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Sobczak, M; Lolkema, Julius; Sobczak, Iwona

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# Structural and mechanistic diversity of secondary transporters

Iwona Sobczak and Juke S Lolkema

Recent reports on the three-dimensional structure of secondary transporters have dramatically increased our knowledge of the translocation mechanism of ions and solutes. The structures of five transporters at atomic resolution have yielded four different folds and as many different translocation mechanisms. The structure of the glutamate transporter homologue $Glt_{Ph}$ confirmed the role of pore-loop structures as essential parts of the translocation mechanism in one family of secondary transporters. Biochemical evidence for pore-loop structures in several other families suggest that they might be common in secondary transporters, adding to the structural and mechanistic diversity of secondary transporters.

**Addresses**
Department of Microbiology, Groningen Biomolecular and Biotechnology Institute, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands

Corresponding author: Lolkema, Juke S (j.s.lolkema@biol.rug.nl)

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## Introduction

Secondary transporters use the free energy stored in ion and/or solute gradients to drive the transport of a solute across the cytoplasmic or internal membranes of biological cells. Accumulation of the solute at one side of the membrane is achieved by coupling the translocation of the solute to the translocation of one or more ions ($H^+$ or $Na^+$) that move down their own gradients, named the proton motive force and $Na^+$-ion motive force, respectively (co-transport) [1]. Secondary transporters are widely spread throughout all kingdoms of life; they are found in every biological cell and can probably be found for every low-molecular weight compound in nature. Their high abundance is reflected in the great diversity of sequences coding for secondary transporters. The transporter classification system (TC system) developed in the Saier laboratory is based on sequence homology and lists some 84 different gene (super)families coding for secondary transporters (class TC 2.A) [2]. Many of these families are likely to be evolutionary related, and the high number of encoded protein families is likely to represent a much smaller number of structures and an even smaller number of translocation mechanisms. We developed the MemGen classification system that clusters families of membrane proteins into structural classes on the basis of hydropathy profile analysis [3]. Hydropathy profiles are proposed to report a specific fold and, therefore, are able to detect distant relationships between protein families. For example, structural class ST[3] (secondary transporter 3) in the MemGen classification contains secondary transporters from 29 different families [4].

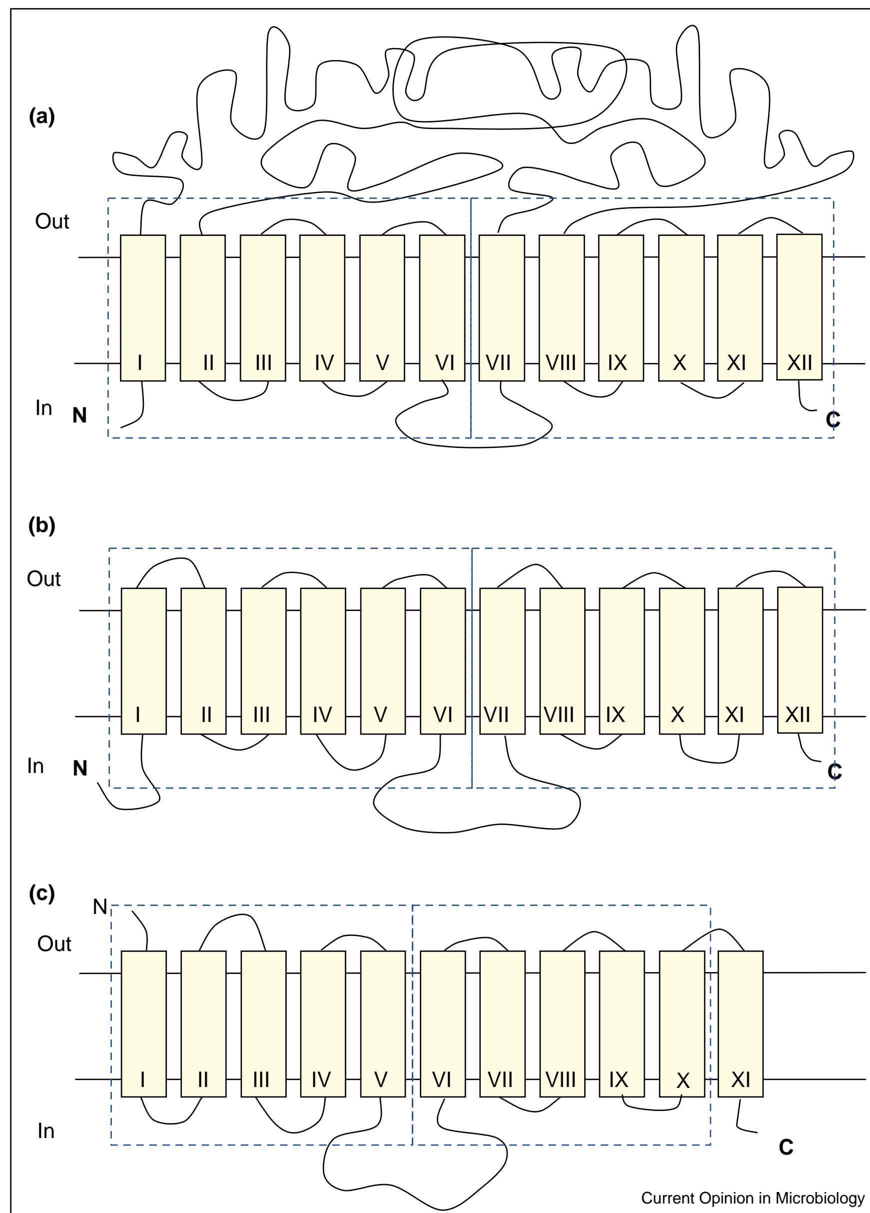
In recent years, the determination of three-dimensional structures at atomic resolution has dramatically increased our knowledge of the structure and molecular mechanisms of secondary transporters. Surprisingly, the five structures obtained to date, AcrB, LacY, GlpT, AmtB and $Glt_{Ph}$, have already revealed four different folds and as many different translocation mechanisms [5,6••–9••]. The structure of $Glt_{Ph}$ confirmed biochemical evidence for the existence of pore-loop structures in secondary transporters, commonly observed in channel proteins. The pore-loops or re-entrant loops are loop regions that fold back between the transmembrane segments. They play an autonomous role in the translocation mechanism.

