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The Na^+/L-proline transporter PutP

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TABLE OF CONTENTS

1. ABSTRACT

The Na^+/L-proline transporter PutP is a member of the Na^+/solute symporter family (TC 2A.21, SLC5), which contains several hundred proteins of pro- and eukaryotic origin. Within the family, the capability of L-proline uptake is restricted to proteins of prokaryotes. PutP contributes to the use of L-proline as a nutrient. In addition, the transporter may supply cells with compatible solute during adaptation to osmotic stress. Based on these and other functions, PutP is of significance for various bacteria-host interactions including the virulence of human pathogens. A homology model of *Escherichia coli* PutP was generated based on the crystal structure of the *Vibrio parahaemolyticus* Na^+/galactose symporter. According to the model, PutP has a core structure of five plus five transmembrane domains forming an inverted repeat similar as originally revealed by the crystal structure of the Na^+/leucine transporter LeuT. The homology model is experimentally verified by Cys cross-linking and site-directed spin labeling in combination with electron paramagnetic resonance spectroscopy. The putative sites of Na^+ and L-proline binding are described, and a putative transport mechanism is discussed.

2. INTRODUCTION

L-Proline is an amino acid of particular significance. It is the only proteinogenic amino acid in which the nitrogen attached to C_{alpha} forms a secondary amine with an aliphatic side chain. In proteins, the resulting rigid ring system has a specific impact on structure and may serve, for example, as alpha-helix breaker (1). Also free L-proline carries out important functions in cells of all branches of life. It may serve as i) source of carbon, nitrogen and energy (2-4); ii) compatible solute during adaptation to conditions of high osmolality (5, 6); iii) regulator of cell metabolism (7); iv) scavenger of reactive oxygen species (8, 9); and v) modulator of the intracellular redox environment (10). The free amino acid does not only occur intracellularly, but is also found on inner and outer surfaces of higher eukaryotes, for example, in the gastric juice, urine, the gut of mammals and nematodes, the insect fat body, and plant exudates in concentrations ranging from micromolar to millimolar (11-15). In view of the abundance and different functions of L-proline, it is not surprising that bacteria employ various L-proline-specific transport systems and enzymes allowing utilization of external L-proline. These systems play important roles in

adaptation of single cell organisms to steadily changing environmental conditions as they occur in soil, water and during interactions with eukaryotic hosts. For example, enteric bacteria like *Escherichia coli* contain three L-proline transport systems (PutP, ProP, ProU) which differ in substrate specificity, mechanism of energy coupling, and physiological role. PutP is a high affinity transporter specific for L-proline and involved in L-proline catabolism (16-18). ProP is less specific and catalyzes the uptake of L-proline and betaines with low affinity. It functions both as an osmosensor and transporter (19-21). ProU (ProWXV) transports L-proline and betaines, and is like ProP involved in cell adaption to osmotic stress (5, 22). PutP and ProP are secondary transporters, while ProU is an ABC-type transporter. The transport and enzymatic functions of these proteins are conserved among different bacteria and archaea, whereas the genetic organization and regulatory mechanisms are diverse, and the physiological significance varies. This review focuses on the properties and physiological roles of PutP in bacteria, and summarizes the current state of knowledge of the transporter structure and molecular mechanism of function. Since PutP and other Na^+/solute transporters are assumed to share a structural fold originally described for the bacterial Na^+/leucine transporter LeuT (23), known structure-function relationships of PutP are discussed in relation to the information obtained from crystal structures of LeuT and structurally similar proteins.

3. PUTP – A MEMBER OF THE NA^+/SOLUTE SYMPORTER FAMILY

The Na^+/L-proline transporter PutP is a member of the Na^+/solute symporter (SSS) family (TC 2A.21, SLC5), which contains several hundred proteins of pro- and eukaryotic origin (4, 24, 25). These proteins have the capability to couple an electrochemical Na^+ gradient with the transport of solutes like glucose, nucleosides, L-proline, pantothenate, or iodide. Within the SSS family, the capability of L-proline uptake is restricted to proteins of bacteria and archaea.

PutP of *E. coli* is the best characterized bacterial L-proline transporter of the SSS family. It catalyzes the symport of Na^+ and L-proline with a stoichiometry of 1:1 (17). Na^+ can be substituted by Li^+, however despite earlier assumptions H^+-driven L-proline uptake by PutP could not be demonstrated (17, 26, 27). Kinetic and ligand binding analyses identify PutP as a high affinity L-proline transporter with a $k_{d(Pro)}$ and $k_{m(Pro)}$ of 2 µM (16, 17, 28). Modifications of the pyrrolidine ring or the carboxyl group of proline reduce affinity significantly, an observation which is in agreement with the high specificity of PutP for L-proline. Known competitive inhibitors are 3,4-dehydroproline (k_i = 9 µM), azetidine-2-carboxylate (k_i = 125 µM), and thioproline (k_i = 190 µM) (29). A Na^+ concentration required for half-maximum stimulation of L-proline uptake ($k_{0.5(Na+)}$) of 30 µM suggests a high affinity also for Na^+ (26, 30). However, the $k_{d(Na+)}$ of Na^+ binding is with about 10 mM more than two orders of magnitude higher than, $k_{0.5(Na+)}$ (30). The discrepancy between kinetic and binding parameters is explained by a functional asymmetry of the transporter i.e., the Na^+ affinity is high on the periplasmic side and low on the cytoplasmic side of the transporter (17, 30). This idea is supported by a significantly increased $k_{0.5(Na+)}$ when uptake is measured with PutP in an inside-out orientation (28). The lower Na^+ affinity on the inside of the transporter may facilitate ligand release into the cytosol under physiological conditions.

Besides transport proteins, the SSS family comprises proteins containing a domain similar to PutP at the primary and secondary structure level, fused to domains typically found in bacterial sensor kinases (4). Proteins exhibiting such a domain composition are found in proteobacteria of the subgroups alpha, gamma, delta (for example the putative proline sensor PrlS of *Aeromonas hydrophila*, CbrA of *Pseudomonas aeruginosa*). CbrA appears to be a global regulator that modulates metabolism, virulence and antibiotic resistance in *P. aeruginosa* (31). The signal(s) sensed by this type of sensor kinases remains to be elucidated. Based on the similarity of the N-terminal domain of CbrA to PutP one may speculate that the domain functions as an amino acid (L-proline) sensor. However, an analysis of the functional properties of CbrA did neither reveal a transport activity for L-proline or other amino acids, nor an influence of these compounds on the autokinase activity (Kerstin Schipper and Heinrich Jung, unpublished information).

