The transport mechanism of the sodium/aspartate symporter Glt_{Ph} is novel and different from LeuT

Re-uptake of neurotransmitters from the synaptic cleft is essential for functional neurotransmission. In recent years, some of the transporters that mediate this uptake have been crystallized and this has improved our understanding of the mechanism by which these transporters function. Two of these transporters are Glt_{Ph} and LeuT, prokaryotic homologs of glutamate and leucine transporters, respectively. In eukaryotes, glutamate is an excitatory neurotransmitter and eukaryotic homologs of LeuT transport some neurotransmitters that are usually inhibitory, like GABA. These transporters are assumed to function via the alternating access mechanism, in which the substrate binding site is alternately exposed to the inside and the outside of the membrane. Several proteins from distinct families have been crystallized with a similar structure as LeuT, but in different conformations, revealing new insights in their transport mechanisms. In this review we will compare the structures and mechanisms of this LeuT-family with the structure of Glt_{Ph}, that has been crystallized in only two conformations. In this way we will examine whether the structures of LeuT could be used to learn more about the transport mechanism of Glt_{Ph}.

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Contents

Introduction

Membranes are the boundaries of life. They separate the cell content from the environment, forming a lipid bilayer with a hydrophobic core that is impermeable for most polar and charged solutes. For the communication with the outer world, membranes are packed with integral proteins like receptors and transporters. The latter catalyze the coordinated movement of molecules across the membranes of cells and organelles, enabling the cell to tightly regulate the concentrations of solutes in these compartments.

For transport across this hydrophobic barrier different energy sources are used. Primary active transporters convert the energy from light, ATP hydrolysis or other chemical reactions to the thermodynamically unfavorable transport of the substrates. Passive and secondary active transporters, on the other hand, mediate downhill transport of respectively the substrate or a second molecule. Passive transporters function as gated channels where substrates, like ions and small molecules, can diffuse down their concentration gradient. Active transporters can facilitate transport 'against the current' and establish very steep gradients, usually via the alternative access mechanism. Upon ligand binding these proteins undergo a conformational change, from the outward facing to the inward facing state or vice versa, releasing the substrate on the other side of the membrane.

Table 1: Schematic summary of primary and secondary transport *(with their energy sources)*

	Active	**Passive**
Primary transport	*(several energy sources)*	-
Secondary transport	Symport/antiport *(gradient of secondary molecule)*	Uniport (or symport/ antiport) *(own gradient)*

Secondary transport can be divided into symport, antiport and uniport (summarized in Table 1). In uniport one species is transported uni-directional, driven by its own electrochemical gradient. Symport and antiport are both active (usually): a secondary molecule (usually sodium, potassium or a proton) is co-transported or counter-transported, respectively, to harvest the energy from the pre-existing ion gradient. Often this transport is electrogenic, resulting in a net transport of charge over the membrane. In some cases symport and antiport can be passive, when the gradient of the substrate is the driving force, but still some ions are co- or counter-transported. A special case of antiport is the precursor/product exchange, where the imported molecule is the precursor for the reaction in which the exported molecule is produced.

To reset the protein for the next cycle of transport, the protein flips back into its original conformation, with or without the help of a counter-transported molecule. But despite these large conformational changes, secondary transporters can achieve a turnover rate of about 1400 min^{-1} (in the case of GltT, Auer 2001).

A process that requires fast and steep concentrative uptake is the reuptake of neurotransmitters from the synaptic cleft. An action potential promotes the release of neurotransmitter, increasing its concentration in the synaptic cleft by 10^3-10^4 fold (Clements 1996). This ligand could open ion channels in the post-synaptic neuron or activate a signaling path via G-proteins. Neurotransmitters

could have an excitatory or an inhibitory effect, depending on the receptors in the post-synaptic cell. Activated excitatory receptors induce depolarization of that neuron and generate an action potential while inhibitory receptors have the opposite effect. The response of the neuron depends on the integration of all inhibitory and excitatory signals. To prevent neural excitotoxity or over-inhibition, the transmitters are transported into the cytoplasm of surrounding neuronal and glial cells (Clements 1996).

A common excitatory neurotransmitter is glutamate. This amino acid is involved in normal development and function of the brain, like learning and memory formation and higher cognitive function (Dingledine 1999) . Dysfunction of glutamate transporters can lead to a wide range of nervous system diseases, like schizophrenia and depression. Therefore, glutamate transporters are of high interest and they have been studied quite intensely over the past years. However, the transport mechanism is still under investigation.

Mammalian glutamate transporters are part of the large excitatory amino acid transporter (EAAT) family (Slotboom 1999). They co-transport the substrate with three sodium ions, followed by the counter transport of one potassium (K^+) ion. Prokaryotic homologs mediate the uptake of glutamate (or aspartate) as a nutrient. Both eukaryotic and prokaryotic glutamate transporters are part of the dicarboxylate/amino acid : sodium symporter (DAACS) family of transporters. In this family, three functional groups can be distinguished: C_4-dicarboxylate transporters, glutamate/aspartate transporters and neutral-amino acid transporters. Many of these transporters from different organisms have been characterized, but only one has been crystallized: Glt_{Ph}, an aspartate transporter from the archea *Pyrococcus Horikoshii*, that shows approximately 36% sequence similarity with his mammalian homologues (Boudker 2007).

