ser493TM288) and the D-loop aspartate (Asp523TM288) of NBD2 (TM288) is formed, and conformational differences are also observed in residues following the Q-loop of NBD2 (Fig. 4.3B and Fig. 4.7F). These residues directly interact with the coupling helix of TM287 and, thereby, would allow for conformational communication between the degenerate site and the TMDs. However, we did not observe structural changes at the coupling helix. When Asp523TM288 was mutated to alanine, the ATPase activity of TM287/288 increased approximately twofold, suggesting that the Ser493TM288-Asp523TM288 interaction slows down the catalytic cycle (Fig. 4.3C and Fig. 4.7). However, other contacts made by the Asp523TM288 side chain during the catalytic cycle may also play a role. In addition, the apparent affinity of ATP hydrolysis was decreased from 20.3 ± 1.3 µM to 71.8 ± 4.0 µM (SEM derived from nonlinear regression analysis). Asp523TM288 is preceded by Glu517TM288 of the Walker B motif, which is essential for ATP hydrolysis at the consensus site (Fig. 4.4B), suggesting that the D-loop of NBD2 allosterically couples the degenerate and the consensus site.

4.2.4 Conformational coupling mediated by a flexible consensus site D-loop.

The biggest structural differences are observed at the D-loop of NBD1, which is part of the consensus site (Fig. 4.2C). When AMP-PNP binds to the degenerate site, the D-loop aspartate (Asp501TM287) forms two hydrogen bonds with the Walker A motif of NBD2 (Fig. 4.3A). These hydrogen bonds are broken in the apo state, and the D-loop and the associated D-loop helix undergo major conformational rearrangements (Fig. 4.3 A and 1http://movie-usa.glencoesoftware.com/video/10.1073/pnas.1400485111/video-1
Figure 4.2: Conformational changes within the NBDs in response to nucleotide binding. (A) Top view on the NBD dimer (NBD1, Gly330-Phe569; NBD2, Gly353-Leu593) with bound AMP-PNP and structural changes accentuated as in Fig. 4.1. (B) NBD dimer with conserved motifs highlighted in colors. (C and D) The absolute backbone carbon distances between each residue of the nucleotide-bound and the apo state of NBD1 (C) and NBD2 (D) are plotted against the residue number. Key residues of conserved NBD motifs are labeled.

B). In the apo structure, the temperature factors of the D-loop residues are elevated, suggesting conformational flexibility (Fig. 4.7). To investigate the functional importance of the Asp501<sub>TM287</sub>-mediated hydrogen bonds, this highly conserved residue was mutated to alanine. The mutation caused a 16-fold decrease of the maximal ATPase activity and a fivefold increase of the apparent ATP affinity (Fig. 4.3C and Fig. 4.9).

Each D-loop mutation was combined with the intracellular spin label pair 131<sup>TM288</sup>/248<sup>TM288</sup> for DEER analysis. The distances in the apo and AMP-PNP-bound states were found to be similar to those obtained in the wild-type background (Fig. 4.7C and D and Table 4.2). However, the reproducible distance decrease observed in the original 131<sup>TM288</sup>/248<sup>TM288</sup> pair upon AMP-PNP binding could not be detected in any of the D-loop mutants, indicating allosteric interference in the rearrangement of the side chains in the intracellular region of the transporter.
Figure 4.3: **NBD–NBD interactions mediated by the D-loops.** (A) and (B) Hydrogen-bonding networks (≤ 3.6 Å) at the degenerate (Upper) and the consensus site (Lower) of the AMP-PNP-bound (A) and the apo (B) structure are depicted. (C) ATPase activities of D-loop mutants D501A<sub>TM287</sub> and D523A<sub>TM288</sub> introduced at the consensus and the degenerate site of TM287/288 were determined at varying ATP concentrations. The scale between 0 and 200 µM ATP is expanded. (D) Transport of BCECF-AM mediated by the D-loop mutants D507A<sub>LmrC</sub> and D593A<sub>LmrD</sub> was measured in *L. lactis* ΔlmrA ΔlmrCD. The inactive E587Q<sub>LmrD</sub> mutant served as negative control.

Both D-loop mutations were additionally introduced into LmrCD (D507A in LmrC and D593A in LmrD), a well-characterized TM287/288 homolog from *L. lactis* sharing a sequence identity of 36%<sup>50,124</sup>. When expressing the D507A<sub>LmrC</sub> consensus site mutant in *L. lactis* ΔlmrA ΔlmrCD, transport of the dyes Hoechst 33342 and BCECF-AM was found to be severely affected (Fig. 4.3D and Fig. 4.11). In contrast, the D593A<sub>LmrD</sub> mutation introduced at the degenerate site only mildly affected LmrCD-mediated transport, confirming the asymmetric nature of the D-loops in heterodimeric ABC exporters.
4.2.5 Restriction of the flexible consensus site D-loop inhibits ATP hydrolysis.

