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Secondary Transport of Amino Acids in Prokaryotes

H. Jung, T. Pirch, D. Hilger
Bereich Mikrobiologie, Department Biologie I, Ludwig-Maximilians-Universität München D-80638, München, Germany

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Abstract. Amino acid transport is a ubiquitous phenomenon and serves a variety of functions in prokaryotes, including supply of carbon and nitrogen for catabolic and anabolic processes, pH homeostasis, osmoprotection, virulence, detoxification, signal transduction and generation of electrochemical ion gradients. Many of the participating proteins have eukaryotic relatives and are successfully used as model systems for exploration of transporter structure and function. Distribution, physiological roles, functional properties, and structure-function relationships of prokaryotic α-amino acid transporters are discussed.

Key words: Amino acid transport — Secondary transport — Sodium/solute symport

Introduction

Prokaryotic cells need to adapt to steadily changing environmental parameters including availability and concentration of nutrients. As a means to adapt to these changes, the cells have the potential to employ a large number of transporters differing in substrate specificity, substrate affinity, turnover number, and accumulation power. The genome of the soil bacterium *Pseudomonas putida* KT2440, for example, encodes about 370 cytoplasmic membrane transport systems out of which about 60 are suggested to function as amino acid transporters (dos Santos et al., 2004). Transport of amino acids is carried out by ATP binding cassette (ABC)-type transport systems and secondary transporters. Every ABC-type system appears to be made of two hydrophobic membrane-spanning domains that are thought to constitute the translocation pathway and two hydrophilic nucleotide binding domains. In addition, prokaryotic ABC systems utilize specific binding proteins for high-affinity solute binding. Transport is driven by ATP hydrolysis (*see* Davidson & Chen, 2004 for a recent review). Secondary transporters usually contain only one polypeptide chain and function as monomers or oligomers. Solute transport is here driven by transmembrane electrochemical gradients of another solute or ion. Depending on the directions of transport of both solutes, symport and antiport processes are discriminated. Prokaryotic amino acid symporters usually employ electrochemical proton or sodium gradients to drive the translocation process. But also antiport of two amino acids or of amino acids with other organic substrates occurs.

Many of the known transporter families contain members of pro- and eukaryotic origin and so most of the currently 46 human genome-based solute carrier (SLC) gene families have prokaryotic relatives. Prokaryotic systems are in many cases easier to manipulate at the genetic level and to obtain in larger quantities than their eukaryotic counterparts. In addition, transport proteins of extremophilic prokaryotes may be more stable and rigid under laboratory conditions than proteins of mesophilic origin, a feature which may facilitate crystallization. Therefore it is without surprise that many important insights into the structural basis of membrane transport processes come from prokaryotic model systems (e.g., Doyle et al., 1998; Fu et al., 2000; Chang and Roth, 2001; Locher et al., 2002; Abramson et al., 2003; Huang et al., 2003). Along this line, the recent crystallization of a glutamate transporter homolog of the hyperthermophilic archaeon *Pyrococcus horikoshii* (Glt_{Ph}) (Yernool et al., 2004) and a sodium/leucine transporter of the bacterium *Aquifex aeolicus* (Yamashita et al., 2005) represent important steps

Correspondence to: H. Jung, LMU Munich, Department Biology, Microbiology, Maria-Ward-Strasse 1a, D-80638 Munich ; email: heinrich.jung@lrz.uni-muenchen.de

Abbreviations: EPR, electron paramagnetic resonance; TC, transporter classification according to Saier, Jr. (2000c); TM, putative transmembrane domain.

towards understanding secondary amino acid transport at the molecular level. Furthermore, several bacterial amino acid transporters participate in virulence and information on structure and mechanism may help to develop new drugs for the treatment of bacterial diseases.

This review starts with an overview on distribution, physiological roles and functional properties of prokaryotic α-amino acid transporters. The main focus is on recent achievements in research of structure and structure-function relationships of prokaryotic proton- or sodium ion-coupled amino acid symporters.

Distribution and Phylogeny

Amino acid transport is not a unique function of members of a specific protein family (Saier, Jr., 2000b). Instead, prokaryotic α-amino acid transporters are found in sixteen out of the eighty six families assigned to uniporters, symporters, and antiporters (Table 1) (*see* also http://www.tcdb.org/tcdb/). The number of families may further grow as more and more genomes are disclosed and functions to gene products are assigned. Based on current assignments, there are families containing solely amino acid transporters [e.g., the branched-chain amino acid/cation symporter (LIVCS) family; the lysine exporter (LysE) family], and families whose members differ quite dramatically in substrate specificity ranging from ions to sugars to various organic acids to specific amino acids [e.g., the sodium/solute symporter (SSS) family]. Some families contain exclusively bacterial members (e.g., LIVCS and LysE family) while others most likely have much older common ancestors and unite members of archaeal, bacterial and eukaryotic origin including man [e.g., SSS and dicarboxylate/amino acid/cation (DAACS) family].

Since amino acids are valuable carbon and nitrogen sources and may also serve other functions (*see* below), most cells have the potential to employ several different transporters for one amino acid. For example, there are at least five different uptake systems for glutamate and aspartate present in *E. coli*, differing in kinetic properties as well as in regulation.

Physiological Role

Amino acid uptake systems have to fulfill several functions in prokaryotic cells. The most prominent function is the supply with C- and N-sources for catabolic and anabolic purposes. Uptake of L-amino acids can serve its intracellular pool directly, so that the amino acids can enter protein biosynthesis, homoserine synthesis (aspartate), or purine/pyrimidine synthesis via orotate (asparagine, glutamine). Alternatively, amino acids can be oxidized via different pathways after deamination to the corresponding oxo-acid. L-Lysine and L-arginine can also be decarboxylated to the polyamines cadaverine and agmatine, respectively.

There exists a variety of amino acid/polyamine antiporters that are involved in acid stress response of enteric bacteria [*cad*, *gad*, *adi* systems; transporters belong to the amino acid-polyamine-organocation (APC) super family] (Lin et al., 1995; Jack et al., 2000). The common principle of function of these systems involves amino acid uptake followed by decarboxylation in the cytoplasm, a proton-consuming reaction that counteracts internal acidification at low external pH. During acid stress response, participating transporters catalyze antiport of the amine and its precursor amino acid. However, at neutral external pH, at least CadB can also act as a proton-coupled cadaverine uptake system (Soksawatmaekhin et al., 2004). The extent of pH stress and the availability of amino acids determine which system is used for protection of the cells. The glutamate/γ-amino-butyrate (*gad*) and arginine/agmatine (*adi*) systems participate in the extreme acid response (''XAR'') of *E. coli*, while the lysine/cadaverine system (*cad*) is preferred at moderate pH stress conditions (Iyer et al., 2003).