A transporter family that also transports neurotransmitters is the NSS family (neurotransmitter : solute symporter). This family has been studied extensively, since it contains transporters that are physiologically important, like those specific to dopamine, norepinephrine, glycine and GABA. Therefore it is no wonder that this protein class is associated with many disorders, like depression and Parkinson's disease and that they are common drug targets (Gether 2006). A bacterial homolog of the mammalian leucine transporters that has been crystallized is LeuT, from *Aquifex aeolicus,* mediating leucine transport (Yamashita 2005). LeuT and Glt_{Ph} are not structurally related, although they share some common features.

In general, integral membrane proteins can adopt two different basic architectures: α-helical bundles and β-barrel proteins. In both structures the polar groups of the backbone and polar side chains are facing inwards, so they are saturated with internal hydrogen bonds. On the surface of the protein, hydrophobic side chains are exposed to the hydrophobic membrane core. This ensures the thermodynamic stability of the membrane protein. Usually the helices are membrane-spanning, but in some cases they are interrupted by a non-helical part (Screpanti 2007). This irregular structural motif is unfavorable because the polar C- and N-termini of the helices are then buried in the membrane. Therefore these discontinuous helices must be stabilized by hydrogen bonds. Glutamate and leucine transporters both are α-helical proteins that contain such a discontinuous motif.

Table 2: General characteristics of the transporters

Fold	Family	Protein	Organism	Substrate*	Assumed stoichiometry	Quaternary structure	# TMs	Symmetry	Main reference
LeuT	NSS	LeuT	*Aquifex aeolicus*	Leucine	$2Na^+$: 1 Leu	Dimer	12	TMs 1-5 *vs* TMs 6-10	Yamashita (2005)
	SSS	vSGLT	*Vibrio parahaemolyticus*	Galactose	$1Na^+$: 1 Gal	Monomer	14	TMs 2-6 *vs* TMs 7-11	Faham (2008)
	NCS1	Mhp1	*Micobacterium liquefaciens*	hydantoin	$1Na^+$: 1 hydantoin	Monomer	12	TMs 1-5 *vs* TMs 6-10	Weyand (2008)
	BCCT	BetP	*Coryenebacterium glutamicum*	Glycine betaine	$2Na^+$: 1 Bet	Trimer	12	TMs 3-7 *vs* TMs 8-12	Ressl (2009)
		CaiT	*Escherichia coli*	L-carnitine/ γ-butyro-betaine antiport	1 Car : 1 But	Trimer	12	TMs 3-7 *vs* TMs 8-12	Tang (2010)
	APC super family	AdiC	*Escherichia coli*	Arginine/ agmatine antiport	1 Arg: 1 Agm	Dimer	12	TMs 1-5 *vs* TMs 6-10	Gao (2009), Fang (2009), Gao (2010)
		ApcT	*Methanocaldococcus jannaschii*	Amino acids (broad specificity)	H^+ : amino acid	Monomer	12	TMs 1-5 *vs* TMs 6-10	Shaffer (2009)
Glt_{Ph}	DAACS	Glt_{Ph}	*Pyrococcus horikoshii*	Aspartate (and glutamate)	$3Na^+$: 1 Asp	Trimer	8**	TMs 1-3 *vs* TMs 4-6 and TM7+HP2 *vs* TM8+HP1	Yernool (2004)

*substrates are symported with the ions mentioned in the next column, unless indicated otherwise

**Glt_{Ph} contains 8 TM helices plus two re-entrant hairpin regions (HP1 and HP2)

Table 3: Details about the crystal structures

Protein	Conformation	molecules bound	Resolution (Å)	PDB ID	Reference
LeuT	Outward-facing (occluded)	Leu, Na^+, Cl^-	1.65	2A65	Yamashita (2005)
vSGLT	Inward-facing (occluded)	Gal, Na^+	2.70	3DH4	Faham (2008)
Mhp1	Outward-facing (open)	-	2.85	2JLN	Weyand (2008)
	Outward-facing (occluded)	benzylhydantoin	4.00	2JLO	
	Inward-facing (open)	-	2.80	2X79	Shimamura (2010)
BetP	Intermediate (occluded)	Glycine betaine	3.35	2WIT	Ressl (2009)
CaiT	Intermediate (open to inside)	Carnitine, Hg^{2+}	3.15	3HFX	Tang (2010)
AdiC	Outward-facing (open)	-	3.61	3LRB	Gao (2009)
	Outward-facing (open)	-	3.20	3HQK	Fang (2009)
ApcT	Inward-facing (occluded)	(apo)	2.32	3GIA	Shaffer (2009)
Glt_{Ph}	Outward-facing (closed)	L-Asp, $2Na^+$	3.50	1XFH	Yernool (2004)
	Outward-facing (open)	TBOA	3.20	2NWW	Boudker (2007)
	Inward-facing (occluded, cross-linked)	L-Asp, $2Na^+$	3.51	3KBC	Reyes (2009)

In the past years several transporters from different genetic families have been crystallized with the same fold as LeuT, although no similarity was observed in their amino acid sequence. LeuT represents a large group of transporters from different families and structural data is available for many of them. Glt_{Ph}, on the contrary, represents a small structural class, with only one structure available in a few conformations.

In this review we compare the transport mechanism of Glt_{Ph} with that of LeuT-folded proteins. We will focus on the global structural transitions and compare the proteins for which a crystal structure is available, summarized in Table 2 and 3. It would be useful if we could use the wealth of structural and functional data already available for the LeuT family to learn more about the transport mechanism of Glt_{Ph}. But the question is, to what extend are those structures comparable?

