Single-molecule studies of the conformational dynamics of ABC proteins

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General introduction

1.1 ABC proteins

ATP-binding cassette (ABC) proteins form one of the largest families of proteins, which are present in all prokaryotic and eukaryotic cells[1, 2]. In humans, 49 genes encode for ABC proteins[3], whereas in the bacterium *Escherichia coli*, 79 genes for ABC proteins are found[4]. ABC proteins hydrolyse adenosine triphosphate (ATP) into adenosine diphosphate (ADP) and inorganic phosphate (Pi) and use the released energy of approximately 20 k_BT in a range of physiological processes[1, 2]. Most ABC proteins, known as ABC transporters, use the energy to translocate various compounds across cellular or organelle membranes. Other ABC proteins, hereinafter collectively referred to as soluble ABC proteins, are not membrane associated, but are part of cellular complexes that use the energy of ATP hydrolysis for DNA mismatch repair, chromosome organisation, double-strand DNA break repair and mRNA translation[5].

Despite their functional diversity, all ABC proteins contain two nucleotide-binding domains (NBDs) that bind and hydrolyse ATP (Figure 1.1). In addition to the NBDs, ABC transporters contain two transmembrane domains (TMDs), which form the translocation pathway for the substrate. The two NBDs and two TMDs together constitute the translocator unit. Soluble ABC proteins do not contain TMDs, but have other domains linked to their NBDs[6-8]. Based on the direction of transport, ABC transporters can be subdivided into importers and exporters. These importers and exporters can be further classified as Type I, II and III importers and Type I and II exporters based on structural and mechanistic distinctions[9, 10]. Type III importers are also known as energy-coupling factor (ECF) transporters[11]. Type I and II importers (the canonical importers) require an additional substrate-binding protein (SBP) to bind and deliver the substrate to the translocator unit. Some ABC transporters have additional domains fused to their NBDs, which are involved in the regulation of transport activity and/or the stabilization of the NBD dimer[12, 13].

1.2 Nucleotide-binding domains

A pair of NBDs is at the core of every ABC protein. The crystal structures of isolated NBD dimers and full-length ABC proteins reveal that all NBDs share common structural and mechanistic features (Figure 1.2A)[14]. Each NBD consists of two subdomains, a RecA-like and an α-helical subdomain, which both contain highly conserved sequence motifs. The RecA-like subdomain contains the Walker A and Walker B sequence motifs. Residues of the Walker A motif interact with the phosphate groups of ATP, whereas the Walker B motif coordinates a Mg^{2+} ion and an H_2O molecule for ATP hydrolysis. The RecA-like subdomain is also present in the Helicase superfamily I, II and III, and the AAA+, V-type and F-type