In this review, we argue that pore-loop structures are a common structural motif in secondary transporters, adding to the structural and mechanistic diversity of secondary transporters.

## Three-dimensional structures of secondary transporters

The first three-dimensional crystal structure of a secondary transporter was reported in 2002 [5]. The *Escherichia coli* AcrB transporter, of the resistance-nodulation-cell division superfamily, forms a complex with the membrane fusion protein AcrA and the outer membrane pore TolC, which functions as a proton motive force-driven multidrug exporter. The transmembrane part of AcrB shows pseudo-twofold symmetry; six N-terminal helices are symmetrically arranged with six C-terminal helices to form two structural homologous domains (Figure 1a). Two long periplasmic loops, one per domain, form a large extramembranous part of the protein that extends up to the TolC pore in the outer membrane. AcrB is organized as a trimeric complex with a threefold symmetry axis perpendicular to the membrane in which the periplasmic parts form a central channel ending in a funnel at the side of TolC. At the opposite side, the channel is connected to a central cavity, which contains the substrate binding sites. The cavity is at the level of the outer leaflet of

**Figure 1**

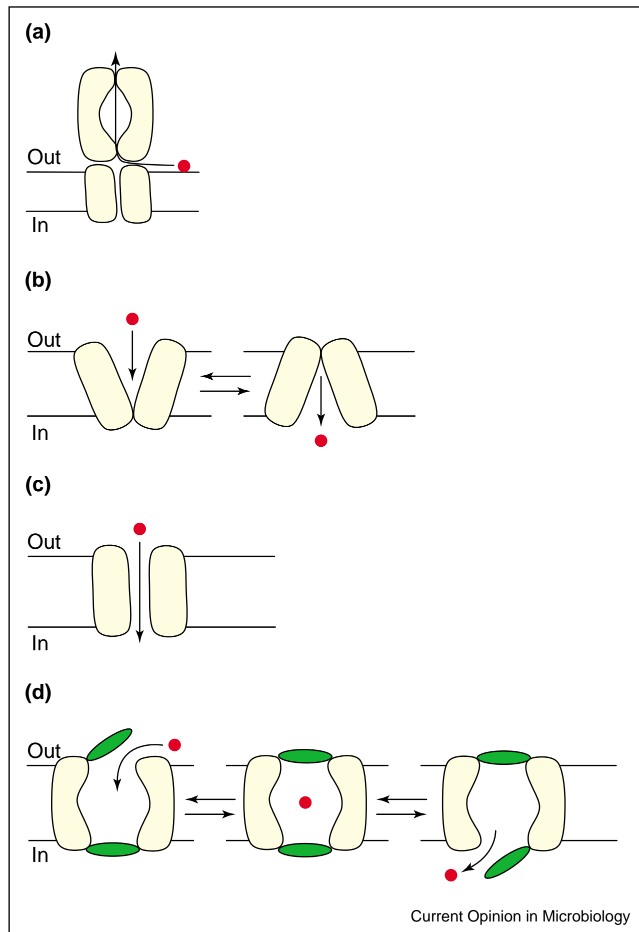



Topology models representing three different structures of families of secondary transporters with no pore-loop structures. Transmembrane segments are represented as yellow boxes. Homologous domains are indicated by blue dashed boxes. **(a)** AcrB of *E. coli*, a member of the resistance-nodulation-cell division superfamily (transporter classification [TC] 2.A.6), **(b)** LacY and GlpT of *E. coli*, members of the major facilitator superfamily (TC 2.A.1) and **(c)** AmtB of *E. coli*, a member of the ammonia transporter family (TC 2.A.49).

the bilayer. Three vestibules at the interfaces of the AcrB protomers provide the access pathway through which the substrates diffuse in by way of lateral movement from the lipid bilayer [5,10–12]. The mechanism of substrate extrusion and the involvement of conformational changes in the energy coupling mechanism, if any, remain obscure (see Figure 2a).

The three-dimensional structures of *E. coli* LacY and GlpT both belonging to the major facilitator superfamily were simultaneously reported in 2003 [6••,7••]. LacY is the well-studied $H^+$/lactose symporter while GlpT catalyzes glycerol-3-P/$P_i$ exchange. The transporters are believed to be functional as monomers. Like AcrB, the proteins consist of two homologous domains each comprising six transmembrane segments (TMSs) but with different tertiary structure, indicating a different genetic origin (Figure 1b). The structures of LacY and GlpT show a large hydrophilic cavity inbetween the two domains that is opened up to the cytoplasm and closed to the

**Figure 2**




Schematic representation of four different types of translocation mechanisms deduced from currently available transporter structures at atomic resolution. The arrows represent the pathway followed by the transported species (indicated by the black dot). In **(a)**, **(b)** and **(c)**, similar shapes represent homologous structures. (a) The elevator mechanism of the multidrug transporter AcrB. The solute is taken up from the outer leaflet of the membrane and is, subsequently, transported out of the cell through a funnel that is formed by protein components that bridge the periplasm. The solute(s) are delivered at a pore in the outer membrane. Conformational changes, if any, during the catalytic cycle are unknown. (b) The alternating access mechanism of the major facilitator superfamily transporters. The solute and co-ions bind to the binding sites on the transporter that are exposed to the outside of the cell (left), after which the binding site reorients and the solute and co-ions dissociate into the cytoplasm (right). (c) Facilitated diffusion through a channel, such as that of AmtB. No major conformational changes are involved. **(d)** The sluice-type of mechanism involving pore-loop structures (green), as in $Glt_{Ph}$. The pore-loop at the external face of the membrane opens up the binding pocket to the solute(s) in the external medium (left), after which the pore-loop closes the entrance thereby occluding the solute(s) within the protein (middle). Dissociation into the cytoplasm follows upon opening of the pore-loop at the inner face of the membrane (right).

periplasm. The cleft contains the substrate binding site in the middle of the membrane. The structure supports the alternating access model for substrate translocation in which the two domains move relatively to one another, thereby opening the hydrophilic cleft that contains the substrate alternately to the two sides of the membrane [13,14] (see Figure 2b).

