



Recent advances in understanding proton coupled peptide transport via the POT family

Simon Newstead

The POT family of membrane transporters use the inwardly directed proton electrochemical gradient to drive the uptake of essential nutrients into the cell. Originally discovered in bacteria, members of the family have been found in all kingdoms of life except the archaea. A remarkable feature of the family is their diverse substrate promiscuity. Whereas in mammals and bacteria they are predominantly di- and tri-peptide transporters, in plants the family has diverged to recognize nitrate, plant defence compounds and hormones. This promiscuity has led to the development of peptide-based pro-drugs that use PepT1 and PepT2, the mammalian homologues, to improve oral drug delivery. Recent crystal structures from bacterial and plant members of the family have revealed conserved features of the ligand-binding site and provided insights into post-translational regulation. Here I review the current understanding of transport, ligand promiscuity and regulation within the POT family.

Address

Department of Biochemistry, University of Oxford, Oxford OX1 3QU, United Kingdom

Corresponding author: Newstead, Simon
 (simon.newstead@bioch.ox.ac.uk)

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Introduction

Peptide transport is an important route for the cellular acquisition of nitrogen from the environment [1,2]. In animals and bacteria the POT family play an important role in the uptake of peptides for both metabolism and growth [3,4]. In plants however the POT family, also known as the NPF or NRT1/PTR family, have evolved to recognize a more diverse range of molecules [5]. These include nitrate for metabolism, glucosinolates for seed defense and phytohormones for growth and developmental control [6–8]. POT family transporters therefore represent

one of the most promiscuous nutrient transport systems in biology.

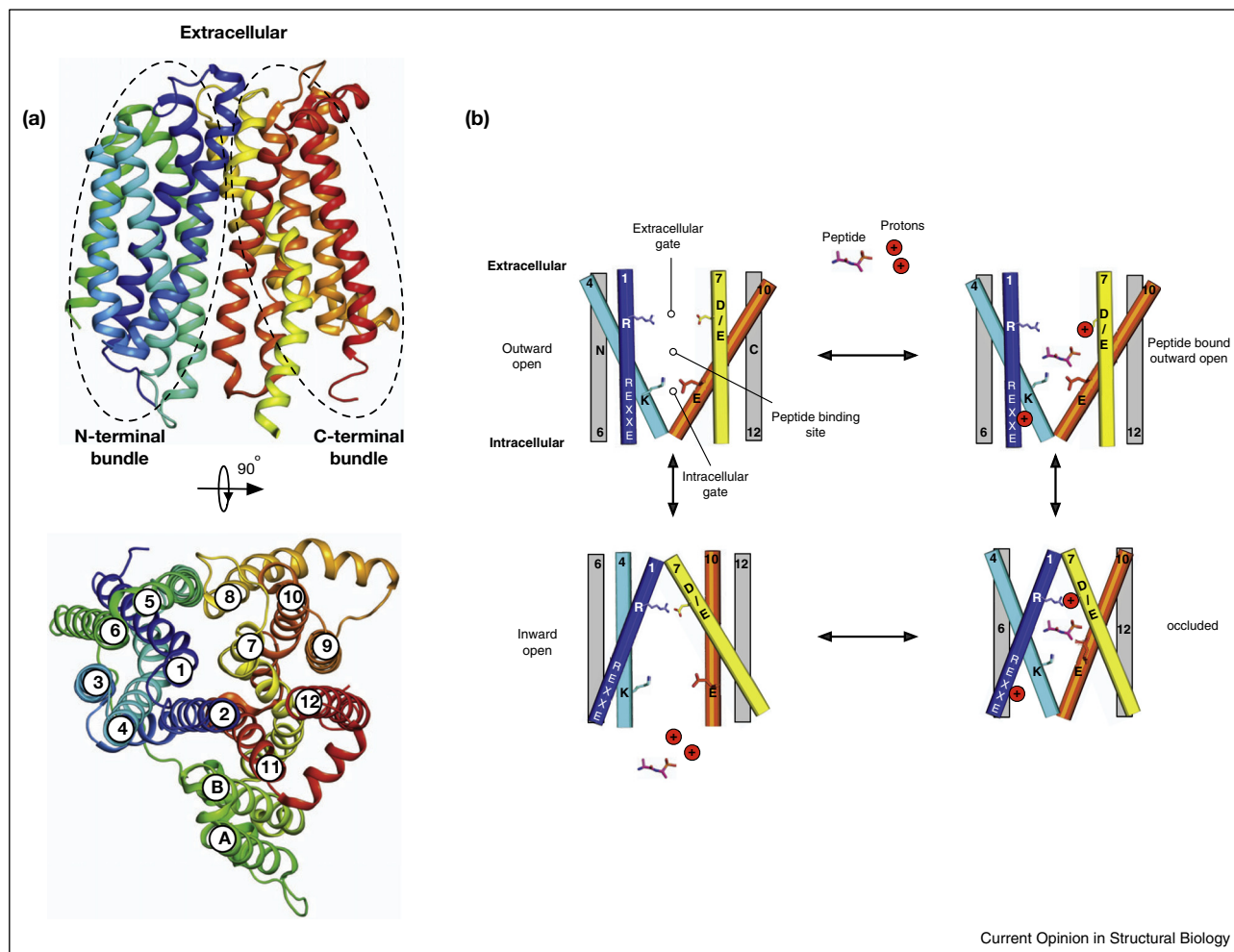
The POT family was first identified and cloned from *Lactococcus lactis* in the 1990s and shown to couple di- and tri-peptide uptake to the inwardly directed proton electrochemical gradient [9,10]. Following this discovery, related proteins were identified in mammalian brush border and renal membranes and named PepT1 and PepT2 respectively [11^{••},12]. Intriguingly none have been discovered in archeal species, possibly due to the lack of peptides in harsh environments or efficient amino acid uptake systems negating their use in this kingdom. Interest in the mammalian members increased substantially following the discovery that both PepT1 and PepT2 are able to recognize and transport a large number of orally administered drug molecules, including beta-lactam antibiotics, antiviral and anticancer drugs [13[•],14,15]. In later studies it became clear that PepT1 and PepT2 are important mediators of intestinal and renal drug absorption and distribution [16[•]].