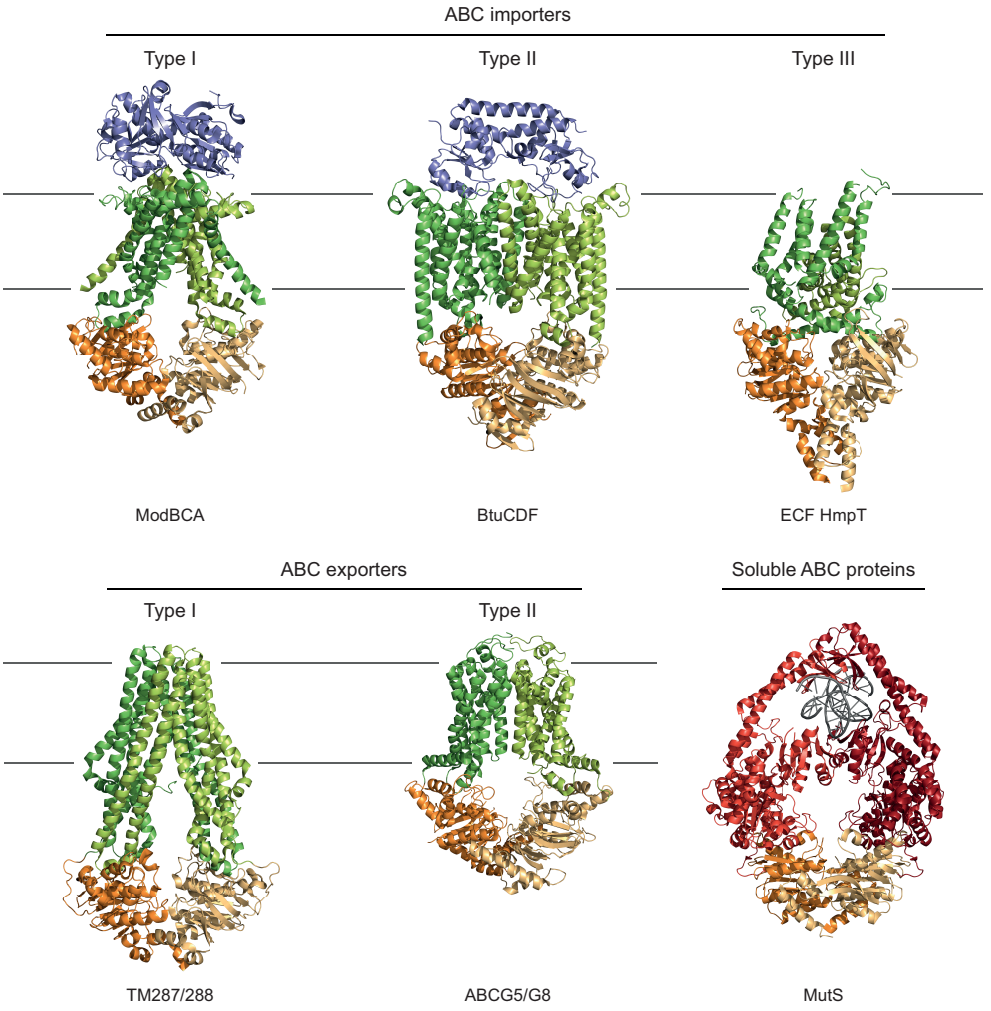

Figure 1.1. The structure and domain organisation of ABC proteins. All ABC proteins contain two NBDs (dark and light orange). ABC transporters contain four domains: two NBDs and two TMDs (dark and light green). ABC transporters can be further subdivided as Type I, II and III importers and Type I and II exporters. Type I and II ABC imports use an SBP (purple) to deliver the substrate to the TMDs. The S component (light green) of Type III importer binds the substrate and the T component (dark green) is associated with the two NBDs. The DNA mismatch repair protein MutS (PBD ID: 1W7A) form *E. coli* contains two NBDs and an additional domain (light and dark red) that binds to DNA (grey). The molybdate transporter ModBCA (PBD ID: 2ONK) from *Archaeoglobus fulgidus*, the *E. coli* vitamin B_{12} transporter BtuCDF (PBD ID: 2QI9) and ECF HmpT (HmpT has also been named PdxU2) transporter (PBD ID: 4HZU) from *Lactobacillus brevis* are Type I, II and III importers, respectively. The heterodimeric exporter TM287/288 (PDB ID: 3QF4) from *Thermotoga maritima* and the human sterol transporter ABCG5/ABCG8 (PBD ID: 5DO7) are Type I and II exporters, respectively.

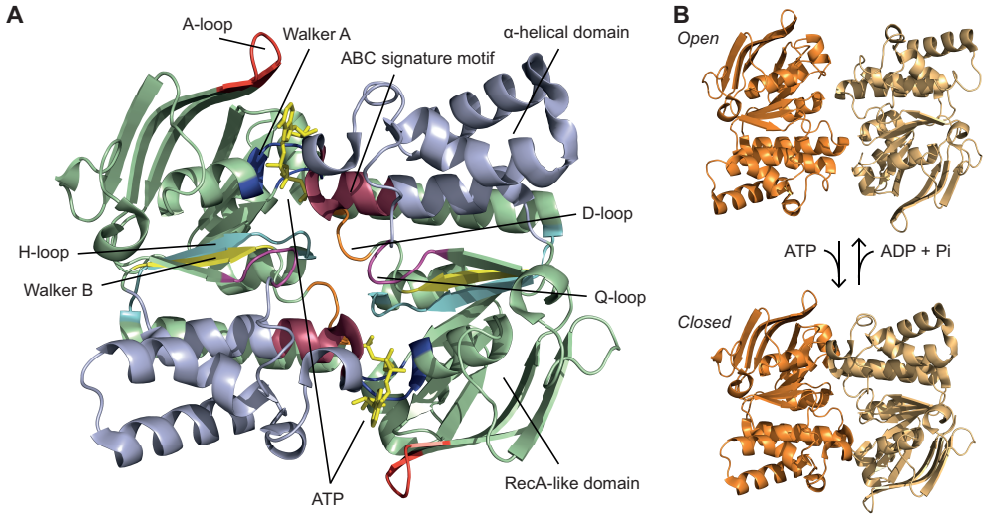

Figure 1.2. The dimer of the nucleotide-binding domains $MalK_2$. (A) X-ray crystal structure of the NBDs $MalK_2$ (PBD ID: 3RLF) with two ATP molecules bound (yellow). The RecA-like and α-helical domain are shown in green and purple, respectively. (B) X-ray crystal structure of nucleotide-free $MalK_2$ (top, PBD ID: 3PV0) and with two ATP molecules (not shown) bound (bottom, PBD ID: 3RLF). NBDs are believed to switch between open and closed conformations by the binding and hydrolysis of ATP. The C-terminal regularity domain and the MalF, MalG and MalE domains are not shown.

ATPase families, whereas the α-helical subdomain is uniquely found in ABC proteins[15]. The α-helical subdomain contains the ABC signature motif, which interacts with the γ-phosphate group of ATP. The Q-loop is located on a loop connecting the RecA-like subdomain to the α-helical subdomain. The Q-loop is crucial to couple the NBD conformational changes to changes in the TMDs[16-18]. Other sequence motifs that are part of the ATP binding site are the A-loop, D-loop and H-motif.

The two NBDs are arranged in a 'head-to-tail' manner, with two ATP binding sites located at the dimer interface (Figure 1.2A). Each ATP binding site is formed by the RecA-like subdomain of one NBD and the α-helical subdomain of the other NBD[6, 19]. The two NBDs associate as homo- or heterodimer, and in the latter case this can lead to an asymmetry between the ATP binding sites[20]. Asymmetry can also originate from other domains of the ABC protein or interacting ligands[21]. Binding of ATP causes conformational changes in the dimer, switching the NBDs from an extended (open) to a more packed (closed) dimer configuration (Figure 1.2B)[22-25]. ATP hydrolysis triggers the reverse reaction, switching the NBDs from the closed to the open conformation. The common mechanistic feature of every ABC protein is that the opening and closing of the NBDs are used to drive conformational changes in the other domains of the ABC protein. This process is termed the 'power stroke' of the NBDs and converts the energy of ATP hydrolysis into mechanical work.

1.3 ABC Exporters

1.3.1 Structure and function of ABC exporters

ABC exporters are located in the plasma membrane and transport substrates from the cytoplasm into the extracellular or periplasmic space. ABC exporters are also found in organelles of eukaryotes and transport substrates from the cytoplasm into the organelle. In case of chloroplast and mitochondria, substrates are transported from the stroma or matrix space into the intermembrane space[26]. Substrates transported by ABC exporters include peptides, proteins, lipids, sterols and polysaccharides[1]. Some ABC exporters transport antibiotics or (anticancer) drugs out of the cell[27, 28].

Every ABC exporter consists of two NBDs and two TMDs. An NBD and TMD are fused as a single polypeptide chain that assembles into a homodimeric (e.g., Sav1866[29]) or heterodimeric (e.g., TM287/288[30]) complex. All four domains are encoded on a single polypeptide chain in some eukaryotic ABC exporters (e.g., the human exporter P-glycoprotein[31]). ABC exporters can be subdivided as Type I and II based on structural features of the TMDs. Only little structural information is available for Type II exporters[32], compared to the wealth of structural[17, 29-31, 33] and spectroscopic[34-36] data that is available for Type I exporters. It is seen that in all ABC exporters, the TMDs consist of 6 transmembrane (TM) helices, resulting in a total of 12 TM helices per exporter. However, the TM helices of Type I exporters are longer than in Type II exporters, and, in contrast to TM helices of Type II exporters, extend further into the solution (Figure 1.1).