The D-loop of NBD1 is preceded by the Walker B motif harboring the Walker B aspartate (Asp494\textsuperscript{TM287}). In the AMP-PNP–bound structure, but not in the apo state, Asp494\textsuperscript{TM287} establishes a hydrogen bond to Ser373\textsuperscript{TM287} of the Walker A motif, that, in turn, coordinates the catalytically essential magnesium ion (Fig. 4.4A and Fig. 4.7D). In this manner, the Walker B motif senses the presence of the nucleotide–magnesium complex at the degenerate site and transmits this information in a long-range interaction via the D-loop to the Walker A motif of the consensus site. To further support this structural observation, we engineered a disulfide cross-link between the two D-loops by mutating Ser498\textsuperscript{TM287} and Ser520\textsuperscript{TM288} into cysteines in a cysteine-free background. Despite distances of 7.7Å and 10.1Å between the thiol groups in the nucleotide-bound and the apo state, respectively, a cross-link between these two cysteines was spontaneously formed during protein purification (Fig. 4.4C). Crystals of the cross-linked mutant diffracted to 3.2Å, and the resulting structure confirmed the expected tethering of the flexible NBD1 D-loop to the...
comparatively solid NBD2 D-loop, whose structure remains largely unchanged (Fig. 4.4D). Although AMP-PNP was added for crystallization of the cross-linked mutant, no additional electron density at the degenerate site was observed. The ATPase activity of the cross-linked mutant was reduced to 15% of cysteine-free TM287/288 and was fully restored by the addition of DTT (Fig. 4.4C). Hence, D-loop flexibility in NBD1 is a requirement for nucleotide binding at the degenerate site and for ATP hydrolysis at the consensus site.

4.3 Discussion

The involvement of the D-loop in NBD-NBD interdomain communication has first been postulated based on structures of isolated, homodimeric NBDs[98,131]. However, there are only a few reports that describe its functional role. A systematic cysteine-scanning study of residues lining the NBD interface of the sulfonylurea receptor SUR1 highlighted D-loop residues to play a key role in MgADP stimulation of its associated potassium channel subunit[140]. Of special note, substituting the degenerate site D-loop aspartate by a cysteine (D1513C) completely abolished MgADP stimulated potassium gating, whereas the functional impact of the corresponding consensus site mutant (D861C) was less severe. Although these results stand in contrast to the transport assays performed with LmrCD in which the consensus site D-loop mutant was most severely affected, they highlight the functional importance of the D-loops in heterodimeric ABC exporters. Mutations in the D-loop (L511P and D512G) of the homodimeric lipid A transporter MsbA, which abolish the function of this essential transporter in Escherichia coli were initially discovered by random mutagenesis[85]. Biochemical analysis of these mutants revealed that the L511P mutant is defective in ATP hydrolysis, which could explain its lacking transport function[141]. The D512G mutant, however, exhibited a threefold increased ATPase activity, reminiscent to the D523A TM288 mutation introduced at the degenerate site D-loop of TM287/288. The inability of the D512G mutant to transport lipid A was explained with a coupling defect to the transmembrane domains. In light of the structural observations presented in this study, we speculate that cross-talk between the two ATP binding sites of MsbA is likely to be disturbed in the D512G mutant and might explain the observed loss of transport function.

Comparison of the apo and the AMP-PNP-bound structures of TM287/288 allow for the first time, to our knowledge, an analysis of allosteric cross-talk between ATP binding sites in the context of a full-length ABC exporter. We provide structural and functional evidence that both D-loops of TM287/288 and LmrCD play a role in allosteric coupling, although in an asymmetric manner. The D-loop of NBD2 is integral part of the degenerate ATP binding site and, thereby, ties the NBDs together even in the absence of nucleotides. By contrast, the D-loop of NBD1 is highly flexible and contacts NBD2 via its conserved aspartate side chain only if the degenerate site is occupied with a nucleotide. Importantly, the consensus site D-loop aspartate was found to be critical for ATP hydrolysis in TM287/288 and substrate transport in LmrCD.

In contrast to other inward-oriented ABC exporters in which the NBDs are separated or twisted (Fig. 4.5), the arrangement of the NBDs as seen in TM287/288 allows for allosteric communication between the two ATP binding sites during the entire transport cycle. Our structural observations are likely to be of functional relevance to understand well-studied heterodimeric ABC exporters of eukaryotic origin. For example, the generally accepted mechanistic model of CFTR envisages the degenerate site to be constantly closed during the entire gating cycle, a notion supported by the fact that nucleotides bind more tightly to the degenerate than to the consensus site of CFTR[142]. Despite some disputes
whether the degenerate site changes its conformation as the channel progresses through its states\cite{1,43,144}, there is unanimous agreement that inter-NBD contacts need to be established at all times to explain the experimentally observed cross-talk between the degenerate and the consensus site. As suggested recently\cite{1,44}, a partially opened degenerate site as seen in TM287/288 does not contradict current functional models of CFTR, but rather could explain how the ATP binding sites sustain their ability to cross-communicate while the TMDs adopt an inward-facing closed-channel state.