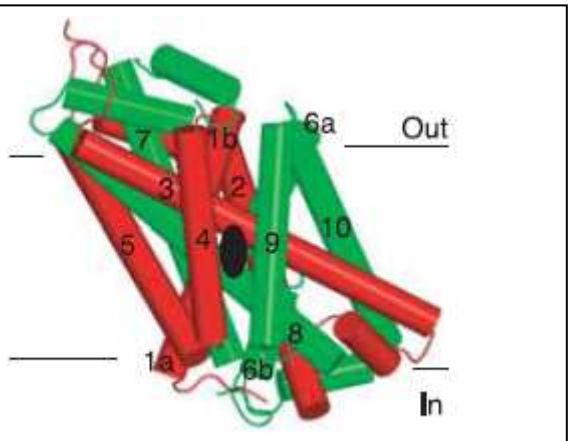

Fig. 1: LeuT structure, taken from Yamashita et al. (2005). The two two structural repeats in LeuT are colored red (TMs 1-5) and green (TMs 6-10), respectively, to emphasize the pseudo-twofold symmetry. The rotation axis is shown as an ellipsoid.

Structure and function of LeuT and structural homologs

Protein architecture

LeuT consists of 12 transmembrane (TM) helices and the structure contains an inverted repeat, relating the first five transmembrane helices (TM1-TM5) to the subsequent five (TM6-TM10) (Yamashita 2005). This symmetry is inverted, because the N-terminus of the first repeat is located on the cytoplasmic side, while the N-terminus of the other unit is located on the extracellular side of the membrane (Fig.2a). In the tertiary structure these units show a pseudo-twofold symmetry, with the symmetry axis located in the membrane plane (Fig. 1). Despite the structural homology, there is no significant sequence homology between the two repeats (Yamashita 2005). Together these helices (TM1-TM10) form the functional part of the transporter. The other two helices, TM11 and TM12, are flanking the outer surfaces of TM9 and TM10. The number and position of these flanking helices varies among the different LeuT-like transporters and also the length of the internal and external loops shows a great diversity.

A remarkable common feature of the LeuT-like structures is the presence of two discontinuous membrane helices. The α-helices of TM1 and the symmetry-related TM6 are interrupted by an irregular sequence of 3-5 extended residues. These breaks are located approximately halfway across the membrane bilayer, dividing TM1 in TMs 1a and 1b and TM6 in TMs 6a and 6b (Yamashita 2005). TM1 and TM6 are lining next to each other, in an anti-parallel orientation. These two helices,

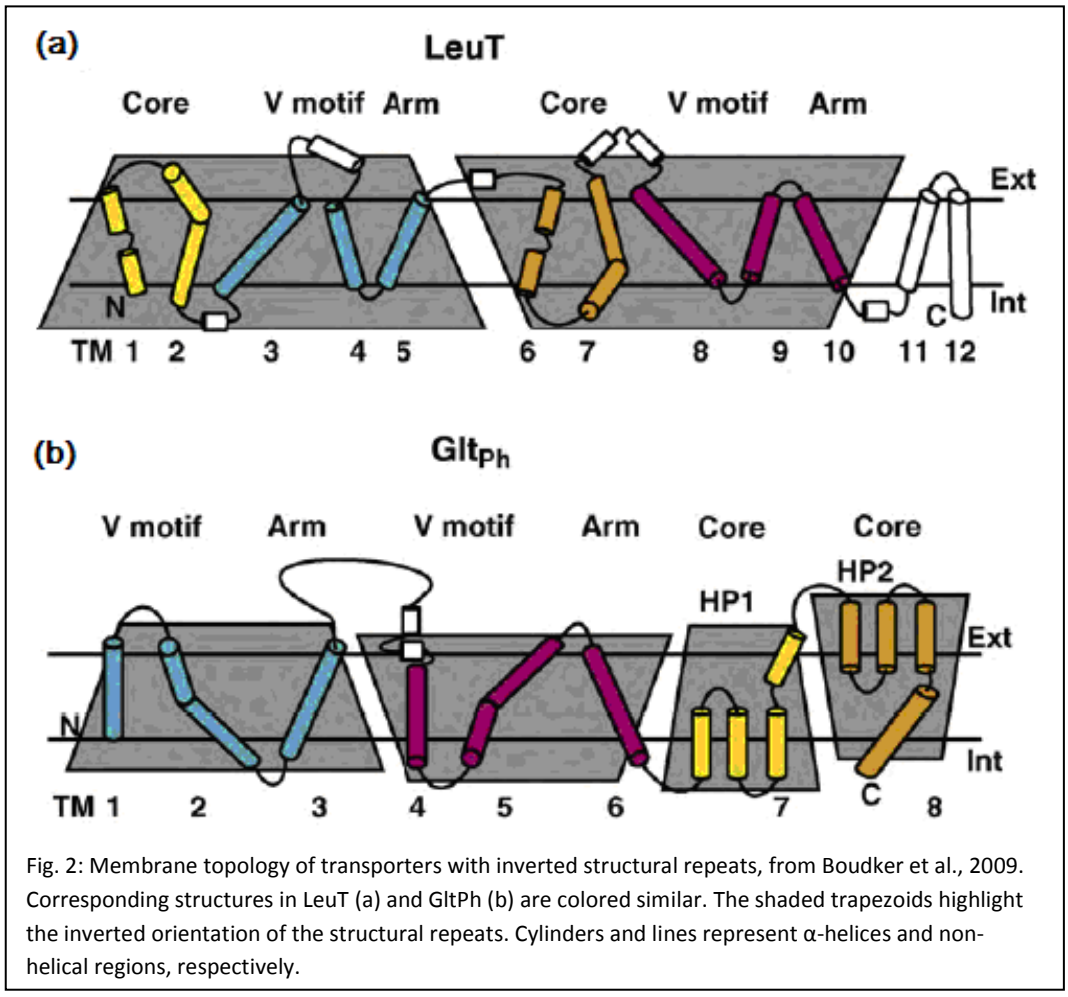

Fig. 2: Membrane topology of transporters with inverted structural repeats, from Boudker et al., 2009. Corresponding structures in LeuT (a) and GltPh (b) are colored similar. The shaded trapezoids highlight the inverted orientation of the structural repeats. Cylinders and lines represent α-helices and non-helical regions, respectively.

