Chapter 1

General introduction
Abstract

Cells from all organisms are surrounded by a selectively permeable membrane, which acts as a barrier between the external environment and the intracellular milieu. This membrane consists of a lipid bilayer through which ions and most solutes cannot penetrate. Molecules that cannot freely diffuse through the lipid bilayer are transported into the cell by membrane-embedded proteins. Passive and facilitated diffusion allows solute movement across cell membrane down the (electro)chemical gradient. On the other hand, transport and accumulation of a solute against its (electro)chemical gradient requires input of energy (e.g. ATP). The ATP-Binding Cassette (ABC) transporters are ATP-driven transporters that catalyze the import or export of small and large molecules across the cell or organelle membrane. This chapter will give an overview of what is known about ABC transporters, especially the bacterial ABC systems.
1.1 ABC transporters in prokaryotes

ABC (ATP-Binding Cassette) transporters form one of the largest protein families, which are present in all kingdoms of life and perform various important functions, ranging from nutrient transport, drug extrusion, antibiotic resistance, antigen presentation to protein export. ABC transporters are characterized by the presence of ATP-hydrolyzing domains (also referred to as the nucleotide-binding domains [NBDs]), which provide a power-stroke by converting chemical energy into mechanical energy. The majority of ABC transporters play roles in translocation (import or export) of a wide variety of substrates across biological membranes. A smaller group of ABC transporters are associated with soluble (supra) molecular complexes and developed non-transport functions such as DNA repair, recombination, chromosome condensation and segregation, and translation elongation.

The basic architecture of the ABC transporters comprises two NBDs and two transmembrane domains (TMDs), together they form one translocation pore. The TMDs and NBDs of the ABC exporters can be fused in multiple ways resulting in one-, two-, three- or four-chain transporters, in which the order of the domains may vary. For ABC importers, the TMDs and NBDs are often separate protein subunits, in which TMDs can assemble to form homo- or heterodimers that bind to homodimeric NBDs.

Based on the directionality of transport, ABC transporters are classified into exporters and importers. ABC importers translocate substrates into the cytoplasm, whereas ABC exporters work in the opposite direction. ABC exporters bind their substrates directly within the TMDs, without the need for accessory proteins. It is thought that the binding pocket can be accessed either from the membrane (lipophilic substrates) or from the cytoplasm (hydrophilic substrates), depending on the system. Upon binding and hydrolysis of Mg-ATP, substrates are transported across the membrane and released from the outward-facing conformation of the protein. Prototypical examples of ABC exporters are P-glycoprotein (Pgp), the transporter for antigen presentation (TAP), and the bacterial putative drug efflux system Sav1866. While ABC exporters are found in both prokaryotes and eukaryotes, ABC importers are exclusively present in prokaryotes. In this chapter we focus on ABC importers, which, based on structural and mechanistic features, are further subdivided into Type I, II and III (Figure 1).
In addition to NBDs and TMDs, Type I and Type II importers include additional soluble substrate-binding proteins (SBPs; also referred to as substrate-binding domains [SBDs]) to perform their functions. SBPs capture the transported substrate and deliver it to the translocation pore of the ABC importers. Type III importers, also referred to as Energy-coupling factor (ECF) transporters, capture their substrates via a small integral membrane protein (S-component), which associates with one transmembrane coupling protein (T-component) and two ATPases subunits (termed A and A').

A subset of Type I importers has an additional domain attached to the highly conserved NBDs. This additional domain controls the function of these importers, and is therefore called regulatory domain (RDs). RDs are found in MalFGK$_2$\cite{11}, ModBC\cite{12}, MetNI\cite{13}, and OpuA\cite{14}. Among these systems, MalFGK$_2$ from \textit{E. coli} and OpuA from \textit{L. lactis} are probably the best-studied systems in terms of regulation of transport. In the maltose transporter MalFGK$_2$, the C-terminal domain is involved in carbon catabolite repression, which determines the hierarchy of sugar utilization\cite{15}. In OpuA, an osmoregulatory transporter, two cystathionine-β-synthase (CBS) domains are fused in tandem to the C-termini of the NBDs. Mutational studies have shown that the CBS module controls transport activity and senses the internal ionic strength through reversible binding to an anionic membrane surface\cite{14}. Moreover, structural analyses suggest that the CBS1 domain

Figure 1. Structural diversity of ABC transporters. The basic components of ABC transporters are: two NBDs (orange and light orange) and two TMDs (blue and light blue). In ABC importers, the SBP is present to capture the transported substrate (magenta). The SBP of Type I and Type II is a soluble protein located in the periplasm (Gram-negative) or external space (Gram-positive) of bacteria, whereas in Type III a membrane embedded binding protein (S-component) is used instead.
is largely unstructured and the ionic regulation of OpuA is mediated by CBS2, while CBS1 serves as a linker\textsuperscript{16}.