In contrast to the well-studied homodimeric ABC transporter MsbA\cite{90,139}, AMP-PNP did not lead to NBD closure and the transition to the outward-facing state in TM287/288, suggesting that major differences exist between the two ABC exporters in the response to this nucleotide analog. Studies on CFTR and SUR1 revealed that AMP-PNP is a poor ATP analog in heterodimeric ABC exporters. For CFTR, a 20-fold decreased opening rate was observed for AMP-PNP compared with ATP\cite{1,45} and in SUR1, AMP-PNP fails to support NBD dimerization and conformational switching\cite{1,46}.

In conclusion, our analysis provides unprecedented mechanistic insight into the cross-communication between asymmetric nucleotide binding sites of heterodimeric ABC exporters and underscores the importance of nucleotide binding at the degenerate site to modulate the catalytic activity of the consensus site and, thus, substrate transport. The presented work offers a structural rationale for future studies on mammalian ABC exporters with asymmetric ATP-binding sites including the medically important transporters MRP1, SUR1, TAP1/2, and CFTR\cite{1,47,150}.

### 4.4 Methods

TM287/288 was purified as described\cite{51} and yielded apo state crystals, which diffracted anisotropically to 2.53 Å. For DEER measurements in detergent solution, double cysteine mutants of TM287/288 were spin-labeled with MTSL [(1-Oxyl-2,2,5,5-tetramethyl-Δ3-pyrroline-3-methyl) methanethiosulfonate] and incubated in the presence or absence of 2.5 mM AMP-PNP and 2.5 mM MgCl$_2$ at 25 °C prior to flash freezing in cold liquid pentane. DEER traces were recorded at Q band with all pulses set to 12 ns and frequency separation of 100 MHz\cite{28}. Data analysis and simulation of the MTSL rotamers on the X-ray structures were performed with the softwares DeerAnalysis2013\cite{26} and MMM2013.2\cite{9}. The D-looop aspartates of TM287/288 and LmrCD were substituted by alanines using site-directed mutagenesis. ATPase activities of TM287/288 mutants were measured by determining liberated phosphate. Transport of the fluorescent dyes Hoechst 33342 and BCECF-AM by LmrCD was measured in Lactococcus lactis. Experimental details are described in SI Methods.

### 4.5 Supporting information

#### 4.5.1 SI methods

**Mutagenesis**

Cys-less TM287/288\cite{51} served as a template to introduce the S498C\textsuperscript{TM287} and the S520C\textsuperscript{TM288} mutations and to generate the double electron-electron resonance (DEER) mutants S150C\textsuperscript{TM287}/T295C\textsuperscript{TM288}, T131C\textsuperscript{TM288}/S248C\textsuperscript{TM288}, T131C\textsuperscript{TM288}/S248C\textsuperscript{TM288}/D501A\textsuperscript{TM287}, T131C\textsuperscript{TM288}/S248C\textsuperscript{TM288}/D523A\textsuperscript{TM288}/D501A\textsuperscript{TM287}, S350C\textsuperscript{TM287}/K475C\textsuperscript{TM288}, and D460C\textsuperscript{TM287}/S363C\textsuperscript{TM288}. Wild-type TM287/288 was used to make the mutants D501A\textsuperscript{TM287} and D523A\textsuperscript{TM288}. The inactive LmrCD mutant E587Q\textsubscript{LmrD} carrying the
Walker B glutamate-to-glutamine substitution in the consensus site was described\cite{51}. The D-loop mutants D507A\textsubscript{LmrC} and D593A\textsubscript{LmdD} were cloned into the \textit{L. lactis} expression vector pNZ8048 by using the vector-backbone exchange (VBEx) cloning method\cite{151}.

Functional analysis of TM287/288 and LmrCD mutants

ATPase activity assays with detergent-purified TM287/288 were performed by determining liberated phosphate using molybdate/malachite green detection. All ATPase reactions were carried out in 20 mM Tris-HCl at pH 7.4, 150 mM NaCl, 10 mM MgSO\textsubscript{4}, and 0.03 % (wt/vol) \textbeta-DDM (n-Dodecyl \textbeta-D-Maltopyranoside) with three technical replicates for each ATP concentration. The ATPase assays shown in Fig. 4.3C and Fig. 4.7 were performed at 70°C for 10 min. To detect phosphate, reaction solution (90 \textmu L) was mixed with filtrated malachite green detection solution (160 \textmu L) consisting of 10.5 mg mL\textsuperscript{-1} ammonium molybdate, 0.15 M H\textsubscript{2}SO\textsubscript{4}, 0.34 mg mL\textsuperscript{-1} malachite green, and 0.1 % Triton X-100, and absorption was measured at 640 nm. ATP mixed with assay buffer was used for background subtraction. To identify the appropriate equation for nonlinear regression analysis, we performed an F-test analysis in which the Michaelis-Menten equation represented the null hypothesis and the Hill equation was the alternative hypothesis. For wild-type TM287/288, the analysis resulted in a P value of 0.0037 and an F value of 12.13, therefore clearly favoring the fit by using the Hill equation (SigmaPlot12.5). For the ATPase activity measurements with the S498C\textsubscript{TM287}/S520C\textsubscript{TM288} mutant and the DEER mutants, the ATPase activity was measured at 50°C in the presence of 1 mM ATP. DTT was added at 1 mM final concentration where indicated.

