Gating Topology of the Proton-Coupled Oligopeptide Symporters

Highlights

- New higher-resolution structure of PepTSo
- Salt bridges stabilizing outward-facing conformations are identified
- The conserved prolines in helix 8 are shown to be important
- The first two helices in each inverted-topology repeat form part of a gate

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In Brief

Fowler et al. use biophysical and modeling approaches to identify salt bridges in two peptide transporters that stabilize their outward-facing conformations. Their results also suggest that the first two helices in each inverted-topology repeat unit forms part of either of the two gates.

Accession Numbers

4UVM
Gating Topology of the Proton-Coupled Oligopeptide Symporters

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http://dx.doi.org/10.1016/j.str.2014.12.012
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SUMMARY

Proton-coupled oligopeptide transporters belong to the major facilitator superfamily (MFS) of membrane transporters. Recent crystal structures suggest the MFS fold facilitates transport through rearrangement of their two six-helix bundles around a central ligand binding site; how this is achieved, however, is poorly understood. Using modeling, molecular dynamics, crystallography, functional assays, and site-directed spin labeling combined with double electron-electron resonance (DEER) spectroscopy, we present a detailed study of the transport dynamics of two bacterial oligopeptide transporters, PepTSo and PepTSt. Our results identify several salt bridges that stabilize outward-facing conformations and we show that, for all the current structures of MFS transporters, the first two helices of each of the four inverted-topology repeat units form half of either the periplasmic or cytoplasmic gate and that these function cooperatively in a scissor-like motion to control access to the peptide binding site during transport.

INTRODUCTION

Peptide transport is the main route through which the body absorbs and retains dietary protein and hence plays an important role in human physiology (Steinhardt and Adibi, 1986). The combined action of acid hydrolysis in the stomach and nonspecific peptidases in the small intestine breaks down ingested protein into peptide fragments and free amino acids. The resulting di- and tripeptides are then actively transported across the intestinal brush border membrane by the integral membrane peptide transporter, PepT1 (Fei et al., 1994; Leibach and Ganapathy, 1996). PepT1 recognizes a diverse range of small peptides and is also responsible for the absorption of many orally administered drugs, including β-lactam antibiotics and a growing number of peptidic prodrugs (Luckner and Brandsch, 2005; Pieri et al., 2009; Brandsch, 2009). We do not yet fully understand the mechanism by which PepT1 recognizes and transports molecules into the cell, and this lack of knowledge is hampering the modification of drugs to improve their pharmacokinetic profiles.

PepT1 is a member of the POT family of proton-dependent oligopeptide transporters (TC 2.A.17), which itself belongs to the much larger major facilitator superfamily (MFS) of secondary active transport proteins (Reddy et al., 2012). POT family transporters contain either 12 or 14 transmembrane α helices. Structures of four bacterial members of the POT family have been determined: these are PepTSo (Newstead et al., 2011), from the bacterium Shewanella oneidensis; PepTSt (Solcan et al., 2012; Lyons et al., 2014), from the thermophilic mesophile Streptococcus thermophilus; and, more recently, GkPOT from the bacterium Geobacillus kaustophilus (Doki et al., 2013); PepTSo2, also from Shewanella oneidensis (Guettou et al., 2013). Biochemical studies on the bacterial POT family have revealed that these proteins operate in a similar way to their mammalian counterparts, with many of the functionally important residues conserved (Figure S8) (Daniel et al., 2006; Harder et al., 2008).

The MFS is the largest superfamily of secondary active transporters, containing over 70 different families (Reddy et al., 2012). Structures of members belonging to several MFS families reveal a common fold consisting of two bundles of six transmembrane (TM) α helices that come together to form a “Y”- or “V”-shaped transporter with a central substrate binding site (Figure S1) (Hirai et al., 2002; Abramson et al., 2003; Huang et al., 2003; Yin et al., 2006; Dang et al., 2010; Newstead et al., 2011; Solcan et al., 2012; Sun et al., 2012; Yan et al., 2013; Pedersen et al., 2013; Quistgaard et al., 2013; Guettou et al., 2013; Doki et al., 2013; Jiang et al., 2013; Deng et al., 2014). Not only are the two six-helix bundles structurally similar but there are also conserved sequence motifs between helices 2 and 3 in the N-terminal domain and between helices 7 and 8 in the C-terminal domain (Pao et al., 1998; Saier et al., 1999).