The three types of importers described above have overlapping substrate specificities; it remains unclear why three different types of importers have evolved, each with a unique mechanism\textsuperscript{7}. In general but not exclusively, Type I importers transport substrates that are required in large amounts such as sugars and amino acids\textsuperscript{2}. On the other hand, Type II and Type III importers are more often specific for compounds needed in small quantities such as metal ions and vitamins\textsuperscript{2,17}.

1.2 Nucleotide-binding domain

NBDs contain sequence motifs that are conserved throughout the ABC transporter families\textsuperscript{2}. There are three general sequence motifs found in all NBDs of ABC transporters\textsuperscript{18}; these motifs are the Walker A, Walker B, and the signature motif. Walker A consists of the sequence GXXGXGKS/T where X can be any amino acid, and Walker B consists of the sequence ΦΦΦΦDE, where Φ is any hydrophobic residue\textsuperscript{19}; together these motifs form a large part of the nucleotide-binding site\textsuperscript{20}. The signature motif (LSGQQ) is unique for ABC proteins and is located upstream of the Walker B motif\textsuperscript{21}. In addition to these three sequences, there are other characteristic motifs that usually contain one highly conserved residue, for example: the Q-loop, H-loop, A-loop and SALD motif (or D-loop)\textsuperscript{7}. The biological significance of these motifs is summarized in Table 1.

Early structural data on isolated NBDs such as HisP\textsuperscript{22}, Rad50cd\textsuperscript{23}, and MalK\textsuperscript{24} revealed the presence of two subdomains (referred to as a large and a small subdomain) [Figure 2]. The large subdomain is similar to the core structure of the Rec-A like motor found in other ATPases, which also possess Walker A and Walker B motifs. The small subdomain is a more diverse α-helical domain that contains the LSGGQ signature motif\textsuperscript{25}. The two subdomains are linked by two flexible loops, one of which contains the Q-loop. Moreover, crystal structures also revealed symmetrical head-to-tail arrangement of NBD dimers, forming two ATP-binding sites, related by two fold (pseudo) symmetry\textsuperscript{26}. The two NBDs in the ABC transporters can adopt different positions relative towards each other – tightly pack against each other (closed conformation) or partly moved away from each other (open conformation).
Chapter 1

Table 1. The conserved motifs in the NBD of ABC transporters.

<table>
<thead>
<tr>
<th>Conserved motif</th>
<th>Description</th>
<th>Function</th>
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<tbody>
<tr>
<td>Walker A (P-loop)</td>
<td>Highly conserved lysine residue in motif GXXGXGK(S/T)</td>
<td>The lysine residue interacts with the phosphate of ATP.</td>
</tr>
<tr>
<td>Walker B</td>
<td>Sequence motif ΦΦΦΦDE in which Φ are hydrophobic residues</td>
<td>Coordinates a magnesium ion via the conserved aspartate. The acidic residue after aspartate, usually glutamate, is the general base that polarizes the attacking water molecule.</td>
</tr>
<tr>
<td>Signature motif (C-loop)</td>
<td>The LSGGQ motif is the defining feature of ABC proteins</td>
<td>Forms the binding site for ATP, together with the Walker A motif.</td>
</tr>
<tr>
<td>A-loop</td>
<td>Contains aromatic residue (usually tyrosine)</td>
<td>Positioning of ATP via stacking with the adenine ring.</td>
</tr>
<tr>
<td>D-loop</td>
<td>Contains SALD motif</td>
<td>Involved in the coordination of ATP in the binding sites of the NBD dimer and plays a role in the directionality of the transport process 27.</td>
</tr>
<tr>
<td>H-loop</td>
<td>Contains histidine residue</td>
<td>Acts as “linchpin” that forms interactions with the catalytic dyad and is involved in the network interactions of ATP, Mg2+ and water molecules 28. May determine the kinetic of certain steps of the catalytic cycle 29.</td>
</tr>
<tr>
<td>Q-loop</td>
<td>A conserved glutamine residue at the N-terminus, located between RecA-like subdomain and the α-helical subdomain</td>
<td>Part of the active site when Mg-ATP is bound. Facilitates interaction between the NBD and the TMDs 30. MD simulations suggest that conformational changes in the Q-loop are coordinated with the coupling of ATP hydrolysis and changes in the TMD 31.</td>
</tr>
</tbody>
</table>
Residues from both monomers form the two ATP-binding sites in the NBD dimer. The P-loop from the first NBD monomer and the signature motif from the second NBD monomer coordinate the binding of the first ATP molecule; and vice versa for the binding of the second ATP molecule 32. During ATP binding, the large and the small subdomains rotate toward each other and close the dimer, and after hydrolysis the two subdomains move away from each other. In this way, the chemical energy of ATP hydrolysis is converted into mechanical energy to power substrate translocation.