Biochemically POT family transporters represent a number of unique puzzles. Principal among these is how ligand promiscuity evolved within a single binding site and how transport is linked to proton movement. Recent structural and biochemical studies on several pro- and eukaryotic transporters have played a major role in addressing this question and in this short review I will focus on the molecular basis for promiscuity and regulation.

Alternating access and the role of salt bridges

The POT family belongs to the Major Facilitator Superfamily of secondary active transporters [17]. The first crystal structure was determined in 2011, from the bacterium *Shewanella oniedensis*, PepT_{So} (Figure 1a) [18^{••}]. There are now five additional structures of bacterial POT family transporters [19[•],20^{••},21[•],22,23^{••}] all of which reveal the canonical 12 TM MFS fold, with two additional helices (A & B) inserted between the two six helix bundles (1–6; 7–12). PepT_{So} was trapped in an inward occluded conformation and allowed the identification of the extracellular and intracellular gate helices that control access to a central peptide-binding site (Figure 1b). Later structures of POT transporters from *Streptococcus thermophilus* (PepT_{St}) [19[•]] and *Geobacillus kaustophilus* (GkPOT) [20^{••}] further identified two pairs of salt bridge interactions that appeared to mediate the opening and closure of these gates in response to proton

Figure 1

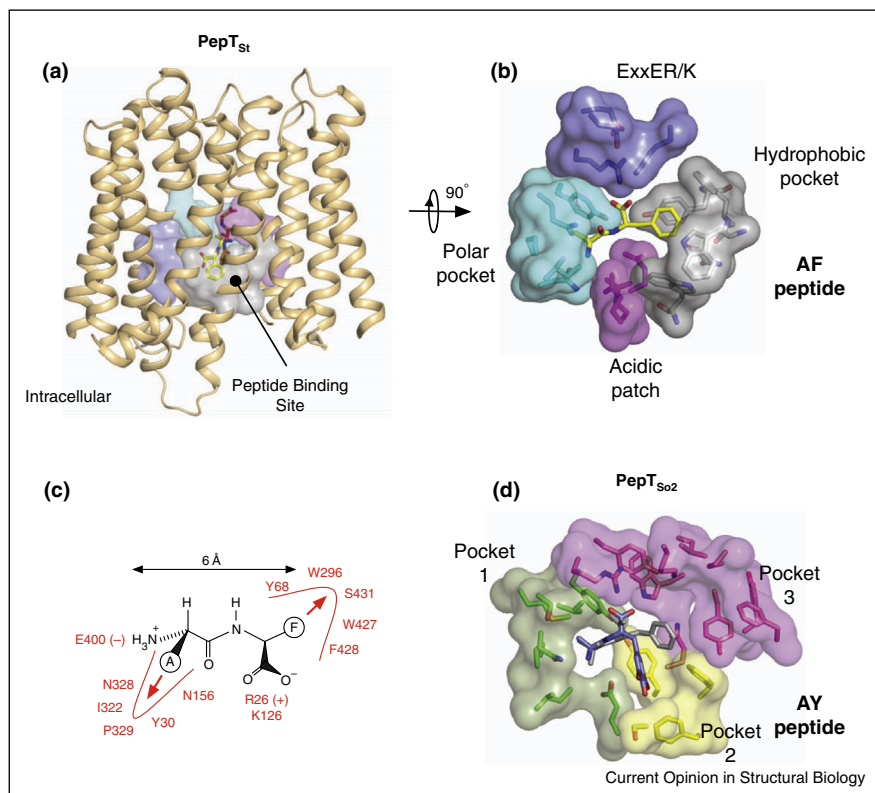


Structure of POT family transporters and transport mechanism. **(a)** Crystal structure of PepT_{So} (PDB:2XUT) showing the core 12 TM domains arranged equally into an N- and C-terminal bundle and the additional helical hairpin, HA & HB, inserted between TM6 and TM7. **(b)** Current model of proton coupled transport by the POT family. Transport is predominantly controlled through the opening and closing of two gates that sit on either side of a central binding site. The gates are made helices 1,2,4,5 from the N-terminal bundle and 7,8,10,11 from the C-terminal bundle. In many POT family members salt bridge interactions are seen coordinating the open/closed state of the gates. Proton binding/release from conserved proton binding sites act to drive the transport cycle in the forward direction, thereby concentrating peptides inside the cell.