together with the TM2 and TM7, form a tightly packed four-helix bundle (Forrest 2009), that is important for protein function, as discussed below.

The other six helices of the functional core structure form a scaffold around the four-helix bundle, on the side of TM1 and TM6. This scaffold consists of two symmetry-related V-shaped structures, consisting of TM4+TM5 and TM9+TM10 respectively (Yamashita 2005). TM3 and the symmetry-related TM8 are lined next to each other, tilted by an angle of about 50°C from the membrane normal. This assembly is tweezed between the V-shaped structures, as visible in figure 2, and these helices are facing directly toward TMs 1 and 6.

LeuT has first been crystallized in the occluded, outward-facing conformation (Yamashita 2005). The access to the binding site from the extracellular solvent is blocked by only a few hydrophobic residues. A large, liquid-filled cavity penetrated the membrane from the extracellular side. Access from the intracellular side, on the other hand, was blocked by tightly-packed protein density. Therefore it is assumed that the protein is crystallized in the outward-facing conformation.

Within the LeuT crystal structure, the two inverted repeats could be superimposed on each other, revealing a clear similarity (Forrest 2009). Interestingly, the difference between these units existed only in the orientation of the bundle-helices (TM1+2 and TM6+7) in respect of the scaffold helices (TM3-5 and TM8-10). The scaffold appeared to have the same orientation in the membrane, so only the orientation of the bundle-helices were asymmetrical (Forrest 2009).

This observation was used to predict the inward-facing conformation of LeuT, by simply swapping the orientations of the two pseudo-symmetrical repeats. In this computational model, an inward-facing cavity became visible and the substrate binding site was separated from the extracellular fluid by a ~20Å barrier, similar to the protein density between the substrate and the cytoplasm, as observed in the X-ray structure (Forrest 2009). Comparison of the model with the crystal structure indicated an alternating-access mechanism, implicating a cycle of distinct protein conformations that expose the substrate-binding site alternately to the cytoplasmic and extracellular solutions (Fang 2009).

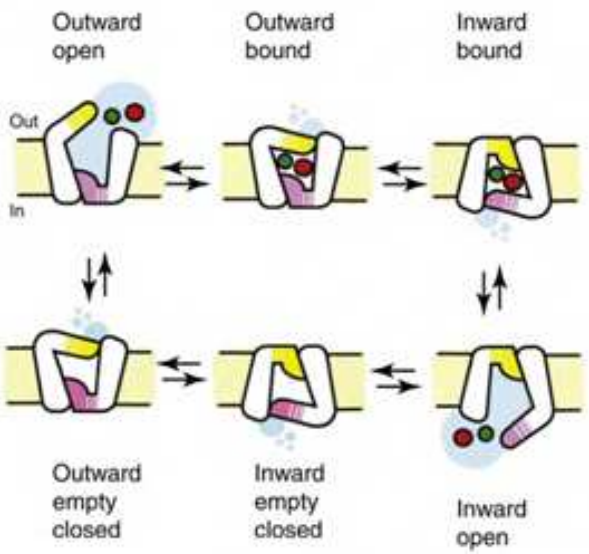

Fig. 3: Alternating access mechanism for anion/substrate symport, taken from Boudker et al. (2010). Schematic view of one cycle of outward and inward conformations, intermediate states between these conformations are not shown. Substrate and coupled ions are shown as spheres and the gates are shown in red and yellow. When a transporter is in the outward conformation, the intracellular gate cannot open and vice

Conformational changes

After the crystallization of LeuT by Yamashita and co-workers (2005), several structures have been determined of transporters that assume the same tertiary fold as LeuT. This was surprising, because the proteins originated from distinct genetic families and showed no sequence homology. These proteins were captured in

different conformational states (summarized in Table 3 and Fig. 3), namely 'outward-facing open' (LeuT, Mhp1, AdiC), 'outward-facing occluded' (LeuT, Mhp1), 'intermediate, occluded from both sides' (BetP, ApcT), 'inward-facing occluded' (vSGLT) and 'inward-facing open' (CaiT, Mhp1), see also Table 3. Since the structures of these transporters have identical topologies, the different conformations can be interpreted as static 'snapshots' of one dynamic structure (Vangelatos 2009). Comparison between these snapshots could reveal new insights into the transport mechanism.

The first of these proteins to be crystallized (after LeuT) was vSGLT, a sodium:galactose symporter, captured in the inward-facing conformation (Faham 2008). This structure was consistent with the model of the inward-facing LeuT, as described above. This confirmed the hypothesis of a symmetrical alternating access mechanism, in which the protein isomerizes from the outward- to the inward-facing conformation via an anti-clockwise rotation of the four-helix bundle around the fixed substrate-binding site (Fig. 4; Forrest 2009).