Because there are two ATP binding sites per one functional unit of ABC transporter, it is tempting to assume that two molecules of ATP are hydrolyzed during the translocation cycle. This 2:1 stoichiometry is in agreement with the in vitro experiments of the glycine betaine transporter OpuA 33, however different stoichiometries have been reported for other ABC transporters. Technically, the stoichiometry of the transport reaction is difficult to determine. The reason is that ABC transporters that are purified and reconstituted in lipid vesicles often have a poor coupling between ATP hydrolysis and substrate translocation, e.g. in some ABC transporters a basal ATPase activity is detected in the absence of substrate 34. Importantly for OpuA, there is hardly any ATP hydrolysis below threshold levels of

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**Figure 2.** Structure of a liganded-NBD dimer. The structure of a MalK homodimer with two molecules of the ATP analogue AMP-PNP bound is shown (PDB code 3RLF). The TMDs, SBP, and regulatory domain (RD) attached to the NBD have been removed for clarity. Each NBD contains two subdomains: a RecA-like subdomain (blue) and α-helical subdomain (red). Two AMP-PNP molecules in the binding sites are shown as stick model in yellow.
ionic strength and at “physiological” lipid composition (>25 mol % of anionic lipids, 40-50% non-bilayer lipid). At present the 2:1 stoichiometry in OpuA is probably the best estimate of the ATP/substrate ratio for ABC transporters.

1.3 Transmembrane domain

In contrast to the well-conserved NBDs, the TMDs are not well conserved, but they do share a similar topology within the transporter types. The diversity within the TMDs is likely due to the diverse nature of the transported substrates. Typically the TMD monomer has 6-10 transmembrane α-helices, resulting in a total of 12-20 transmembrane segments per functional transporter. The two TMDs could be either identical (homodimer), which is generally found in Type II importers, or different (heterodimer) but structurally related (found in Type I importers). Particularly for Type III importers, the two TMDs are structurally and functionally unrelated. The first TMD (termed as T-component) contains 4-8 transmembrane α-helices. The second TMD is termed the S-component, which binds substrates with high affinity and together with the T-component facilitates their transport. Regardless of the variation in the TMDs structure, they are dedicated to form a translocation pathway, which in Type I and II transporters is alternately accessible from either side of the membrane.

Some ABC transporters have a second substrate-binding site located within the TMDs along the translocation pathway. The Type I maltose transporter MalEFGK2 from *E. coli* has the additional maltose binding site located at one of the TMDs. A crystal structure of MBP-MalFGK2 in the pre-translocation state shows that maltose is trapped in a large solvent-filled cavity located at the interface of MBP, MalF and MalG. A recent crystal structure of the Type I amino acid transporter Art(QN)2 from *Thermoanaerobacter tengcongensis* reveals the presence of a negatively charged pocket located at the interface of the two TMDs (ArtQ). Surprisingly, this pocket contains two polar amino acids when the protein is in the inward-facing conformation. Up to date, no additional binding sites have been found in the translocation pathway of Type II importers. The vitamin B12 transporter BtuCDF structure shows a hydrophobic cavity which can be blocked from both sides of the membrane. It has been proposed that the translocation pathway of the Type II importers act as “teflon-like” (inert) pocket with little or no affinity for the transported substrate.