LmrCD-mediated transport of Hoechst 33342 and BCECF-AM was performed in \textit{L. lactis} ΔlmrA ΔlmdC\cite{109} at 25°C. Cells were grown in M17 medium incubated at 30°C, and expression was induced at an OD\textsubscript{600} of 0.4–0.6 with 5 \textmu g mL\textsuperscript{-1} nisin A for 2 h. The fluorescence measurements were performed with cells diluted to an OD\textsubscript{600} of 0.5 in 50 mM potassium phosphate at pH 7.0, 5 mM MgSO\textsubscript{4}, and 0.5 % glucose by using a Jobin-Yvon Fluoromax-4 spectrofluorimeter (Horiba Scientific). Hoechst 3334 transport was measured at a final concentration of 0.5 \mu M. Excitation and emission wavelengths were set at 355 nm and 457 nm, with 4 nm slit widths. BCECF-AM transport was performed as described\cite{124}. Fluorescence measurements were independently carried out twice and repeated by using the same batch of cells. Representative results of one complete experimental dataset are shown.

Protein crystallization

Production and purification of TM287/288 was performed as described\cite{51}. Crystals of TM287/288 in its nucleotide-free (apo) state were obtained by the vapor diffusion method in sitting drops at 20°C against a reservoir containing 26 % (wt/vol) polyethylene glycol (PEG) 400, 50 mM Na-cacodylate at pH 5.5, and 50 mM CaCl\textsubscript{2}. The crystallization setups were performed in the presence of 2.5 mM ADP and 3 mM MgCl\textsubscript{2}. Electron density for ADP was not observed in the solved structure. Crystals of TM287/288 were also obtained in the absence of nucleotides and MgCl\textsubscript{2} and grew against a reservoir containing 25 % (wt/vol) PEG400, 50 mM N-(2-acetamido)iminodiacetic acid at pH 6.5, and 50 mM Zn acetate. These crystals diffraction to 3.5 Å, and the resulting structure was indistinguishable to the high-resolution apo structure presented in this manuscript. The S498C\textsubscript{TM287}/S520C\textsubscript{TM288} mutant was purified in the absence of reducing agents, which led to an almost complete formation of the desired disulfide cross-link. Crystals were obtained in 32 % (wt/vol) PEG 400, 50 mM Na-Acetate at pH 5, and 500 mM KCl. Although 2.5 mM AMP-PNP and
3 mM MgCl₂ were included for crystallization, electron density for the nucleotide was not observed.

Data collection and processing

All crystals were frozen without further cryoprotection. Diffraction data were collected at the protein crystallography beamline X06SA at the Swiss Light Source of the Paul Scherrer Institut and processed by using the program XDS[152]. Apo TM287/288 was crystallized in the same space group as the previously solved AMP-PNP-bound TM287/288[51]. Diffraction data exhibited strong anisotropy and was therefore truncated by using the online anisotropy server of the University of California, Los Angeles using default settings[153], which lead to an improvement of the electron density map. Our previous AMP-PNP dataset[51] was reprocessed and cut at a resolution of 2.6 Å. The models were built in Coot[154] and refined by using PHENIX[155]. Side-by-side comparison of constant parts of the apo and the nucleotide-bound structures allowed for an iterative improvement of both models. Data collection and refinement statistics are listed in Table 4.1. Superimpositions were performed with the CCP4 program Superpose.

Spin labeling of the cysteine mutants for DEER

The cysteine mutants were Ni²⁺-NTA−purified in the presence of 2 mM DTT. Before spin labeling, DTT was removed on a PD-10 column equilibrated with 20 mM Tris-HCl at pH 7.4, 150 mM NaCl, 0.03 % n-Dodecyl β-D-maltopyranoside (β-DDM). MTSL [(1-oxyl-2,2,5,5-tetramethyl-3-pyrroline-3-methyl) methanethiosulfonate, Toronto Research] was added in a 1:10 (cysteine to label) fold molar excess to the transporter sample and incubated at 4°C overnight (for labeling efficiencies and ATPase activities, see Table 4.2). Excess of free spin label was removed by size exclusion chromatography in 20 mM Tris-HCl at pH 7.4, 150 mM NaCl, and 0.03 % β-DDM on a Superdex-200 10/300 GL column. The spin-labeled pair 131⁰M288/248⁰M288 was reconstituted into lipid membrane vesicles consisting of polar E. coli lipids and egg phosphatidylcholine mixed at a ratio of 3:1 as described[110].

