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Energy Coupling Factor-Type ABC Transporters for Vitamin Uptake in Prokaryotes

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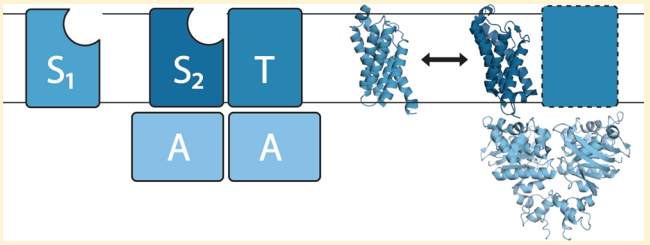
ABSTRACT: Energy coupling factor (ECF) transporters are a subgroup of ATP-binding cassette (ABC) transporters involved in the uptake of vitamins and micronutrients in prokaryotes. In contrast to classical ABC importers, ECF transporters do not make use of water-soluble substrate binding proteins or domains but instead employ integral membrane proteins for substrate binding (named S-components). S-components form active translocation complexes with the ECF module, an assembly of two nucleotide-binding domains (NBDs, or EcfA) and a second transmembrane protein. In some cases, the ECF module is dedicated to a single S-component, but in many cases, the ECF module can interact with several different S-components that are unrelated in sequence and bind diverse substrates. The modular organization with exchangeable S-components on a single ECF module allows the transport of chemically different substrates via a common route. The recent determination of the crystal structures of the S-components that recognize thiamin and riboflavin has provided a first clue about the mechanism of S-component exchange. This review describes recent advances and the current views of the mechanism of transport by ECF transporters.



ATP binding cassette (ABC) transporters form a large superfamily of membrane protein complexes involved in the transport of a wide variety of substrates at the expense of ATP hydrolysis.[1] The architecture of ABC transporters is conserved and consists of a core of four subunits: two transmembrane domains (TMDs) that form a membrane pore through which the substrate is transported and two nucleotide binding domains (NBDs) that provide the energy for substrate translocation by means of ATP hydrolysis (Figure 1). Both the amino acid sequence and tertiary structure of NBDs are well-conserved, and for this reason, NBDs are considered to be a hallmark of the ABC transporter family. The amino acid sequence and structure of the TMDs are not conserved in all ABC transporters and likely have a polyphyletic origin.[2]

Depending on the direction of substrate translocation, ABC transporters are classified as importers or exporters. Importers are abundantly present in prokaryotes and usually rely on an additional substrate binding protein (SBP) for the recognition of their substrates (Figure 1). In Gram-negative bacteria, the SPBs are present in the periplasm as water-soluble proteins, but in Gram-positive bacteria, the SBPs are associated with the membrane through a lipid anchor or fused to the TMDs.[3,4] On the basis of the fold of the TMDs, two types of SBP-dependent importers have been distinguished[5] [types I and II (Figure 1)]. Exporters are found in prokaryotes as well as eukaryotes and do not require SBPs.

ECF transporters have recently been discovered as a new class of ABC importers in prokaryotes.[6] Like all ABC transporters, they contain two identical or homologous NBDs (named EcfA and EcfA′ in ECF transporters), which are associated with the transmembrane domains. The transmembrane assembly consists of two sequence-unrelated membrane proteins: the EcfT subunit and the S-component.[6] Instead of employing SBPs, substrate binding in ECF transporters is exclusively performed by the S-component. S-components are also stable as separate entities (not in complex with the other subunits), which suggests that they may also have a function as solitary proteins.[7−11]

On the basis of the organization of the genes encoding the subunits of ECF transporters, a distinction has been made between ECF transporters of subclass I and subclass II. In the case of subclass I ECF transporters, the genes for the subunits of an ECF module are organized in an operon that also encodes one S-component. It has been suggested that subclass I transporters form a "dedicated" complex: the ECF module and the S-component encoded in the same operon exclusively interact with each other.[6] The ECF transporters of subclass II are characterized by a genetic separation between the operon encoding the ECF module and the genes for one or more S-components. There are usually more S-component genes (up to 12, scattered around the genome) than ECF module operons, and the ECF module is shared by the different S-components.[12] Many organisms encode subclass I as well as subclass II transporters in their genomes. The question of whether in subclass I transporters the ECF modules indeed are dedicated to a single S-component remains to be answered.