Recently it was proposed that each bundle can be further divided into two inverted-topology repeat units (four in total, labeled A–D, Figure 1) (Hvorup and Saier, 2002; Radestock and Forrest, 2011), revealing a more fundamental level of symmetry. The first three helices of the protein form repeat unit A; its structure is related to the second three helices (repeat...
The structures of PepTSo and PepTSt further suggested that as forming the periplasmic gate of GlpT (Huang et al., 2003). (H1 & H7), one from each six-helical bundle, were identified units (Radestock and Forrest, 2011), suggesting that an “asymmetry by swapping the structures of each pair of repeat conformations of LacY can be converted to an outward-open LacY, such that the two six-helix inverted repeats are asymmetric (in that state). This feature means that the inward-open conformation of LacY can be converted to an outward-open conformation by swapping the structures of each pair of repeat units (Radestock and Forrest, 2011), suggesting that an “asymmetry-exchange” mechanism underlies the overall conformational change. This method has been shown to reproduce key features of alternate conformations for several other transporter architectures (Schushan et al., 2012; Crisman et al., 2009; Liao et al., 2012; Radestock and Forrest, 2011).

Peptide transporters are proton-coupled symporters and use the inwardly directed proton electrochemical gradient to drive peptide uptake into the cell. The alternating-access mechanism (Jardetzky, 1966) predicts that they (1) facilitate transport by moving between outward- and inward-open conformations, (2) can only exchange between these conformations when in either the apo state or when both a substrate and one or more protons are bound, and (3) cannot form a continuous pore across the bilayer. Peptide transporters must therefore prevent access to the binding site(s) from at least one side of the membrane at all times. The rocker-switch mechanism of MFS transport extended the alternating-access mechanism by proposing that the bound ligand becomes alternately exposed to either side of the membrane by the rocking of the two symmetry-related six-helical bundles around the central binding site (Huang et al., 2003; Law et al., 2008).

To occlude the binding site to either side of the membrane, MFS transporters form gates; these are transient constrictions formed by the close packing of several transmembrane helices. These gates block entry or exit to the central cavity and are stabilized by interactions between transmembrane helices, specifically through key salt bridges, which in turn are controlled by substrate binding and release. Two transmembrane α helices (H1 & H7), one from each six-helical bundle, were identified as forming the periplasmic gate of GlpT (Huang et al., 2003). The structures of PepTSo and PepTSt further suggested that the periplasmic gate is formed by helices H1 & H2 packing against H7 & H8 (Newstead et al., 2011; Solcan et al., 2012).

To investigate the gating of the proton-coupled oligopeptide symporters, we needed representative structures of members of this family in both inward- and outward-facing conformations. The experimental structures of PepTSo, PepTSt, PepTSo2, and GkPOT, however, are all inward facing. We therefore pursued two independent approaches to generating plausible models of both PepTSo and PepTSt in outward-facing conformations. The first approach was to build outward-facing models of both proteins using the repeat-swapping method (Radestock and Forrest, 2011). Instead it was suggested that a dynamic movement of helices within the two six-helical bundles may be required for the central cavity to be alternately exposed to both sides of the membrane.

Here we present a mechanism for alternating access within the POT family. By systematic analysis of available MFS structures, we show that the cytoplasmic gate is formed by helices H4, H5, H10, and H11, as predicted previously (Newstead et al., 2011; Solcan et al., 2012) and also that the periplasmic gate is formed from the equivalent helices by symmetry, H1, H2, H7, and H8. The first two helices in each of the four repeat units therefore participate in either the periplasmic or the cytoplasmic gate while the third helix is less dynamic. Furthermore, we show that within the POT family, the inward-open and outward-open conformations are stabilized by salt bridges and that kinks introduced into the transmembrane helices by conserved prolines are important in transport. We propose that transport in the POT family can be described by an asymmetric scissor-type motion of the helices in the repeat units, thereby linking the structural symmetries and the alternating-access mechanism in this family of MFS transporters. This provides a more complete picture of the gating topology of the MFS superfamily.