4. PHYSIOLOGICAL SIGNIFICANCE OF PUTP

4.1. PutP in L-proline catabolism and osmoprotection

In enteric bacteria *putP* is genetically associated with *putA* in the *put* operon (2, 3, 32). The *putA* gene encodes a multifunctional enzyme catalyzing the sequential oxidation of L-proline and delta^1-pyrroline-5-carboxylate to L-glutamate (3, 33, 34). The *put* operon is responsible for the use of L-proline as source of carbon, nitrogen and energy. Expression of the operon is induced by L-proline and subject to catabolite repression. Induction involves the chemical reduction of PutA, which in its oxidized state functions as a transcriptional repressor of the *put* operon (34, 35). Other bacteria such as *Vibrio, Pseudomonas,* and *Helicobacter* species control expression of the *put* operon by separate proteins. In *P. aeruginsosa*, the regulator PruR mediates *put* gene expression, while *P. putida* PutA regulates L-proline catabolism similar as enteric bacteria (36). In *Vibrio vulnificus* the regulator PutR and a cAMP binding protein (CRP) coactivate *put* expression (37). Remarkably, oxidation of L-proline to L-glutamate is stimulated under high salt conditions in *V. vulnificans*. The resulting L-glutamate is accumulated as a compatible solute and contributes to osmoprotection (38, 39). However, the *putP* gene and its orthologs are not always genetically linked with *putA*, and the transporter may not be functionally associated with a L-proline degradation pathway. For example OpuE, a PutP ortholog of *Bacillus subtilis*, is involved in cell adaptation to osmotic stress (40). The *opuE* gene is expressed from two osmoregulated promoters: *opuE* P-1 recognized by the vegetative sigma factor A and *opuE* P-2 dependent on the stress-induced transcription factor sigma B (41). Consistent with the temporary pattern of sigma^B activation after sudden

Table 1. Significance of *put* and related genes for bacteria-host interactions

Bacterium	Gene	Encoded Protein	Significance	Reference
Helicobacter pylori	*putP*	Na^+/proline symporter*	essential for stomach colonization	(49)
	putA	proline dehydrogenase	essential for bacterial motility and stomach colonization	(50, 53)
Helicobacter hepaticus	*putA*	proline dehydrogenase	modification of bacterial redox status, impact on host inflammatory response	(52, 53)
Staphylococcus aureus	*putP*	Na^+/proline symporter	transcriptional activation by low proline concentrations and osmotic stress, contribution to *in vivo* survival of *S. aureus* in various infection models	(45-47)
Vibrio cholerae	*putP*	Na^+/proline symporter	important for initial steps in biofilm formation (monlayer formation)	(58, 59)
	sssA	Na^+/solute symporter*	important for initial steps in biofilm formation (monlayer formation)	(58, 59)
Vibrio vulnificus	*putP*	Na^+/proline symporter	adaption to changing osmolalities during host infection	(39)
Francisella novicia	*putP*	Na^+/proline symporter*	required for pulmonary and systematic infection of mice	(56)
Yesinia pestis	*putP*	Na^+/proline symporter*	induced upon temperature shift from 26 to 37°C	(57)
Photorhabdus luminescens	*putP*	Na^+/proline symporter*	regulation of alternative lifestyles during adaptation to nematode and insect hosts	(7)
Pseudomonas putida	*putP*	Na^+/proline symporter	facilitation of the colonization of plant rhizosphere	(12)
	putA	proline dehydrogenase	facilitation of the colonization of plant rhizosphere	(12)
Escherichia coli	*putP*	Na^+/proline symporter	expressed in clinical isolates, performs housekeeping functions important for survival of *E. coli* in different environments including the human host	(108)

*Protein function not experimentally verified

environmental challenges, activity of the $sigma^B$-dependent *opuE* P-2 promoter rises transiently upon osmotic upshock. The promoter allows the input of other typical $sigma^B$-inducing stimuli such as heat and ethanol stress into the genetic control of *opuE*. In contrast, transcription initiating from the $sigma^A$-dependent *opuE* P-1 promoter increases in proportion to the external osmolarity and is maintained at high levels (41). Differing from other uptake systems for osmoprotectants such as ProP or BetP, OpuE activity is not controlled at the protein level (19, 41, 42). *B. subtilis* contains another PutP ortholog, YcgO, as well as genes predicted to encode proline oxidizing enzymes. Whether or not the gene products are involved in L-proline catabolism remains to be experimentally tested.

4.2. Role of PutP in bacteria host interactions

In addition to ensuring bacterial survival and growth in soil and aqueous environments, PutP and its substrate L-proline play a role in various bacteria host interactions (Table 1). Most extensive investigations have been performed for *Staphylococcus aureus* infections. The L-proline transporter PutP does specifically contribute to *in vivo* survival of *S. aureus* in various animal models, for example, murine abscess, urinary tract and systemic infection models (43-45). Here, PutP supports adaptation of the bacterium to high osmolarity. Similar to *opuE* of *B. subtilis,* the *putP* gene of *S. aureus* is not genetically linked with a gene coding for a L-proline oxidizing enzyme. Furthermore, the *putP* gene is transcriptionally activated by low-proline and high osmotic environments both in growth media and in murine or human clinical specimens (44, 46, 47). However, in high-proline and high osmotic environments, *putP* expression is down-regulated. Under these conditions, proline uptake is likely to be taken over by the osmoresponsive, low affinity proline and betaine transporter ProP which is also present in *S. aureus*. Down-regulation of *putP* expression depends on $sigma^B$ which acts here as a transcriptional repressor (46). PutP activity of *S. aureus* is not known to be regulated at the protein level.

L-proline transport and metabolism is also important for interactions between Gram-negative bacterial pathogens and hosts. Growth of *Helicobacter pylori*, the causative agent of type B gastritis, peptic ulcer and a risk factor for the development of gastric carcinoma and mucosa associated lymphoma in humans, is reported to be enhanced by amino acids released by gastric epithelial cells (48). A genome-wide screen for genes involved in the virulence of *H. pylori* identified the *putP* gene as essential for colonization of the stomach in the Mongolian gerbil infection model (49). *H. pylori putP* forms together with the L-proline dehydrogenase gene *putA* the *put* operon. Remarkably, a *putA* mutant does neither accumulate L-proline, show motility in response to amino acids, nor display swarming activity. Flagella seem to exist but full length sheathed flagella are rarely observed. Finally, the *putA* mutant proves incapable to colonize the stomach of mice (50). The precise role of *putP* and *putA* in *H. pylori* metabolism and virulence is not known. The respective proteins may be required to utilize L-proline as a nutrient ensuring, for example, the supply of energy for transmembrane transport processes and bacterial cell motility. In fact, L-proline is suggested to be the predominant amino acid in the gastric juice of humans infected with *H. pylori* reaching concentration of up to 10 mg per g gastric juice (11, 50, 51). In addition, L-proline is shown to function as respiratory substrate in *H. pylori* (11, 52). But it cannot be excluded that the PutP-catalyzed accumulation of L-proline contributes also to the resistance of *H. pylori* against osmotic or oxidative stress. In fact, L-proline appears to be the most abundant free amino acid in *H. pylori* cells (accumulation to up to 600 nmol/g wet weight of cells during growth on Brucella agar plates (50). While L-proline accumulation may protect the bacterium from oxidative stress in the host, there is also the potential that L-proline oxidation via PutA affects the redox status of the bacterium by the transfer of electrons from reduced flavin to molecular oxygen leading to the generation of reactive oxygen species (52, 53). Clearly, further investigations are necessary to fully understand the significance of PutP and PutA in *H. pylori*. Finally, it is not known whether PutP is solely responsible for L-proline accumulation in *H. pylori*. The genome analysis predicts

the existence of minimum two additional transporters potentially catalyzing L-proline accumulation: ProP and ProU (ProV/ProWWX) (54). Since L-proline is not an essential amino acid for *H. pylori* (55), L-proline biosynthesis may also contribute to intracellular L-proline accumulation. The significance of the latter transporters and of L-proline biosynthesis for *H. pylori* is not known.