TMDs are connected to the NBDs via so-called coupling helices, which have been identified in the TMDs of ABC exporters as well as in the Type I and Type II importers. Coupling helices are short α-helices located in cytoplasmic loops.
between transmembrane segments that are embedded in a groove of the NBDs surface. The energy provided by ATP binding and hydrolysis is transformed to mechanical movement of NBDs and subsequently transferred to the TMDs via these coupling helices.

1.4 Substrate-binding proteins

In addition to NBDs and TMDs, a soluble substrate-binding protein (SBP) is also part of ABC importers Type I and II. SBPs are responsible for substrate identification, capture, and eventually substrate release to the translocator of the ABC importer. SBPs were first discovered in the periplasm of E. coli \(^{39}\), and soon after their discovery the first crystal structure of L-arabinose-binding protein was solved \(^{40}\). Up to date, more than 100 crystal structures of SBPs are available. Despite a general lack of sequence similarity, SBPs share a highly conserved structural fold consisting of two globular domains (lobes), connected by a linker or hinge region. Both domains are built by a common \(\alpha/\beta\) fold with internal core \(\beta\)-sheets surrounded by \(\alpha\)-helices. In the absence of substrate the two lobes are well separated \(^{8}\) and flexible, rotating around the hinge \(^{41}\). Upon substrate binding the two lobes undergo a major conformational change from open to closed that involves a rotation at the hinge region \(^{42,43}\), subsequently the two lobes move toward each other and engulf the substrate; a mode of substrate capture akin that of a “Venus Flytrap” \(^{8}\).

SBPs are not limited to ABC transporters but also found in other membrane protein complexes. Examples of such proteins are the tripartite ATP-independent periplasmic (TRAP)-transporters, two-component regulatory systems, guanylate cyclase-atrial natriuretic peptide receptors, G-protein coupled receptors (GPCRs), and ligand-gated ion channels \(^{44}\). In addition, SBP domains are part of prokaryotic DNA-binding proteins involved in gene regulation. Despite low sequence similarity, the structural folds of all SBPs are remarkably similar, which has been used to cluster the SBPs based on structural similarity \(^{9}\). Based on pairwise structural alignment of more than 100 SBPs structures, SBPs were classified into six different clusters (A-F), three of which (cluster A, D, and F) were further subdivided based on the substrate specificity (Table 2). Apparently there is little or no correlation between the structural clustering and functional classification, i.e. SBPs with very different substrate specificities are present in a cluster and SBPs of different clusters can contain similar substrates. Furthermore, the SBPs in the individual clusters are not necessarily homologous, as judged from the absence of significant sequence similarity.
Table 2. The SBP classification

<table>
<thead>
<tr>
<th>Cluster</th>
<th>Membrane system</th>
<th>Features</th>
<th>Substrates</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>Type II ABC importer</td>
<td>The two lobes of SBP are connected by a single rigid α-helix hinge</td>
<td>(A.I) Metal ions, (A.II) Siderophores (including vitamin B12)</td>
</tr>
<tr>
<td>B</td>
<td>Type I ABC importer, two-component histidine-sensory complexes, and guanylate cyclase-atrial natriuretic peptide receptors</td>
<td>The hinge that connects the two lobes is formed by three distinct protein segments</td>
<td>Carbohydrates, branch chain amino acids (Leu, Ile, Val), Autoinducer-2</td>
</tr>
<tr>
<td>C</td>
<td>Type I ABC importer</td>
<td>The proteins have an extra domain, which can extend the binding pocket of the SBPs</td>
<td>Di- and oligo-peptides, Arginine, nickel ions and cellubiose</td>
</tr>
<tr>
<td>D</td>
<td>Type I ABC importer</td>
<td>The hinge consists of two relatively short stretches</td>
<td>(D.I) Carbohydrates (D.II) Putrescine, thiamine (D.III) Tetrahedral oxyanion as well as ferric or ferrous iron (D.IV) Iron ions</td>
</tr>
<tr>
<td>E</td>
<td>TRAP transporter</td>
<td>Contains a large single β-strand and a long α-helix which are spans on both of the two lobes</td>
<td>Ectoine, pyroglutamic acid, lactate, 2-keto acids and sialic acid</td>
</tr>
<tr>
<td>F</td>
<td>Type I ABC importer</td>
<td>The hinge consist of two relatively long segments compared to cluster D</td>
<td>(F.I) Trigonal planar anions (e.g. nitrate, bicarbonate) (F.II) Methionine (F.III) Compatible solutes (e.g. glycine betaine) (F.IV) Amino acids</td>
</tr>
</tbody>
</table>
SBPs of Type I importers are present in large excess over the translocator complexes in the membrane, allowing efficient capture of substrates and initiation of the translocation reaction (Heinemann et al., unpublished). On the other hand, the SBPs of Type II importers appear to be stoichiometric with the translocators. The SBP can be either a soluble periplasmic protein or connected to the cell membrane via a protein anchor, a lipid moiety or a membrane-embedded peptide (the latter has only been observed in Archaea), or can be fused to the TMDs resulting in two substrate-binding domains (SBDs) per functional complex e.g. the osmoprotectant uptake A (OpuA) protein from *L. lactis*. In some cases, two or even three SBDs are fused together and linked to the TMDs, resulting in a total of four or six extracytoplasmic substrate-binding sites e.g. the amino acid transporter GlnPQ from *L. lactis*. Transporters with SBDs fused to the TMDs are predominantly found in Gram-positive bacteria but are also present in Gram-negative bacteria however less frequently.