A similar scheme is found for histidine and aspartate uptake systems of *Lactobacillus buchneri* and *Lactobacillus* subsp. M3, respectively. The amino acids are taken up and converted by decarboxylation to histamine and alanine (Molenaar et al., 1993; Abe et al., 1996). In both reactions the carboxylate of the precursor leaves the cells after decarboxylation as neutral carbon dioxide or dihydrogen carbonate, thereby generating an electrochemical proton gradient across the cytoplasmic membrane similar as described for malolactic fermentation in *L. plantarum* (*L. lactis*) (Poolman et al., 1991). The proton gradient is then used by the F-type -ATPase to generate ATP. Especially under energy-limiting conditions (e.g., fermentation) this type of transport/energy generation is used.

Amino acid transporters are also involved in cell adaptation to osmotic stress. So, some transporters (e.g., OpuE of *B. subtilis*; ProP of *E. coli*) accumulate proline as a compatible solute, thereby participating in the ''salt-out'' strategy of osmoregulation (Wood, 1999). OpuE (SSS family, TC 2.A.21) transports proline probably in symport with sodium, and its expression is osmotically activated (von Blohn et al., 1997). ProP [major facilitator superfamily (MFS)] mediates proton-coupled proline uptake and simultaneously acts as a sensor and regulator (Racher et al., 1999; MacMillan et al., 1999). The high-affinity sodium/proline transporter PutP (SSS family) of *Staphylococcus aureus* contributes to in vivo survival

Table 1. Transport families containing prokaryotic amino acid transporters

Family name	TC number (SLC relation)	Origin of family members	Energy coupling	Examples	Membrane topology	References
Major facilitator superfamily (MFS)	2.A.1	Archaea, bacteria, yeast, fungi, plants, animals	Solute /proton symport	ProP of *E. coli*	12 TMs iN, iC[a)], coiled-coil domain	Racher et al., 1999
Amino acid-polyamine-organocation (APC) superfamily	2.A.3 (SLC7)	Archaea, bacteria, yeast, fungi, plants, animals	Solute/proton symport,	PheP of *E. coli* (AAT family)	12 TMs, iN, iC, reL[b)]?	Dogovski et al., 2003
			Aa/polyamine antiport	CadB of *E. coli* (APA family)		Soksawatmaekhin et al., 2004
Drug/metabolite transporter (DMT) superfamily	2.A.7.	Archaea, bacteria, yeast, fungi, plants, animals	Drug (metabolite)/ proton antiport	YdeD of *E. coli* (DME family)	10 TMs	Dassler at el., 2000
Solute/sodium symporter (SSS) family	2.A.21 (SLC5)	Archaea, bacteria, yeast, animals	Solute/sodium symport	PutP of *E. coli*,	13 TMs, eN, iC,	Jung, 2002
				OpuE of *B. subtilis*		Spiegelhalter and Bremer, 1998
Neurotransmitter/sodium symporter (NSS) family	2.A.22 (SLC6)	Archaea, bacteria, animals	Solute/sodium symport	LeuT of *A. aeolicus*	12 TMs, iN, iC, reL, *trans* reLs? iH[c)]	Yamashita et al., 2005
Dicarboxylate:amino acid/cation symporter (DAACS) family	2.A.23 (SLC1)	Archaea, bacteria, animals	Solute/proton (or sodium) symport	Glt of *P. horikoshii*	8 TMs, iN, iC, *trans* reLs, iH	Yernool et al., 2004
Alanine or glycine/cation symporter (AGCS) family	2.A.25	Archaea, bacteria	Alanine, glycine/ sodium (or proton) symport	DagA of *Alteromonas haloplanktis*	8 TMs	Reizer et al., 1994
Branched chain amino acid/cation symporter (LIVCS) family	2.A.26	Bacteria	Branched chain amino acid/ sodium (or proton) symport	BraB of *P. aeruginosa*	12 TMs	Reizer et al., 1994
Glutamate/sodium symporter (ESS) family	2.A.27	Bacteria	Glutamate/ sodium symport	GltS	10 TMs, eN, eC, *trans* reLs	Deguchi et al., 1990
Hydroxy/aromatic amino acid permease (HAAAP) family	2.A.42	Bacteria	Amino acid/proton symport	Mtr of *E. coli*	11 TMs, iN, eC	Sarsero et al., 1991
Tripartite ATP-independent periplasmic transporter (TRAP-T) family	2.A.56	Archaea, bacteria	Solute/proton (or sodium) symport	GtrABC of *Synechocystis* strain PCC68003	10 TMs + 4 TMs (iN, iC), SBP, heterooligomer	Kelly and Thomas, 2001
L-Lysine exporter (LysE) family	2.A.75	Bacteria	Lysine/proton symport (or lysine/ hydroxid anion antiport)	LysE of *C. glutamicum*	5(6) TMs, iN, eC?	Eggeling and Sahm, 2003
Resistance to homoserine/ threonine (RhtB) family	2.A.76	Bacteria	Amino acid/proton antiport	RhtB	5(6) TMs (LysE)	Aleshin et al., 1999
				YfiK	6 TMs	Franke et al. 2003
Branched chain amino acid exporter (LIV-E) family	2.A.78	Archaea, bacteria, protozoa	Branched chain amino acid/ proton antiport	BrnEF of *C. glutamicum*	4+7 TMs heterodimer	Trotschel et al., 2005

Table 1. Continued

Family name	TC number (SLC relation)	Origin of family members	Energy coupling	Examples	Membrane topology	References
Threonine/serine exporter (ThrE) family	2.A.79	Archaea, bacteria, eukarya	Threonine (serine)/proton antiport	ThrE of *C. glutamicum*	10 TMs ?	Simic et al., 2001
Aspartate/alanine exchanger (AAE) family	2.A.81	Archaea, bacteria	Aspartate/alanine antiport	AspT of *Tetragenococcus halophila* D10	8 TMs, eN, eC, 183 aa comprising cytoplasmatic loop	Abe et al., 2002

[a]iN, intra-cytoplasmatic N-terminus; iC, intra-cytoplasmatic C-terminus; eN, extra-cytoplasmatic N-terminus; eC, extra-cytoplasmatic C-terminus
[b]reL, re-entrant loop
[c]interrupted helices.

of this causative organism of a wide variety of human infections (Schwan et al., 1998; Schwan et al., 2004). It could be shown that insertional inactivation of the *putP* gene of *S. aureus* leads to significantly reduced virulence in experimental endocarditis (Bayer et al., 1999). This contribution to virulence is also attributed to the function of proline as an osmoprotective substance.

Besides transport, members of the SSS family show a distant similarity to the N-terminal domains of some sensor kinases of a yet uncharacterized type of bacterial two-component signal transduction system and may function as sensors and signal transducers (Jung, 2002).

Taken together, secondary transporters with specificity for amino acids participate not only in the scavenging of nutrients, but are also involved in pH-stress response, osmoprotection, detoxification, signal transduction and the generation of energy.