1.3.2 Transport mechanism of ABC exporters

X-ray crystallography[17, 29-31, 33, 37], cryo-electron microscopy (cryo-EM)[38], electron paramagnetic resonance (EPR) spectroscopy[35, 36] and recent single-molecule Förster resonance energy transfer (smFRET)[34] data show that ABC exporters adopt different conformations. The TMD interior is exposed to the cytoplasm in the inward-facing (IF) conformation and is open to the extracellular space in the outward-facing (OF) conformation. In the OF conformation, the NBDs are closed and contain two molecules of ATP or ATP analogues. In the IF conformation, the NBDs are open and contain ADP or are nucleotide free. Some exceptions are the crystal structures of the IF conformations of TM287/288[30] and ABCB10[39] that are formed with certain ATP analogous and the OF conformations of Sav1866 and PgKl that are formed with ADP[29, 40].

A popular model for the transport mechanism of ABC exporters is the ATP-switch model[41] or the alternating access mechanism (Figure 1.3)[42]. In this model, a substrate binds to the IF conformation from the cytoplasm or, in case of highly hydrophobic substrates, from

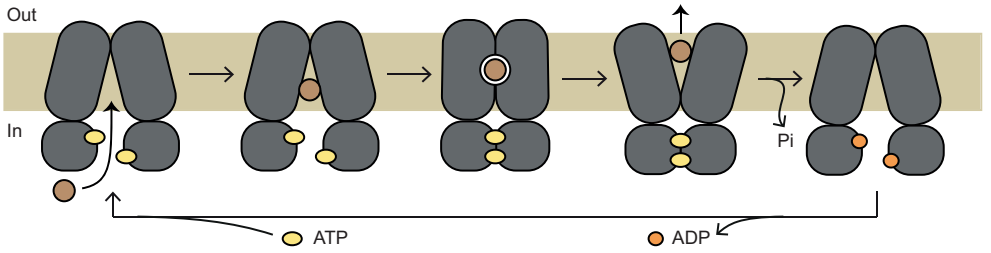

Figure 1.3. Mechanism of transport by ABC exporters. Schematic representation of the proposed transport mechanism of ABC exporters. The mechanism involves switching between inward- and outward-facing conformations, which is regulated by ATP binding and hydrolysis and substrate binding. The membrane is shown in light brown, the substrate in brown, ATP in yellow and ADP in orange. The exporter consists of two NBDs (located on the inside) and two TMDs (located in the membrane) and are all shown in dark grey.

the inner leaflet of the membrane. In the IF conformation, the NBDs are open and free of nucleotide. However, the ATP concentration in the cytoplasm might be sufficiently high so that the ATP free state is only short-lived. Thus, before the substrate binds, the exporter might already contain ATP, but closing of the NBDs is prevented because the substrate is absent[34]. Once the substrate and ATP are bound, the exporter goes via an outward-occluded intermediate state to the OF conformation. In the outward-occluded state, the substrate is trapped within the TMD interior and the NBDs are closed[37, 38]. The substrate is released on the other side of the membrane once the OF conformation is formed. Finally, ATP hydrolysis opens the NBDs, leading to the formation of the IF conformation.

Some heterodimeric ABC exporters contain only a single ATP binding site that can hydrolyse ATP[30, 38, 43]. The site that cannot hydrolyse ATP might always contain an ATP molecule and prevents thereby the complete opening of the NBDs[30]. This could lead to further complexity of the transport mechanism that is not included in the ATP-switch model[35].

1.4 ABC importers

1.4.1 Function of ABC importers

ABC importers are only found in prokaryotes and transport substrates from the extracellular space into the cytoplasm. ABC importers of Gram-negative bacteria are located in the inner membrane and transport substrates from the periplasm into the cytoplasm. Type I ABC importers mediate the uptake of nutrients, such as amino acids, peptides, sugars, ions and oligosaccharides[44]. In addition, some Type I ABC importers, such as OpuA of *Lactococcus lactis*[45], control the volume of the cell via the uptake of so-called compatible solutes (e.g., glycine betaine). Type II and III importers are involved in the uptake of metal ions, metal-

siderophore complexes and vitamins[11, 44]. In general, Type II and III importers facilitate the uptake of substrates that are present in low concentrations in the external environment and are needed by the cell in low quantities, whereas Type I importers transport substrates that are present in higher concentrations and are needed in larger amounts. The substrate availability in the environment is reflected by the dissociation constants (K_D) for substrate binding: Type I importers have typical K_D values of 0.1-10 μM, whereas K_D values of 0.1-10 nM are common in Type II and III importers[11, 44].

Prokaryotes use multiple distinct ABC importers for the uptake of many essential nutrients. Most of these importers are specific for only a few chemically similar substrates. For instance, GlnPQ of *L. lactis* transports the related amino acids asparagine and glutamine[46] and $MalFGK_2$ of *E. coli* transports unmodified, linear maltodextrins ranging in length from two to seven glucosyl units[47, 48]. Other ABC importers have a broader transport specificity. This is possible because some SBPs can bind substrates that are chemically less related. For instance, the SBP OppA of *L. lactis* can bind peptides ranging in size from 4 to 35 residues with only minor sequence preference[49]. The transport specificity of some other ABC importers is broadened because different SBPs with non- or partially overlapping substrate specificities can deliver their substrates to the same translocator. For instance, the SBP HisJ is specific for histidine and the SBP LAO binds lysine, arginine and ornithine, with both SBPs donating their substrates to the translocator $HisQMP_2$ of *Salmonella typhimurium*[50].

1.4.2 Transmembrane domains of ABC importers

In ABC importers the NBDs and TMDs are separate proteins that assemble into a tetrameric complex. In this complex, either (i) all proteins are different (e.g., OppBCDF[49]), (ii) the NBDs are identical but the two TMDs are different (e.g., $MalFGK_2$[51]) or (iii) the NBDs are identical and the TMDs are identical (e.g., GlnPQ[52]). Contrary to ABC exporters, the number of TM helices per TMD varies in ABC importers. TMDs of Type I importers consist minimally of 6 TM helices per TMD. In case of the *E. coli* maltose importer, the TMDs MalF and MalG contain 8 and 6 TM helices, respectively, resulting in a total of 14 TM helices per translocator[18]. The TMDs of Type II importers consist of 10 TM helices per TMD and are more densely packed than in Type I importers. In both Type I and II importers the two TMDs together form the translocation pathway for the substrate, which faces towards the cytoplasm in the IF conformation and towards the external environment or periplasm in the OF conformation[13, 16, 18, 51, 53-55].