and peptide binding. Using a combination of proton driven and counterflow transport assays a conserved ExxER/K motif on TMs 1 & 4 and conserved acidic residues on TM7 and TM10 were identified as important sites of proton/peptide binding [19^{*},20^{**},24,25]. Based on these studies a model for alternating access transport by the POT family was proposed (Figure 1b) [26], which is consistent with other secondary active transporters [27]. This suggests that transport is initiated through the binding of protons to both the ExxER/K and TM7 carboxylate in the outward open conformation. Peptide entry causes a large conformation change in the gate helices such that the extracellular gate closes to occlude the peptide. In this state proton movement from the extracellular gate salt bridge to the intracellular gate salt

bridge facilitates opening of the intracellular gate and release of peptide and protons into the interior of the cell. Resetting of the cycle is likely the rate-determining step and involves reforming the intracellular gate salt bridge, thereby closing the intracellular gate and subsequent opening of the extracellular gate to accept peptide. This model makes the assumption that the main movements in a POT family transporter are made by the eight helices that form the core of the transporter, TMs 1,2,4,5 and 7,8,10,11 with the remaining four helices, TMs 3,6 and 9,12 acting as scaffolds to coordinate and possibly regulate the gate helices [28^{**}]. Strict coupling must occur through the ability of the bound ligand to coordinate the proton relay, although precise details of this mechanism still remain to be determined.

Figure 2



Specificity pockets coordinate peptide binding. **(a)** Crystal structure of PepT_{St} shown in the plane of the membrane with the di-peptide Ala-Phe (PDB:4D2C) (yellow) and tri-peptide Ala-Ala-Ala (PDB:4D2D) (orange) shown as sticks. **(b)** Close up view of the specificity pockets formed around the Ala-Phe peptide. **(c)** Schematic detailing the interactions made to the Ala-Phe peptide shown in B. **(d)** Equivalent view of (b) in PepT_{So2} (PDB:4TPJ), showing the specificity pockets identified in this POT family transporter. The Ala-Phe peptide from PepT_{St} is shown overlaid in grey.