The shared use of a single ECF module by multiple S-component subunits in subclass II provides a mechanism for

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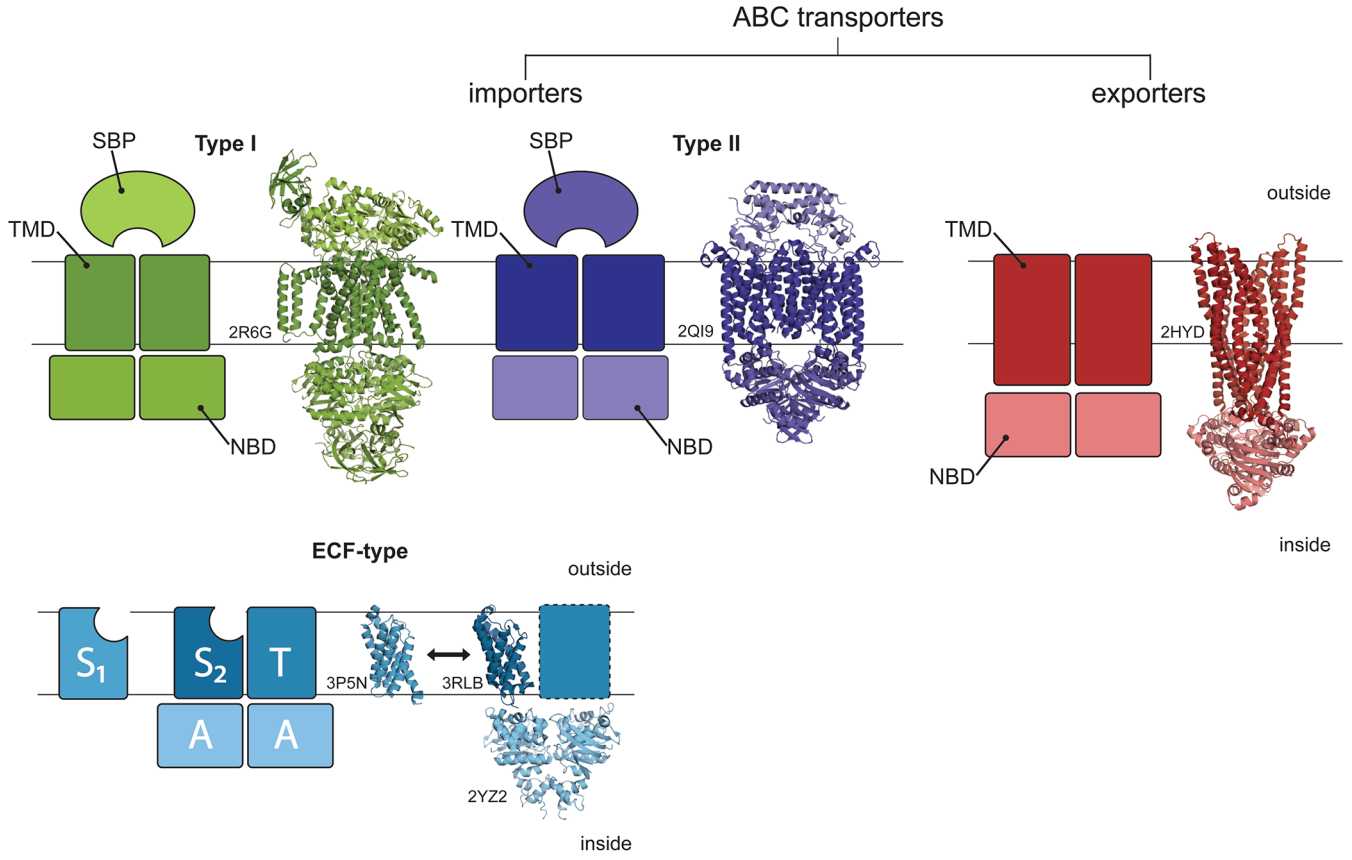