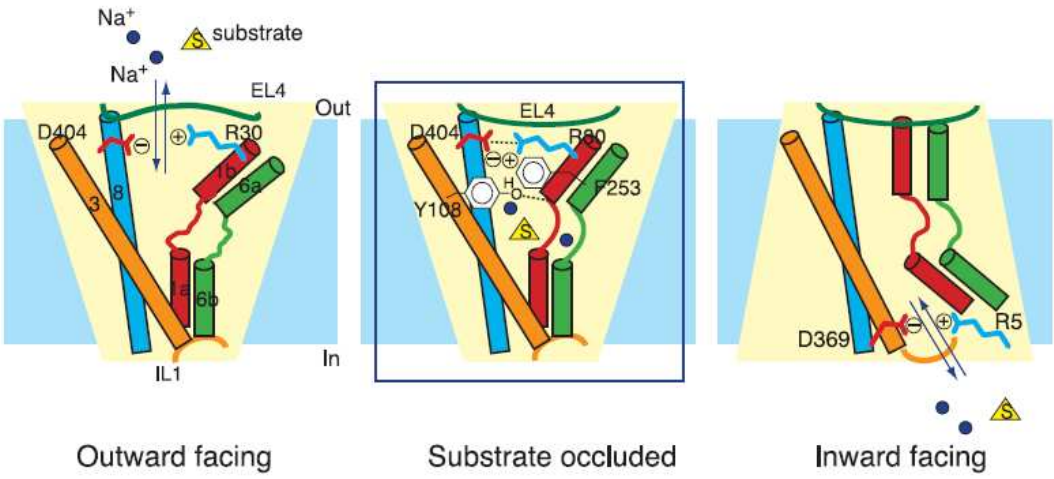

Fig. 4: A simplified view of the transport mechanism of LeuT, as hypothesized by Yamashita et al. (2005). This figure aims to illustrate the movement of TM1 and TM6 in respect of TM3 and TM8. Not the whole transport cycle is described. The middle panel shows the substrate-occluded state, which corresponds to the crystal structure presented in the paper (Yamashita 2005).

For Mhp1, a sodium-hydantoin symporter, two distinct outward-facing conformations (substrate free and substrate bound; Weyand 2008) have been crystallized, revealing detailed information about the conformational change upon substrate binding from the outside of the membrane. Recently, also an inward-facing conformation of Mhp1 has been crystallized (Shimamura 2010). The isomerization steps could now be compared within one protein instead of the combination of different proteins. Therefore more detailed information about the transitions is available, like about the structural changes in the binding sites. From this, Shimamura et al. (2010) concluded that the Na^+ binding site is intact in the outward-facing and occluded forms and disrupted in the inward-facing form. The residues that shaped the binding site had changed position in the inward conformation compared to the outward state, so the interactions of these residues with the ion were changed.

BetP provided a unique intermediate structure, crystallized by Ressl and colleagues (2009). This Na^+/betaine symporter was occluded from both sides, leaving only very thin funnels open to both sides of the membrane. The core structure was symmetrical in the membrane plane and

superimposition of the structural repeats showed no difference in their conformations. In other words, the helices adopted intermediate positions between the outward- and inward-facing conformations of LeuT and vSGLT (Ressl 2009).

AdiC and ApcT belong to the large APC (amino acid, polyamine and organocation) superfamily. AdiC was crystallized by two research groups in the outward-facing open conformation, similar to the open conformation of Mhp1 (Gao 2009, Fang 2009). The ApcT structure was situated in the apo conformation, most similar to that of the substrate-bound, occluded conformation of the BetP structure (Shaffer 2009). No substrate was bound, only a water-filled cavity was present at the position of the binding site. This conformation possibly shows us the final step in the transport cycle, where the inward-facing transporter switches back to the outside to bind new ligand. That would mean that water molecules are counter-transported when the protein is completing its transport cycle. More research should be done to examine whether the counter-transport of water molecules is a common principle among these transporters.

Interesting about the CaiT crystals, is that this structure contained four L-carnitine molecules, indicating the presence of multiple substrate binding sites (Tang 2010). CaiT is a carnitine/ butyrobetaine antiporter, from the same BCCT family as BetP. Because this transporter doesn't make use of an ion-gradient, it is hyporthesized that the transport is 'pushed' by binding of the substrates in the other binding sites (Tang 2010). Intererstingly, also for LeuT a secondary substrate binding site has been characterized (Shi 2008).

Taken together, these proteins show us a sequential 'movie' of the alternating access mechanism. The orientations of the core varies relative to the V-motifs in a 'rocking-like' movement of the domains pivoting approximately around the substrate-binding site (Fig. 4; Boudker 2010). This mechanism seems to be common in the LeuT superfamily.