Peptide recognition is achieved through specificity pockets

Recently three bacterial POT family members have been crystallised in complex with physiological peptides [29^{••},30^{••}]. PepT_{St} was crystallised in complex L-alal-phe (AF) and L-alal-L-alal-L-alal (AAA) (Figure 2a). The peptides can be seen sitting in the central peptide-binding site observed previously for PepT_{So} and GkPOT. GkPOT was captured with the antibacterial peptide alafosfalin and was recently reviewed [26], so will not be discussed further here. The PepT_{St} structures however revealed four distinct regions of the binding site that accommodate the peptides (Figure 2b). There is the polar pocket, which forms around the side chain of the first amino acid and composed mostly of asparagine, glutamine and serine side chains. The phenyl group of the second amino acid can be seen accommodated in a hydrophobic pocket, formed by the movement of the C-terminal helices towards the peptide. A movement previously observed in the structure of PepT_{St} [19[•]]. Clamping the peptide at either end are the conserved acidic and basic charges of the intracellular gate salt bridge, R26 and K126, which also form

part of the polar pocket and E400, the conserved acidic group on TM10 (Figure 2c). Interestingly, an acidic patch of residues that contains the conserved carboxylic group on TM7 does not interact with the AF peptide, but as discussed below, interacts with the AAA tri-peptide in PepT_{St}. These structures and additional studies [23^{••},25] clearly support an important role for electrostatic interactions in orientating the peptide.

At a similar time the structures of PepT_{So2} in complex with three peptides were also reported [30^{••}]. In this study the transporter was captured with L-peptides AY, AYA and AAA. The authors identified three pockets that accommodated the peptides (Figure 2d). As all three peptides bound in the same manner only one is shown here, with the AF peptide of PepT_{St} shown for comparison. Comparing Figure 2b and d it is easy to see the similarities between the two structures with respect to the polar pocket in PepT_{St} and pocket 1 in PepT_{So2}, and likewise the ExxER/K pocket and the top half of pocket 3. However, differences do exist and the most notable is the absence of the hydrophobic pocket in the PepT_{So2} structures. However, both pocket 3 and pocket 2 in

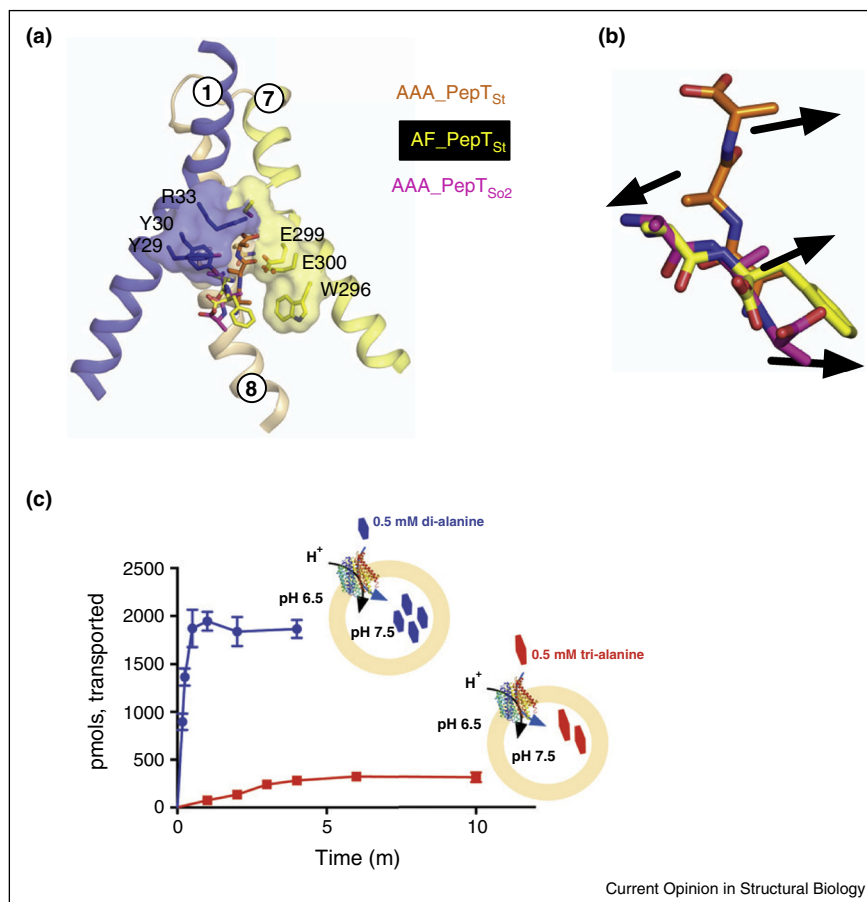
PepT_{So2} contain several large hydrophobic groups and it may simply be that in PepT_{So2} the hydrophobic pocket is not as clearly defined in this transporter.