Figure 1. Subunit architecture of the different ABC transporter subclasses. The horizontal lines indicate the approximate positions of the lipid bilayer. Next to the schematic representations, example structures of ABC transporters are depicted. In the top row from left to right are shown maltose transporter $MalEFGK_2$ [Protein Data Bank (PDB) entry 2R6G], vitamin B_{12} transporter BtuCDF (PDB entry 2QI9), and P-glycoprotein (PDB entry 2HDY), respectively. In the bottom row are shown the S-component for riboflavin (RibU) (PDB entry 3P5N), the S-component for thiamin (ThiT) (PDB entry 3RLB), and the EcfA−EcfA′ dimer from *Thermotoga maritima* (PDB entry 2YZ2).

the translocation of chemically diverse substrates by the same ECF module. The molecular basis of the modular S-component−ECF module interactions is unclear because the S-components for different substrates lack significant sequence similarity (average levels of amino acid identity between 10 and 20%). The recent elucidation of the crystal structures of two different S-components (RibU specific for riboflavin[13] and ThiT specific for thiamin[14]) has provided the first atomic-level insight into the architecture and substrate selectivity of this class of proteins. In addition, the structures have provided tantalizing clues about the mechanism of modular interaction with the ECF module. In this review, we discuss how the recent advances in ECF transporter research shed light on the mechanism of the exchangeable interactions between ECF transporter subunits.

S-COMPONENTS ARE MEMBRANE PROTEINS THAT SPECIALIZE IN SUBSTRATE SCAVENGING

The substrate specificity of ECF transporters is determined by the S-component subunit. S-components are small (~20 kDa) membrane proteins that are not related to any other membrane protein of known function. There is a large variation in the chemical nature of the substrates that can be recognized by S-components, which ranges from divalent cations[11,15] to vitamins[8−10,16] and amino acids.[6] On the basis of amino acid sequence analysis, 21 different S-component families have so far been defined, but substrate specificity has been confirmed for only a subset. Table 1 gives an overview of the S-component families and their (predicted) substrate specificity. The pairwise sequence identity between S-components from different families is on average only 10−20%. Despite the differences, S-components share a number of common characteristics. The consensus (predicted) membrane topology consists of six transmembrane helices with the C- and N-termini located on the cytoplasmic side of the membrane. Because of their relatively small size, S-components are thus particularly hydrophobic proteins.

In the absence of the ECF module, S-components are stable both in the membrane and in a detergent solution. The stability of solitary S-components had already been noted long before the molecular identities of the proteins were known: the S-component for folate (FolT) could be purified from wild-type *Lactobacillus casei* cells grown under folate limiting conditions without apparent copurification of the ECF module.[17] It seems reasonable to assume that there is a biological function associated with the presence of solitary S-components. Possibly, the lone S-components are high-affinity substrate scavengers (see below); once their substrates are bound, the ECF module can subsequently recognize the loaded S-component and translocation takes place.