In 2004, the structure of *E. coli* AmtB was reported at an astonishing resolution of 1.35 Å [8••]. AmtB belongs to a family of ammonia transporters to which, also, the Rhesus proteins of humans belong. The proteins form trimers, although the functional unit is believed to be the monomer. Again, the structure of a single protomer revealed two structurally homologous domains, but in this case each domain contained five TMSs causing the two domains to have opposite orientations with respect to the plane of the membrane (inverted topology; Figure 1c) [8••]. Thus, the N-termini of the N- and C-terminal domains are in the periplasm and cytoplasm, respectively. TMS XI is not part of this domain structure. Interestingly, the crystal structure did not reveal any major conformational changes upon binding of the substrate, suggesting that it functions as a channel rather than as a transporter. The protein binds ammonium ions ($NH_4^+$) and subsequently allows passage of ammonia ($NH_3$) while leaving the proton at the same side of the membrane (see Figure 2c).

The issue of transporters versus channels bares also some relevance in relation to the structure of a glutamate transporter homologue $Glt_{Ph}$, from the archeon *Pyrococcus horikoshii*, which was reported in 2004 [9••]. Prominent members of the family of glutamate transporters are responsible for the re-uptake of the neurotransmitter glutamate from the synaptic cleft in the central nervous system. In addition to ion-coupled glutamate transport, the proteins are known to catalyze the activity of a glutamate gated chloride channel [15,16]. Like AcrB and AmtB, $Glt_{Ph}$ forms a trimeric complex with a three-fold symmetry axis perpendicular to the membrane [9••], but has a completely different structure. The transporter proteins consist of eight TMSs with no internal homology. The N-terminal six TMSs of each monomer form a rim around a 'bowl'-shaped indentation in the center of the trimer at the external side of the membrane, the bottom of which is halfway along the membrane. The water-filled bowl is the access pathway for the substrate and co-ions to the translocation sites, with one being present in each monomer. The binding site is inbetween two re-entrant loops formed by helical hairpins that enter the membrane from opposite sides (*trans* re-entrant loops). Translocation is achieved by opening and closing access to the substrate binding site by movement of the re-entrant loops, possibly in a sluice-type of mechanism (see Figure 2d).

## Transporters and channels

Pore-loops, or re-entrant loops, are commonly observed in channel proteins that, like secondary transporters, are

universal to biological cells. In potassium ion channels, four pore-loop structures of as many monomers enter the membrane from the same side (*cis* re-entrant loops) into the pore formed by the tetramer. In the family of aquaporins, two re-entrant loops in two homologous domains consisting of three TMSs each, enter the membrane from opposite sides (*trans*). The pore-loops situated in the pores of the rigid channel protein structures act as selectivity filters [17–19].

When pore-loop structures were identified in glutamate transporters by biochemical experiments [20–22], they were associated with the accessory chloride ion ($Cl^-$) channel function observed for the neuronal counterparts mentioned above [23,24]. However, the structure of $Glt_{Ph}$ suggests that in secondary transporters the re-entrant loops have a different, more dynamic, function. They form gates that open and close in response to substrate and co-ion binding [9••]. In addition, the AmtB structure shows that re-entrant loops are not essential for channel function [8••] and, recently, it was demonstrated that a prokaryotic member of the chloride channel family catalyzes co-transport, an activity typical of transporter type proteins [25,26]. It follows that in terms of structural architecture the difference between channels and transporter proteins is very small and, for both, quite diverse.

## Pore-loops in secondary transporters

The demonstration of pore-loops as an essential part of the translocation mechanism in the $Glt_{Ph}$ structure was preceded by biochemical evidence for the existence and the functional importance of these structures in members of the glutamate transporter family. This evidence was based on the accessibility of loop regions via the protein structure from the opposite side of the membrane, and the modulation of this accessibility by substrates and co-ions. Substantial evidence has also been documented for transporters from at least two other families of secondary transporters, the 2-hydroxycarboxylate transporter (2HCT) family and the cation/calcium ion ($Ca^{2+}$) exchanger superfamily, suggesting that pore-loops could be a feature of many secondary transporters.