Similar to *H. pylori*, a recent genome-wide screen in *Francisella novicida,* a Gram-negative facultative intracellular bacterium and a causative agent of the potentially life-threatening disease tularemia in a large number of mammals, identified *putP* as one of the genes required for pulmonary and systemic infection of mice (56). The molecular basis of the effect of the *putP* deletion is not known. Furthermore, in *Yersinia pestis*, eleven genes required for efficient catabolism of amino acids are rapidly induced upon temperature shift from 26 to 37 °C, including those encoding the L-proline transporter PutP and the proline dehydrogenase PutA. It is speculated that these and other regulatory events allow a profligate catabolism of numerous metabolites available in the mammalian host (57). In *Vibrio cholerae*, a halophilic bacterium found in estuarine and coastal waters and an infectious agent of the diarrheal disease cholera, utilizes PutP and the ABC-type transporter OpuD for accumulation of L-proline and betaine under osmotic stress conditions. In this context it is hypothesized that these transport processes support the association of V. cholerae with the surfaces of algae, phytoplankton, and zooplankton, which in turn may lead to cholera outbreaks during plankton blooms (58). In addition, PutP and its homolog SssA are shown to play a role in the transition from transient to permanent attachment of *V. cholorae* to surfaces (59). L-proline is also involved in regulating alternative lifestyles in entomopathogenic bacteria (7, 60). Bacteria of the genera *Photorhabdus* and *Xenorhabdus* participate in trilateral interactions in which they enable their nematode hosts to parasitize insect larvae. Differential metabolomic profiling in *Photorhabdus luminescens* and *Xenorhabdus nematophila* revealed that L-proline in the insect's hemolymph initiates a metabolic shift leading to adaptation of the bacterial metabolism to the host environment. Thereby, PutP, PutA, and the ProU system are proposed to regulate the metabolic shift and to maintain the bacterial proton motive force that ultimately regulates the downstream bacterial pathways affecting virulence and antibiotic production (7).

L-Proline uptake and metabolism may also support symbiotic interactions between bacteria and plants. Almost all of the twenty proteinogenic amino acids can be detected in plant exudates, with L-proline being one of the most abundant (12, 13). For root colonizing *Pseudomonas* species it is assumed that the PutP and PutA-dependent L-proline catabolism supports interactions of the soil bacteria with the plant rhizosphere (12). In *Sinorhizobium meliloti*, L-proline catabolism contributes to root colonization as well as to the establishment of symbiotic interactions with roots (61, 62). However, the latter strain does not contain a PutP homolog, and the role and mechanism of proline transport in the strain remains to be elucidated.

5. PUTP AND THE LEUT-TYPE STRUCTURAL FOLD

In view of the significance of PutP for bacterial physiology and interorganismic interactions, the molecular mechanism of Na^+/solute symport catalyzed by the transporter is investigated. Clearly, information of the three dimensional structure of the protein is a prerequisite for understanding of this mechanism. However, despite the growing number of available crystal structures of secondary transporters (23, 63-72), a high resolution structure of PutP or any other L-proline transporting integral membrane protein is not available yet. A first insight into the fold of a member of the SSS family was gained by the crystallization of the Na^+-dependent galactose symporter vSGLT of *Vibrio parahaemolyticus* (65). It turned out that vSGLT shares a characteristic core architecture with genetically unrelated gene families which are integrated in a structural family of LeuT-like transporters (23, 65, 67, 68, 70-72). The common core architecture is in good agreement with former hydropathy profile alignments (73). The core contains ten transmembrane domains (TMs) in which TMs 1-5 are related to TMs 6-10 by a pseudo-two-fold axis located in the plane of the membrane. This finding suggests that these two domains arose by gene duplication and evolved from an ancestral transporter whose functional unit consisted of multiple identical domains (74). With the increasing number of high resolution structures of LeuT-like proteins it became obvious that the ten TM core can be divided into two subdomains. TMs 3, 4, 8, and 9 form a so called hash motif, and a four helix bundle is built up by TMs 1, 2, 6, and 7 (67). These two domains do not only built up substrate and ion binding sites. They are also involved in structural rearrangements which allow sequential opening and closing of extra- and intracelluar cavities during the transport cycle. Beside this unexpected internal structural repeat, the first TMs of both repeats (TMs 1 and 6) adopt an "alpha-helix – extended peptide - alpha-helix" motif. As a consequence of these breaks in the alpha-helical structures approximately in the middle of the membrane, main chain carbonyl oxygen and nitrogen atoms are exposed and serve as hydrogen bonding partners for ions and substrates. This interruption of the secondary structure is accompanied by the exposition of positive and negative partial charges of helix dipoles in a low dielectric environment in the middle of the membrane which can be saturated either by tertiary contacts or by interaction with ions or substrates. The antiparallel oriented TMs 1 and 6 which are in close proximity in LeuT-like proteins are not only major determinants for the formation of polar microenvironments of binding pockets but also for transporter dynamics with the unwound regions used as a hinge (65, 75).