The fusion of (multiple) SBDs near the TMD increases the effective concentration of the substrate-binding sites near the translocator and enhances the rate of substrate delivery (Schuurman-Wolters & Poolman, unpublished). A mutational study on OpuA, with two SBDs present per functional complex, has shown that one SBD is absolutely required for transport, whereas the presence of the second SBD enhances the activity in a cooperative manner. SBDs are connected to TMDs by relatively short (10-20 amino acids) flexible linkers. This unique architecture not only allows the SBDs to probe a small volume around the translocator but also lowers the degrees of freedom for the SBDs to interact with the translocator.

While the affinity of the fused SBDs for the translocator is not known, the dissociation constants might be in the millimolar range or higher. In case of soluble or lipid-anchored SBPs, the affinity of the closed-liganded SBPs for the translocator is ~0.1 mM. The low affinities of SBPs toward TMDs may dictate a high concentration of SBPs in the periplasm; i.e. maltose-binding protein reaches a concentration of ~1 mM. Given that the periplasm is highly crowded and diffusion is slow (diffusion coefficients for proteins of the size of SBPs in the periplasm are even lower than in the cytoplasm; van den Berg et al., unpublished) and synthesis of a large excess of SBPs is costly, it is perhaps surprising that the covalent linking of SBDs is not more widespread in ABC importers.

Most ABC importers are specific for a limited set of substrates, typically structurally-related molecules. A broader substrate specificity is possible if the transporter can interact with multiple SBPs with different specificities or if the SBPs can bind multiple substrates such as the multiple-sugar transporter Msm from *Streptococcus mutans*, the amino acid transporter LivJ and LAO.
Generally SBPs bind substrates with dissociation constants ($K_D$) often in the range of 0.01-100 µM. Some of SBPs have low affinity for their substrates such as OpuBC from *Bacillus subtilis* ($K_D$ for choline = 30 µM)\(^\text{58}\), MolA from *Haemophilus influenzae* [$K_D$ for molybdate and tungstate = 100 µM]\(^\text{59}\), and the SBD1 of GlnPQ from *Streptococcus pneumoniae* with a $K_D$ for glutamine ~ 700 µM (chapter 2). SBPs fused to the TMDs (in this case also referred to as SBDs) such as found in the amino acid transporter GlnPQ, have been shown to have both multiple substrate specificities and affinities (chapter 2). The substrate affinity of SBD is driven by enthalpy and/or entropy changes upon substrate binding. In case of enthalpy driven, the substrate affinity is mostly dictated by the strength and quantity of hydrogen bonding between the substrate and the residues in the active site. The entropy contribution is provided by conformational dynamics of the protein, e.g. the domain reorientation of the two lobes, or and the release/binding of water molecules\(^\text{60}\). NMR studies of MBP have shown a linear correlation between the apparent binding energy of maltose and the rotational angle of the hinge region\(^\text{43}\).