DEER analysis

Samples (40 µl final volume, protein concentration 30–40 µM) containing 10 % (vol/vol) d8-glycerol (Sigma Aldrich) were inserted in 3mm outer-diameter quartz tubes. To populate the AMP-PNP state, 2.5 mM AMP-PNP and 2.5 mM MgCl₂ were added before adding d8-glycerol. Tubes were pre-incubated at room temperature and then quickly frozen in a mixture of n-pentane and liquid nitrogen (−131°C). The frozen tubes were inserted into the precooled home-made Q-band cavity[28,156] at 50 K. DEER measurements were performed at Q-band frequencies (34–35 GHz) on a Bruker ELEXSYS E580 spectrometer equipped with a 200 W traveling-wave tube amplifier (Applied System Engineering). Dipolar time evolution data were acquired by using the four-pulse DEER experiment. All pulses were set to 12 ns, and deuterium nuclear modulations were averaged by increasing the first interpulse delay by 16 ns for eight steps. The ELDOR frequency was set at the maximum of the echo-detected field swept spectrum, 100 MHz higher than the observer frequency[28]. The background of the normalized DEER primary data [V(t)/V(0)] was fitted with the software DeerAnalysis2013[28]. Data were recorded for two different protein batches and found to be highly reproducible. The simulation of interspin distances on the X-ray structures was performed with the software MMM2013.2[9] by using the MTSL rotamer library at 175 K. In Fig. 4.1B, the most populated rotamer at each spin-labeled site is presented (Table 4.3).
Figure 4.5: **Structural comparison of all crystallized ABC exporters.** (A) The structures are shown from the side as ribbons. Inward-facing homodimers are colored dark blue and light blue. Inward-facing heterodimers are colored dark blue and green. Outward-facing homodimers are colored black and gray. The distances between the coupling helices (red dotted lines) were measured between Cα positions of residues corresponding to Gly201 in TM287 and Gly225 in TM288 (highlighted as red spheres). The distances are depicted next to the protein name and PDB ID code. Bound nucleotides are shown in purple (for TM287/288 one; for ABCB10, *Salmonella typhimurium* (S.t.) MsbA and Sav1866 two each). (B) Nucleotide binding domain (NBD) twisting analysis of TM287/288, ABCB10, and *Vibrio cholerae* (V.c.) MsbA. Chain A of the Sav1866 NBD dimer (gray) was taken as a reference for superimpositions. Displacement of the nonaligned chain is shown relative to chain B of the Sav1866 NBD dimer (black). For clarity, chain A of Sav1866 is omitted in the superimpositions. E.c., *Escherichia coli*; M.m., *Mus musculus*; C.e., *Caenorhabditis elegans*; V.c., *Vibrio cholerae*; S.t. *Salmonella typhimurium*. 
Figure 4.6: **Primary double electron-electron resonance (DEER) traces related to the data presented in Fig. 4.1.** Primary Q-band DEER data $V(t)/V(0)$ (black and red lines) with the corresponding 3D background fits (gray lines) obtained with DeerAnalysis2013 are shown for the four pairs in the extracellular (A) and intracellular region of the exporter (B–D). All spin-labeled samples were measured after incubation at room temperature followed by flash freezing in cold liquid pentane in the presence (red) or absence (black) of adenosine 5’-($\beta$, $\gamma$-imido)triphosphate (AMP-PNP) and MgCl$_2$. 
Figure 4.7: Effects of protein reconstitution in liposomes and D-loop mutations on the distance in the intracellular spin-label pair $^{131}_{\text{TM288}}/^{248}_{\text{TM288}}$. Primary DEER data $V(t)/V(0)$ (black and red lines) with the corresponding background fits (gray lines) are shown in left graphs. The background corrected Formfactors $F(t)/F(0)$ (black and red lines) with the corresponding fits (gray lines) obtained with DeerAnalysis2013 are shown in Center graphs. Distance distributions obtained with a Tikhonov regularization parameter of 100 are shown in Right graphs. All spin-labeled pairs were measured after incubation at room temperature followed by flash freezing in cold liquid pentane in the presence (red) or absence (black) of AMP-PNP and MgCl$_2$. (A) Reference data in detergent solution identical to the ones shown in Fig. 4.1 and Fig. 4.6B. (B) DEER data obtained in proteoliposomes. (C) DEER data obtained for the $^{131}_{\text{TM288}}/^{248}_{\text{TM288}}$ pair containing the D501A$^{\text{TM287}}$ mutation. (D) DEER data obtained for the $^{131}_{\text{TM288}}/^{248}_{\text{TM288}}$ pair containing the D523A$^{\text{TM288}}$ mutation. A vertical dashed line guides the eye for the comparison between the different sets of distance distributions.
Figure 4.8: Close-up views highlighting local differences seen between the nucleotide-bound (cyan/pink) and the apo structure (light gray/dark gray). The smaller picture in the second line is given as an overview. For clarity, conserved motifs are only colored in the AMP-PNP structure. AMP-PNP is shown as sticks in A–E, Mg\(^{2+}\) as a green sphere in B–E and hydrogen bonds and Mg\(^{2+}\) coordination as dashed lines. (A) Structural changes in the A-loop (blue) of NBD1. Tyr\(^{341}_{\text{TM287}}\) of the A-loop is highlighted as sticks. (B) Differences of the Walker A motif (red) in NBD1 and residues following Walker A. Walker A Lys\(^{372}_{\text{TM287}}\), Ser\(^{373}_{\text{TM287}}\), Leu\(^{375}_{\text{TM287}}\), and Met\(^{376}_{\text{TM287}}\) are shown as sticks. (C) Structural differences of the Q-loop (yellow) of NBD1. Gln\(^{414}_{\text{TM287}}\) (yellow) is shown in stick representation and is involved in Mg\(^{2+}\) coordination. (D) Deviations observed in the Walker B motif (orange) of NBD1. Asp\(^{494}_{\text{TM287}}\) and Asp\(^{495}_{\text{TM287}}\) of the degenerate site are shown as sticks. (E) Large conformational changes observed in the D-loop (black) of NBD1. Asp\(^{501}_{\text{TM287}}\) is highlighted as sticks. (F) Deviations seen in NBD2 associated with nucleotide binding to NBD1. The ABC signature motif is colored in green (Ser\(^{493}_{\text{TM288}}\)), the D-loop in black (Asp\(^{523}_{\text{TM288}}\)), and the Q-loop in yellow (Gln\(^{436}_{\text{TM288}}\)).
Figure 4.9: ATPase activity measurements of the D-loop mutants of TM287/288. The data are identical to those shown in Fig. 4.3C. ATPase activities were determined at various ATP concentrations at 70 °C for wild-type TM287/288 (A), the consensus site D-loop mutant D501A<sup>TM287</sup> (B), and the degenerate site D-loop mutant D523A<sup>TM288</sup> (C). The error bars shown in the graphs correspond to the SD of three technical replicates. The data were fitted by nonlinear regression by using the Hill equation. \( V_{\text{max}} \), \( K_{\text{M,app}} \), and the Hill coefficient are indicated along with the SEMs obtained from nonlinear regression analysis.
Figure 4.10: **B-factor analysis of the three TM287/288 structures.** (A) B factors are shown for the nucleotide-bound structure, the full-length transporter viewed from the side (Left), and the NBDs from the top (Right). (B) and (C) Same analysis as in (A) for the apo structure (B) and the cross-linked S498C\textsuperscript{TM287}/S520C\textsuperscript{TM288} structure (C). The disulfide bond in C is highlighted as sticks. The D-loop of NBD1 is indicated with a red circle.
Figure 4.11: **Hoechst 33342 transport mediated by LmrCD D-loop mutants.**