Based on the crystal structure of vSGLT, a homology model of PutP was generated which contains amino acids 38–481 (76) (Figure 1). In the model the core of PutP exhibits the characteristics described above for a core of LeuT-like proteins. The 3D model does not contain the N-terminal extension of the core (TM -1) which is also missing in the vSGLT structure. Two additional TMs are

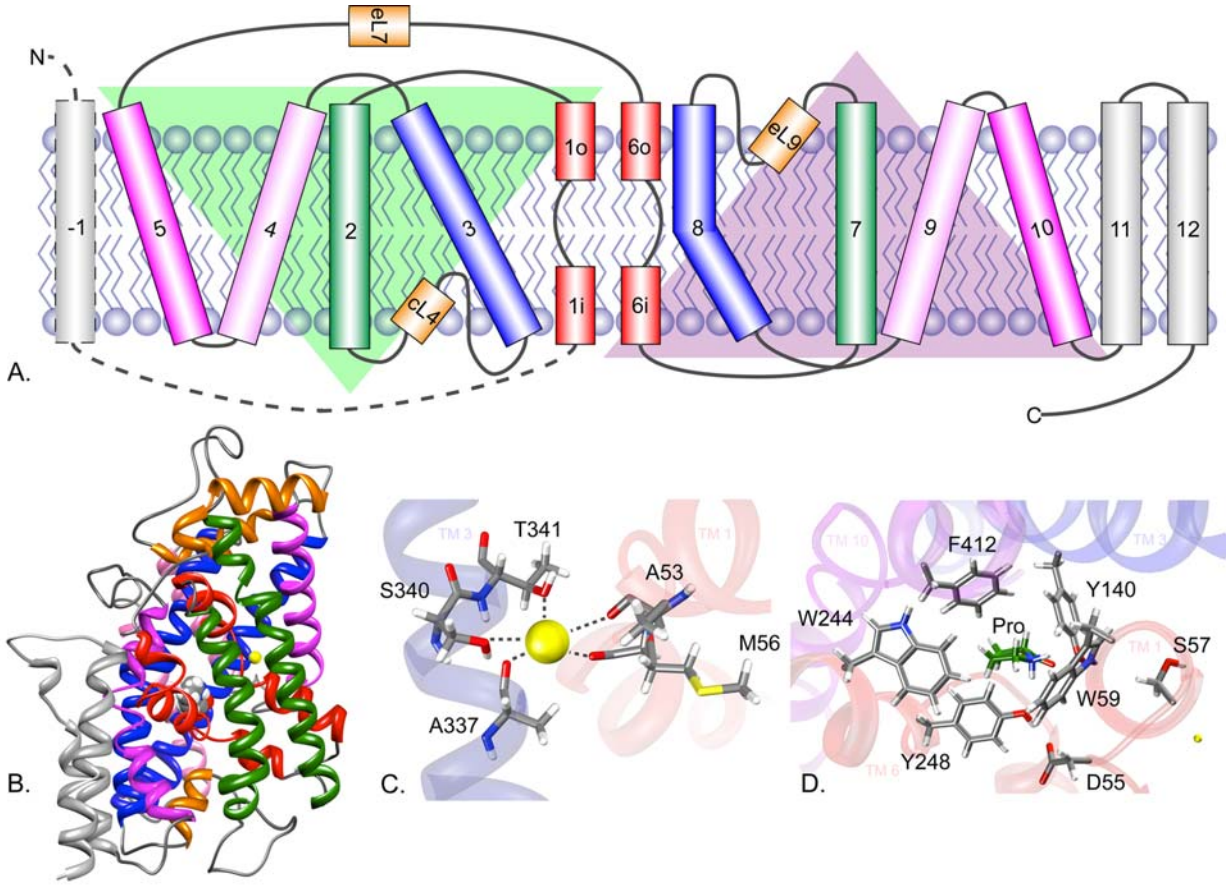

Figure 1. Architecture of the Na^+/proline symporter PutP of *E. coli*. A. Topology model of PutP. To avoid confusion the 10 TM core is numbered as in LeuT. TM 2 of PutP corresponds to TM 1 in LeuT. Additional peripheral TMs (N-terminal: -1; C-terminal: 12 and 13) are colored in grey. The five TM repeats related by a pseudo-two-fold symmetry axis are overlapped by a green and purple triangle respectively. TMs corresponding in the two repeats are highlighted in the same color. B. Homology model of PutP based on the crystal structure of vSGLT. The structure represents PutP in an inward-facing, occluded conformation. TMs are colored as in A. L-Proline (shown as spheres) is located in the center of the 10 TM core domain approximately in the middle of the membrane. Na^+ (yellow sphere) is placed in a binding site corresponding to Na_2 of LeuT ~11 Angstrom away from the substrate. C. Close up view on the Na^+ binding site. Residues in TM 1 and 8 involved in ion coordination are displayed as sticks colored by atom type. D. Close up view on the L-proline binding site. Residues in TMs 1, 3, 6, and 10 predicted to be close to or part of the L-proline binding pocket are depicted as sticks colored by atom type. L-Proline is highlighted in green. The figure was prepared with the program UCSF Chimera (109).

located C-terminal to the ten TMs core (Figure 1AB). The model shows PutP in an inward-facing occluded conformation (Ci_cS). In this conformation PutP contains a large negatively charged cavity extending from approximately the middle of the membrane to the cytoplasmic space. It is built by TMs 1, 3, 6, 8, 10, and the internal loop cL4 (76). Direct access from the cytoplasm to the putative sites of Na^+ and L-proline binding located at the apex of the cavity is blocked by an intramolecular gate.

Is the homology model of PutP supported by experimental data? Topological analyses of PutP by hydropathy profile analysis, Cys-accessibility, LacZ/PhoA-fusions and electron paramagnetic resonance (EPR) spectroscopy revealed a thirteen TM motif with the N-terminus facing the periplasm and the C-terminus facing the cytoplasm (28, 77, 78). This motif is in good agreement with the homology model except that boarders of some TMs had to be shifted. Furthermore, biochemical and biophysical approaches support the tertiary structure of PutP. Previous Cys scanning mutagenesis of TMs 1 and 8 suggested already a close proximity of both TMs and an involvement in the formation of a cytoplasmic cavity which spans approximately from the middle of the membrane to the cytoplasm (79-81). A more detailed view on TM 8 was gained by a combination of site-directed spin labeling and double electron electron resonance (DEER) measurements of distances and subsequent modeling of the membrane spanning domain. The results suggest that TM 8 exhibits a pronounced kink near residue T341 which is important for Na^+ binding (79-82). Breaking up interhelical H-bonds in this region of the protein exposes backbone oxygen atoms which may allow complexing of the cation. Also A337 which is located near this non-helical interruption is

supposed to be involved in the formation of the Na^+ binding site (76, 81). Taken together, the currently available experimental data strongly support the PutP homology model.

6. STRUCTURE-FUNCTION RELATIONSHIPS IN PUTP

6.1. The Na^+ binding site

Despite the increasing number of available transporter structures with the LeuT-type structural fold, only the LeuT diffraction data provide direct information of Na^+ coordinating amino acids. In LeuT two binding sites, Na_1 and Na_2, were identified (23). The Na^+ binding sites of structurally related Na^+ dependent transporters were assigned only indirectly by sequence and structure alignments with LeuT and inclusion of functional analyses.