Substrate binding site

In all LeuT-folded structures known thus far, the substrate binding site is located approximately in the middle of the membrane, between TMs 3, 8 and the unwound regions of TMs 1 and 6 (LeuT numbering). Thanks to these breaks in the helix structures (Fig.5b), the substrates can interact with the backbone of the extended residues. In the case of the zwitter-ionic leucine substrate, the α-carboxy groups of the bound leucine interacts with NH-groups of the exposed backbone, while the α-

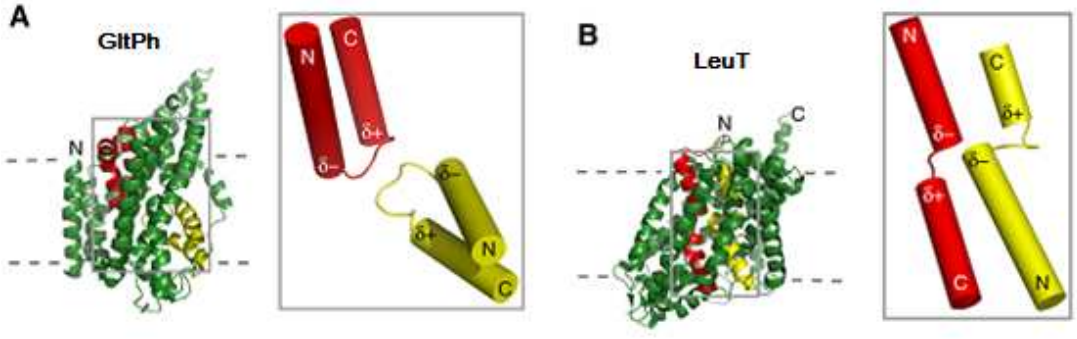

Fig. 5: Discontinuous membrane helices in transport proteins (Screpantie & Hunte, 2007). Crystal structures of GltPh (A) and LeuT (B), with the discontinuous helices magnified in cylindrical presentation on the right side. The partial charges at the termini are indicated and the estimated membrane position is shown by broken lines.

amino group interacts with the carbonyl oxygens. Also the partial charges of the dipole ends of TMs 1a (δ+), 6a (δ+) and 1b (δ-) are stabilized by the substrate (Yamashita 2005).

Besides these H-bonds and electrostatic interactions, leucine is also 'sandwiched' between a few non-polar residues stacked below and on top of the substrate. In the occluded crystal structures, these hydrophobic residues form 'gates', preventing the substrate to escape to the extracellular or intracellular cavities in the outward-facing or inward-facing proteins, respectively. These gates prevent the formation of a continuous channel during the movement of the surrounding helices in the isomerization steps.

Ion binding sites

Except for CaiT, AdiC and ApcT, all LeuT-like transporters are sodium-coupled. In the LeuT structure two sodium binding sites have been characterized in the proximity of the substrate binding site, referred to as Na1 and Na2. The first sodium binding site (Na1) was directly coupled to the bound leucine molecule, while the secondary site (Na2) was located in a neighboring site, about 7Å away from Na1 (Caplan 2008). Na1 makes contact with the substrate and helix 1, 6 and 7 and Na2 is bound to TM1a and TM8, on the opposite side of TM1.

Molecular dynamics studies indicated that Na1 is important for substrate affinity, while Na2 stabilizes the binding pocket (Caplan 2008). This insight provides a mechanism for the co-operative binding of ions and substrates, important for ion-substrate coupling.

Similarities between LeuT and Glt_{Ph}

Protein architecture

Although the overall-folding of Glt_{Ph} is very different from LeuT, these proteins contain similar structural motifs, most evident in the secondary structure (Fig. 2). They show inverted structural repeats of 5 (LeuT) or 3 (Glt_{Ph}) helices, with an anti-parallel orientation and a pseudo-symmetry axis parallel to the membrane (Boudker 2010, Yernool 2004). The repeat in Glt_{Ph} is followed by a second repeat of a re-entrant helical hairpin and a broken helix.

Although the order of the membrane topology in the three protein sequences is different, the similarity between the structural elements is striking. The yellow and orange helices in figure 2 form the inner core or 'four-helix bundle' of the protein and contain two symmetrical discontinuous helices. In Glt_{Ph} the

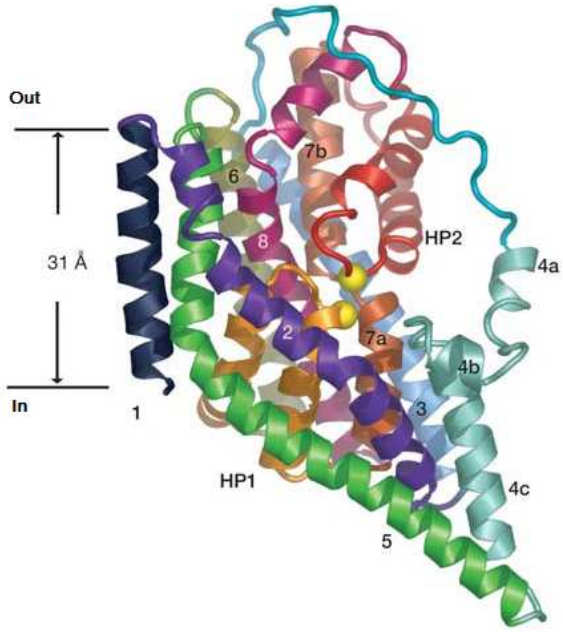

Fig. 6: Fold of GltPh in the outward-facing conformation, from Yernool et al. (2004). The protomer is viewed in the membrane and transmembrane helices (1-8) and hairpin regions (HP1 and HP2) are labeled.

re-entrant loops are also discontinuous and in the occluded conformation these helices point towards each other with the substrate in between (Yernool 2004). The orientations of the discontinuous helices are summarized in figure 5. The core of Glt_{Ph}, however, also consists of helices 3 and 6, while the similar helices in LeuT are part of the scaffold. In all three proteins the core can be distinguished from the 'scaffold', formed by the other six helices, as described for LeuT. Also the 'V-motif' and the 'arm' are comparable in these topologies (Boudker 2010). These similarities would suggest a common ancestor, after which divergent evolution resulted in the rearrangement of the structural motifs. However, the differences indicate something else, as we will see in the next section.