### Glutamate transporter family

Early on, it was recognized that the C-terminal half of the transporters had an unusual structure. Evidence for the pore-loop located inbetween TMS VI and TMS VII was based on independent results obtained for the neuronal glutamate transporter GLT-1 and the glutamate transporter GltT of *Bacillus stearothermophilus* (Figure 3a) [21,22]. Cysteine residues in a serine-rich part of the loop were accessible from both sides of the membrane while neighboring residues were only accessible from the cytoplasmic side. The water-filled bowl in the $Glt_{Ph}$ structure explains how bulky thiol reagents could reach the residues in the loop. Protection of the cysteine residues against labeling suggested the involvement of the re-entrant loop in substrate binding and that it has functional importance. Cysteine mutagenesis of residues located in the extracellular loop between TMS VII and TMS VIII of the neuronal glutamate transporter GLT-1 revealed one residue, Ala431, which was accessible from the cytoplasm but was separated at each side, by a stretch of only 8–11 residues, from residues that were accessible from the external water phase. This observation could only be explained if the region forming a loop was folded back into the cytoplasmic membrane [20]. Altered accessibility of residues in the presence of co-ions and the substrate showed functional importance of the region [27], and cross-linking of residues in the loop with certain positions in TMS VII further confirmed the existence of the re-entrant loop [28].