Functional Properties

Uptake of amino acids for catabolic purpose is mostly associated with the dissipation of an electrochemical sodium or proton gradient and is usually a symport (co-transport) mechanism enabling accumulation of amino acids inside the cell against a concentration gradient. Thereby ion/amino acid stoichiometries of 1:1 or 2:1 are most frequently found in prokaryotic systems. The affinity for amino acids of these transporters is mostly in the micromolar range (PutP, GltP, GltS). Amino acid transporters are highly selective for size, polarity, and charge of the amino acid side-chain. There are transporter families specific for small (AGCS), acidic (DAACS, ESS), basic (APA and CAT of the APC superfamily, LysE family), aliphatic (LIVCS, LIV-E), and aromatic (HAAAP, ArAE) amino acids. However, there are also transporter families containing members with quite different substrate specificities (SSS, AAT of the APC superfamily).

Structure

Knowledge of the structure of amino acid transporters is a basic prerequisite for understanding the molecular mechanism of translocation. Attributed to the difficulty of crystallizing integral membrane proteins for high-resolution structural studies, 3-D structures of only two amino acid transporters, the sodium/leucine transporter $LeuT_{Aa}$ of *A. aeolicus* and the glutamate transporter Glt_{Ph} of *P. horikoshii*, have been solved to date (Yernool et al., 2004, Yamashita et al., 2005). The crystal structures demonstrate that transporter architecture can differ considerably. The latter conclusion is also supported by the results of secondary structure analyses of various amino

acid transporters which — besides the structural diversity – also reveal common features and allow classification into different structural groups. In the following part, we provide an overview of the secondary, tertiary, and quaternary structures of prokaryotic proton- or sodium ion-coupled amino acid symporters and attempt to present characteristic features of the different structural groups.

Secondary Structure

The topological arrangement of membrane proteins can be determined by hydropathy profile analysis in combination with different experimental approaches, such as Cys accessibility analysis, generation of hybrid proteins containing topological reporters such as LacZ or PhoA, site-specific proteolysis and electron paramagnetic resonance (EPR) spectroscopy. Application of one or more of these techniques to several amino acid symporters suggests a membrane topology according to which the proteins possess 8–14 transmembrane domains (TMs) (Table 1). The most common number of hydrophobic membrane spans is twelve and is described for proteins of various TC families (Table 1). It is suggested that these transporters arose by gene duplication events of an ancestral 6 TMs coding unit (Saier, Jr., 2000a).

The proposed topologies can be classified into few basic classes of secondary structures that are distinguished, e.g., by their domain architectures and loop arrangements (Fig. 1) (cp. also Lolkema & Slotboom, 1998; Sobczak & Lolkema, 2005). The first secondary structure class of prokaryotic amino acid symporters is represented by the proton/osmoprotectant (e.g., proline, glycine betanine and ectoine) symporter ProP of *E. coli*, which belongs to the MFS superfamily (Fig. 1*A*). The characteristic feature of the topology model of the osmoregulated ProP is that it consists of N- and C-terminal domains with analogue topology but low sequence homology. Each domain encloses six helices. They are related by an approximate two-fold symmetry and connected by a large cytoplasmic loop (Wood et al., 2005). This structural arrangement of ProP, shown by homology modeling, gene fusion analysis and site-directed fluorescence labeling, reflects the membrane topology of the crystallized LacY, GlpT and EmrD of the MFS superfamily, with the exception that ProP exhibits a cytoplasmic C-terminal extension (Huang et al., 2003; Abramson et al., 2003; Wood et al., 2005; Yin et al., 2006). This extension displays an α-helical coiled coil domain, which is important for modulation of osmotic activation, dimerization and interaction with ProQ, an amplifier of osmotic activation (Hillar et al., 2005; Tsatskis et al., 2005).

The second class of secondary structures of prokaryotic amino acid symporters is represented by $LeuT_{Aa}$, a member of the NSS family (Yamashita et al., 2005) (Fig. 1*B*). Here, only the first 10 TMs of the total 12 TMs containing protein show an internal symmetry. The first five TMs are related to the next five TMs by a pseudo-two-fold axis in the plane of the membrane. But in contrast to the proposed internal structural repeat of ProP, these domains are tilted against each other by approximately 180° and are connected via a long periplasmatic loop. Although the structural repeats share the same fold and are likely to have the same genetic origin, homology of the amino sequences of the two domains could not be detected. A second feature of this secondary structure class is the existence of a re-entrant loop between the second and the third TM of $LeuT_{Aa}$, but in contrast to the re-entrant loops of the glutamate transporters of the DAACS family, a role in substrate binding for this structure is not proposed. Due to the symmetry between the first and the subsequent five TMs of this protein, one would expect a second re-entrant loop between second and third TM of the C-terminal domain (dotted line in Fig. 1*B*). However, in the crystal structure this loop does not enter the bilayer but lies on the outer surface of the membrane. Since the current structure resembles the substrate-occluded

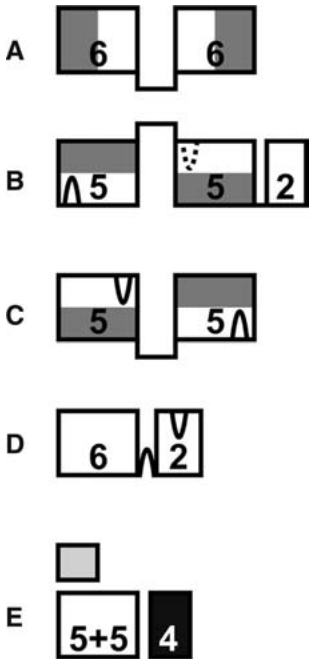

Fig. 1. Topological classes of ion-coupled amino acid symporters. Transporter domains are drawn as boxes. Numbers correspond to number of membrane-spanning segments typically found per domain. Gray and white colors reflect the orientation of symmetric domains relative to each other. Loops connecting domains as well as re-entrant loops are shown as lines. Representative members of the topological classes are: (*A*) ProP (MFS family). (*B*) $LeuT_{Aa}$ and TnaT (NSS), PheP, LysP, GabP and AroP (APC), PutP (SSS), BraB and BrnQ (LIVCS), Mtr, TnaP and TyrP (HAAAP). (*C*) GltS (ESS) and GtrB of GtrABC (TRAP-T). (*D*) GltP, GltT and Glt_{Ph} (DAACS). (*E*) GtrABC (TRAP-T).

state, it is possible that this loop re-enters the membrane when the conformation of the symporter is changed during the transport cycle. Furthermore, the crystal structure of $LeuT_{Aa}$ shows interrupted, expanded and to the membrane normal tilted helices, thereby demonstrating that the hydrophobic membrane-spanning helices of the prokaryotic amino acid symporters are by no means as regular as it is supposed in several topology models.

For the sodium/glutamate symporter GltS of *E. coli* (ESS family, TC 2.A.27), no experimental structural information is published, but hydropathy profile analysis and family hydropathy profile alignments propose a fold similar to the better structurally characterized citrate/cation symporter family (CSS, TC 2.A.24) (Lolkema & Slotboom, 2003; Lolkema et al., 2005). However, in contrast to the CSS family the suggested topology of GltS misses the first TM present in the CSS family and therefore exhibits ten TMs.