For PutP, site-directed mutagenesis studies and biochemical characterization identified various residues important for Na^+ binding (79-81, 83, 84). Combining these data with the vSGLT-based homology model and LeuT led to the proposal of a Na^+ binding site corresponding to Na_2 of LeuT (23, 65, 76). Accordingly, Na^+ is coordinated by A53 and M56 of TM 1, and A337, S340 and T341 of TM 8 in a trigonal bi-pyramidal manner (Figure 1C). Since A53 and M56 are located in the unwound region of TM 1 and A337 at the kinked part of TM 8 the residues are able to bind Na^+ by main chain carbonyl oxygen atoms. In contrast, S340 and T341 are coordinating Na^+ by hydroxyl oxygens (76). Interestingly this cation binding site can be found in all crystallized symporters within the LeuT structural family up to now. A fascinating aspect is the observation, that even in Na^+-independent transporters a positive charge at the according positions seems to be essential. In the H^+-coupled amino acid transporter ApcT, Na^+ is replaced by K158 and in the L-carnitine/γ-butyrobetaine antiporter CaiT by R262 (70, 85).

What is the functional role of the bound coupling ion? It was postulated that Na^+ bound to the Na_2-site stabilizes unwound TM 1, thereby increasing the substrate binding affinity of the transporter (86, 87). In vSGLT, N64 is part of the unwound region of TM 1 near the Na_2 site and thought to mediate crosstalk between the sites of Na^+ and substrate binding and an intracellular gate formed by Y263. The first structure showed vSGLT in an inward-facing, occluded conformation (65). Here a loosely coordinating Na^+ binding site is described which is readily releasing the ion to the intracellular space (65, 88, 89). A follow up structure shows vSGLT in an inward-facing, open conformation. The release of Na^+ leads to a reorientation of gating residue Y263 and minor helical movements, which open the substrate binding site towards the cytoplasm and allows dissociation of galactose (90).

6.2. The L-proline binding site

Putative L-proline coordinating sites in PutP were identified by ligand docking experiments with the PutP homology model and experimental analyses (76) (Figure 1D). The L-proline binding pocket is located at the apex of the inward-facing cavity, approximately in the middle of the membrane bilayer. This is in good agreement with the location of substrate binding sites observed in crystal structures of other transporters with a LeuT-like structural fold. Residues located in TMs 1, 3, 6, 8, and 10 of PutP are involved in L-proline coordination. The interrupted helices of TMs 1 and 6 play an important role in the formation of the L-proline binding pocket. Main chain hydrogen bonding partners are exposed in the unwound stretch of the respective TMs and in addition helix dipoles contribute to a polar environment that allows substrate coordination. These polar features of the binding site may be important for recognition of the imino and carbonyl groups. However, the role of the helix dipoles of TMs 1 and 6 and of backbone carbonyl H-bonding partners remains elusive since these are difficult to track. Also a hydrophobic pocket seems to be involved in L-proline binding (29). Recently the two conserved amino acids Y140 (TM 3) and W244 (TM 6) were shown to be crucial for PutP function and are proposed to play a role in L-proline coordination. Substitution of the residues led to a decrease of the apparent L-proline affinity by two or three orders of magnitude (76). For Y140, both the apolar benzene side chain and the hydroxyl group are required for optimum L-proline binding. Y140 of PutP aligns with Y108 of LeuT which builds together with other residues of TMs 6 and 8 a hydrophobic pock*et al*lowing accommodation of the aliphatic tale of the LeuT substrate leucine. In PutP, Y140 and W244 may not only be involved in L-proline binding but may also fulfill a gating function. Further discussions of the subject can be found below (paragraph 7.3).

Kinetic analysis suggested either a direct involvement of S57 in substrate binding or at least close proximity to the binding site (91). In the homology model, S57 is localized within the unwound stretch of TM 1 but does not seem to directly participate in L-proline coordination. It is shown for other LeuT-like transporters that residues localized in this discontinuous segment are involved in substrate binding (68). The exact localization of S57 between the Na^+ and the proline binding site rather suggests an involvement in coupling Na^+ and L-proline binding.

7. MOLECULAR MECHANISM OF NA^+/SOLUTE SYMPORT

7.1. Transport via alternating access

Based on available information on membrane topology, structure, conformational alterations, and kinetics, PutP and other secondary transporters are thought to operate via an alternating access mechanism (92-94). According to this mechanism, the transporter can adopt two major alternating conformations in which the opening of the translocation pathway is outward- (Co) or inward-facing (Ci) (Figure 2). Transitions between these two conformational states expose the substrate-binding site alternately to the *cis* or *trans* side of the lipid bilayer, and thereby allow the translocation of substrates across the membrane without formation of a continuous transmembrane pore. By comparison of the currently available X-ray crystal structures of members of the LeuT