It remains unknown if all TMDs of Type I and II importers contain a substrate-binding site. In the crystal structures of the Type I importers $MalFGK_2$[56] and $Art(QM)_2$[55] well-defined substrate-binding sites are present. Mutational[57] and homology modelling[55] studies indicate

that some other Type I ABC importers also contain a binding site in their TMDs. In contrast, no specific binding sites have been identified in the crystal structures of the Type II vitamin B_{12} importer BtuCDF[58], suggesting that the SBP BtuF determines the transport specificity and that the TMDs BtuC simply form an inert cavity for vitamin B_{12}.

Type III importers are structurally and mechanistically completely different from other ABC importers[9, 10]. The two TMDs of Type III importers are termed the T and S component. The T component consists minimally of 4 TM helices and interacts with the two NBDs via two long, crossed α-helices (the coupling helices)[59-62]. The complex formed between the T component and the two NBDs is termed the ECF (energy coupling factor) module. The S component consists minimally of 6 TM helices and contains the binding site for the substrate[63, 64].

1.4.3 Substrate-binding proteins

Type I and II ABC importers require an SBP to bind and deliver the substrate to the translocator. In Gram-negative bacteria, SBPs freely diffuse in the periplasm, whereas in prokaryotes that lack a periplasm, they are attached to the membrane via either a lipid or protein anchor or are directly linked to the TMDs (Figure 1.4)[65]. In the latter case, importers have been identified with a minimum of one and a maximum of six SBPs attached to the translocator (Figure 1.4)[65, 66]. For instance, the Type I importer GlnPQ from *L. lactis* has two SBPs (called SBD1 and SBD2) linked to each TMD, leading to a total of four SBPs per translocator (Figure 1.4D)[52]. SBPs that are directly linked to the TMDs are sometimes called substrate-binding domains (SBDs)[67].

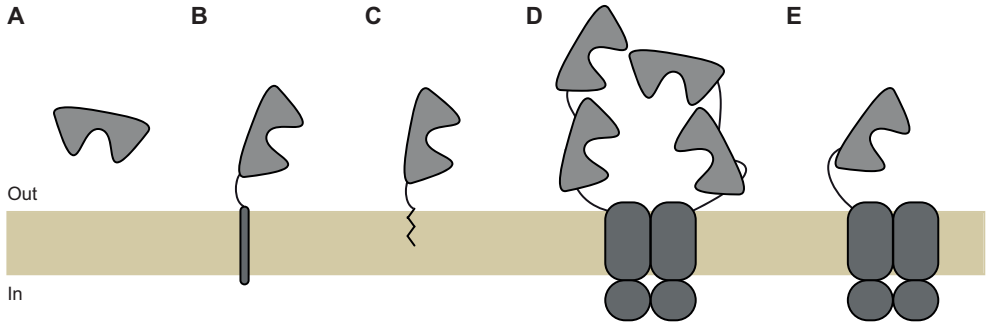

Figure 1.4. Schematic representation of the domain organisation of SBPs. Type I and II ABC importers require an extra-cytoplasmic SBP for function. SBPs can be free proteins that are located in the periplasm (A). In prokaryotes that lack a periplasm the SBPs are attached to the outer membrane via a protein (B) or lipid anchor (C) or are directly linked to the TMDs (D-E). In the latter case, ABC importers with a total of one to six SBPs fused have been identified[68]. A homodimeric and a heterodimeric transporter complex with four (e.g., GlnPQ of *L. lactis*) (D) and one (e.g., $MalEGK_2E$ of *Dellovibrio bacteriovorus*) (E) SBP(s), respectively, are depicted.

SBPs are not only associated with Type I and II ABC importers, but are also part of other protein complexes. For example, tripartite ATP-independent periplasmic (TRAP) transporters, tripartite tricarboxylate transporters (TTT) and G-protein coupled receptors (GPCRs) employ SBPs[68, 69]. Moreover, some SBPs are part of more than one protein system. For instance, the *E. coli* SBP MalE is the receptor domain of both the ABC importer $MalFGK_2$ and the chemotactic signal transducer Tar[70]. SBPs are not only associated with membrane proteins, but are also involved in transcription regulation in bacteria. For example, LysR-type transcriptional regulators (LTTRs) use SBPs[71]. SBPs of the LTTR family have a DNA-binding domain fused to its N-terminus[71].

To date, around 400 crystal structures of SBPs are solved, started with the firstly solved structure in 1974 of arabinose-binding protein[72, 73]. This tremendous amount of structural information revealed that all SBPs have a common structural fold[44]. SBPs consist of two structurally conserved subdomains, both having a so-called α/β fold, i.e., an internal β-sheet surrounded by α-helices. The two subdomains are connected via a hinge region[44]. SBPs can be classified into six structural clusters (cluster A to F), wherein the hinge region is the most defining feature of each cluster[44, 72]. SBPs of Type II importers are solely found in cluster A and contain a single rigid α-helix as hinge. The hinges of Type I SBPs are structurally more diverse, containing multiple connections of β-strands and/or α-helices, and are found in four of the six structural clusters (cluster B, C, D and F). Cluster E contains only SBPs that are part of TRAP transporters.