A general feature of ECF transporter S-components is their high binding affinity. Picomolar to nanomolar dissociation constants have been reported for the S-components for biotin,[18] riboflavin,[7] folate[9] and thiamin.[8,9] The occurrence of

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Table 1. Overview of S-components and Their Substrate Specificities

protein	substrate	confirmed	ref
ThiT	thiamin (vitamin B_1), TMP, TPP	yes	8, 9
RibU	riboflavin (vitamin B_2), FMN	yes	7, 16
FolT	folic acid (vitamin B_9), (6*S*)-folinic acid	yes	9
BioY	biotin (vitamin B_7)	yes	10
PanT	pantothenic acid (vitamin B_5)	yes	42
QueT	queuosine precursor	no	
NiaX	niacin (vitamin B_3)	yes	12
PdxU2	pyridoxine-related	no	
YkoE	hydroxymethylpyrimidine (thiamin precursor)	no	
ThiW	thiazole (thiamin precursor)	no	
MtsT	methionine precursor	no	
TrpP	tryptophan	no	
LipT	lipoate	no	
CblT	cobalamine (vitamin B_{12}) precursor	no	
CbrT	cobalamine (vitamin B_{12}) precursor	no	
QrtT	queuosine precursor	no	
PdxU	pyridoxine (vitamin B_6)	no	
MtaT	methylthioadenosine	no	
NikMN	nickel ions	yes	15
CbiMN	cobalt ions	yes	15
HtsT	unknown	–	

S-component genes in the genomes of bacteria has been linked to a lack of biosynthetic genes for ECF substrates.[6,19] Many of the substrates of ECF transporters are compounds needed in small amounts (micronutrients), which often are present in low concentrations in the environment. Excess amounts of solitary S-components would allow bacteria to scavenge these valuable compounds when resources are limited. The expression of most S-components is regulated by the intracellular concentration of their substrates, for instance by riboswitches.[20] As a consequence, the level of expression of S-components is increased when the intracellular concentration of their substrate decreases, thus ensuring an upregulation of those S-components needed to meet the specific demands. The solitary S-components may keep their substrates tightly bound until a complex is formed with the ECF module to transport the substrate.

Two families of S-components appear to have additional features. First, some S-components form bipartite complexes with an extra membrane protein, as has been observed for the Co^{2+}- and Ni^{2+}-specific S-components CbiMN and NikMN, respectively.[11] The exact function of this additional component is unknown, but it is required for substrate transport or binding in the absence of the ECF module. Second, the *bioY* gene (which encodes the biotin-specific S-component BioY) is present in several organisms that lack genes encoding an ECF module.[6] Intriguingly, BioY from *Rhodobacter capsulatus* is the only S-component that has been attributed a transport function in the absence of the ECF module,[10] which may suggest that it is functionally distinct, because all other S-components that have been studied require the ECF module for substrate transport.[12−14] However, it must be noted that the evidence of biotin transport by solitary BioY is based solely on *in vivo* translocation assays. Performing experiments with living cells is highly relevant, but interpretation of the data is often complicated by the complexity of the system. The ultimate proof of whether BioY can transport biotin in the absence of other proteins should come from *in vitro* experiments such as translocation assays with purified proteins reconstituted in liposomes.

STRUCTURE−FUNCTION RELATIONSHIPS OF S-COMPONENTS

Recently, crystal structures of the S-components for riboflavin (RibU) from *Staphylococcus areus*[13] (PDB entry 3P5B) and thiamin (ThiT) from *Lactococcus lactis*[14] (PDB entry 3RLB) have been determined. With these structures available, it is now possible to explain some of the functional characteristics associated with ECF transporters. The level of sequence identity between ThiT and RibU is only 15%, which is too low to establish homology.[21,22] Nevertheless, the two proteins have a similar fold (Figures 1 and 2). In line with the membrane