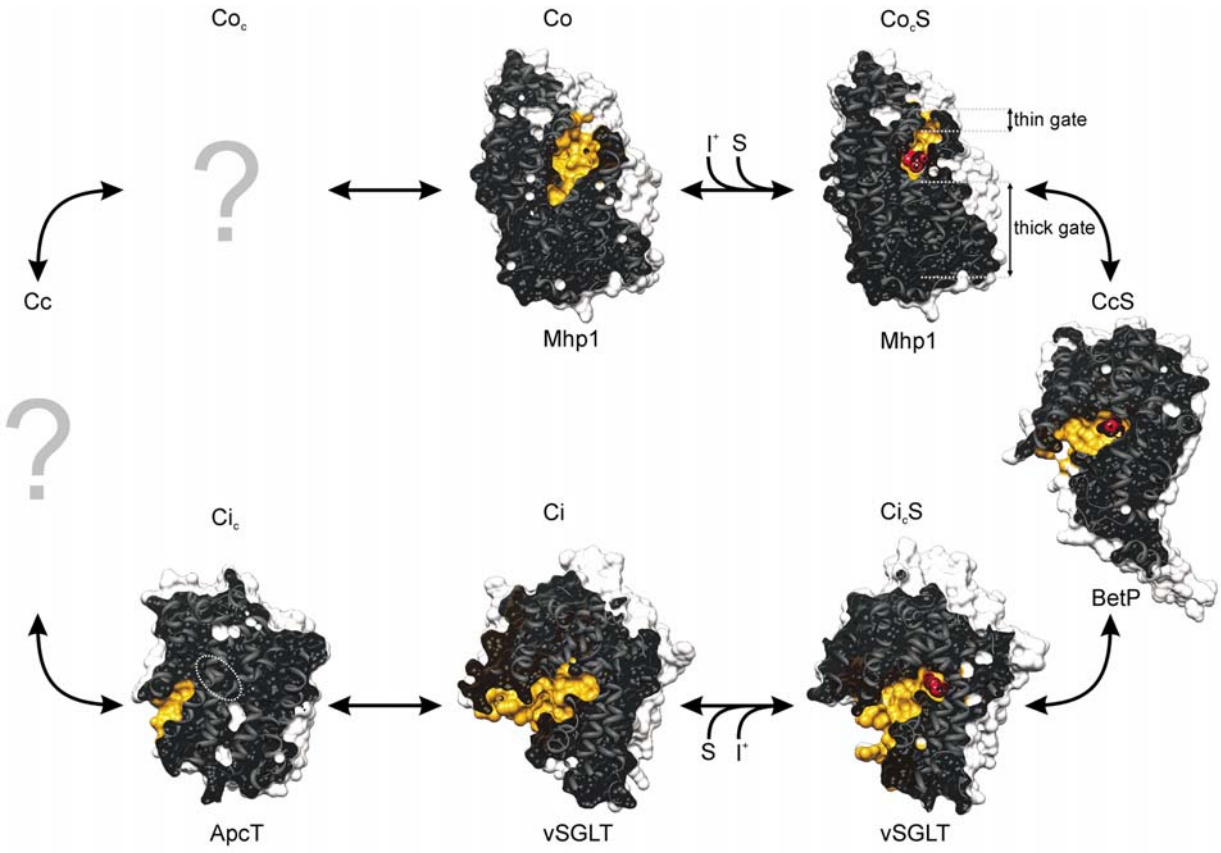

Figure 2. Alternating access mechanism for symport. The individual conformational states appearing throughout the transport cycle are shown based on currently available crystal structures of secondary transporters of the LeuT structural family. Shown are slices through surface-models of the transporters, viewed approximately parallel to the membrane with the extracellular side at the top. The surface of residues lining the cavities and substrate binding sites is colored in yellow. Respective bound substrates are shown as van der Waals spheres, highlighted in red and the backbone of the proteins is illustrated as ribbon models colored in dark grey. The position of the thin and thick gates is exemplarily shown for Mhp1 in the Co_cS state by grey dashed lines. For ApcT, the position of the putative ligand-binding site is highlighted by a white dashed ellipse. Predicted transport intermediates for which no crystal structure is available yet are represented by grey question marks. S, substrate; I^+, coupling ion; Co, open, outward-facing; Co_c, occluded, outward-facing; Cc, occluded; Ci_c, occluded, inward-facing; Ci, open, inward-facing. The figure was prepared with the program UCSF Chimera (109).

structural family, the alternating access mode of transport can be extended to at least eight different conformational states that emerge throughout the transport cycle (23, 65, 67, 68, 70, 71, 90, 95, 96) (Figure 2). Since PutP shares the same overall fold of the ten-helix core domain with the LeuT structural family, it is suggested to show similar conformational states during substrate transport. In the following, the individual steps of the alternating access mechanism are described in detail based on the structures of Mhp1, AdiC, LeuT, BetP, vSGLT, CaiT, and ApcT, that all share the LeuT structural fold.

In the first state of the transport cycle, the protein adopts an open, outward-facing conformation (Co), as represented by the substrate-free structures of Mhp1 (PDB: 2JLN) and AdiC (PDB: 3HQK, 3LRB). This state exposes a water-filled cavity to the extracellular aqeous solution and allows access to the ligand binding sites from the outside of the membrane. Subsequently, the translocation pathway becomes partly occluded due to small conformational alterations induced by solute binding. Such an occluded, outward-facing conformation (Co_cS) can be found in the substrate-bound structures of Mhp1 (PDB: 2JLO), LeuT (PDB: 2A65), and AdiC (PDB: 3L1L). Here, the binding pocket is shielded from the extracellular, aqueous bulk by relatively small structural elements that build up a thin gate, while the access to the intracellular solvent in the Co and the Co_cS state is blocked by around 20 Angstrom of tightly packed protein residues, known as a thick gate (Figure 2). The transporter then converts from the outward- to the inward-facing state via an intermediate, occluded conformation (CcS) that is suggested to be represented by the BetP structure (PDB: 2WIT). In this state, the ligand-binding site is protected from the extracellular aqueous bulk by a thick gate, but an initial stage of an evolving intracellular cavity is observed. However, it can be clearly distinguished from the next, inward-facing, occluded state (Ci_cS) by the fact that opening of the cytoplasmic cavity

will require considerable conformational changes of the protein backbone. An additional argument for the correct assignment of the BetP structure as a CcS state is given by the structural symmetry of the two 5 TM repeats that was observed to be significantly higher than in the inward- and outward-facing conformations (71). The already mentioned Ci_cS state can be described by the structure of vSGLT (PDB: 3DH4) in its substrate-bound form. This conformation exposes an intracellular cavity to the inner aqueous bulk. Similar to the Co_cS state, the substrate resides in the binding pocket at the apex of the cavity, and is separated from the translocation pathway by a thin gate, whereas the accessibility from the extracellular environment is blocked by a thick gate. Interestingly, MD simulations of this Ci_cS state of vSGLT indicates that this structure already represents an ion-releasing state facilitating the diffusion of Na^+ towards the cytoplasm (88-90). The transporter then proceeds through the cycle by opening the small gate to connect the substrate binding site with the water-filled cavity. This open, inward-facing conformation (Ci), which is shown in the ligand-free structures of vSGLT (PDB: 2XQ2), CaiT (PDB: 2WSX, 2WSW), and Mhp1 (2X79), allows dissociation of the substrate to the intracellular solvent and thus completes the translocation process. However, to enable several rounds of substrate translocation, the empty transporter has to switch back to the Co state to restart the transport cycle. Such a reorientation of the protein may occur through three additional substrate-free conformations: an occluded, inward-facing (Ci_c), occluded (Cc), and occluded, outward-facing (Co_c) state. While there is no crystal structure available yet for the Cc and Co_c conformations, the apo state of ApcT (PDB: 3GIA) is proposed to represent the Ci_c state, appearing after substrate release and closure of the intracellular thin gate that shields the binding pocket from the intracellular oriented cavity.

For PutP, no direct evidence for one of the eight conformations is available due to missing three-dimensional structural information. Nevertheless, analyses of changes in Cys accessibility, spectroscopic properties of site-specifically attached fluorescent or paramagnetic groups and intramolecular distances have led to the identification of an inward-facing, polar cavity (76, 80, 81, 83, 97, 98). This finding is in good agreement with the homology model of PutP, representing an inward-facing conformation (76). In addition to topological analyses, it is shown that binding of Na^+ and/or L-proline to the transporter decreases the accessibility to Cys-specific labels and the mobility of paramagnetic groups attached to positions located in the identified crevice. From these findings, it is concluded that binding of ligands induces conformational alterations of PutP that may lead to a closure of the intracellular permeation pathway and thus to an occluded state of the transporter. Alternatively, it can be envisaged that the conformational changes switch the protein from an inward-facing to an outward-facing conformation. Although the latter idea remains highly speculative, it is supported by a Na^+-induced increase in the accessibility to thiol-specific ligands observed at positions located above the putative L-proline-binding site on the periplasmic side of the protein (76, 80, 83). Still, whether these accessibility changes are really due to opening of an outer cavity remains to be demonstrated.