Washed and preenergized *L. lactis ΔlmrA ΔlmrCD* cells expressing LmrCD wild type and mutants were supplemented with Hoechst 33342 (indicated by arrow) whose fluorescence signal increases upon binding to the membrane and chromosomal DNA. Active efflux mediated by LmrCD results in a slower increase of fluorescence. The inactive E587Q\textsuperscript{LmrD} mutant served as negative control.
### 4.5.2 Table 4.1. Data collection and refinement statistics

#### Table 4.1: Data collection and refinement statistics

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<tr>
<td>Water</td>
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<td>rms deviations</td>
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<td>Bond lengths, Å</td>
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<td>0.008</td>
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<tr>
<td>Bond angles, ○</td>
<td>0.856</td>
<td>0.862</td>
<td>1.201</td>
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</table>

<sup>*</sup>Conventionally scaled data using XSCALE.

<sup>†</sup>Ellipsoidal truncation and anisotropy scaling was performed by using the University of California, Los Angeles Molecular Biology Institute-Diffraction Anisotropy Server (ref).

<sup>‡</sup>Improved model of the previously deposited PDB ID code 3QF4.

<sup>§</sup>Highest resolution shell is shown in parenthesis.
Table 4.2: Simulated and measured DEER distances. Comparison between mean experimental distances and those simulated on the two crystal structures. All distances were measured by using detergent-solubilized transporters, unless otherwise stated. The mean distances and the SD ($\sigma$) of the distributions are presented as obtained from DeerAnalysis2013 [26] in the range 1.58 nm (experimental data) or from MMM2013.2 in the same range [9] (simulated data). The difference between experimental and simulated mean distances is shown (Delta).

