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review

ABC transporters: one, two or four extracytoplasmic substrate-binding sites?

Tiemen van der Heide & Bert Poolman+

Department of Biochemistry, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

Received June 3, 2002; revised July 31, 2002; accepted August 1, 2002

Two families of ATP-binding cassette (ABC) transporters in which one or two extracytoplasmic substrate-binding domains are fused to either the N- or C-terminus of the translocator protein have been detected. This suggests that two, or even four, substrate-binding sites may function in the ABC transporter complex. This domain organization in ABC transporters, widely represented among microorganisms, raises new possibilities for how the substrate-binding protein(s) (SBPs) might interact with the translocator. One appealing hypothesis is that multiple substrate-binding sites in proximity to the entry site of the translocation pore enhance the transport capacity. We also discuss the implications of multiple substrate-binding sites in close proximity to the translocator in terms of broadened substrate specificity and possible cooperative interactions between SBPs and the translocator.

Functions of substrate-binding proteins (SBPs)

ABC transporters, found in all pro- and eukaryotic species, use the hydrolysis of ATP to translocate solutes across cellular membranes. The translocator component of the ABC transporters is composed of two multi-transmembrane and two intracellular ATP-binding subunits (Higgins, 1992), with the individual subunits expressed as separate polypeptides or fused to each other in any possible combination. In addition to these ubiquitous components, prokaryotic ABC transporters involved in solute uptake into the cell employ a specific ligand-binding protein to capture the substrate (Figure 1). These SBPs, which are the main determinants of SBP-dependent ABC transporter specificity, were first identified in Gram-negative bacteria, where they reside in the periplasmic space (Neu and Heppel, 1965). Gram-positive bacteria and Archaea, organisms without a periplasm, anchor the proteins to the outer surface of the cell membrane via an N-terminal lipid moiety (Figure 1A) (Gilson *et al.*, 1988; Sutcliffe and Russell, 1995) or, in the case of Archaea, use an N-terminal transmembrane segment to anchor the protein to the cytoplasmic membrane (Albers *et al.*, 1999).

The majority of SBPs involved in ABC transport consist of two domains (C- and N-lobes) that are connected by a flexible hinge (Quiocho and Ledvina, 1996; Lanfermeijer *et al.*, 2000). This enables the SBP to assume an 'open-unliganded' conformation with a high affinity for the substrate and a 'closed-unliganded' state with a low affinity. Upon binding of a substrate molecule, the two lobes of the SBP close around the ligand. The protein in its closed conformation then interacts with the translocator, which is located in the cytoplasmic membrane. Although there is evidence that periplasmic SBPs exist in a monomer–dimer equilibrium, the fact that the monomer has the higher affinity for the ligand (Richarme, 1983) has led to the assumption that a single SBP interacts with a translocator (Liu *et al.*, 1999; Chen *et al.*, 2001). However, the actual evidence for this is scarce. Even the high-resolution structure solution of the vitamin-B_{12} ABC transporter does not resolve the issue, as the complex was crystallized without SBP (Locher *et al.*, 2002).

One of the proposed functions of the liganded SBP is to transmit a signal via the transmembrane subunits to the ATPase subunits on the other side of the membrane, which is thought to increase the transporter's affinity for ATP. The subsequent binding and hydrolysis of ATP leads to the opening of a translocation pore and the concomitant release of the substrate from the SBP (Davidson *et al.*, 1992). An intermediate step in this translocation has been probed using vanadate as an analogue of the γ-phosphate of ATP in the transition state for ATP hydrolysis (Chen *et al.*, 2001). Vanadate inhibition of the *Escherichia coli* maltose ABC transporter ($MalFGK_2$) results in tight binding of

+Corresponding author. Tel: +31 50 3634190; Fax: +31 50 3634165; E-mail: b.poolman@chem.rug.nl