GltS represents the third class of secondary structures of prokaryotic amino acid transporters and is characterized by two homologous N- and C-terminal domains with inverted membrane topology (Fig. 1*C*). Each of these domains contains five helices and one re-entrant loop or pore loop. The domains are connected by a large cytoplasmic loop, and the re-entrant loops are arranged in *trans* while entering the membrane from opposite directions. In GltS, the membrane-entering loops are proposed to be located between the fourth and fifth TMs of each domain respectively.

Re-entrant loops were also found in glutamate transporters of the DAACS family (TC 2A.23) (e.g., GltP of *E. coli*, GltT of *Bacillus stearothermophilus*, Glt_{Ph} of *P. horikoshii*). These transporters represent the fourth class of topological assembly of prokaryotic amino acid symporters (Fig. 1*D*). In comparison to the other secondary structure classes, this group has the smallest number of membrane-spanning α-helices. They are arranged in two parts: the N-terminal domain consisting of six TMs and the C-terminal domain containing two TMs (Slotboom et al., 2001). Interestingly, the domains of this topology class show neither any sequence homology nor a structural symmetry, and hence differ remarkably from the other classes. The recently resolved 3-D crystal structure of the glutamate transporter homologue Glt_{Ph} of *P. horikoshii* confirms this topology arrangement and the existence of the two previously suggested *trans* re-entrant loops (Yernool et al., 2004). These loops are located after the sixth and seventh TM in the C-terminal domain of Glt_{Ph} and are composed of a helix-turn-helix motif. Like $LeuT_{Aa}$, the high-resolution structure of Glt_{Ph} shows nonregular TMs with interrupted, kinked and/or tilted helices.

In contrast to the previously described TC families, which can be easily classified into the mentioned secondary structure classes, the prokaryotic amino acid transporters of the APC (TC 2.A.3), SSS (TC 2.A.21), LIVCS (TC 2.A.26), and HAAAP (TC 2.A.42) families are not so well structurally characterized, and therefore, the classification of these symporters is much more difficult. Based on similarities of hydropathy profiles of different secondary transporters, Lolkema and Slotboom developed the MemGen classification system, that clusters amino acid symporters of these five TC families into one structural class (class 2) together with transporters of the NSS family (Lolkema and Slotboom, 1998) (Fig. 1B). But it is not yet experimentally proven whether the topologies of these families are indeed similar to the secondary structure of $LeuT_{Aa}$. Analysis of the human cysteine/glutamate antiporter xCT (TC 2.A.3.8) by biotinylation and MTS reagent labeling hint at a structural motif similar as described for $LeuT_{Aa}$ (Gasol et al., 2004). The studies indicate that xCT contains a re-entrant loop-like structure between the second and third TM similar as described for $LeuT_{Aa}$, but it is not known whether any prokaryotic member of the APC and the other families also possess this pore-loop structure. What we can clearly say is that the experimentally determined number of transmembrane domains among this group differs from 11 (e.g., Mtr, tryptophan transporter of *E. coli*, TC 2.A.42.1.2) to 13 (PutP, sodium/proline symporter, TC 2.A.21.2.1). The 13-TM motif is proposed to be the common structural motif of members of the SSS family (TC 2.A.21) and was experimentally established by a number of genetic and biochemical approaches (Jung, 1998; Jung et al., 1998; Wegener et al., 2000).

A further remarkable amino acid transporter is the sodium/glutamate symporter system GtrABC of *Synechocystis* (TRAP-T family, TC 2.A.56). It consists of three components, a membrane protein with ten putative TMs (GtrB); a small membrane protein with 4 TMs (GtrA) and a periplasmatic binding protein (GtrC) (Quintero et al., 2001; Thomas et al., 2006; Kelly and Thomas, 2001) (Fig. 1*E*). Although GtrB belongs like GltS to structural class 3 (Fig. 1*C*) according to the MemGen classification system, we assign this kind of transport system to a fifth topology class because of its exceptional heterooligomeric composition that represents one functional unit (Fig. 1*E*).

Tertiary Structure

The crystal structure of $LeuT_{Aa}$ shows a pseudo-symmetry in its tertiary structure which is based on the N-terminal ten TMs that display two internal structural repeats with inverted topology (Yamashita et al., 2005). Thereby, the helices are arranged in such a symmetrical manner that the folds of the α-carbon backbone of these two domains show an approximate

concordance by rotation of about 180°. The core of the protein comprises the ligand binding site and consists of four helices of which two are interrupted helices that are oriented antiparallel to one another and two are tilted by about 50° from the membrane normal and are also related by a pseudo-two-fold axis. The remaining TMs surround this core in support of this helix arrangement and are fractionally involved together with the protein core and two α-helix-containing loops in the formation of an extracellular aqueous cavity that protrudes into the bilayer but does not penetrate it. On the bottom of this hydrophilic cleft lies the ligand-binding pocket that is presumed to be occluded in the current structure, while the extracellular and intracellular gates are closed.

Compared to $LeuT_{Aa}$, the high-resolution structure of Glt_{Ph} shows a completely different fold (Yernool et al., 2004). The N-terminal six TMs of Glt_{Ph} form a cylindrical coat, surrounding the C-terminal part of the protein that establishes the substrate binding site and consists of one interrupted and one continuous α-helix and of two re-entrant loops. The tips of the *trans* re-entrant loops interact halfway across the membrane at the bottom of an extracellular hydrophilic cavity that is formed by the crystallized trimer of Glt_{Ph}. Due to this aqueous basin the re-entrant loops and therefore the ligand binding sites are exposed to the solvent and can be easily accessed by substrate and co-ions when the protein is not in the occluded state.

Information on the tertiary structure of other amino acid transporters is only fragmentary and comes from protein chemical and spectroscopic studies. For example, Cys scanning mutagenesis of the sodium/proline symporter PutP of *E. coli* in combination with Cys accessibility analyses of two TMs (TM II and TM IX) probably involved in ligand binding suggest a hydrophilic cleft that is open to the cytoplasmic side of the membrane and extends from the cytoplasmic ends of TM II and IX to the middle of the domains (Pirch et al., 2002; D. Hilger, M. Böhm, A. Hackmann and H. Jung, manuscript in preparation) (*see* Fig. 3). This inward-facing conformation was also shown in the crystal structures of the lactose permease LacY and the glycerol 3-phosphate transporter GlpT of *E. coli* and represents probably the predominant conformation in the absence of ligands and a membrane potential (Abramson et al., 2003; Huang et al., 2003). Analyses of the tertiary structure of PutP by Cys cross-linking revealed an at least temporal proximity of TM II and IX (D. Hilger, M. Böhm, A. Hackmann and H. Jung, manuscript in preparation). Furthermore, intramolecular distance determination by site-directed spin labeling in combination with pulse EPR techniques provides for the first time information on distances between amino acid positions in the tertiary structure of the protein (Jeschke et al., 2004). The studies reveal distances between positions in cytoplasmic and periplasmic loops that are typical for a location on opposite sides of the membrane (∼ 5 nm) and distances between intracellular loops, that are in agreement with a location on the same side of the membrane (∼ 2 nm).