<table>
<thead>
<tr>
<th>Distance (Å)</th>
<th>Experimental (Å)</th>
<th>Simulated (Å)</th>
<th>Delta, Å</th>
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<td>4.00 (0.88)</td>
<td>3.19 (0.88)</td>
<td>0.80</td>
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<td>1.00</td>
</tr>
</tbody>
</table>

In all cases, the mean distance of the labeled mutant in liposomes is shown (Δ).
4.5.3 Table 4.2: Simulated and measured DEER distances

4.5.4 Table 4.3. Spin-labeled rotamers and partition function

Table 4.3: Values were calculated with the software MMM2013.2[9].

<table>
<thead>
<tr>
<th>Spin-labeled site</th>
<th>Apo state (4Q4H)</th>
<th>AMP-PNP-bound state (4Q4A)</th>
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<tr>
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<td>No. of rotamers</td>
<td>Partition function</td>
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</tr>
<tr>
<td>248^{TM288}</td>
<td>62</td>
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<tr>
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<tr>
<td>295^{TM288}</td>
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<td>0.19</td>
</tr>
<tr>
<td>350^{TM287}</td>
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<td>2.39</td>
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<tr>
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<td>466^{TM287}</td>
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<td>1.19</td>
</tr>
<tr>
<td>365^{TM288}</td>
<td>25</td>
<td>0.32</td>
</tr>
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</table>

Author contributions

M.H., M.G.G., E.B. and M.A.S. designed research and provided funding; M.H., L.M.H., J.S. and M.A.S. performed protein expression and purification, functional analysis, crystallization and diffraction experiments; S.B. performed EPR and shock freezing experiments; M.H. and M.A.S. analyzed diffraction data; S.B. and E.B. analyzed EPR data; M.H., S.B. and M.A.S. prepared figures; and M.H., E.B. and M.A.S. wrote the paper.
5 Summary and outlook

Although there had been crystal structures available in the apo-, AMP-PNP-bound- and vanadate-trapped state of the type I ABC transporter MalFGK₂, little was known of how the transporter reacts to the availability of maltose (substrate) and/or its corresponding substrate binding protein MalE. By using distance measurements in the nanometer range performed with EPR, allowing to probe protein domain movements, it could be shown that the transporter binds both liganded and unliganded MalE. Furthermore, its periplasmic site adopts distinct conformations depending on the presence of maltose. Strikingly, MalE alone suffices to induce a closure of the NBDs, which is a requirement for the hydrolyzation of ATP. Additionally, it is shown that dissociation of the substrate binding protein is not a prerequisite for substrate translocation. Existing data proposing a distinct structure of the ADP state are strongly supported by probing EPR, which clearly shows a new conformation of the periplasmic site as well as of the NBDs in the post-hydrolytic state. The EPR study shed light on the conformational plasticity of this ABC importer.

For the homodimeric bacterial exporter MsbA, the 1:1 stoichiometric binding to an *in vitro* selected DARPin was structurally investigated by EPR. It could be shown that the binding of the DARPin doesn’t affect the transporter’s ability to hydrolyze ATP and to switch from an inward to an outward facing conformation, thereby suggesting that the DARPin is not binding close to the C₂ symmetry axis of the molecule, or that it moves during the nucleotide cycle. Using different spin-labeled sites within the transporter as well as in the DARPin, the binding epitope could be mapped to the TMDs, away from the C₂ symmetry axis of the transporter, thus leading to the conclusion that DARPin traps or triggers MsbA in an asymmetric conformation. Moreover, binding of DARPin stimulates the basal ATPase activity of the transporter without impairing the substrate induced conformational transition, suggesting a stimulation of the catalytic cycle when MsbA is in an asymmetric state.

Many heterodimeric ABC transporters carry NBDs where one site deviates from the consensus sequence and is thus called the degenerate site. ATP is mainly hydrolyzed at the consensus site, therefore these transporters operate in an asymmetric manner. Hints for asymmetric behavior of homodimeric ABC transporters had been published before, e.g. crystal structures of the vanadate trapped states of P-glycoprotein and BmrA, which carry two consensus ATP binding sites, show that vanadate trapping occurs only at one of the sites. As a conclusion, a mechanism of alternating catalytic sites was proposed, which implies that the homodimer switches to an asymmetric conformation before ATP is being hydrolyzed. Additionally, the crystal structure of ATP-bound HlyB showed an asymmetric NBD-dimer.

In addition to the findings concerning the MsbA-DARPin complex, another transporter named TM287/288 was investigated by EPR. It is a heterodimeric transporter with one consensus and one degenerate NBD site. As a starting point, the crystal structures in the apo- and AMP-PNP-bound state were known, both showing an inward-facing conformation with only slight structural differences. The nucleotide analog AMP-PNP was found to bind only to one NBD, namely to the degenerate site. By using EPR, these findings were confirmed in solution by comparing measured DEER distances with those simulated with a rotamer library approach (MMM) based on the crystal structures. Differences of the
NBD's structure mainly manifest within the D-loops, which are directly connected to the catalytically important Walker B motif. The D-loop of the degenerate site ties the two ATP binding sites tightly together (as mentioned above even in absence of nucleotides), and as shown by biochemical experiments as well as EPR, substitution of its aspartate by alanine is well tolerated. However, if the aspartate of the D-loop of the consensus site is mutated to alanine, a drastic decrease of the ATPase activity and an increase of the ATP affinity are observed. Based on the crystal structures and the verification of the conformational and physiological behavior in solution by EPR and biochemical experiments, the structural basis for an allosteric cross-talk model between the two ATP binding sites was presented.