In the crystal structures of the SBPs, the two subdomains are separated (open conformation) in the absence of substrate and are closer together (closed conformation) when a substrate is present at the interface of the two subdomains (Figure 1.5)[74, 75]. The switching between the open and closed conformation has been called the 'Venus-flytrap' mechanism[76], as the process is reminiscent to a Venus flytrap catching a fly. The crystal structures show that the degree of opening varies between different SBPs, ranging from openings angles of

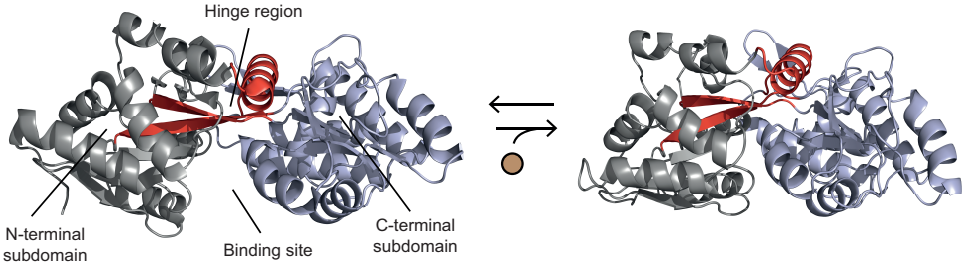

Figure 1.5. Open and closed conformations of the SBP MalE. X-ray crystal structure of *E. coli* MalE without (left; PDB ID: 1OMP) and with (right; PDB ID: 1ANF) maltose bound. The maltose molecule is not shown for clarity. The two subdomains are shown in grey and purple and the hinge in red.

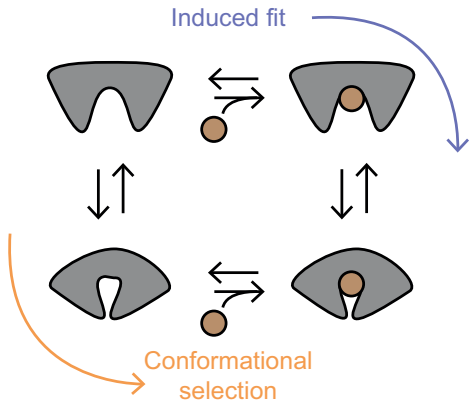

Figure 1.6. The induced-fit and conformational selection mechanism. Two basic mechanisms that connect the open and closed conformations of the SBP with the substrate-free and substrate-bound states are the induced-fit and the conformational selection mechanism. In the induced-fit mechanism, the substrate binds to the open conformation and triggers subsequently closing of the SBP. In the conformational selection mechanism, the substrate is directly bound by the intrinsic closed conformation of the SBP.

almost 60° in the SBP LivJ[77], to only a few degrees for many SBPs that belong to Type II importers[78]. Crystal structures of the same SBP, but with different substrates bound, generally report the same degree of closing[79-82]. For instance, the crystal structures of MalE with maltose, maltotriose or maltotetraose are virtually identical[82]. An exception is the substrate β-cyclodextrin, which induces only a minor conformational change in MalE[83, 84]. Despite the fact that β-cyclodextrin is bound by MalE, it cannot be transported by the maltose importer[85]. This suggests that only substrates that induce closing of the SBP are transported.

There are also indications that SBPs undergo conformational changes in the absence of substrate[67, 86-88], thereby questioning the precise mechanism of substrate binding by the SBP. Two basic mechanisms that describe how substrate binding is coupled to SBP conformational changes are the induced-fit[89] and conformational selection mechanism[90] (Figure 1.6). In the induced-fit mechanism, the open conformation binds the substrate and subsequently triggers closing of the SBP. In the conformational selection mechanism, the apo SBP already exists in an equilibrium between open and closed conformations. Contrary to the induced-fit mechanism, in the conformational selection mechanism, the substrate binds directly to the intrinsic closed conformation.

1.4.4 Transport mechanism of Type I importers

The model for the transport mechanism of Type I importers is largely based on the crystal structures[91] and the spectroscopic[22, 92] and functional[47, 93] data of the *E. coli* maltose importer (Figure 1.7A). The model starts from the IF conformation, with the NBDs open and free of