Other experimentally determined data of tertiary structures of amino acid transporters are rare. Intramolecular cross-linking of TM VII and TM VIII of the tryptophan transporter TnaT of *Symbiobacterium thermophilum* suggests a close spatial proximity of these transmembrane domains, which is in accordance with the crystal structure of $LeuT_{Aa}$ of the same TC family (NSS, 2.A.22) (Kniazeff et al., 2005). Second-site suppression studies by replacement of highly conserved small residues with larger side chains and complementary changes in other transmembrane domains of the phenylalanine and tyrosine transporter PheP of *E. coli* indicate an interhelical interaction between the functionally important TMs I and II and also between TMs I and III (Dogovski et al., 2003). To our knowledge, the latter data are the sole information on the tertiary structure of prokaryotic amino acid transporters of the APC superfamily.

Quaternary Structure

Protein dimerization or oligomerization is a common structural feature of many different types of membrane proteins (e.g., receptors, channels, transporters), but due to the difficulty to rigorously address the oligomeric state of secondary transporters only little information is available to date about oligomeric states of amino acid transporters (Veenhoff et al., 2002).

For many proton- or sodium ion-coupled amino acid symporters it is presumed that they function as monomers [e.g., PutP of *E. coli* (Jung, 2002)], but there are also several examples of proteins that exhibit a higher oligomeric state. $LeuT_{Aa}$, for example, forms dimers in the crystal with a two-fold axis perpendicular to the membrane (Yamashita et al., 2005). The dimer interface between two parallel orientated protomers is composed of one α-helix-containing loop that is also involved in the formation of the hydrophilic cleft, and two helices of the C-terminal part of $LeuT_{Aa}$. Thereby, the two transmembrane helices of each protomer interact together to form a four-helix bundle. Oligomer formation has also been reported for eukaryotic relatives of $LeuT_{Aa}$ (NSS family), thereby a dimer of dimers may be formed under physiological conditions (Sitte et al., 2004; Just et al., 2004)

Another amino acid transporter that presumably forms a homodimer is ProP of the MFS superfamily (Hillar et al., 2005). ProP contains a C-terminal extension that is characteristic of α-helical coiled coil-

forming proteins. EPR and NMR measurements of a peptide corresponding to this extension confirm the formation of a homodimeric, antiparallel α-helical coiled coil stabilized by salt-bridges (Hillar et al., 2003; Zoetewey et al., 2003). Furthermore, in vivo cross-linking analysis of ProP suggests that ProP dimerizes by coiled-coil formation by the C-terminal extension and/or by interactions between other parts of the protein facilitating C-terminal coiled-coil formation (Hillar et al., 2005). Interestingly, the dimerization is required for activation of ProP at low osmolarity and therefore has probably a stabilizing effect on the active conformation of ProP under these conditions (Hillar et al., 2005).

The best investigated prokaryotic amino acid transporters with respect to the quaternary structure are the glutamate transporters of the DAACS family. The crystal structure of Glt_{Ph} shows that the transporter forms a trimer with a triangular shape and a three-fold symmetry axis perpendicular to the membrane (Yernool et al., 2004). The three protomers of Glt_{Ph} form a large extracellular basin that protrudes from the membrane up to 15 Å. The interface between individual Glt_{Ph} molecules in the trimer is composed of three TMs of the N-terminal helix bundle. Thereby, one transmembrane domain concatenates the intersubunit contacts between the two remaining TMs in one protomer and with a helix of the same TM motif in an adjacent protomer. The trimeric quaternary structure is proven by cross-linking analysis of Glt_{Ph} in a non-crystalline environment (Yernool et al., 2003; Yernool et al., 2004). Trimer formation has also been shown for other glutamate transporters of the DAACS family. So, the trimeric state of GltT of *Bacillus caldotenax* and *B. stearothermophilus* and GltP of *E. coli* was determined by several methods, including, e.g., cross-linking analysis, mass spectrometry, size-exclusion chromatography, and blue native polyacrylamide gel electrophoresis (Gendreau et al., 2004).

The homooligomeric organization of amino acid symporters may fulfill several functions, including stabilization of distinct conformations, modulation of transporter activity and formation of an aqueous "waiting area" for transported solutes (Glt_{Ph}) (Hillar et al., 2005; Kavanaugh, 2004). However, each protomer contains a ligand binding site and is most likely capable to form a translocation pathway, suggesting that each monomer constitutes a functional unit (Yernool et al., 2004; Yamashita et al., 2005).

In contrast to the homooligomers of $LeuT_{Aa}$, ProP and the glutamate transporters of the DAACS family, the GtrABC transport system of the TRAP-T family shows a heterooligomeric structure of two integral membrane units and one periplasmatic solute-binding protein (SBP) (Kelly and Thomas, 2001; Driessen et al., 2000). Thereby, the larger membrane protein (GtrB) and the smaller integral membrane subunit (GtrA) probably build up a two-domain structure. Due to its homology to other secondary transporters, GtrB could be responsible for the actual transport reaction and energy coupling, whereas GtrA might be important for the interaction with GtrC, the substrate binding protein (SBP). The special heterooligomeric structure of the GtrABC system may facilitate control of the directionality of the transport process and allows scavenging of low-concentration substrates due to the high affinity of the SBP (Kelly and Thomas, 2001; Driessen et al., 2000).

Structure-Function Relationships

Besides information on the different structural levels, understanding membrane transport proteins at work requires the assignment of structural elements to specific tasks and the analysis of the dynamics of interactions of these elements during the transport cycle. The best effort was made in the last decade to relate structure to function despite the fact that high-resolution structures had not been available for a long time. The studies include, for example, random and site-directed mutagenesis, comprehensive analyses of functional consequences of amino acid substitutions, affinity and site-directed labeling techniques in combination with a set of powerful spectroscopic methods. The following part attempts to summarize the current knowledge on structural elements participating in binding of coupling ion and/or substrate in prokaryotic α-amino acid transporters, and reports on recent developments regarding investigation of transporter dynamics relevant for function.