### 1.5 Mechanism of substrate (ligand)-binding

Based on the available crystal structures, SBPs exist in several conformations: open-unliganded (O), closed-unliganded (C), open-ligated (OL) and closed-ligated (CL) forms. These structural snapshots combined with other biophysical techniques have provided an insight into the mechanism of substrate binding by SBPs. In the absence of substrate, SBPs are present in both open and closed conformation with the major population (>95%) in the open conformation (O)\(^\text{61}\). In the presence of substrate the equilibrium is shifted towards the closed-ligated form. Mutational and structural analyses have shown that the closed-ligated form interacts efficiently with the translocator complex, with each lobe of the SBP binding to one of the TMDs\(^\text{2,6}\).

There are two opposing models for substrate binding, namely the conformational selection and the induced fit mechanism [Figure 3]\(^\text{60-62}\). In the induced fit model, the ligand actively reshapes the binding site by interacting with the open-unliganded form of the SBP, which subsequently leads to the formation of the closed-ligated conformation. In contrast, the conformational selection model involves fast dynamic transition from open to semi-closed or closed without the participation of a ligand. Ligand binding further stabilizes the closed state, and therefore shifts the equilibrium to the closed form. Additionally, a third model which combines both mechanisms has been suggested\(^\text{63}\). It has been proposed that
conformational selection is the more dominant mechanism of ligand binding by enzymes and receptors \textsuperscript{64}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3}
\caption{Different mechanisms of substrate binding to the SBP. Different conformations of the SBP are shown: open (O), partially-closed (PC), closed (C), open with ligand bound (OL), and closed with ligand bound (CL). Modified from Gouridis et al \textsuperscript{61}.}
\end{figure}

In case of the induced fit, the closing rate from open to closed increases with the ligand concentration. On the contrary, in the conformational selection model it is the life-time of the closed state that increases with the ligand concentration. It is very difficult to discriminate both mechanisms using ensemble measurements, because the differences in conformations are averaged out. However, discrimination of both mechanisms is possible by employing single-molecule techniques. Single molecule Föster resonance energy transfer (smFRET) studies on the maltose-binding protein (MalE) \textsuperscript{65,66}, and the tandem SBDs of the amino acid transporter GlnPQ \textsuperscript{61} have shown that these SBDs follow the induced fit mechanism to bind their substrates. It was found that apo SBD visits open and closed conformations in the absence of substrate, and in the presence of ligand the SBDs visit the closed-state more frequently. Remarkably the lifetime of the closed-liganded state is similar to that of the closed-unliganded state. On contrary, the life-time of the closed-liganded state shortens when low-affinity ligands are bound. Thus, ligands do not stabilize the closed form of SBPs. Overall, the data suggest that a special form of induced fit and intrinsic closing (in the absence of ligand) is operative in these ABC transporter-associated substrate-binding proteins.
1.6 Transport mechanism of ABC transporters

The structural diversity among ABC transporters suggests differences in the transport mechanism; see \(^3,^7\) for the recent reviews. Type I and Type II are classified based on differences in size and the overall architecture of the core transporters \(^67,^68\). Comparison of Type I and II ABC transporters show that Type I proteins undergo wide rigid body movements, whereas conformational changes in Type II are limited to the core helices and the loop between TM 2 and TM 3 \(^3\). Regardless this difference, both Type I and Type II obey the so-called alternating access mechanism, in which the transporter cycles between outward- and inward-facing conformations, thereby allowing the substrate to be transferred from the external medium to the cytoplasm.

The transport mechanism of Type I is exemplified by the maltose transporter MalE-MalFGK\(_2\) from \textit{E. coli}. The conformations of the maltose transporter have been captured in several states; together with biophysical data allowing deduction of a tentative mechanism of transport [Figure 4A] \(^7,^11,^67,^69,^70\). In the absence of ATP, the transporter is in the resting state in which the two NBDs are well separated. In this state the TMDs are in the inward facing conformation, in which the translocation pathway is open to the cytoplasm and sealed by a hydrophobic gate on the periplasmic side. Docking of the substrate-loaded maltose binding protein (MalE) to the inward-facing tranlocator (MalFG) triggers the movement of the two NBDs (MalK) towards each other. Full closing of the NBDs, requires ATP binding, which is accompanied with conformational changes of the TMDs to the outward facing conformation. Subsequently, the substrate is transferred from the SBP to the TMD. Subsequent ATP hydrolysis and release of inorganic phosphate (Pi) and ADP completes the translocation cycle and resets the system to the resting state (inward-facing conformation). In an alternative model, binding of ATP triggers the outward-facing conformation of MalFG to which unliganded MalE binds with high affinity \(^71\). Binding of maltose to MalE-MalFGK\(_2\) would then initiate substrate translocation. This model however, finds little support in the work on other Type I ABC importers.