The nature of the binding site in these two transporters supports a model for peptide recognition that relies heavily on interactions with the side chains of the ligand, rather than relying solely on interactions with the amino and carboxy termini as previously proposed [31,18^{**}]. The importance of the polar pocket in determining peptide specificity in PepT_{St} was recently demonstrated through computational docking and supported by *in vitro* transport assays [32^{**}]. The importance of specificity pockets would also explain the preferences for different peptides observed between members of the POT family that have been reported [33^{*},34,19^{*},20^{**},23^{**},30^{**}].

A dual binding mode for peptides may explain polyspecificity

The co-crystal structure of PepT_{St} in complex with AAA revealed the tri-peptide sitting in a vertical position, as opposed to the more horizontal orientation observed for the AF peptide in PepT_{St} and the AYA, AAA peptides in PepT_{So2} (Figures 2a and 3a). The interactions between PepT_{St} and the AAA peptide were also different, mainly contributed by the conserved tyrosines on TM1 (Y29, Y30) and the glutamate on TM7 (E300) [29^{**}]. Unlike the AF peptide, which we could see interacting with the intracellular gate salt bridge residues, the AAA peptide was clearly interacting more with the extracellular gate helices (Figure 3a). However, although the orientation of the two peptides was different, the direction into which the side chains were pointing between them is very similar. Indeed, it seems likely that the same specificity

Figure 3



Peptide can adopt different conformations in the binding site. (a) Crystal structure of PepT_{St} bound to tri-peptide Ala-Ala-Ala (orange). The specificity pockets are made predominantly from TM1 and TM7 and differ slightly between the di-peptide AlaPhe (yellow). (b) Overlay of the peptides bound to PepT_{St} and PepT_{So2}. For clarity only the AAA peptide is shown for PepT_{So2}. Arrows indicate similar directions for the side chains. (c) Steady state uptake of di- and tri-alanine by PepT_{St} under the same proton electrochemical gradient. The increased uptake of di-ala confirming the dual stoichiometry transport mechanism in this POT transporter.

pockets observed for the AF peptide will accommodate the first and second side chains from a tri-peptide, with a third pocket opening up to accommodate the final residue. Indeed, selected mutations in these pockets in PepT_{St} supported this hypothesis [29^{••},32^{••}].

The different positions occupied by both the AF and AAA peptides in the binding site of PepT_{St} suggested something rather interesting. If the transporter could interact with peptides in different ways, could it also transport them with different numbers of protons? The transport model in Figure 1b shows two protons per peptide, which was suggested for PepT2, although similar studies in PepT1 suggest a proton:peptide stoichiometry of 1:1 for di-peptides [35,36]. It is unclear if this stoichiometry holds for tri-peptides and drug molecules.

Using a reconstituted liposome based transport assay the stoichiometry of proton:peptide transport for PepT_{St} was however determined to be 3:1 for tri-peptides and 5-6:1 for di-peptides [37^{••}]. It is likely, given the similar orientation of side chains observed for the AF and AAA peptides in PepT_{St} that both di- and tri-peptides are 'handled' in similar ways, using the specificity pockets identified, but are clearly moved across the membrane with different numbers of protons. This result creates a surprising realization, that PepT_{St} has two different mechanisms for transporting peptides.

This result also suggested that given the same pH gradient across a membrane, di-peptides should be concentrated to higher steady state levels than tri-peptides. Indeed, this was found to be the case, with di-alanine being concentrated to far higher levels than tri-alanine for the same pH gradient (Figure 3c). PepT_{St} can therefore not only biochemically select for peptides based on side chain interactions with the specificity pockets but also thermodynamically 'select' di-peptides over tri-peptides due to its ability to concentrate these several fold higher than tri-peptides in the cell. This flexibility in binding and stoichiometry would be extremely difficult to achieve in a sodium-coupled system and may explain why peptide transport has remained proton coupled in bacteria, plants, fungi and mammals [4].