Sites Involved in Binding of the Coupling Ion

The most comprehensive information on sites liganding coupling ions is provided by the 3-D structure of $LeuT_{Aa}$ which shows the protein with two sodium ions bound (Yamashita et al., 2005) (Fig. 2A). Both ions are buried inside the protein in cavities located in about the middle of the membrane. The cavities are each formed by five polarized oxygen atoms which make direct contact to the sodium ions. The oxygen originates from main-chain carbonyl groups, side-chain hydroxyl and amide groups. In addition, one of the two sodium ions is liganded by a sixth group, which is the negatively charged carboxylate of the substrate leucine (Gouaux and MacKinnon, 2005). The described arrangement is similar to the composition of the sodium binding sites of the structurally and functionally unrelated c subunits of sodium-dependent ATPases (Meier et al., 2005; Murata et al., 2005) (Fig. 2B). The described sodium binding sites share the following common features: i) each sodium ion is in direct contact with four to six oxygen atoms originating from main-chain carbonyl and polarized

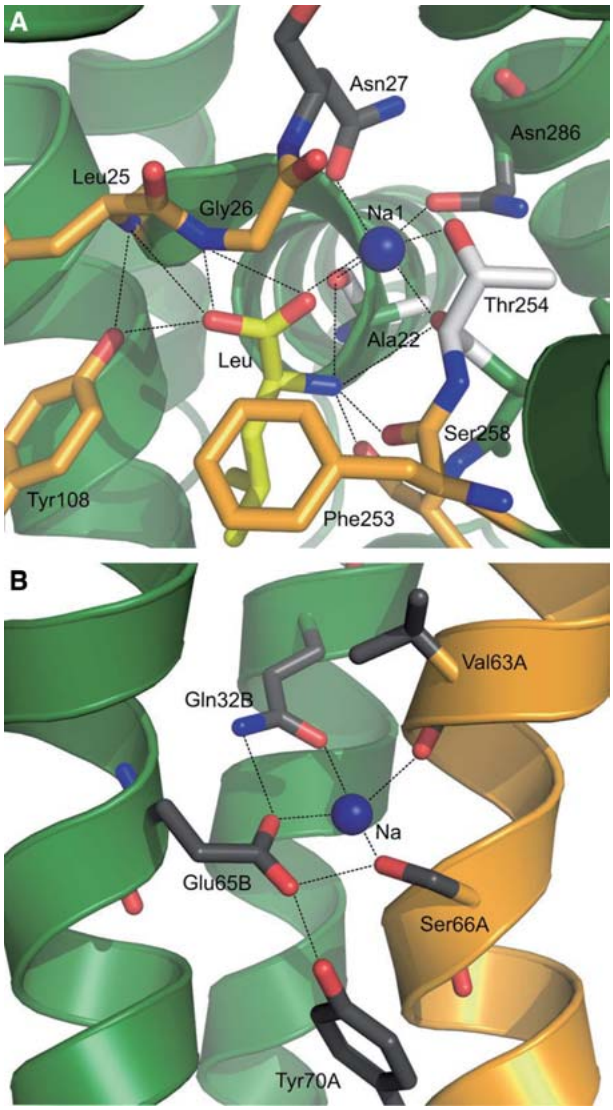

Fig. 2. Known structures of sodium binding pockets. (*A*) Structure of the sodium binding site 1 of $LeuT_{Aa}$, viewed along the membrane normal from the extracellular site. The helices in the ribbon representation of the Na1 binding site are colored in green. The blue sphere represents the bound Na^+ ion. For clarity, bound leucine and the residues involved in sodium binding are shown as stick models. Residues involved in sodium binding are colored in grey, residues exclusively participating in leucine binding in orange, and residues involved in ion and solute binding are colored in white. Hydrogen bonds and ionic interactions are depicted as dashed lines. (*B*) Structure of the sodium binding site between two sub-units of the c ring of the F-type Na^+-ATPase from *Ilyobacter tartaricus*. Subunits that build up a three-helix bundle representing a functional unit responsible for sodium binding are shown in different colors. The view is parallel to the membrane from the external surface of the c ring. The blue sphere represents the bound sodium ion and the residues involved in sodium binding are shown as stick models. Hydrogen bonds and ionic interactions are depicted as dashed lines. Structures were generated with program PyMOL (DeLano, 2002) and are based on the crystal structures of $LeuT_{Aa}$ of *Aquifex aeolicus* (Yamashita et al., 2005) and the c ring of the sodium ion–translocating ATP synthase of *Ilyobacter tartaricus* (Meier et al., 2005).

side-chain groups or substrate; ii) the binding cavities match the size of the dehydrated sodium ion with sodium to oxygen distances between 2.2 and 2.4 Å; iii) a negative charge may contribute to sodium binding but is not essential; and iv) when negatively charged side-chains participate in binding they are kept deprotonated at physiological pH by additional hydrogen bonds.

Does this sodium binding scenario also apply to other transporters? Structure-function analyses of the sodium/proline transporter PutP of *E. coli* (SSS family) provide evidence for a sodium binding scenario similar to that described for $LeuT_{Aa}$. PutP has a sodium to proline stoichiometry of 1 to 1 and is consequently supposed to possess only one sodium binding site (Jung, 2001; Wilson & Ding, 2001). Residues proposed to directly participate in sodium binding are Asp55, Ser57 (TM II), Ser340 and Thr341 (TM IX). The residues are conserved within the SSS family. Individual substitution of these residues reveals that the side chains at these positions are particularly important for binding of the coupling ion (Quick et al., 1996; Quick & Jung, 1997; Pirch et al., 2002). Thus, removal of the hydroxyl group at positions 57, 340 or 341 led to a decrease of the apparent sodium affinity by two to three orders of magnitude. The carboxylate of Asp55 proved to be essential for transport, and significant, albeit highly reduced, activity was detected only with Glu at this position (Quick & Jung, 1997). The latter substitution caused an about 50-fold decrease of the apparent affinity of the protein for sodium. Furthermore, pairs of Cys residues placed at various positions in TM II and IX could efficiently be crosslinked, indicating an at least temporal proximity of both domains. Finally, analysis of the accessibility of single Cys individually placed at different positions in TM II and IX indicates a participation of both TMs in the formation of a hydrophilic pore or cleft (Pirch et al., 2002; D. Hilger, M. Böhm, A. Hackmann and H. Jung, manuscript in preparation) (Fig. 3). Taken together, the data strongly support the idea that amino acids of TM II and IX of PutP contribute to the formation of the ion translocation pathway of PutP. So far, convincing evidence for a participation of main chain carbonyl groups of PutP in sodium binding is not available. The latter groups are more difficult to identify as the side-chains of corresponding amino acids are not necessarily highly conserved and are not required to possess specific properties (e.g., charged or polar groups). In $LeuT_{Aa}$ the main-chain carbonyl groups belong to Ala, Gly, Thr, and Val (Yamashita et al., 2005) suggesting a preference for main-chain carbonyl groups of small and apolar amino acids for ion liganding. In PutP, replacement of conserved Ala48 or Gly63 (TM II) impairs transport. Thus it is tempting to speculate that the main-chain carbonyl