7.2. Impact of Na^+ on the outward-facing cavity and substrate binding

During the transport process, the transitions between different conformational states must be tightly regulated to avoid uncoupling of ion and substrate transport. In the case of PutP and other symporters, such a regulation has to prevent interconversion of the outward- and inward-facing state from occurring when only one of the ligands is bound. Otherwise, each solute could traverse the membrane independently, and coupling would be abolished. Furthermore, the opening and closure of the extracellular and intracellular gates has to be strictly regulated to assure that only one gate is open at a time in order to prevent the formation of a continuous membrane-spanning pore. To understand the molecular principles of solute transport including the coupling and gating mechanism, the sequence of ligand binding, the translocation pathway of the solutes and conformational changes have to be considered. For PutP kinetic and electrophysiological measurements show that transport at low L-proline concentrations occurs according to an ordered binding mechanism (30, 99). In this scheme, Na^+ binds to the empty transporter first, thereby inducing a conformational change, which increases the affinity of the transporter for L-proline. At high L-proline concentrations, binding of Na^+ and L-proline is random (99). Evidence for the Na^+-induced conformational change of PutP comes from analyses of Cys-accessibility to thiol-specific reagents in the presence or absence of Na^+ (76, 79-81). In particular, the accessibility of positions in the periplasmic halves of TMs 1, 3, and 6 was shown to increase in the presence of Na^+, while positions in the middle and periplasmic half of TM 8 was partially protected by Na^+ binding to the protein. As mentioned above these findings can be interpreted as a Na^+-induced conformational change that opens an extracellular cavity, which is formed minimum by TMs 1, 3, 6, and 8 of the core structure of PutP. Notably, similar observations have been made by site-directed spin labeling in combination with EPR measurements and MD simulations on LeuT (100). The latter study provides evidence for a Na^+-dependent opening of the extracellular permeation pathway of LeuT that is lined by TM 1a, TM 3, TM6b, TM8, and TM 10, in full agreement with the results obtained for PutP. The conformational alteration leading to this open conformation of LeuT is based on a movement of eL4 (eL9 in PutP) out of the extracellular crevice. The movement of eL4 allows the entrance of substrate to the binding pocket and thus facilitates solute binding. Furthermore, MD and free energy perturbation simulations on LeuT suggest that Na_2 is important for structural stabilization of the substrate binding pocket leading to a slight increase in binding affinity (86). Although it is not clear whether general conclusions can be drawn from these observations for other members of the structural family, they provide the opportunity to speculate that the ion-based opening of the outer cavity and stabilization of the substrate binding pocket permit cooperativeness of ion and substrate binding.

7.3. The outer thin gate and substrate binding

As described before, substrate binding from the periplasmic site of the membrane induces closure of the outer thin gate and switches the transporter to a Co_cS configuration (Figure 2). The importance of this gate in preventing substrate diffusion back to the extracellular aqueous milieu raises the question how these structural elements open and close. In PutP, accessibility measurements provide evidence that the positions in TMs 1, 3, and 6, which are exposed to the periplasmic cavity in the presence of Na^+, are protected upon addition of L-proline (76, 80, 83). Since the putative gating residues Y140 and W244 of PutP are located in two of these helices (TM 3 and TM 6), and found to be accessible in the Na^+- but not in the Na^+/L-proline bound state, it is tempting to speculate that these residues build up a thin gate that closes due to movement of TMs 3 and 6 towards each other. Notably, both residues are also predicted to be involved in substrate binding, so that closure of this aromatic lid may simultaneously allow improvement of substrate binding. Although these ideas of PutP function are speculative, they parallel results obtained from crystallization, EPR spectroscopy, MD simulations, and accessibility measurements of other transporters of the LeuT family (68, 72, 100, 101). For AdiC, structural alignment of the two available X-ray structures in the Co and Co_cS state revealed a pronounced structural rearrangement in TM 6 and, to a lesser extent, in TMs 2 and 10 (72). Due to the movement of TM 6, the gating residue W202 (W244 in PutP) is positioned directly above the bound substrate, blocking its diffusion back to the extracellular solution. With Mhp1, in comparing the Co and Co_cS state, the most substantial conformational change was observed in the N-terminal part of TM 10 (68). This structural element moves into the outward-facing cavity and occludes the substrate-binding site. Thereby, the side-chain of the corresponding residue W220 in TM 6 is repositioned to interact with the bound substrate, akin to W202 of AdiC. Similar conformational changes are proposed based on accessibility measurements of TM 10 of human SGLT1 (101). For LeuT, EPR measurements and MD simulations show that binding of the substrate leucine alters the conformation of eL4, TM 6 and TM 10 (100). In agreement with the observed changes of the orientation of aromatic residues in other transporters, Y108 and F253 (Y140 and W244 in PutP), engage different rotamer states in the presence of substrate that lead to shielding of the binding pocket from the extracellular vestibule. Besides the aromatic lid, LeuT also exhibits a salt bridge formed by residues R30 and D404 (23). As this salt bridge is proposed to close and open depending on ligand binding, the salt bridge is believed to be part of the extracellular thin gate together with Y108 and F253 (87, 102). Taken together, the ligand-induced conformational changes occurring during closure of the extracellular thin gate are relatively small. Although the general principles of gating, for example, the structural elements that undergo conformational changes, seem to be conserved, the extent and the direction of the conformational alteration varies between the individual transporters of the LeuT structural family. Therefore, further experimental studies are required to understand the movements in PutP that describe the opening or closing of the extracellular gate.

7.4. Transition between outward- and inward-facing conformations

In contrast to the relatively small structural rearrangement associated with ligand binding from the extracellular bulk, the transition between the Co and Ci state requires larger-scale conformational alterations that are distributed almost over the entire protein. This is due to the second gating mechanism that includes closing of the outward-facing cavity by a thick extracellular gate and opening of an intracellular thick gate to establish an inward-facing translocation pathway. Evidence for a inwardly-oriented cavity in PutP comes from accessibility analyses at positions in TMs 1 and 8 (80, 81). The results suggest that minimum TMs 1 and 8 participate in the formation of an inner hydrophilic translocation pathway that is closed upon L-proline binding in the presence of Na^+. The latter effect of substrate binding on the inner cavity clearly contradicts the presumption that the bound ligand induces an inward-facing conformation of the transporter that allows releasing of the solute to the cytoplasm. One possible reason for this phenomenon could be that the accessibility measurements were performed in random oriented membrane vesicles in the absence of a membrane potential. Since Na^+-coupled proline uptake is not detected in the absence of such a potential, inclusion of the effect of a membrane potential in the studies might lead to different results (A. Hackmann, M. Nietschke, and H. Jung, unpublished data). Nevertheless, the identification of the inward-facing conformation suggests that PutP undergoes ligand-induced transitions between outward- and inward-facing conformations.