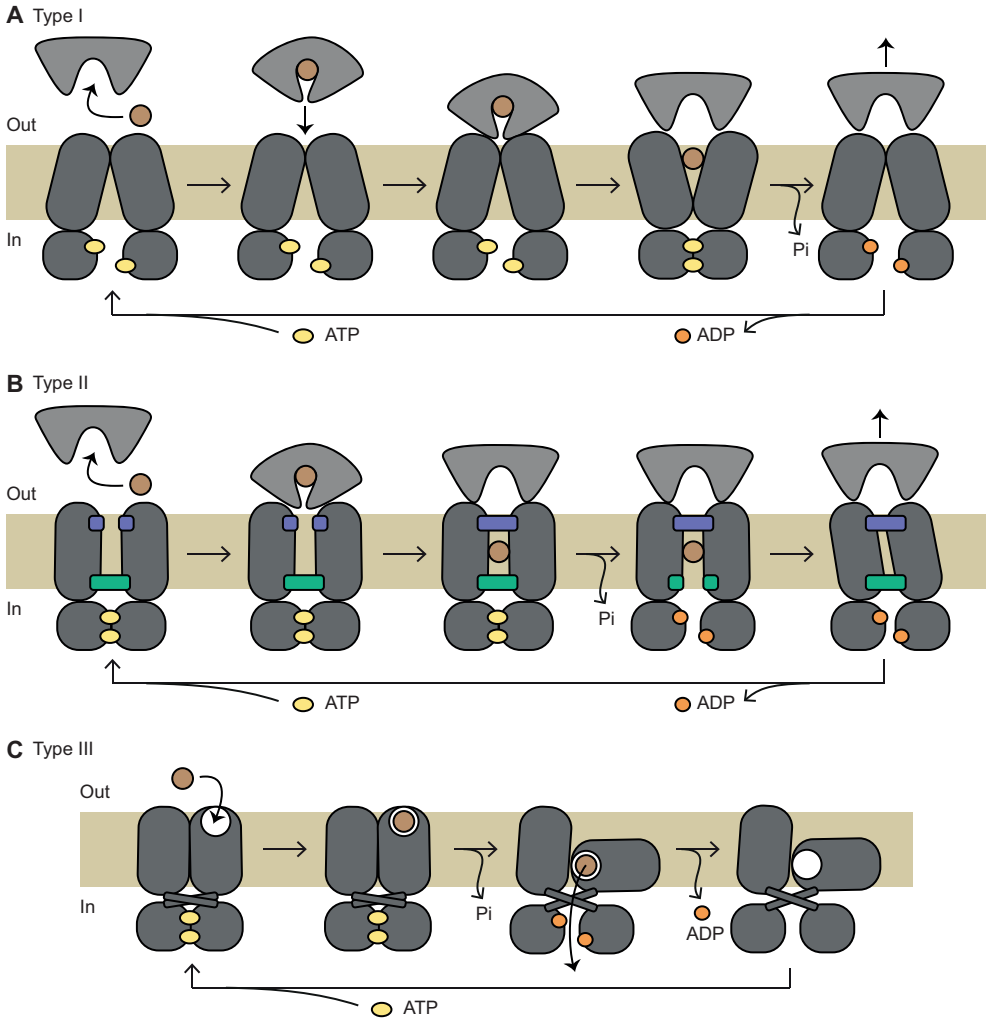

Figure 1.7. Mechanistic diversity of ABC importers. Type I (A), II (B) and III (C) ABC importers use different mechanisms to transport substrates across the membrane. The common features and differences are highlighted in the schematic and are disused in more detail in section 1.4. The membrane is shown in light brown, the substrate in dark brown, ATP in yellow and ADP is shown in orange. The translocator consists of two NBDs and two TMDs, which are shown in dark grey and the SBP is shown in light grey. Both Type I and II importers use an SBP, which are here depicted in an open and closed conformation without and with substrate bound, respectively. The periplasmic and cytoplasmic gates of the Type II importer are depicted in purple and green, respectively. In the schematic of the Type III importer, the S component is shown in the horizontal and vertical orientation. The two large, crossed α-helices of the T component are shown and interact with both NBDs.

nucleotide[94]. The high ATP concentration in the cytoplasm ensures that ATP is rapidly bound by the NBDs. Substrate binding by the SBP switches the SBP from the open to the closed conformation[95]. In the step that follows, the substrate-bound SBP docks onto the IF conformation[51]. Next, closing of the NBDs triggers formation of the OF conformation[18]. Simultaneously with the formation of the OF conformation, the SBP opens and releases the substrate into the TMD interior. The majority of the interactions with the substrate are lost when the SBP is in the open conformation[95], so ensuring that the substrate is rapidly released once the docked SBP opens. Moreover, in the *E. coli* maltose importer, a periplasmic loop of the TMD MalF inserts into the binding site of MalE, thereby preventing rebinding of the substrate[18]. Next, ATP hydrolysis opens the NBDs, leading to the formation of the IF conformation. Finally, the SBP dissociates from the translocator, ADP is released and the substrate diffuses into the cytoplasm.

1.4.5 Transport mechanism of Type II importers

The model for the transport mechanism of Type II importers is largely based on the crystal structures[16, 58, 96-98] and the spectroscopic[99, 100] and functional[101] data of the *E. coli* vitamin B_{12} importer BtuCDF (Figure 1.7B). In contrast to the large rigid-body rearrangements of the TMDs of other ABC transporters, the conformational changes in the TMDs of Type II importers are smaller and are more localized. In Type II importers, the conformational changes are centred around the so-called periplasmic and cytoplasmic gates[16, 58, 98], which are located on the periplasmic and cytoplasmic faces of the TMDs, respectively.

The transport model starts from the OF conformation with the periplasmic gate open and the cytoplasmic gate closed[58]. In this state, the NBDs are closed and contain ATP. An SBP captures a substrate and subsequently docks onto the translocator. The docked SBP opens and releases the substrate into the TMD interior. In the following step, the periplasmic gate closes[98], which results in an occluded state with the substrate trapped within the TMD interior. ATP hydrolysis opens the NBDs, leading to the opening of the cytoplasmic gate and the subsequent diffusion of the substrate into the cytoplasm[54]. Next, an asymmetric importer state is formed, which is characteristic for Type II importers[96, 97]. In this state, both the periplasmic and cytoplasmic gates are closed. In the final step, the NBDs bind ATP, the SBP dissociates from the translocator and the OF conformation is formed by opening of the periplasmic gate.

1.4.6 Transport mechanism of Type III importers

The study of the transport mechanism of Type III importers has been complicated by the fact that all crystal structures of Type III importers have been solved in the same conformational

state[59-62]. Moreover, the crystal structures of isolated S components are only solved with substrate bound and no apo structure has been solved to date[63, 64]. Nevertheless, based on the combined functional[102], structural[59-62] and spectroscopic[103] data on various Type III importers, a transport model has been proposed, termed the toppling mechanism (Figure 1.7C)[10, 11].

The model starts from the nucleotide-free state, the state that has been captured by X-ray crystallography[59-62]. In this state, the NBDs are open and the S component lies almost horizontally in the membrane. ATP binding induces closing of the NBDs. This pushes the coupling helices together, resulting in a rotation of the S component to a more vertical orientation. Now the S component can bind a substrate. In the next step, ATP hydrolysis opens the NBDs, leading to the toppling of the S component to the more horizontal orientation. Now the substrate can leave the S component and diffuse into cytoplasm[62, 104, 105]. In this model, transport is mediated by Jardetzky's alternating access model[106]. However, contrary to other ABC transporters, transport in Type III importers does not involve switching between IF and OF conformations, but involves a toppling of the S component to expose the substrate-binding site on alternate sides of the membrane.

1.5 Soluble ABC proteins

1.5.1 Function and domain architecture

ABC proteins are arguably best known for their function in solute transport across membranes. However, some ABC proteins are soluble proteins and serve other functions[5]. For instance, soluble ABC proteins play crucial roles in functions such as DNA mismatch repair[107], double-strand DNA break repair[108] and mRNA translation[7]. In humans, 4 out of the 49 ABC genes encode for soluble ABC proteins and are the only members of ABCE and ABCF subfamilies[3].

In soluble ABC proteins, the two NBDs are fused together (e.g., in ABCE1[109]) or two separate proteins that each contain an NBD subunit assemble together (e.g., in MutS[8]). In addition to the NBDs, soluble ABC proteins require additional domains for function. For instance, the *E. coli* DNA mismatch repair protein MutS (Figure 1.1) contains a DNA-binding domain, a DNA mismatch recognition domain and a third domain that is important for the interaction with the endonuclease MutL[110]. Other examples are the HEAT domain and chromodomain of the yeast specific elongation factor eEF3. The HEAT domain is important for ribosome association and the chromodomain removes the tRNA from the ribosmome[111]. The additional domains of soluble ABC proteins can be fused to the termini of the NBDs or they can be inserted directly within the NBD structure. For instance, the HEAT domain is fused to the N-terminus of eEF3, whereas the chromodomain is inserted

within one of the NBDs[111]. One remarkable example of a domain insertion is the 50 nm long coiled-coil helix that is inserted in both NBDs of the ABC protein Rad50[112].