POT family: regulation and adaptation

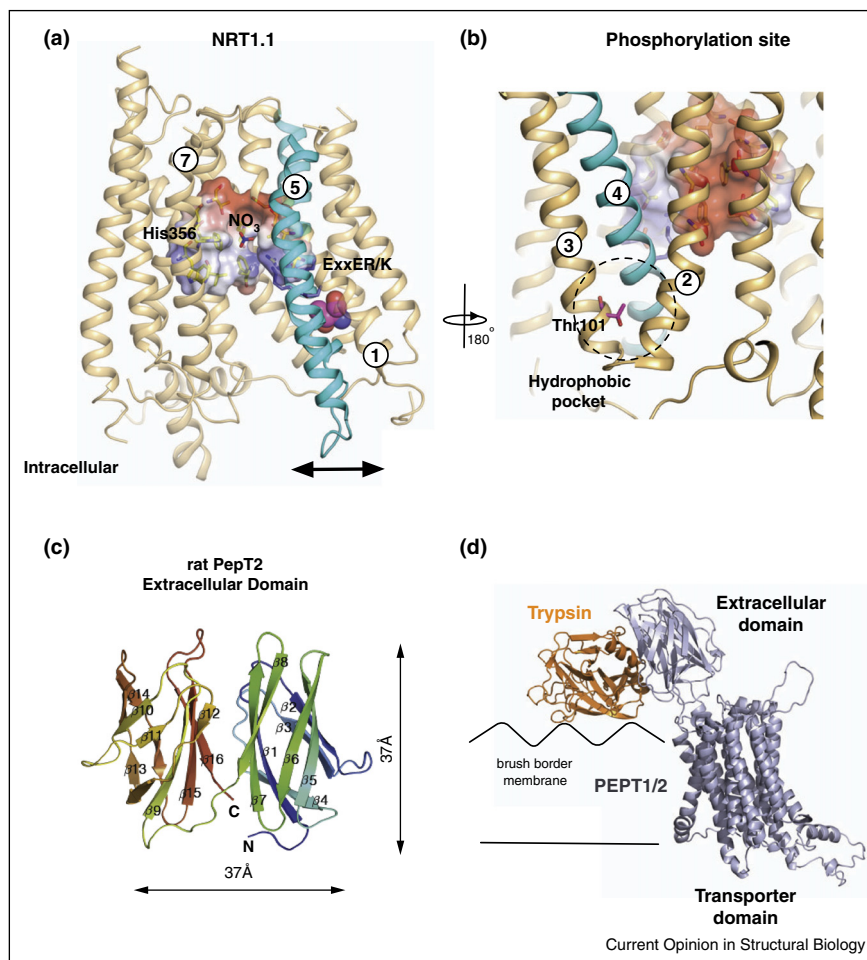
The crystal structure of the plant nitrate transporter NRT1.1 allowed for a unique insight into how this family has changed substrate specificity [38^{••},39^{••}]. The individual specificity pockets that we observed for the side chains in the peptide transporters have disappeared, being replaced by one larger pocket formed by TM1 and 7 (Figure 4a). Remarkably the binding site in NRT1.1 is actually larger than observed in the bacterial peptide transporters, even though we observe only a single nitrate molecule in the binding site. A significant change in the binding site appears to be the replacement

of the acidic patch with a histidine on TM7 (His356). Given the pK_a of histidine (~6.0), it is likely this requires protonation to form a favorable electrostatic interaction with nitrate under physiological conditions. What is more interesting however is that all of the intracellular gate machinery remains intact in NRT1.1. This includes the ExxER/K motif on TM1 and TM4 and the glutamate on TM10. Interestingly, the ExxER/K motif is also required for glucosinolate transport via NPF2.11 [40[•]]. Taken together the current evidence suggests that specificity within the POT family most likely resides in the extracellular gate region of the binding site, which is different between diverse POT family members. Such a mechanism would enable rapid evolution of different specificity, whilst retaining an efficient proton driven transport motor.