In Type II (\textit{e.g.}, the vitamin B12 transporter, BtuF-BtuCD), the transport is initiated by docking of liganded BtuF to the outward-facing conformation of the transporter [Figure 4B] \(^38\). Binding of ATP closes the NBDs as well as the periplasmic and cytoplasmic gates, and therefore traps the substrate in a translocation cavity (“occluded state”). Subsequent ATP hydrolysis opens the cytoplasmic gate and releases the substrate on the \textit{trans} side of the membrane \(^38\). Unlike Type I, Type II ABC importers bind SBPs with high affinity \(^7,^72\). It is not yet understood what is the
mechanism for SBP dissociation from the transporter complex, however it is thought that basal ATP hydrolysis may play important roles.\textsuperscript{34}

![Figure 4](image-url)

**Figure 4.** The transport mechanism of Type I (A), Type II (B) and Type III (C) ABC importers. The two TMD subunits are colored in blue and light blue, and the two NBD subunits are colored in orange and light orange. The SBP is colored in magenta while the substrate is depicted in yellow. TMDs are connected to NBDs by the so-called coupling helix (grey), which transfers the conformational change in the NBD to the translocator domain. ATP and ADP molecules are colored in red and green, respectively. Modified from Beek et al\textsuperscript{7}, Rice et al\textsuperscript{3} and Slotboom\textsuperscript{10}.

The transport mechanism of Type III importers has recently been reviewed\textsuperscript{10,17,73,74}. Unlike the Type I and Type II, Type III transporters do not employ soluble substrate-binding proteins but use a transmembrane domain (S-component) to capture substrates with high affinity (Figure 4C). Crystal structures of the liganded
S-components combined with molecular dynamic simulations revealed that the substrate-binding site is located near the extracellular surface of the proteins, in which the binding site is occluded from the periplasmic side by the L1 loop. In the structures of the complete transporter complexes, the L1 loop has moved away and the binding site is open to the cytoplasm. This data is in agreement with the electron paramagnetic resonance (EPR) studies of ThiT, which show conformational change of L1 upon substrate binding to the S-component. Furthermore, the crystal structure of the complete transporter reveals an unusual orientation of the S-component, which is lying almost parallel in the membrane as if it has toppled over. On the basis of the available crystal structures, it is proposed that the substrate-loaded S-component undergoes a major rigid body movement around an axis in the plane of the membrane, this movement is called the toppling mechanism.

1.7 Outline of this thesis

This thesis focuses on structural and functional characterization of Type I ABC importers, which have SBDs fused to the translocator domain. ABC transporters with SBDs fused to the translocator domain are present in Gram-positive bacteria including pathogens. The first model system studied here is the amino acid transporter GlnPQ, which has two distinct SBDs fused in tandem to each of its TMDs, resulting in a total of four SBDs per functional transporter. The second target is a bacterial osmoregulatory system OpuA, which has a total of two SBDs in the functional complex.

Chapter 2 and Chapter 3 provide structural and functional analyses of the individual SBDs of GlnPQ from both pathogenic and non-pathogenic bacteria. Our data reveal the residues in the binding pocket that determine the affinity of the SBDs for amino acids.

Chapter 4 describes the implication of amino acid import via GlnPQ for the growth of L. lactis. We show the versatility of GlnPQ in the uptake of the essential (glutamate, glutamine) and growth-stimulating (asparagine) amino acids. Importantly, the composition and concentrations of these amino acids need to be designed carefully to obtain the optimal growth.

In Chapter 5, a strategy to develop drugs against pathogenic Gram-positive bacteria is described using structural knowledge of SBDs (chapter 2 and 3). By combining virtual screening methods of ligand docking with a bioactivity assay
(chapter 4), we were able to identify compounds that affect growth when the target organism is made dependent of transport via GlnPQ.

In Chapter 6, the purification, stabilization of the detergent-solubilized state, and crystallization of the glycine betaine transporter OpuA are described. Our preliminary data on OpuA crystallization provide a first step towards the structure determination of this osmoregulatory transporter.
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