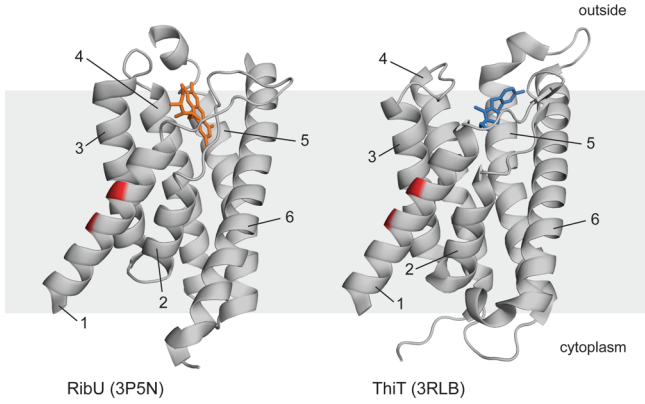

Figure 2. Structures of RibU (left, PDB entry 3P5N) and ThiT (right, PDB entry 3RLB). The numbers indicate transmembrane helices 1−6. The bound substrates are colored orange (riboflavin) and blue (thiamin). The positions of the alanines in the AxxxA motif are colored red. The position of the lipid bilayer is colored gray.

topology predictions, the structures are built of six transmembrane helices, but the lengths and tilts of the helices are variable; helices 5 and 6 are long and cross the membrane at an angle of almost 45°, whereas helix 2 spans only half the membrane and is preceded by a hydrophobic loop (loop L1). The proteins were crystallized with their respective substrates bound. In both RibU and ThiT, a single substrate molecule was found at a site located near the extracellular face of the protein. The 1:1 substrate:protein binding stoichiometry is in line with biochemical experiments.[7,8] The resolution of the RibU structure (3.6 Å) did not allow a detailed analysis of the interactions between the substrate and the protein, but in the ThiT structure (2.0 Å resolution), these interactions can be traced, allowing a detailed description of the high-affinity binding site.[14] The substrate thiamin has a conformation different from that of the catalytic V shape that is found in enzymes with thiamin phosphates as cofactors, which is consistent with the binding and transport function of ThiT (Figure 3). Thiamin is kept in place by numerous hydrogen bonds, as well as ionic and aromatic interactions. The abundance of interactions provides an explanation for the high binding affinity. The residues that interact with thiamin are located almost exclusively in the C-terminal half of the protein (comprising helices 4−6 and the loop between helices 5 and 6). Earlier mutagenesis studies of ThiT had already indicated that residues from helices 5 and 6 and the connecting loop were involved in substrate binding.[8] The only part from the N-

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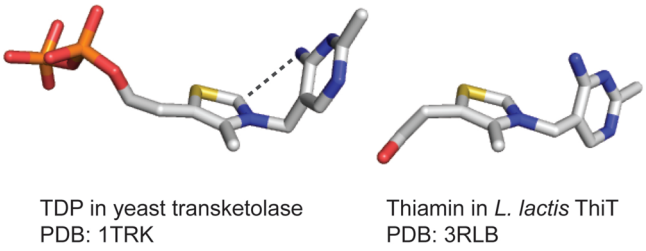

Figure 3. Structures of thiamin pyrophosphate (TPP) bound to yeast transketolase[38] and thiamin bound to *L. lactis* ThiT. TPP adopts the "V-shaped" conformation that stabilizes the molecule during catalysis; the interaction between the two aromatic rings is indicated by the dashed line. In ThiT, thiamin is not bound in the V-shaped conformation.

terminal half of ThiT that interacts with the substrate is loop L1 between helices 1 and 2. On the basis of both the RibU structure and the ThiT structure, a special function has been proposed for the hydrophobic loop L1. Loop L1 forms a lid on the binding site, and a relatively small displacement of this segment would expose the bound substrate to the external environment, allowing it to leave the binding site.

There are currently two speculative models for the mechanism of substrate translocation through ECF transporters. On the basis of the RibU structure, it was proposed that the substrate passes through the interior of the RibU molecule.[13] For riboflavin (M_W = 376 Da) to pass through the proposed channel, very large conformational changes would be required involving a separation of the N- and C-terminal halves of the protein (helices 1−3 and 4−6, respectively), which are tightly packed in the crystal structure. The hypothesis that such a pathway would exist was based on the presence of moderately conserved amino acids that could line the path. In the ThiT structure, we did not find a potential translocation pathway lined with conserved amino acids.[14] In fact, many of the amino acids that were marked as conserved in the RibU structure turned out to be poorly conserved when a larger set of RibU homologues was used for the multiple-sequence alignment. An alternative location for the translocation pathway could be on the interface between the S-component and the EcfT component. Via rearrangement of the L1 loop, such a translocation pathway might become connected with the substrate binding site through a lateral gate. A translocation pathway at the interface of the TMDs is also common to all other ABC transporters.[1]