NRT1.1 is also regulated by phosphorylation [41^{••},42]. To date the only other transporter regulated at the mechanistic level by phosphorylation is the human serotonin transporter SERT [43]. NRT1.1 is able to switch between a high and low K_M state in response to phosphorylation at an intracellular threonine, Thr101. When external nitrate concentrations are high (>1 mM) NRT1.1 is unphosphorylated and held in a high K_M state (K_M^{app} 10 mM), presumably to avoid saturation. However, when nitrate levels in the external environment fall below ~1 mM, NRT1.1 is phosphorylated at Thr101, and switched into a low K_M state (K_M^{app} < 1 mM), to scavenge the remaining nitrate. The recent crystal structure of NRT1.1 revealed that Thr101 sits on TM3 and points into a hydrophobic cavity formed by residues on TM2 and TM4, which make up the extracellular and intracellular gates respectively (Figure 1b). We proposed that phosphorylation of this residue would disrupt the packing between these helices and enable NRT1.1 to cycle faster, possibly lowering the energetic barrier for the return step in the transport cycle [38^{••}]. This destabilization is also linked to a dimer-monomer transition in the membrane [39^{••},44] and may have subsequent effects in regulating the nitrate-signaling cascade that activates the high affinity nitrate uptake system [45^{••}].

NRT1.1 is suggested to coordinate a network of protein-protein interactions in the membrane, regulating the kinase CIPK23 that phosphorylates Thr101 and also transcription factors for the NRT2 family of high affinity nitrate transporters [45^{••}]. Could something similar be happening with PepT1 and PepT2? Unlike other eukaryotic POT family members, PepT1 and PepT2 have a large ~200 amino acid extracellular domain inserted between TM9 and TM10. Recently the crystal structure of the extracellular domain from the mammalian peptide transporters has been reported and shows two IgG-like domains linked in tandem (Figure 4c) [46^{••}]. Removing these domains from PepT2 resulted in no effect on peptide or drug uptake, suggesting they

Figure 4



Eukaryotic POT family transporters. **(a)** Crystal structure of NRT1.1 bound to nitrate (PDB:5A2O & 4OH3). The binding pocket for nitrate is shown coloured according to the electrostatic properties of the side chains. The principle nitrate binding residue, H356, is shown. The intracellular gate helices, TM 4 & 5 are coloured cyan, with Thr101 shown as sphered in magenta. **(b)** Close up view of the position of Thr101 in relation to the intracellular gate. **(c)** Crystal structure of the extracellular domain of rat PepT2 (PDB:5A9H), showing overall dimensions. **(d)** Hybrid model of human PepT1 showing the location of the extracellular and trans membrane domains. A model of trypsin has been docked in the putative interaction site.

were incorporated to add functionality. Biochemical evidence showed a specific interaction with the intestinal protease trypsin through a conserved electrostatic patch on the surface of the domain. Such an interaction would be highly novel and suggest that PepT1 and PepT2 may act within a protein network at the plasma membrane. Using this crystal structure and a homology model of the trans membrane domain built from the bacterial and plant members, a hybrid model of the mammalian transporter can be built and the trypsin molecule modeled at the interaction site (Figure 4d). The model shows how the extracellular domain would sit in relation to the transport domain, and makes a role for the extracellular domain in protein-protein interactions a distinct possibility.

Conclusion

The POT family provides a number of puzzles for biochemists and pharmacologists alike. How has one binding site adapted to recognize such a diverse range of substrates and can we utilize this knowledge to improve drug uptake in the human body? How does the POT family couple transport to protons and is there a conserved mechanism? How are the family regulated in eukaryotes, and are they part of more complicated nutrient uptake systems yet to be discovered? It will take a number of additional studies to address these questions in full, however the current success of structural and biochemical studies have provided fresh impetus into previous physiology based approaches to study these transporters.

The emerging structural and functional data reviewed here support an important role for the interaction of peptide side chains with the specificity pockets in the binding site. Differences in peptide specificity observed between bacterial POT family homologues suggest that in bacteria POT transporters have evolved to preferentially recognize distinct subsets of peptides, be they polar, hydrophobic, acidic or basic. Indeed, many bacterial species contain several POT family homologues, most probably with each adapted to recognize and transport a specific set of peptides. However, mammals contain only two POT family transporters, suggesting that our transporters have found a way to increase specificity over their bacterial counterparts. The trade offs made, in terms of efficiency and mechanism however, will require further study to understand, especially with respect to drug and pro-drug transport in the small intestine.

Finally, the recent elucidation of the crystal structures of NRT1.1 and the extracellular domains of PepT1 and PepT2 demonstrate two different ways that POT family members have adapted to the work in higher eukaryotes. It will be interesting to see if, like the plant NRT1.1, the mammalian members of this family also play a role in nutrient sensing and metabolic regulation.

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