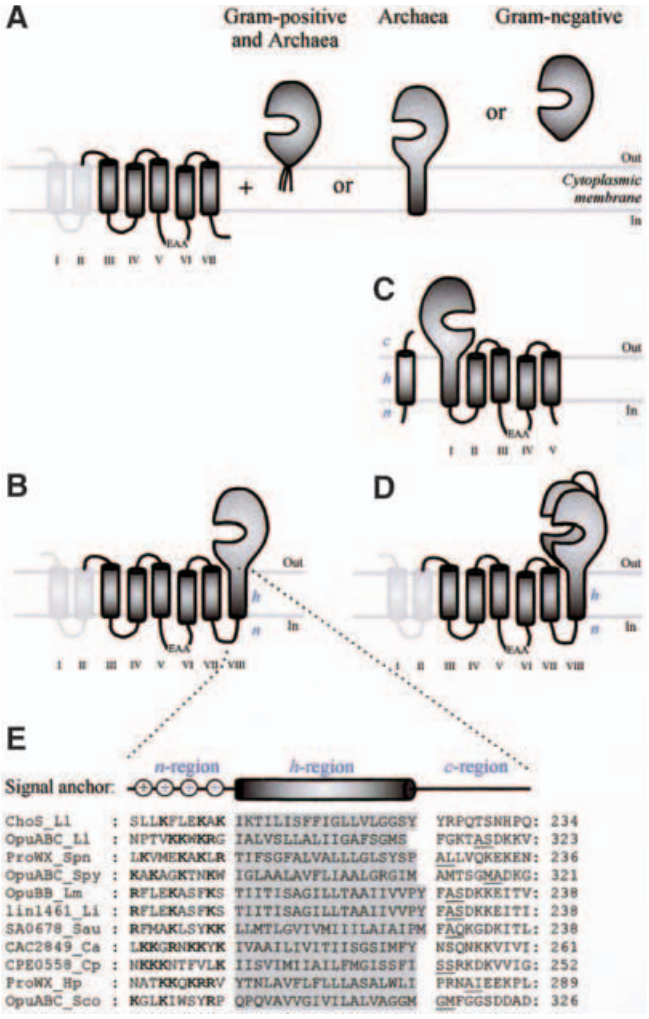

Fig. 1. Schematic representation of the architecture of the substrate-binding and translocator moieties of ABC transporters. Cylinders depict transmembrane segments (TMSs). (**A**) Previously characterized ABC transporter types with independently encoded SBPs. (**B**–**D**) Newly discovered chimeric transporters characteristic of the PAO (C) or OTCN (B and D) subfamilies. Chimeric proteins with the substrate-binding domain(s) at the C-terminus (B and D) have an additional TMS (signal anchor), denoted here as VIII. The signal sequence of the chimera with the substrate-binding domain at the N-terminus could potentially be cleaved off as depicted in (C). Since the first two TMSs of OTCN family members are not always present, they are indicated in light grey (A, B and D). The position of the signature motif (EAA) within the different ABC translocator proteins is also depicted. (**E**) Alignment of the C-terminal signal anchors, which are predicted by the SignalP program (http://www.cbs.dtu.dk/services/SignalP-2.0/); the *n*-, *h*- and *c*-regions characteristic of signal (anchor) sequences are indicated.

ADP, trapping of the maltose-binding protein (MBP) and the transfer of maltose from the binding protein to the translocator. The simultaneous release of ligand and tight binding of the SBP to the translocator opening have been proposed to direct the ligand to the cytoplasm via the translocation pathway; in other words, SBP is thought to act as a plug that blocks the ligand from returning to the external medium (Chen *et al.*, 2001; Davidson, 2002). Furthermore, for the maltose and histidine ABC transporters, there are strong indications that both lobes of the corresponding SBP interact with the translocator (Davidson *et al.*, 1992; Liu *et al.*, 1999). For example, the C- and the N-lobes of MBP interact with the transmembrane proteins MalF and MalG, respectively (Covitz *et al.*, 1994). The triggering of substrate molecule translocation would therefore likely involve (large) rearrangements of the transmembrane α-helical segments of these proteins because of the altered contacts with the two lobes of MBP.