Without further experimental data, we can only speculate about the location of the translocation pathway in ECF transporters. As noted above, the S-component for biotin (BioY from *R. capsulatus*) has been ascribed a transport function in the absence of the ECF module.[10] If substrate translocation could be directly mediated by S-components, an intramolecular transport pathway through these proteins must exist. However, other S-components that have been characterized do not transport their substrate in the absence of the ECF module.[8,12−14]

There is some uncertainty about the oligomeric state of solitary S-components. Static light scattering coupled to refractive index measurements (SEC−MALLS) with the S-component for thiamin ThiT from *L. lactis* and BioY from *R. capsulatus* has unambiguously demonstrated that the proteins are monomeric in a detergent solution.[8,23] In contrast, *in vivo* fluorescence (FRET lifetime) experiments with BioY from *R. capsulatus* (BioY) have indicated the possible existence of higher-order oligomers.[23] For the S-components RibU and ThiT, their monomeric form has been confirmed by the X-ray structures of these proteins.[13,14] It is not known whether the proposed higher-order oligomeric state of BioY is common to all S-components in a membrane environment or whether it is related to the transport function that is associated with the solitary BioY. It is also possible that the detection of higher-order oligomers *in vivo* might reflect the presence of a small fraction of aggregated protein that is commonly observed upon membrane protein overexpression.

MODULAR INTERACTION PLATFORM OF S-COMPONENTS

In ECF transporters of subclass II, several sequence-unrelated S-components interact with the same ECF module to form an active ECF transporter. Therefore, different S-components must provide a structurally conserved interface that is recognized by the ECF module. Because the S-components are almost entirely membrane-embedded, it is likely that interactions between EcfT and the S-component take place largely in the bilayer. A comparison between the structures of ThiT and RibU revealed that N-terminal halves of the proteins (helices 1−3) were structurally similar, whereas the C-terminal halves (which contain the dissimilar substrate binding sites) had very different structures,[14] making the N-terminal half a probable location for the site where the interaction with the ECF module takes place. A conserved alanine motif (AxxxA) is present on the lipid-exposed surface of helix 1, and mutation of either of the alanines resulted in a complete loss of transport activity caused by an inability of ThiT to form a complex with the ECF module.[14] This alanine motif is present at the same position (transmembrane helix 1) in all eight S-components from *L. lactis* that interact with the same ECF module and is conserved in many orthologs (Figure 2).

Helix−helix interactions in membrane proteins are often mediated by GxxxG motifs,[24−26] consisting of two very conserved glycines, separated by three random amino acids. Other amino acids with a small side chain volume (like alanine or serine) are sometimes tolerated at the position of the glycines as well. The GxxxG motif was first recognized in the transmembrane domain of glycophorin A, where it was found to be required for homodimerization.[27] Since then, several studies of model peptides have provided insight into the biophysical parameters and mechanism of the interaction between GxxxG motifs.[25] Because the glycines are spaced by a linker of three amino acids, their side chains end up on the same face of the transmembrane helix. The small size of the side chains allows two helices to pack together closely, and the interactions are stabilized by van der Waals interactions and weak hydrogen bonds donated by the C_α-H groups.[28]

The proposed interaction surface consisting of the AxxxA motif in helix 1 of ThiT is relatively small and could facilitate the dissociation and reassociation that is required for the exchange of S-components. Such dynamic behavior of S-components has indeed been observed: *in vivo* transport experiments showed competition between different S-components for a shared interaction partner (the ECF module).[14,29] Interestingly, the competition depended on the presence of substrate, which indicates that substrate-bound and substrate-free S-components have different affinities for the ECF module. Probably, these differences in affinity reflect a structural difference between the substrate-bound and substrate-free S-