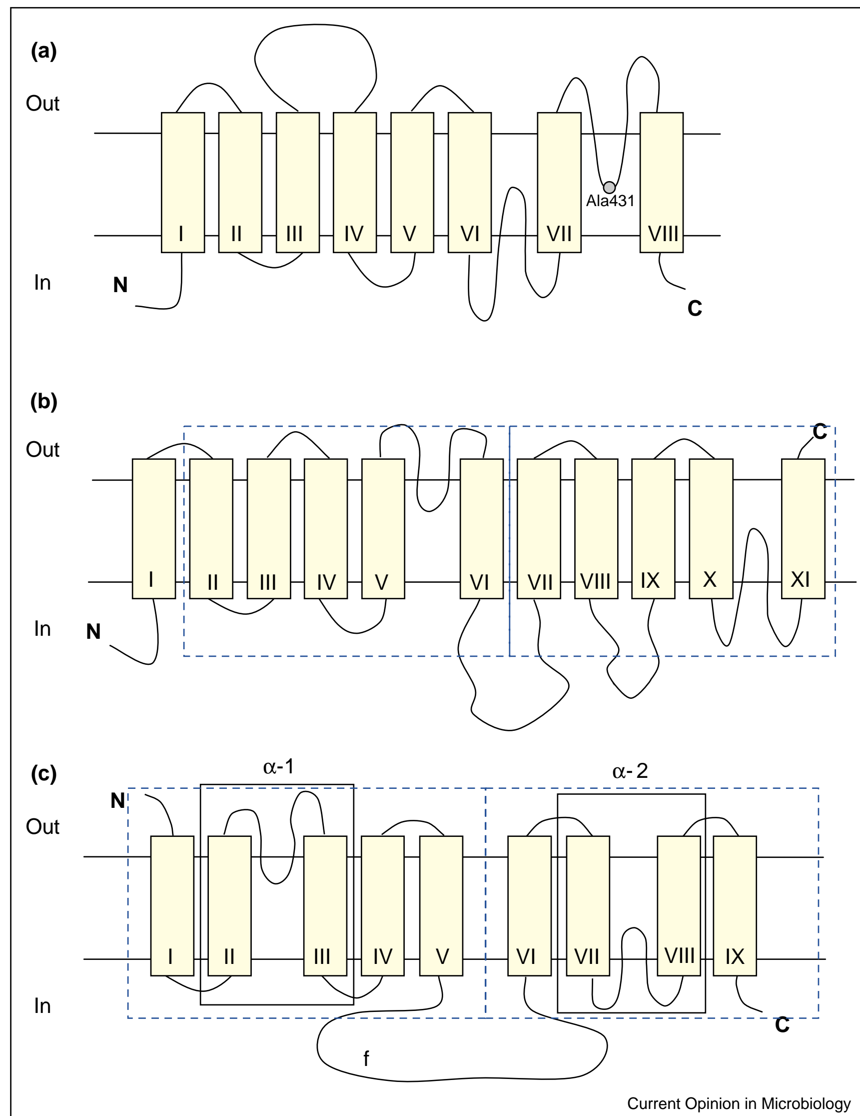
### 2-Hydroxycarboxylate transporters

The 2HCT family is the best-studied family of the structural class ST[3] in the MemGen classification and as such is the paradigm for 29 other families [4]. This family contains, exclusively, bacterial members that transport substrates containing the 2-hydroxycarboxylate motif ($HO–CR_2–COO^-$) such as citrate, malate, lactate, among others [29]. The topology model of this protein family mostly on the basis of studies of the $Na^+$-citrate transporter CitS of *Klebsiella pneumoniae* [30], shows eleven TMSs (Figure 3b). Further studies demonstrated the accessibilities of sites in the cytoplasmic loop between TMS X and TMS XI from both sides of the membrane [31,32•]. In contrast to what was observed for the glutamate transporters, the cysteine residues could only be accessed by small thiol reagents from the periplasmic side of the membrane, suggesting a much more restricted access pathway. The presence of the co-ion $Na^+$ completely blocked this access pathway. Cysteine residues at certain positions in the postulated pore-loop structure were shown to have different properties when accessed from the cytoplasmic or periplasmic side of the membrane, in agreement with an alternate access model for translocation [32•]. Studies of the proton-dependent citrate/malate transporter CimH of *Bacillus subtilis* from the same family provided results that were in line with these observations [33•]. A hydrophobic region inbetween TMS V and TMS VI that was shown to be not transmembrane is proposed to form a second re-entrant loop that enters the membrane from the opposite side.

### Cation/$Ca^{2+}$ exchangers

$H^+/Ca^{2+}$ exchangers and $Na^+/Ca^{2+}$ exchangers have been investigated predominantly in plant and animal cells. Topology models of the five proposed families in the superfamily [34] show a typical two domain structure that consist of N- and C-terminal parts containing four, five or six transmembrane segments each and that are connected by a relatively long cytoplasmic loop (Figure 3c, loop f). The two domains have opposite orientations in the

Figure 3




Topology models representing three different structures of families of secondary transporters that contain pore-loop structures. Transmembrane segments are represented as yellow boxes. (Putative) homologous domains are indicated by blue dashed boxes. **(a)** $Glt_{Ph}$ of *Pyrococcus horikoshii*, a member of the glutamate transporter family (TC 2.A.23) and **(b)** CitS of *Klebsiella pneumoniae*, a $Na^+$-citrate transporter of the 2-hydroxycarboxylate transporter family (TC 2.A.24). The two domain structure and pore-loop structure in the N-terminal domain is based on unpublished results (JS Lolkema, I Sobczak and DJ Slotboom). **(c)** NCX1 of mammals, a $Na^+/Ca^{2+}$ exchanger of the cation/$Ca^{2+}$ exchangers family (TC 2.A.19). α-1 and α-2 represent the two internal repeats in the sequences.