Substrate and sodium binding sites

The substrate binding sites differ between all proteins, since different substrates are coordinated by different protein residues. But still the principles are similar, interactions occur via hydrogen bonds, salt bridges and cation-π interactions (observed in AdiC, BetP and CaiT). In LeuT and Glt_{Ph} the binding pocket is located in the middle of the membrane, in the open spaces created by the extended residues of discontinuous helices.

Both LeuT and Glt_{Ph} bind two sodium ions in close proximity of the amino acid substrate. This is important for the coupling of substrate and ion transport, achieved by cooperative binding between the ions and the ligand via direct (Na1) and indirect (Na2) interactions as described for LeuT. Recently the aspartate-sodium stoichiometry of 1:3 has been determined for Glt_{Ph} (Groeneveld 2010). This implies that there should be a third Na^+ binding site present, although this site has not yet been characterized. Whether this site is also interacting with the substrate is not known.

Global conformational changes

Glt_{Ph} has been crystallized in different conformations (Table 3), making it possible to compare the inward-facing and the outward-facing structures. Like in LeuT, this change depends on the 'conformation swap' between the symmetrical structural elements, resulting in the movement of the core domain in respect to the scaffold. In this way the substrate binding site is alternately exposed to

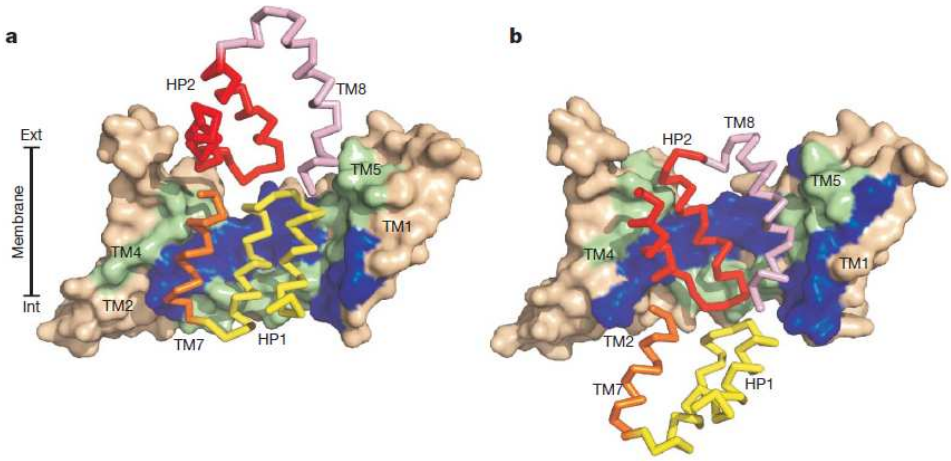

Fig. 7: Conformational changes in GltPh, taken from Reyes et al. (2009). The outward-facing conformation of wild-type GltPh (a) and the inward-facing conformation of GltPh(55C/364CHg) (b) are shown. Scaffold domains are shown in surface representation and the hairpin regions, together with TM7 and TM8 are shown in ribbon representation.

the periplasm and the cytoplasm. Although both transporters make use of the same 'mechanistic tricks', the overall transport mechanism shows remarkable differences, as discussed in the next section.

Differences between the transport mechanisms

The main difference between transport in Glt_{Ph} and LeuT is the translocation of the binding site. In the inward-facing structure of Glt_{Ph}, the substrate has moved as much as 18Å towards the cytoplasm compared to the outward-facing structure. The substrate is moved together with the core region, as shown in figure 7. In LeuT, on the other hand, the binding site is fixed as a pivot and the four-helix bundle turns around this point. In Glt_{Ph} the loops between TMs 2 and 3 and between TMs 5 and 6 function as hinges between the core and the scaffold (visible in Fig. 8a).

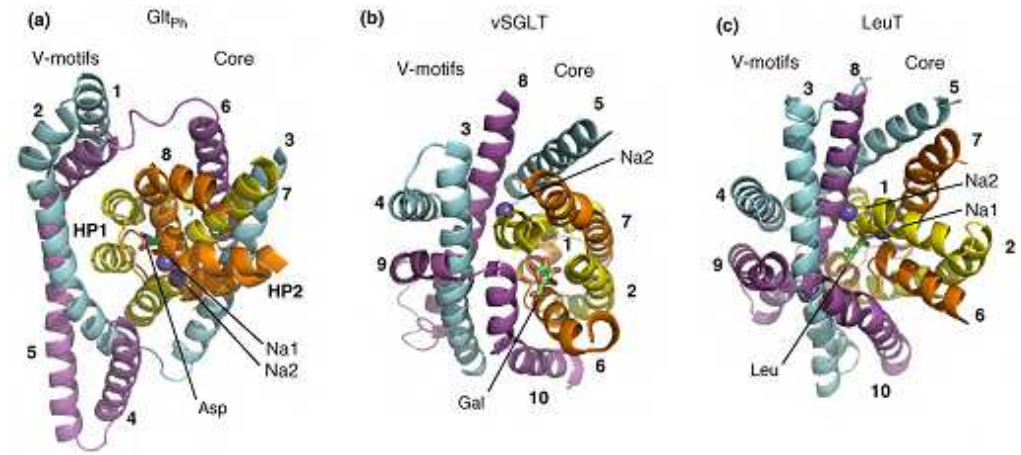

Fig. 8: Position of the binding sites, from Boudker et al., 2010. vSGLT (b) and LeuT (c) are viewed from the extracellular space and GltPh (a) is tilted to reveal the binding site. For clarity only TMs that are part of the inverted repeats are shown. The structures are colored as in figure 1, sodium ions are purple spheres and substrates are in stick representation.