Soluble ABC proteins and ABC transporters share common mechanistic principles. Both use the binding and hydrolysis of ATP to couple the opening and closing of the NBDs to conformational changes in the other domains[7, 112]. For instance, NBD opening and closing of Rad50 displaces the long coiled-coils to organize the DNA[112], whereas in ABC transporters the opening and closing of the NBDs are coupled to conformational changes in the TMDs.

1.5.2 ABCE1

We take ABCE1 as an example to discuss in more detail how ATP hydrolysis, NBD conformational changes and function are linked in a soluble ABC protein. ABCE1 is a highly conserved protein and is essential in all archaea and eukaryotes[109]. The two NBDs of ABCE1 are linked via two flexible loops. In addition, ABCE1 contains a cysteine-rich N-terminal region, which binds two diamagnetic $[4Fe\text{-}4S]^{2+}$ clusters[109, 113]. This domain is termed the FeS cluster domain. These domains are generally involved in electron transfer processes, however, in ABCE1, the structural rigidity and positive charge of the FeS cluster domain are probably used to efficiently bind and split the ribosome[7]. ABCE1 plays a vital role in ribosome splitting, but has also been implicated in other functions, such as innate immunity, tissue homeostasis, HIV capsid assembly, ribosome biogenesis and translation initiation[114].

Translation of the mRNA sequence into a polypeptide chain occurs at the ribosome[114]. The ribosome consists of a small (30S in archaea and 40S eukaryotes) and a large subunit (50S in archaea and 60S eukaryotes). Translation is terminated when a stop-codon in the mRNA sequence is reached. The release factor e/aRF1 subsequently removes the synthesized polypeptide chain from the ribosome. After polypeptide release, a stalled ribosome complex, which consist of the 70S/80S ribosome (70S in archaea and 80S eukaryotes), mRNA, tRNA and e/aRF1, is formed. ABCE1 splits the stalled ribosome complex[109, 115, 116].

The current model of ribosome splitting is shown in Figure 1.8 and is revised in Chapter 7. Free ABCE1 is in an open conformation, with the two NBDs separated[109]. Binding of ATP and a stalled ribosome complex induces formation of an intermediate conformation of ABCE1. This complex is termed the pre-splitting complex and has been observed with cryo-EM[117]. The NBDs are slightly closer together in the intermediate conformation. Next, full closing of the NBDs displaces the FeS cluster domain, resulting in steric classes between the ribosomal subunits[118, 119]. This leads to the release of the large ribosomal subunit, while the small subunit remains bound to ABCE1[109]. This latter complex is termed the post-splitting complex and has recently been observed with cryo-EM[119]. Finally, ATP hydrolysis opens the NBDs, causing ABCE1 and the small ribosomal subunit to dissociate.

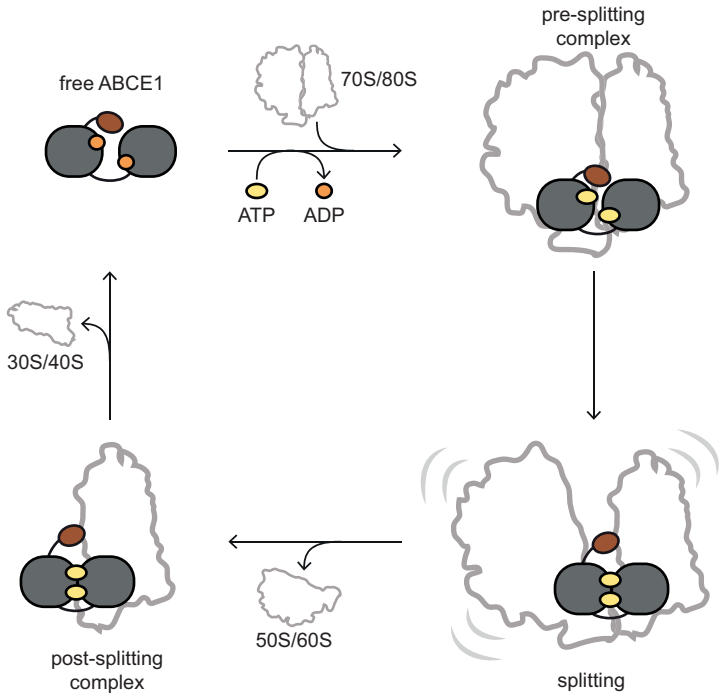

Figure 1.8. Mechanism of ribosome recycling by ABCE1. Schematic representation of the soluble ABC protein ABCE1. The two NBDs of ABCE1 are shown in dark grey. The NBDs are connected via two loops (only one is shown for clarity). The N-terminal FeS cluster domain is shown in brown. ATP and ADP are shown in yellow and orange, respectively. Apo and ADP-bound ABCE1 are in the open conformation. Binding of ATP and a stalled 70S/80S ribosome complex induces formation of an intermediate conformation. This complex is termed the pre-splitting complex. Full closing of ABCE1 displaces the FeS cluster domain and releases the 50S/60S ribosomal subunit. ABCE1 remains bound to the 30S/40S subunit to form the post-splitting complex, which is released upon ATP hydrolysis.

1.6 Outline of the thesis

X-ray crystal structures combined with biochemical data provided valuable insight into the working mechanism of ABC proteins. However, the mechanism is inherently dynamic and is thus difficult to study solely with X-ray crystallography, cryo-EM or any other related method. Methods like NMR and EPR can provide information on the protein dynamics. However, the interpretation of these data is complicated by the fact that the measured signal is averaged over a huge number of molecules. To avoid this problem, single-molecule methods have been developed that measure individual molecules[120, 121]. In this thesis, single-molecule detection in combination with Förster resonance energy transfer (smFRET; Box 1) is used to study the conformational changes of ABC proteins and structurally homologous domains. By using smFRET, it is possible to study the protein conformation, the conformational dynamics and occurrences of rare events as well as any heterogeneity[120-122].