membrane (see the AmtB structure described earlier) [35]. The sequences of the proteins revealed two internal repeats of α-1 and α-2, which correspond to two TMSs and a connecting loop. The two loops, one for each domain face opposite sides of the membrane [36,37]. Detailed cysteine-scanning-based studies of the connecting loops of the mammalian exchanger NCX1 indicated that the loops fold back inbetween the TMSs as pore-loop structures [38,39]. Several residues in the postulated re-entrant loops are directly involved in the binding and the transport of $Ca^+$ [37]. The proximity of the two re-entrant loops of the α-1 and α-2 regions in the three-dimensional structure was demonstrated by cross-linking studies [39].

## Conclusions

Recent three-dimensional determination of the crystal structures of secondary transporters has revealed a surprisingly high diversity of transporter structures and translocation mechanisms (Figure 2). Also, this has shown that structural differences between secondary transporters and channels are less distinct than previously

thought, suggesting that subgroups of both types might have originated from a common ancestor. A recurring common theme is the presence of two homologous domains per transporter protein, suggesting ancient gene duplication events. The only exception appears to be the glutamate transporter family (Figures 1 and 3).

The $Glt_{Ph}$ structure has shown that biochemical experiments can correctly identify pore-loop structures in secondary transporters. The identification of pore-loop structures in other families indicates that they will be found as essential parts in other transporter structures in the future.

The atomic resolution of the five secondary transporters available to date, all of proteins from prokaryotic origin, is a major step forward in our understanding of translocation mechanisms. This emphasizes the importance and power of the study of prokaryotic homologs of eukaryotic (membrane) proteins involved in important physiological processes or their dysfunction in man.

## Acknowledgements

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- •• of outstanding interest

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Together with [7••], these studies determined the first two structures of members of the major facilitator superfamily transporters, which are the most abundant family of transporters in nature. The structures confirm and extend the massive amount of experimental data that is available for this type of transporter. These transporter proteins consist of two homologous domains, each comprising six transmembrane segments connected by a large loop.

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