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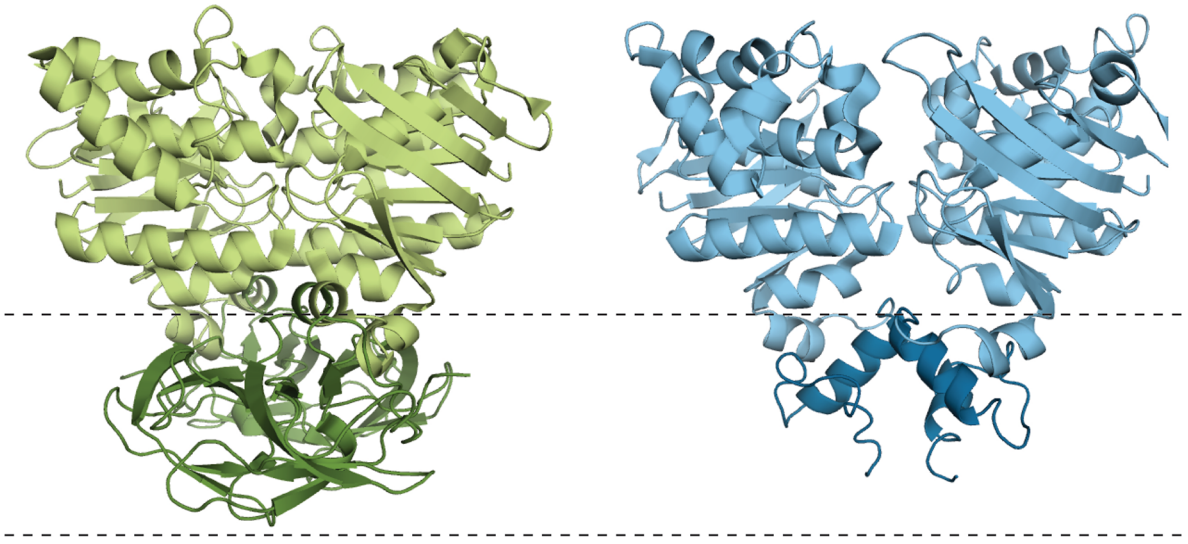

Figure 4. Structures of ABC transporter NBDs. On the left, the NBD dimer from maltose transporter $MalFGK_2$ is depicted; on the right, the ECF transporter NBD (EcfA) dimer from *T. maritima* is depicted. The dashed lines indicate the positions of additional domains. In the MalFGK and other ABC transporters, these C-terminal additional domains often have regulatory functions.[39−41] It is not known whether all EcfA proteins also have such additional domains and whether they are associated with a regulatory function.

component, and it is the substrate-bound conformation that is recognized by the ECF module so that substrate translocation can take place after S-component docking.

The AxxxA motif is located in helix 1, which is adjacent to the mechanistically important L1 loop (see above) that appears to function as a lid on the substrate binding site. Conformational changes in the EcfT subunit (as a result of ATP hydrolysis in NBDs) may lead to repositioning of the L1 loop and open a lateral gate in the S-component through which the substrate can leave and enter into a pathway that is located on the interface between the S-component and EcfT.[14]