Upon completion of the translocation cycle, the unliganded SBP can dissociate from the translocator, but the release does not necessarily have to occur. For the histidine transporter from *Salmonella typhimurium,* there is evidence that liganded and unliganded SBPs interact with the translocator with equal affinity (Ames *et al.*, 1996), challenging the conventional view that substrate transfer switches the affinity of the SBP for the translocator from high to low. However, even though the apparent affinity constants for binding are similar, the liganded and unliganded binding proteins seem to interact differently with the translocator subunits. One possible explanation for this is that the translocator-associated unliganded SBP might serve to capture free substrate for a subsequent cycle of translocation. This ability could be an advantage as the diffusion of proteins through the periplasm is greatly hindered by high viscosity and crowding in this cell compartment, and potentially also by the presence of other binding partners on the surface of the plasma membrane (Brass *et al.*, 1986).

In Gram-negative bacteria, the SBPs are present in the periplasm in submilli- to millimolar concentrations and are in large excess over the translocator proteins (Ames *et al.*, 1996). If it is assumed that a single translocator-associated SBP is sufficient for transport, then what is the role of the excess SBPs? It has been proposed that they aid in the recruitment of substrate and its transfer to the 'open' unliganded SBP bound to the translocator (Ames *et al.*, 1996). If transfer of ligand from one SBP to another, and ultimately to one associated with the translocator, is more rapid than is diffusion of liganded SBP, the excess of SBP indeed would be beneficial. In addition, some SBPs not only form part of an ABC transporter but also participate in chemoreception, interacting with specific membrane-bound signal transducers to carry out these other functions. For instance, MBP and the Tar signal transducer together constitute the maltose chemoreceptor of *E. coli* (Zhang *et al.*, 1999). Finally, there is some evidence that bacterial SBPs have a role in protein folding and protection from stress in the periplasm (Richarme and Caldas, 1997). These chaperone-like functions are observed with the liganded and unliganded oligopeptide-, maltose- and galactose-binding proteins even at concentrations much lower than in the periplasm. All of these additional functions require the SBP to be present in excess of the membrane components of ABC transporters.

Chimeric substrate-binding/translocator proteins

Until recently, it was thought that microorganisms without a periplasm and outer membrane would use a lipid or protein anchor to prevent the escape of SBPs (Figure 1A). The study of the glycine betaine transporter (OpuA) from *Lactococcus lactis*, however, showed that ABC transporters can have the substrate-binding subunit fused to the C-terminus of the translocator (Obis *et al.*, 1999; Van der Heide and Poolman, 2000). In searching

the non-redundant database (NCBI) with the BLAST sequences program and manually inspecting ABC operons in published genome sequences (http://www.ncbi.nlm.nih.gov/PMGifs/Genomes.micr.html), we have discovered that such chimeric proteins are widespread among microorganisms, with members present in several families of low-GC Gram-positive bacteria (Clostridiaceae, Listeriacea, Streptococcaceae, Staphylococcaceae), high-GC Gram-positive Actinobacteria (*Streptomyces*), and the Gram-negative *Helicobacteraceae* within the ε-Proteobacteria (see Table I of Supplementary data, available at *EMBO reports* Online). OpuA from *L. lactis* is the only well-studied representative of this subset of ABC transporters. The topology of the chimeric substrate-binding/translocator protein is schematically depicted in Figure 1B. Two ATPase subunits (OpuAA) and two chimeric substrate-binding/translocator proteins (OpuABC) together constitute the OpuA complex (Obis *et al.*, 1999; Van der Heide and Poolman, 2000; Van der Heide *et al.*, 2001), which belongs to the OTCN family of the ABC superfamily (Dassa and Bouige, 2001). Furthermore, database searches indicate that the PAO family, another branch of the ABC superfamily, comprising putative glutamine/glutamate transporters, includes proteins in which the SBP is fused to the N-terminus of the translocator (Figure 1C). This architecture of ABC transporters was discovered in several families of low-GC Gram-positive bacteria (Clostridiaceae, Listeriacea, Streptococcaceae) and Cyanobacteria (*Nostoc* and *Synechocystis* sp.). Within the PAO family, the homology is evident from comparisons of the substrate-binding as well as of the translocator domains (Figure 2 and Supplementary data). The same holds true for the translocator domains of the OTCN family members, but the corresponding substrate-binding domains in this case fall into two subfamilies (Figure 2). The subdivision of the OTCN substrate-binding domains coincides with the presence/absence of two additional transmembrane segments (light gray domains in Figure 1B).