In figure 9 the complete transport cycle of Glt_{Ph} is described. The substrate binding site is covered by two gates: hairpin region one (HP1) on the inside and hairpin region two (HP2) on the outside of the membrane. It is hypothesized that upon substrate binding, HP2 closes and the substrate binding site moves from one to the other side of the membrane, followed by opening of HP1 and substrate release into the cytoplasm. The prominent role for the gates in Glt_{Ph} is not observed for LeuT.

Although the binding site is moved in Glt_{Ph}, the pocket stays intact during translocation. The sodium and aspartate molecules are buried inside the bundle, without making contact to the scaffold. For LeuT and other outward-facing protiens, on the other hand, the ligands are placed between the moving parts. In the structure of inward-facing transporters like vSGLT, the sodium ion is connected only to the core helices and not to TM8 of the scaffold (Fig. 8). So probably the pockets get disrupted in the isomerization to change their affinity for the substrate, contributing to the ion-releasing states of these transporters, as described for BetP (Shimamura 2010).

These major differences between GltPh and LeuT would suggest convergent rather than divergent evolution. The common features of these classes could be the result of distinct evolution pathways, in which similar solutions were found to establish the most efficient transport.

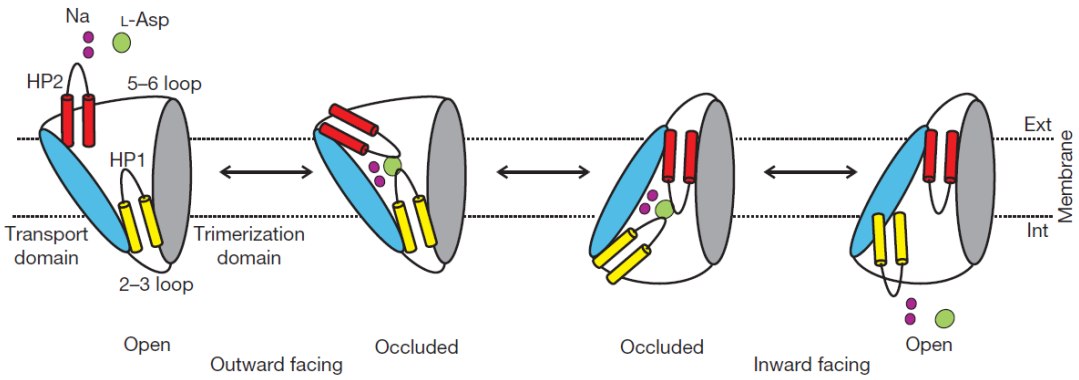

Fig. 9: Schematic transport mechanism of GltPh, taken from Reys et al. (2009). HP2 (red) and HP1 (yellow) close and open upon substrate binding and release, respectively. In between the binding sites move from the outside to the inside of the membrane, due to the movement of the core domain (light blue) relative to the scaffold domain (gray). The inward facing open state is hypothetical because it has not been crystallized.

Discussion and conclusion

Very recently, Boudker et al. (2010) published a review about secondary active transporters, including LeuT and Glt_{Ph}. They focused on the similarities between these proteins, making it tempting to conclude that the global conformational changes are similar as well.

However, we would like to suggest that the global transport mechanism of Glt_{Ph} differs significantly from that of LeuT, because it depends on a different transport principle. Indeed, in LeuT the substrate binding site is fixed in the centre of the membrane, while in Glt_{Ph} the substrate is carried from one side of the membrane to the opposite.

In the 1960s there was a discussion about the transport mechanism of membrane proteins. Before the mechanism of alternating access was proposed (Jardetzky 1966), transporters were suggested to function via a migrating carrier mechanism. In this model, the membrane protein binds his substrate at one side of the membrane and diffuses together with the occluded ligand through the membrane, before releasing the substrate on the other side. This model is still valid for some small ionophores, like valinomycin, but not for 'normal' membrane transporters.

Now, more than 40 years later, the migrating-carrier model seems to make his return. In the case of Glt_{Ph}, and probably his homologous glutamate transporters, a hybrid mechanism between the alternating access- and the migrating carrier-mechanisms could be proposed. In this new model, the carrier migrates from one side of the membrane to the other, but is also connected to a scaffold that is fixed in the membrane.

It is conceivable that this guided translocation is faster than random diffusion of the carrier, so the protein can reach a high turnover rate, required for a rapid glutamate uptake in the brain. Of course

the standard alternating access mechanism facilitates high turnover rates as well, so about the advantages of the shuttle model we could only speculate.

In summary, LeuT and Glt_{Ph} are comparable in many respects, like substrate binding and local conformational changes. These proteins use the same 'tools', like discontinuous transmembrane helices, ion-binding site composition and the two-fold pseudo-symmetry. These similarities could have been evolved via convergent evolution and they could serve a better understanding of how these proteins function, when all data is combined.

However, the global transport mechanism relies on different principles. In Glt_{Ph} the substrate binding site translocates from one side of the membrane to the other and in LeuT the domains move around the fixed substrate. This means that the structural data available from LeuT-folded proteins is only partly applicable for Glt_{Ph}.

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