■ INTERACTIONS BETWEEN THE TRANSMEMBRANE AND ATPASE COMPONENTS

How could ATP hydrolysis by the NBDs be coupled to substrate translocation? There is a wealth of structural and biochemical data available about the function of the NBDs in ABC transporters.[1,30] NBDs in ABC transporters form a dimeric arrangement with two active sites for ATP binding and hydrolysis that are formed with contributions from both monomers.[31] Large conformational changes take place during the ATP hydrolysis cycle,[32] and these conformational changes are transmitted to the TMDs through a structurally conserved element named the "coupling helix".[5,33] The coupling helix is an α-helical segment with a length of 8−12 amino acids that is located in a cytoplasmic loop of the TMD. There is little sequence conservation between coupling helices from different ABC transporters, making it hard to predict their location in the amino acid sequences of TMDs. The coupling helices of the two TMDs fit into two grooves of the NBD dimer (one in each NBD). During the ATP hydrolysis cycle, the conformational changes in the NBDs are transmitted to the coupling helices and consequently lead to a structural rearrangement in the TMDs. The TMDs thus alternate between outward- and inward-facing conformations, allowing substrate translocation to take place.[34]

The EcfA subunits associated with ECF transporters possess all the mechanistically important sequence motifs of the NBD superfamily. Also, the structure of the EcfA−EcfA′ dimer from *T. maritima* (PDB entry 2YZ2) confirms that EcfA proteins have the same fold as NBDs from other ABC transporters (Figure 4). Therefore, it is likely that the mechanism of ATP hydrolysis is similar to that of other ABC transporters. However, although the groove of the NBDs that binds to the coupling helix appears to be conserved in the EcfA proteins,[14] the cytoplasmic loops of the S-components do not have structural elements that could be coupling helices. If the EcfA subunits of ECF transporters indeed interact with the membrane domains via coupling helices, these structural elements must be present in EcfT. Cross-linking studies have revealed that a cytoplasmic loop in EcfT (BioN) from the dedicated transporter BioMNY interacts with the EcfA (BioM) subunit.[35] It is possible a single EcfT subunit provides both of the coupling helices needed for interaction with the EcfA dimer.

The subunit stoichiometry of ECF transporter complexes is under debate. On the basis of SEC−MALLS of purified ECF module−S-component complexes, a 1:1:1:1 stoichiometry was proposed,[12] but *in vivo* FRET lifetime experiments with fluorescently tagged ECF transporters indicated the presence of multiple S-components in each complex.[23] A possible explanation for the different results could be that ECF transporters in the membrane environment have a dynamic stoichiometry and may have one or more S-components associated with the ECF module. On the other hand, genetic fusions of the genes for ECF transporter subunits are found in several genomes and point to a 1:1:1:1 subunit stoichiometry.

■ DO ALL S-COMPONENTS SHARE A SIMILAR FOLD?

Because the different S-components can interact with the same ECF module, the proteins must have a certain degree of structural similarity, although their overall level of sequence identity does not indicate such relatedness. The sequence-unrelated S-components ThiT and RibU indeed have a very similar overall fold. In the past few years, other examples have been described of membrane proteins that share the same fold, without significant sequence conservation. These observations raise fundamental questions about protein evolution and structure−sequence relationships in membrane proteins.

It has been pointed out that the specific chemical and structural environment of membrane proteins may put a limit on the structural evolution and diversity of membrane proteins and that unrelated proteins might therefore independently evolve to a similar fold.[36] On the other hand, the absence of sequence similarity between proteins does not exclude a common ancestry. Protein sequences can diverge to a level of sequence identity of <10%, a value that is too low for the detection of homology.[21,37] In those cases, structural similarity (i.e., an identical fold) may remain as the only evidence of a shared ancestry. Further structural studies of S-components and the ECF transporter complex should now be performed to test the hypothesis of a general S-component fold.

■ CONCLUDING REMARKS

With the discovery of ECF transporters, a new subclass has been added to the versatile family of ABC transporters. Although united by the utilization of the NBD "motor domain", ECF and ABC transporters display some striking differences. In particular, the mode of substrate binding by means of integral membrane proteins (S-components) sets ECF transporters apart from other ABC transporters. Intriguingly, in many ECF transporters, multiple S-components can make use of the same ECF module. Further structural and functional analysis will be required to elucidate the transport mechanism of ECF transporters.

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Notes

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