By analogy with other ABC transporters (Figure 3A and B), the chimeric systems are composed of two integral membrane subunits and two ATP-binding subunits. This oligomeric structure implies that two substrate-binding sites are present per functional complex (Figure 3C). For OpuA from *L. lactis*, a protein complex that has been purified and characterized, the presence of two substrate-binding domains has a strong experimental basis (Van der Heide and Poolman, 2000). Although this newly discovered domain organization is not necessarily contradictory to the idea that only a single SBP interacts with the translocator of non-chimeric ABC transporters, it highlights the need for further investigation into this stoichiometry.

Even more surprising than the finding of a single substrate-binding domain fused to a translocator subunit are the discoveries that *opuABC* from *Streptomyces coelicolor A3* codes for a protein with two copies of the substrate-binding domain fused to the C-terminus of the translocator (Figure 1D), and that in *L. lactis, Streptococcus pneumoniae, Streptococcus pyogenes* and *Streptococcus agalactiae,* members of the PAO family have two copies of the substrate-binding moiety fused to the N-termini of the corresponding translocator domains (data not shown). If these systems form homodimeric complexes, four binding domains are present per functional unit (Figure 3D). Alternatively, these proteins could form a heterodimeric complex with a transmembrane subunit lacking a substrate-binding domain,

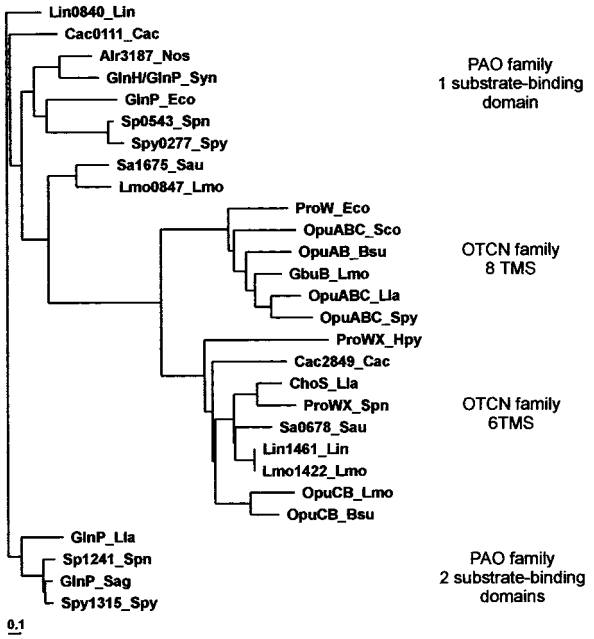

Fig. 2. Phylogenetic tree of the translocator domains of the OTCN and PAO family members. OpuAB_Bsu, OpuCB_Bsu, OpuCB_Lmo, ProW_Eco, GbuB_Lmo (OTCN) and GlnP_Eco (PAO) are translocators without a substrate-binding domain. The others are the newly identified chimeric substrate-binding/translocator proteins of which a detailed description is presented in Table I (Supplementary data). The programs ClustalX (1.8) and Treeview (1.6.6) were used for sequence alignment and generation of the phylogenetic tree, respectively. The horizontal bar indicates the number of amino acid substitutions per site.

and thus contain only two binding domains per complex. Genome searches indicate that each of the genes specifying a chimeric two-substrate-binding/translocator protein is present in an operon that also contains gene(s) for the ATPase subunit(s) but lacks additional translocator components, favoring an architecture with four binding domains.

Evolution of the chimeric proteins

It is possible that the two substrate-binding domains within single polypeptides have evolved through gene duplication. Analysis of the primary sequences of the two-substrate-binding/translocator proteins of the PAO family shows that the substrate-binding domains within the same polypeptide are on average 45% identical. A similar identity score was found when the two substrate-binding domains of the three polypeptides from *S. pneumoniae, S. pyogenes* and *S. agalactiae* were compared with each other, although the translocator domains of these proteins were found to be 84% identical on average. For the one-substrate-binding/translocator proteins, the identity is also highest for the translocator domains. However, when polypeptides with one and two substrate-binding domains from the same organism are compared, as is possible for both *S. pneumoniae* and *S. pyogenes*, the substrate-binding and translocator domains share on average only 25 and 35% identity, respectively. It thus seems that the polypeptides with one and two substrate-binding domains have the same ancestor and most probably transport