In **Chapter 2**, we investigated the effect of substrates on the conformation of six SBPs, namely MalE, OppA SBD1, SBD2, PsaA and OpuAC. These SBPs belong to both Type I and II ABC importers. By using smFRET, we show that a unique, single closed conformation does not exist for SBPs. Instead, SBPs sample a range of conformations that activate transport. Some non-cognate (i.e., non-transported) substrates induce an SBP conformation that cannot initiate transport. In other cases, failure of transport arises from the slow SBP conformational dynamics that is induced by the non-cognate substrate. The results provide a new view on the relationship between SBP conformational changes, SBP-substrate interactions and transport function

In **Chapter 3**, the results of Chapter 2 were combined with mathematical modelling to investigate the effect of non-cognate substrates on the inhibition of transport. We show that, depending on how the non-cognate substrate influences the SBP conformation and the SBP conformational dynamics, drastic differences can exist in the amount of transport inhibition.

In **Chapter 4**, we studied the conformational changes of two SBPs that are *not* part of ABC importers: SiaP from *Vibrio cholerae* and the regulatory domain (RD) of CynR from *E. coli*. SiaP is part of a TRAP transporter and the transcription factor CynR belongs to the LTTR family. We show that ligand binding switches SiaP from an open to a closed conformation, while no such conformational changes could be detected for CynR. Therefore, transcription activation by CynR is probably based on minor and localized structural changes in the SBP, rather than the rigid-body rearrangements that are common to SBPs of membrane protein complexes.

In **Chapter 5**, a single-molecule fluorescence assay and data analysis procedure was developed to simultaneously observe substrate binding and the conformational changes in FeuA. The SBP FeuA is part of the Type II ABC importer FeuABC, which is involved in iron uptake in *Bacillus subtilis*. The conformational changes of FeuA were determined via FRET, whereas the presence of the substrate was probed by fluorophore quenching. We observed that FeuA uses the induced-fit mechanism, although closing without substrate is also possible. The results provide a detailed kinetic and thermodynamic view on how substrate binding and SBP conformational changes are coupled.

In **Chapter 6**, we determined the effect of length and structure of the linkers, which connect the SBPs SBD1 and SBD2 to each other and to the translocator GlnPQ, on transport. By combining transport assays with mathematical modelling, we reveal that varying the linker length impacts transport in a manner that depends on both the conformational dynamics of

the SBP and the substrate concentration in the external environment. Furthermore, we show that not only linker length but also sequence features of the linkers are important for transport.

In **Chapter 7**, we studied the soluble ABC protein ABCE1 and its function in ribosome recycling. Confocal microscopy was used to determine the conformations of the two ATP binding sites via FRET and the association with the ribosome was determined by measuring the diffusion constant of ABCE1. In contrast to the deterministic models of ABC proteins, we found that both sites are always in a dynamic equilibrium between three conformations: open, intermediate and closed. The two sites behave asymmetrically, allowing, for example, one site to close, while the other remains open. Moreover, the interaction of ABCE1 with ribosomes influences the conformational equilibrium of both sites differently. The results reveal a remarkable conformational plasticity and asymmetric behaviour of the highly conserved NBDs of ABCE1.

In **Chapter 8**, we used classical statistical mechanics to describe the conformational ensemble of a protein. We found that changes in the apo conformational equilibrium biases the holo conformational equilibrium in the same direction. Furthermore, the affinity for the substrate is found to be sensitive to changes in the apo conformational equilibrium. These theoretical findings show that an ABC protein, or any other protein, could modify its apo conformational equilibrium to alter its response to substrate.

In **Chapter 9** and **Chapter 10**, we summarize our findings and provide an outlook about future directions.

Box 1. Energy transfer via Förster resonance energy transfer (FRET) occurs between a donor and acceptor fluorophore (Figure 1.9A)[123]. The donor de-excitation rate via FRET is

$$k_{FRET} = k_D \left(\frac{R_0}{r}\right)^6 \tag{1.1}$$

where k_D is the rate of donor de-excitation in the absence of acceptor, r is the distance between the transition dipoles of the donor and acceptor fluorophore and R_0 is a constant that depends on the interaction between the transition dipoles and is equal to

$$R_0^6 = \frac{9000 \ln 10 \, \kappa^2 Q_D J}{128 \, \pi^5 n^4 N_A} \tag{1.2}$$

where Q_D is the donor fluorescence quantum yield, J is the overlap integral between the donor and acceptor density of states, N_A is Avogadro's number, n is the refractive index of the medium and κ^2 is a factor that depends on the orientation of the transition dipoles[124].

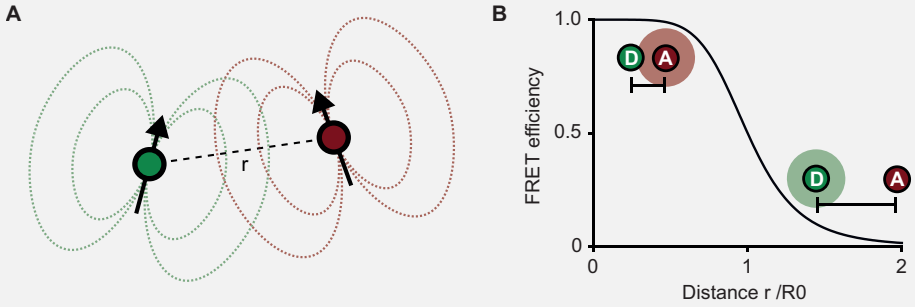

Figure 1.9. FRET. (A) FRET occurs via dipole-dipole resonance interaction. (B) FRET efficiency as function of the distance between a donor and an acceptor fluorophore.

The FRET efficiency is defined as the probability of donor de-excitation via FRET:

$$E = \frac{k_{FRET}}{k_{FRET} + k_D} = \frac{R_0^6}{R_0^6 + r^6} \tag{1.3}$$

The FRET efficiency can be determined experimentally by measuring the donor and acceptor fluorescence intensities during donor excitation[125]. E is most sensitive to distance changes around R_0 (Figure 1.9B). Commonly used fluorophore pairs have R_0 values of 4-6 nm[124]. Thus, the sensitive range of FRET matches with the typical size of proteins, making FRET ideal to use as a 'spectroscopic ruler' to study the conformational changes of proteins[120, 121].

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