Meanwhile, further work on this system provided new insights into its behavior in presence and absence of nucleotides and different nucleotides analogs. Six pairs of different spin labels placed all over the transporter were investigated under ATP-Mg2+, ATP-EDTA, AMP-PNP-Mg, ATPγS-Mg (Adenosine-5′-(γ-thio)-triphosphate) and ADP-Mg conditions. Addition of ATP-Mg in presence of vanadate induced distinct conformational changes in the six spin-labeled pairs in line with MMM simulations on the inward-facing crystal structure and an outward-facing structure based on a homology model of Sav1866. Although the errors from the homology modeling concerning the correct side-chain arrangements add to the errors from the coarse-grained rotamer library approach, the observed changes of distance distributions between the inward and outward facing states corresponds pretty well with the predictions. By incubating the spin-labeled transporter with ATP-Mg alone at 25°C for 20s, a high fraction of the transporter's outward facing state was populated. Since TM287/288 is from a hyperthermophilic bacterium, the ATPase rate at the respective temperature is pretty low and thus mimics the vanadate trapping at 50°C. The conformational transition under both vanadate trapping and hydrolysing conditions could even be observed in one spin pair which was reconstituted into liposomes (131TM288/248TM288).

In agreement with our previous study, AMP-PNP-Mg wasn’t able to switch the transporter into the outward facing state. In contrast, adding of ATPγS-Mg drove a fraction of the transporter into the outward facing state. To further investigate the different behavior of AMP-PNP and ATPγS, the $K_i$-values for AMP-PNP, ATPγS and ADP were determined towards ATP and clearly showed that ATPγS is the strongest inhibitor of ATP-hydrolysis. Furthermore, experiments with ATP in presence of EDTA to chelate remaining Mg2+ showed only a small fraction of transporters switching to the outward facing conformation. Thus, hydrolysing conditions are not strictly required for closing the transporter. Nevertheless, a significant increase even under higher ATP-EDTA-concentrations (up to 14 mM) of the outward facing fraction couldn’t be achieved. To test, whether the switch of the transporter to the outward facing state depends on hydrolysis, the so called E-to-Q mutant (TM287/288_E517QTM288) was investigated after incubation with ATP-Mg for 10min at 25°C. The transporter showed implosable distance distributions to the spin-labeled wild-type transporter. The findings were further confirmed by the results in presence of ATPγS-Mg and AMP-PNP-Mg.

EPR and especially DEER combined with site-directed spin labeling (SDSL) are shown in this work to be suitable techniques for investigating conformational changes of ABC transporters in dependence of nucleotides and/or substrate, and also for answering the questions such as in which state of the nucleotide cycle the substrate or a potential substrate binding protein are bound to the transporter. To monitor substrate binding mechanisms, a spin label should be introduced in the substrate. In case of the MalFGK2/MalE transport complex, spin-labeling of maltose is part of ongoing research to adress several questions. Even in cases where the binding cavity is not yet known, mapping the epitope by different combinations of spin-labeled positions in the transporter with a spin-labeled substrate
may be a successful tool. Furthermore, DEER can be applied to issues concerning possible regulation mechanisms of ABC transporters. One theory involves, e.g., coupling of a regulating enzyme to the NBDs, blocking the transporter’s ability to hydrolyze ATP and thus to transport the substrate through the cell membrane\textsuperscript{[157]}. Ongoing research in the lab is also devoted to the analysis of the energy landscape of TM287/288, testing the effects of different nucleotides, temperature and environment on the conformational transition of this heterodimeric transporter.

In general, DEER measurements have proven to be a powerful tool for probing conformational changes within membrane proteins in recent years, which are usually challenging for crystallization. FRET ( Förster resonance energy transfer) is basically applicable as well for this kind of problems, but requires more complicated labeling strategies, since two different, suitable labels need to be introduced at specific sites. Furthermore, these labels are in general much bigger and thus are potentially more disturbing the native structure than the standard spin label MTSSL, which is mostly used for DEER, being about of the same size like a natural amino acid side chain. On the other hand, FRET is way more sensitive (down to single molecule level) and can be applied at ambient or even physiological temperatures (37 °C) instead of cryogenic temperatures, which may cause other problems, such as disturbing the steady state distribution of conformations. Furthermore, FRET can be applied in cells, so one can measure under real physiological conditions at best. Extensive work on performing in-cell EPR is currently under progress and may lead to a more widespread use of electron spin-labeling techniques for investigating problems in structural biology.
Bibliography


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