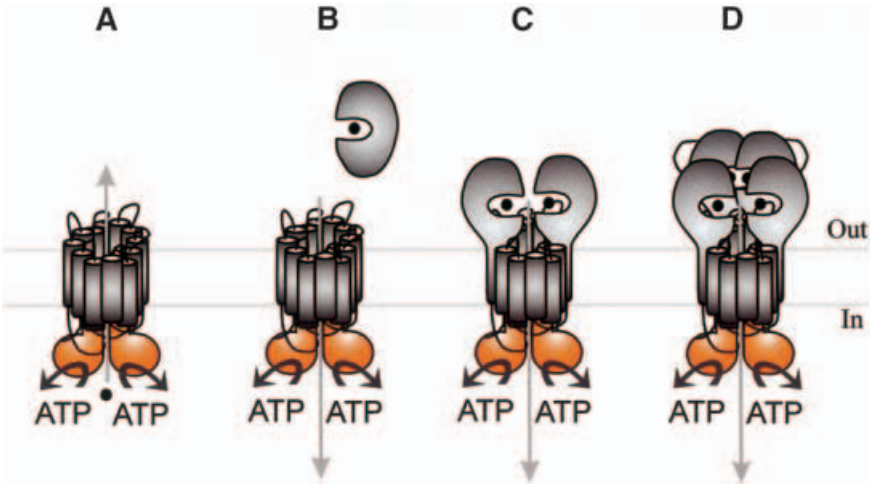

Fig. 3. Schematic representation of the domain organization of ABC transporters. A SBP-independent efflux system is shown in (**A**), and a conventional SBP-dependent uptake system is shown in (**B**). The newly identified chimeric substrate-binding/translocator systems with two and four substrate-binding sites per functional complex are shown in (**C** and **D**), respectively. ATP-binding domains (ABC cassettes) are shown in orange.

similar substrates, but have evolved/adapted differently in order to function with two or four substrate-binding sites.

Signal (anchor) sequences

The SignalP prediction method (Nielsen *et al.*, 1997) indicates that the N- and C-terminal substrate-binding domains are preceded by typical bacterial cleavage signal sequences (Figure 1E). However, a clear distinction between cleaved signal sequences and uncleaved signal anchors is not easily made. The members of the PAO family are expected to anchor the substrate-binding domains irrespective of whether the N-terminal signal sequence is cleaved (at the C-terminus of segment *c*, Figure 1C), because the substrate-binding domain remains anchored and fused to the translocator moiety. Cleavage of these proteins might be advantageous because it would provide the substrate-binding domains with some flexibility rather than leaving them tethered to the membrane from both ends. Given the large structural changes that take place upon substrate-binding/release in SBPs, we speculate that this is important. In contrast, the OTCN family sequences should serve as uncleaved anchors, since cleavage (above segment *h*, Figure 1B and D) would release the ligand-binding domain(s) from the transmembrane portion of the protein. Indeed, OpuABC of the OpuA system from *L. lactis* has been shown not to be cleaved at its signal sequence (Van der Heide and Poolman, 2000).

The presence of a signal (anchor) sequence suggests that the chimera have evolved from the fusion of a gene for a pre-protein to the 3′ (OTCN) or the 5′ (PAO) end of a translocator gene. The sequence identity among the signal anchor sequences (Figure 1E) is low compared with that among the other transmembrane segments (TMSs) within the same family, suggesting that this domain is not an inherent part of the translocon. For the members of the OTCN and PAO families, we speculate that the five related TMSs (dark gray in Figure 1) form the minimal unit for translocation, giving rise to a total of 10 TMSs in the functional translocon. However, some members of the OTCN family will have up to an additional four (two × two) contributing TMSs (light gray in Figure 1B and D).