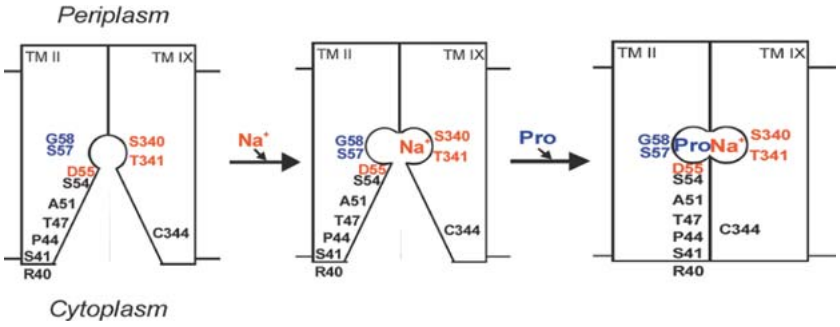

Fig. 3. Model showing the participation of amino acids of TMs II and IX of the sodium/proline transporter PutP of *E. coli* in the formation of a cytoplasmically exposed aqueous cavity. Given amino acid positions are accessible to polar sulfhydryl reagents in the absence of ligand. Colored residues are proposed to directly participate in sodium and/or proline binding. It is suggested that sodium binding induces a conformational alteration that exposes Ser57 and Gly58 to the cavity. Subsequent binding of proline closes the cavity.

group of one of these residues (or both) contributes to sodium binding.

One of the prominent features of the sodium binding sites of secondary transporters described above arises from the fact that sodium binding occurs at the interface of the N- and C-terminal domains. Cys accessibility analyses and double spin-labeling in combination with EPR spectroscopy of PutP suggest that both domains can undergo relatively large conformational alterations, including movements relative to each other. Sodium seems to be required for coordination of both domains and formation of a high-affinity substrate binding site. This scenario may in fact be true for transporters like PutP, which strictly depend on sodium as coupling ion (Tsuchiya et al., 1984). In other transporters, sodium does not appear to play such a central coordinating role, as it can easily be replaced by a proton under certain conditions (e.g., in the sodium/glutamate transporter GltT of *B. stearothermophilus*), implicating that the structural differences between sodium- and proton-binding pockets are small (Gaillard et al., 1996). In the latter case, it can be envisaged that, e.g., protonation of a single negative group within the ion binding pocket is sufficient to reorganize existing hydrogen-bridge and ion-pair networks within the transporter, thereby ensuring coordination of the different functionaly important domains, as suggested for the proton/β-galactoside transporter LacY (Kaback, 2005). Instead of group protonation, hydronium ions could occupy the ion pocket in the absence of sodium and take over the coordination function as suggested by Boyer (1988).

Proton translocation in proton-coupled symport systems appears to involve predominantly His and an acidic amino acid residue, as demonstrated for the lactose permease (LacY) (Kaback, 2005). Conserved acidic amino acid side-chains are also crucial for the function of various proton-dependent amino acid transporters. In the phenylalanine transporter PheP (APC superfamily), for example, two Glu residues proved essential for transport and may participate in intramolecular salt-bridge formation and/or proton translocation (Pi et al., 1998). In general, it is assumed that hydrogen-bonding networks, the so-called ''proton wires'', support proton conduction inside proteins. In addition to negatively charged amino acids, backbone carbonyls and hydroxyl groups may participate in these networks (Nachliel & Gutman, 2001; Abramson et al., 2003; Leiderman et al., 2006).

Regions Important for Amino Acid Binding

All α-amino acids contain an amino group and a carboxyl group bonded to the α-C atom and oppositely charged (zwitterionic) at neutral pH. Both groups are crucial for substrate specificity (e.g., discrimination of amino acids from carboxylic acids or amines) and affinity of respective transporters. However, information on residues involved in binding of this characteristic group is limited and does currently not allow elucidation of common principles of interaction of amino acid transporters with the zwitterionic structure. Most comprehensive insights into amino acid binding come again from the crystal structure of $LeuT_{Aa}$ (Fig. 2*A*). It shows that the charged α-substituents of leucine are predominantly exposed to main-chain atoms of unwound regions of TMs I and VI, thereby exploiting α-helix dipole moments while charged side-chains of the transport protein are not involved in binding (Yamashita et al., 2005). The amino group of leucine is coordinated by main-chain carbonyl oxygen of three amino acids and the side-chain hydroxyl of a Ser residue. The carboxyl group interacts with amide nitrogen of two amino acids, the hydroxyl of a Tyr residue, and with sodium (Yamashita et al., 2005).

Besides the α-substituents, also the side-chains of the amino acid substrates are important for substrate binding. In fact, most amino acid transporters are highly selective for size, polarity, and charge of the amino acid side-chain. As a consequence, the respective substrate binding domains of the individual transporters must contain charged, polar and/or hydrophobic residues, allowing specific interaction of the transporter with the amino acid side-chain. In the case of $LeuT_{Aa}$, a hydrophobic pocket formed by side-chain atoms of Ile, Phe, Ser, Tyr, and Val residues located on TMs III, VI, and XIII accommodates

the hydrophobic side-chain of the substrate leucine (Yamashita et al., 2005). Alteration of size and shape of these residues affects the volume of the binding pocket and is most likely the reason for the different substrate specificities (e.g., leucine, glycine, serotonin) observed within the NSS family (Yamashita et al., 2005).

In the case of PutP, the proline binding site apparently consists of a hydrophobic pocket that accommodates C3, C4, and C5 of the pyrrolidine ring. Both 4- and 5-membered rings fit into the substrate binding pocket, but 6-membered rings are excluded. In addition, carboxyl group and imino groups are essential for high-affinity binding (Liao & Maloy, 2001). The proline binding pocket is most likely formed by distinct regions within the N- and C-terminal domains of PutP, as indicated by the clustering of mutations causing altered substrate specificity (Dila & Maloy, 1986). In fact, site-specific amino acid substitution analyses identified Ser57, Gly58 (TM II) and Thr341 (TM IX) as particularly crucial for proline binding. Alteration of the side chain of either one of these residues decreases the apparent proline affinity by up to two orders of magnitude (Quick et al., 1996; Pirch et al., 2002; D. Hilger, M. Böhm, A. Hackmann and H. Jung, manuscript in preparation). All three residues are accessible from the water phase and most likely located in a hydrophilic cleft in membrane (Fig. 3). Positions 57 and 58 are protected by proline (Pirch et al., 2003). Furthermore, current evidence suggests that binding of sodium and proline occurs in close proximity, therefore the coupling ion and the carboxyl group of proline may directly interact. This idea is supported by the following observations: i) sodium and proline bind cooperatively to PutP, ii) residues at positions 57, 58 and 341 are crucial for proline AND sodium binding, and iii) reduction of the carboxyl group of proline to a hydroxyl group (prolinol) caused an about 500-fold reduction of the apparent proline affinity.

Intimate interaction between ion-binding and substrate-binding sites has been observed also for other secondary transporters independent from substrate specificity and family membership (e.g., for members of the DAACS family (Slotboom et al., 1999) or the MFS superfamily (Pourcher et al., 1991). In fact, close proximity or even direct interaction between coupling ion and substrate may be the key to ensure tight coupling and efficient use of energy stored in electrochemical ion gradients.