The only known member of the LeuT family that was crystallized in three states (Ce, CecS, and Ci) is Mhp1 (67, 68) (Figure 2). Therefore, comparison between the different conformations of Mhp1 may provide important insights into the conformational alterations involved in transitions between the outward- and inward-facing states. During the conformational switch, the hash motif (TMs 3, 4, 8, and 9) undergoes a rigid-body movement with respect to the four-helix bundle (TMs 1, 2, 6, and 7) to close the outwardly- and to open the inwardly-oriented cavity. During this movement, TMs 5 and 10, which participate in building up the extracellular and intracellular thin gates, bend independently near their helix midpoints. This reorientations lead to closure of the extracellular and opening of the intracellular thin gates, followed by dissociation of the ligands into the cytoplasm. Besides this mechanism, two additional mechanistic models have been proposed (23, 103). In the first one, transitions between the Co_cS and Ci_cS conformations of LeuT are suggested to involve flexing of the discontinuous TMs 1 and 6, together with reorientation of TMs 2 and 7 (23). Although, such bending of the pseudo two-fold related TMs 1 and 6 is in agreement with single-molecule FRET analyses of LeuT and with the conformational alterations seen by comparison of the Co and Co_cS of AdiC, it is still not known whether this reorientation is sufficient to cause a switch between the outward- and inward-facing state (68, 104). The second model involves a rocking movement of the four-helix bundle (TMs 1, 2, 6, and 7) against the scaffold (TMs 3, 4, 5, 8, 9, and 10) of the core structure (103). Similar to the

other two proposed mechanisms, this model allows synchronization of the opening and closure of the extracellular and intracellular gates, respectively, to avoid simultaneous opening of both barriers and the formation of a continuous transmembrane pore.

7.5. The inner thin gate and substrate release into the cytoplasm

After adaptation of the inward-facing conformation, the question arises whether release of coupling ion and substrate also occurs in a cooperative manner, as it is proposed for binding. With the recently published vSGLT structure in the Ci state in combination with MD simulations, this appears to be indeed the case (90). In this study, it is proposed that during transition from the Co_cS to the Ci_cS (Figure 2), the distance between the Na^+-binding TMs 1 and 8 increases, resulting in a fast release of the ion from the binding site to the cytoplasm. Upon dissociation, the unwound region of TM 1 undergoes a conformational change that disrupts the H-bonds between the functional important N64 and the gating residue Y263. Subsequently, the side-chain of Y263 becomes reoriented in order to open the cytoplasmic thin gate and to allow dissociation of the substrate. Additionally, the substrate release is facilitated by small rigid-body movements in the hash motif and in the 4-helix bundle that widen the intracellular cavity. As mentioned above, the predicted role of N64 and Y263 in coupling Na^+ and substrate release in vSGLT perfectly fits to the observations made for the corresponding residues, D55 and Y248 in PutP (76, 84). In particular, the carboxylate of D55 proved essential for transport and was previously implicated in Na^+ binding (84). However, in the homology model of PutP, the side-chain of D55 is not found to be part of the Na^+-binding pocket, but it is involved in H-bonding with the hydroxyl group of Y248 in TM 6, similar to the situation found in vSGLT (76, 90). A substitution analysis implicates Y248 of PutP in coupling Na^+ and proline transport and/or gating. These results are consistent with the idea that Na^+ and L-proline release is coupled in PutP in the same way as it is assumed for vSGLT. This assumption is further supported by the observed role of the highly conserved residue D187 of PutP (D189 in vSGLT) (88-90, 105). In both transporters the aspartate residue is located close to the putative Na^+-binding site and forms part of the putative pathway of the coupling ion through the membrane. This finding implies that this residue is important for release of Na^+ on the cytoplasmic side of the membrane. Indeed, computational studies demonstrate that D189 of vSGLT facilitates the diffusion of the ion toward the cytoplasm (88-90).

Besides the Na^+-couled release of the substrate on the inner side of the membrane, LeuT ligand-binding experiments, MD simulations and steered-molecular-dynamics have identified another mechanism potentially important for coupling of ion and substrate transport (106). This mechanism involves a second binding site (S2) in the extracellular vestibule of LeuT, that triggers Na^+ and substrate release from the primary binding site (S1) upon substrate binding. A S2 site has also been found in the extracellular part of CaiT that triggers binding of co-substrate from the inside of this antiporter (70). Since the position and function of the allosterical sites in the different transporter significantly varies, it is not clear wheather this principle is of general impact for the transport mechanism of LeuT-like proteins. Furthermore, it has to be noted, that the existence of a second binding site in LeuT is still discussed and the data are controversial (107). Moreover, kinetic and binding analyses published for PutP so far have identified only one binding site for L-proline (17).

8. CONCLUSIONS AND PERSPECTIVES

Depending on the availability of external L-proline and on environmental conditions, PutP may play important roles for the metabolism and stress response of bacteria and archaea. Clearly, further investigations are necessary to fully understand the molecular basis of the significance of PutP and potentially other L-proline uptake systems. Furthermore, it is expected that L-proline and related transporters and enzymes are crucial for bacteria-host interactions in many more systems than known so far. Because of its physiological significance and as amenable bacterial member of the SSS family, PutP is used as a model system to understand the molecular mechanism of function of SSS family proteins. The described homology model of PutP shows the protein in a LeuT-type structural motif and is in good agreement with the available Cys accessibility, cross-linking, and EPR distance data as well as the previously proposed 13 TMs topology. In addition, the Na^+ and L-proline binding sites are predicted and confirmed based on ligand docking calculations and functional analyses, demonstrating the validity of the model as a guide for further analyses of structure-function relationships. However, it is clear that due to the relatively low sequence identity to the template vSGLT, the homology model has to be considered as a low resolution model and awaits further experimental validation. This can be done by intramolecular distance measurements based on SDSL in combination with DEER spectroscopy. Investigations along this way are currently in progress. Notwithstanding the power of this technique in determining structural data, it does not provide information on side-chain orientation. Therefore, a high-resolution structure obtained, for example by X-ray crystallography, is indispensable for the understanding of molecular interactions and ligand-coordination within the protein. In addition to the structure, information on functional relevant conformational alterations is needed to describe how the ligands are translocated through the protein. Analyses of Cys accessibility and SDSL EPR measurements on PutP in the absence or presence of ligands support a mechanistic model according to which transport follows an alternating access mechanism. With the elucidation of X-ray structures of different LeuT structural family members in different conformations, the mechanistic states and the conformational changes that occur throughout the transport cycle can be predicted. Although the structural alterations observed for PutP fit to this mechanistic model, it is based on structural comparison of transporters with low sequence identity and different substrate specificity. A detailed description of the conformational alterations of an individual transporter, thus require the determination of its structure in each state of the transport cycle. Since the

crystallization of secondary transporters is still highly challenging and the structures only represent static pictures, alternating techniques to investigate the dynamic of transporters are required. For PutP, again SDSL EPR spectroscopy provides an efficient means for obtaining information on the nature and the extent of structural changes. This can be done with high time resolution and, in contrast to X-ray crystallography, under native conditions in a lipid bilayer.

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Abbreviations: DEER spectroscopy, double electron electron resonance spectroscopy; SSS family, Na^+/solute symporter family; SDSL, site-directed spin labeling; TM, transmembrane domain;

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