Biological significance and concluding remarks

Given the general assumption that, mechanistically, a single SBP interacts with the non-chimeric translocators, the presence of two or perhaps four integral substrate-binding sites per chimera complex raises intriguing questions about the functional roles of these proteins. Rather than being redundant, it is possible that the multiple binding domains broaden the specificity of the system (only when substrate-binding domains with different primary sequences are fused in tandem; systems exemplified by Figures 1D and 3D), increase the translocation capacity or influence the kinetics; the latter two options are also possible with two identical substrate-binding domains per functional complex (Figures 1B, 1C and 3C).

A case for a role of multiple binding sites in broadening specificity can be made by analogy with the non-chimeric histidine ABC transporter from *S. typhimurium*, for which it has been shown that the periplasmic binding proteins for histidine (HisJ) and arginine (ArgT) deliver their substrates to one and the same translocator (Higgins and Ames, 1981). Similarly, GlnPQ, the PAO family glutamate transporter from *L. lactis*, has two different substrate-binding domains in tandem. The finding that glutamate transport is competitively inhibited by arginine suggests that the two structurally very different substrates are bound by its distinct substrate-binding domains (Poolman *et al.*, 1987; G. Schuurman-Wolters and B. Poolman, unpublished results).

Two other models could explain the benefit of having two (or more) substrate-binding domains in terms of translocation activity. First, the second binding domain could deliver the substrate to the one directly engaged by the translocator, in which case the domains would have different functions. This would be equivalent to the situation in Gram-negative bacteria where most, if not all, SBP-dependent ABC transporters seem to

have the SBP in excess of the translocator (Ames *et al.*, 1996). Secondly, the binding domains could alternately interact with the translocator. In this view, the second (and/or third and fourth) domain could capture a substrate molecule for a subsequent cycle of translocation while the first binding domain is still docked onto the membrane-embedded translocator complex. Under conditions in which substrate binding is rate limiting (low substrate concentration), the rate of transport would be increased by the presence of additional substrate-binding sites. One could argue that two or four substrate-binding domains per functional complex would only be a small excess compared with the ratio of periplasmic SBPs over translocator complexes in Gram-negative bacteria. However, the chimeric proteins would have the added advantage of keeping the substrate-binding sites in close proximity to the translocator rather than allowing them to disperse throughout the periplasmic compartment. The concentration of substrate-binding sites near the translocator would be approximately 100 mM, which is much higher than the maximal concentration of periplasmic SBPs in the other system. Since the diffusion of low molecular weight substrates through the cell wall of Gram-positive bacteria would be much less restricted than the diffusion of liganded SBPs in Gram-negative bacteria, the substrate-binding/donation capacity certainly would be increased with multiple fused substrate-binding domains.

An alternative function of multiple SBPs could relate to the kinetics of the systems. It is possible that more than one SBP or substrate-binding domain is actually required for delivery of substrate to its translocator. For the maltose transporter from *E. coli*, there is evidence that the transport rate increases sigmoidally with the amount of periplasmic SBP (Manson and Boos, 1985). This observation on apparent cooperativity has a bearing on the findings that SBPs form dimers, but the cooperative behavior of the system has never been supported by biochemical data. It should be possible to address this issue by constructing heterodimers of chimeric proteins, with one functional and one defective substrate-binding domain in the transport complex.

It is interesting to note that chimeric substrate-binding/translocator proteins have been found in only two families of the ABC superfamily, and that these systems are predicted to transport glycine betaine (or related compounds) or glutamate/glutamine to extraordinarily high levels, e.g. glycine betaine can be present at molar levels (Poolman *et al.*, 2002). This requires high rates of transport in a system operating far from thermodynamic equilibrium because of the demands of basic cellular processes that dissipate the concentration gradient (i.e. substrate utilization, substrate leakage and substrate redistribution during cell division) and kinetic regulation of transport. It will indeed be exciting to unravel the secrets of these amazing machines and possibly uncover mechanistic principles of translocation that generally hold for ABC transporters.

Supplementary data

Supplementary data are available at *EMBO reports* Online. Supplementary material is also available at http://www.chem.rug.nl/enzymology/ABC

Acknowledgements

This research was supported by grants from the Netherlands Foundation of Life Sciences (ALW) and the Foundation for Chemcial Sciences (CW), which are subsidized by the Netherlands Organization for Scientific Research (NWO).

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DOI: 10.1093/embo-reports/kvf201