Conformational Alterations Related to Transporter Function

Known 3-D structures show individual proteins only in a single conformation which corresponds to the transporter either in a state open to the cytoplasm (LacY, GltP) (Abramson et al., 2003; Huang et al., 2003) or in a state with the ligand binding pocket occluded (access from cytoplasm or periplasm closed) ($LeuT_{Aa}$, Glt_{Ph}) (Yernool et al., 2004; Yamashita et al., 2005;). However, it is clear that function of secondary transporters by alternating access of ligand binding pockets requires comprehensive conformational changes allowing opening of the gate at one or the other side of the membrane. So, based on the crystallographic snap shots, these changes have been proposed to involve rotation of the N- and C-terminal domains relative to each other in the plane of the membrane and/or changes of structure and tilt of individual TMs (Abramson et al., 2003; Yamashita et al., 2005) or movement of re-entrant loops (helical hairpins) and surrounding structures (Yernool et al., 2004).

Physical evidence for the tremendous conformational flexibility of secondary transporters comes from protein chemical and spectroscopic studies. Stimulated by the pioneering work on LacY (cp. e.g., Guan & Kaback, 2006), Cys labeling and spectroscopic techniques are now being applied for the investigation of structure and structural dynamics of a wide variety of transport proteins. The following paragraph summarizes results obtained with the model system PutP using some of these techniques.

Analyses of changes of Cys accessibility or spectroscopic properties of site-specifically attached fluorescent or paramagnetic groups have led to the identification of sites directly involved in sodium and/or proline-induced conformational alterations (Wegener et al., 2000; Pirch et al., 2002; Pirch et al., 2003; Pirch und H. Jung, unpublished information). These sites are distributed almost over the entire protein, indicating that ligand binding does not only affect intramolecular interactions in the immediate vicinity of the ligand binding pocket but induces also long-range structural alterations. The functionally important TMs II and IX of PutP are involved in these alterations. More precisely, Cys accessibility analyses suggest that both TMs participate in the formation of an aqueous cavity in the membrane, which is open to the cytoplasm under the conditions of the experiment (e.g., in the absence of a membrane potential). The cavity allows sodium binding to residues located in the middle of the membrane (e.g., Asp55 of TM II, Ser340, Thr341 of TM IX) (Fig. 3). Sodium binding in turn induces a conformational alteration which makes Ser57 and Gly58 accessible to the aqueous phase and leads to high-affinity proline binding at or close to these residues. In the ternary complex the hydrophilic cavity appears to be closed, as suggested by the inaccessibility of Cys residues placed along the cytoplasmic halves of TMs II and IX (Pirch et al., 2002; Pirch et al., 2003; Pirch and H. Jung, unpublished information) (Fig. 3). An opening of a new cavity, e.g., at the periplasmic side of the membrane, could not be detected yet. So it is

possible that the investigated ternary complex represents an occluded state of the transporter similar as shown in the crystal structure of $LeuT_{Aa}$.

Ligand-induced conformational alterations of PutP involve also intramolecular charge movements, as suggested by the results of solution exchange protocols combined with solid-supported membranes (Zhou et al., 2004). It was demonstrated that sodium and/or proline individually or together induce a displacement of charge. This was assigned to an electrogenic sodium and/or proline binding process at the cytoplasmic face of the enzyme with a rate constant of $k > 50\ s^{-1}$, which proceeds at the rate-limiting translocation step. Kinetic analysis of the electrical signals indicates that sodium and proline binding sites interact cooperatively with each other. Furthermore, binding of the electroneutral (zwitterionic) proline proceeds in a two-step process that involves an electrogenic conformational transition.

Intriguing yet unanswered questions concern the extent and directions of domain movements in secondary transporters. Clearly, crystallization of individual transporters in different conformational states would help a lot to answer these questions. However, all secondary transporters crystallized only in a single conformation so far. A powerful, emerging alternative in the investigation of protein dynamics is provided by double spin labeling in combination with continuous-wave (cw) (distances between 0.8 and 1.8 nm) or pulsed electron paramagnetic resonance (EPR) techniques (distances between 2 and 6 nm) (Pannier et al., 2000; Columbus & Hubbell, 2002; Jeschke, 2002; Steinhoff, 2004). Cys residues modified by commonly used nitroxide spin labels have about the size of tryptophan and are in general relatively well accommodated by proteins. Still, sites of labeling of secondary transporters have to be carefully selected to minimize inhibition of the conformational flexibility of the proteins. Using PutP and the sodium/proton antiporter NhaA as model systems it is demonstrated that four-pulse double electron-electron resonance (DEER) can successfully be applied to determine intra- and intermolecular distances in secondary transporters and their oligomers (Jeschke et al., 2004; Hilger et al., 2005). Cw EPR and DEER are now being used to systematically investigate interhelical distances of PutP and the influence sodium and proline have on these distances.

Common Mechanistic Principles

Available information on membrane topology, structure and structural dynamics of secondary transporters strongly supports an alternating access mechanism according to which ion and amino acid binding site(s) are alternately exposed to one side of the membrane or the other via structural rearrangements within the transport proteins. In this process there is no open amino acid channel going all through the membrane at any time. Instead, at minimum one gate is closed at all steps of the transport cycle. Furthermore, kinetic analyses suggest an ordered binding mechanism according to which sodium binds to the empty transporter first, thereby inducing a conformational alteration that increases the affinity of the transporter for the solute. The ternary complex reorientates in the membrane, leading to release of coupling ion and substrate on the other side of the membrane. This scenario does not exclude low-affinity binding of substrate in the absence of the respective coupling ion, as recently demonstrated for PutP (Zhou et al., 2004). Both N- und C-terminal halves of the transporter appear to be required for the formation of the translocation pathway. Ligand binding pockets are located in about the middle of the membrane and are reached via hydrophilic cavities that extend to either one of the membrane surfaces. Ion selectivity is ensured by polar binding pockets which perfectly match the size of the dehydrated coupling ion. Finally, tight coupling of ion and substrate transport appears to be ensured by close vicinity or even overlapping of respective binding sites.

Perspectives

The recent success in crystallizing secondary transporters, including amino acid transporters, in combination with structure-function analyses have tremendously stimulated research on molecular mechanisms underlying amino acid transport. Nonetheless, we are still far away from a complete understanding of ion/substrate symport at the molecular level. There are large families such as APC, SSS and others for which crystal structures are not available yet. Furthermore, and most importantly, more comprehensive and detailed information on protein dynamics relevant for function needs to be obtained. Besides trials to crystallize individual transporters in different conformational states, site-directed labeling in combination with spectroscopic measurements such as EPR and FTIR spectroscopy are very powerful techniques to obtain information on the nature and the extent of the changes in high time resolution. Further development of the latter techniques may also allow further exploration of the role of membrane potential for stabilization of conformational states. Finally, even with crystal structures at hand, it is obvious that structure-function analyses have to be extended in order to understand the role of individual protein domains.

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