RESULTS

To investigate the gating of the proton-coupled oligopeptide symporters, we needed representative structures of members of this family in both inward- and outward-facing conformations. The experimental structures of PepTSo, PepTSt, PepTSo2, and GkPOT, however, are all inward facing. We therefore pursued two independent approaches to generating plausible models of both PepTSo and PepTSt in outward-facing conformations. The first approach was to build outward-facing models of both proteins using the repeat-swapping method (Radestock and Forrest, 2011).
The Cα RMSD, excluding the HA and HB motif, between both structures is 1.7 Å (394 residues). Some differences can, however, be seen. One of these is the positions of the residues that make up the thin gate; in the new structure these are such that the peptide binding site is accessible to the cytoplasm and hence this structure is inward-open. Additional detail can be found in Figure S2.

(C) An outward-open model of PepTSo built using the repeat-swapping method. An image of the outward-open model of PepTSt is shown in Figure S2.

Figure 2. An Inward-Open Structure of the Bacterial Oligopeptide Transporter PepTSo

(A) The structure of PepTSo in an inward-open conformation solved to 3.0 Å using X-ray crystallography. The transmembrane helices are colored from red (H1) to blue (H12) as in Figure 1. The two additional helices found in the bacterial proton oligopeptide transporters, HA and HB, are colored light gray. A lateral helix (LH) found between H6 and HA and not seen in the previous structure is highlighted. The data collection and refinement statistics can be found in Table 1.

(B) This new structure of PepTSo is broadly similar to that of the lower-resolution inward-occluded structure of PepTSt (PDB: 2XUT) (Newstead et al., 2011) is a biophysical method for determining the distance distribution between two labeled cysteines in a protein. PepTSt, however, formed dimers in solution, complicating the interpretation of the spin-spin distances, and hence was less suitable. Eight pairs of cysteine residues were introduced into PepTSo and labeled with the nitroxide spin label (1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl)methanethiosulfonate (MTSL) (Figure 3 A); these pairs were designed to measure three periplasmic distances and five cytoplasmic distances on the protein (Figure 3B). The transport activity of all eight double cysteine mutants was tested using a proton-coupled assay and all eight were competent at active transport, although some mutants were less active than wild-type (Figure 3C). The experimental DEER distance distributions are broad, often covering ~30 Å, and are typically multimodal, usually with two or three distinct peaks (Figure 3D). This suggests that PepTSo is highly dynamic and is present in several different conformations during the experiment.

Since the MTSL label has a flexible linker, it can adopt a wide range of conformations when bound to a cysteine residue. The DEER distance distributions are hence convolutions of all the protein conformations present with all possible conformations of the spin label. Determining if a structure is consistent with the distance distributions derived from the DEER data is therefore complicated. To estimate the resulting broadening attributable to the flexibility of the MTSL linker, we mapped a rotamer library of spin labels onto each pair of residues, thereby estimating the spin-spin distance distribution that would arise from a single, specified structure (Stelzl et al., 2014; Polyhach et al., 2011). This method allows stronger inferences to be made than either simply calculating the distances between the Cα atoms of the labeled residues, or determining if, for a given structure, there are spin label rotamers consistent with the most likely distances as represented by the positions of the maxima observed in the DEER distance distributions (Madej et al., 2012).
Since the POT family comprises proton-coupled symporters, the apo protein can move between inward- and outward-open conformations, and hence we expect both conformations to be populated in the DEER experiment in the absence of substrate. Comparing the distance distributions predicted from the inward-open crystal structure and the repeat-swapped outward-open model with the experimental DEER data therefore provides a route to validate the PepTSo outward-open model. If the experimental data fit the peaks in both sets of predicted distance distributions then that would be a strong validation of the model. Not being able to explain all the features of the predicted distance distributions, discrepancies in the position or width of the peaks or variation between the different residue pairs studied would weaken the level of validation. Finally, predicted distance distributions that simply lie within the bounds of the experimental data but whose peaks do not exactly match the experimental DEER data would constitute a weak form of validation.

The correspondence between the experimental DEER data and the distributions predicted from the inward-open PepTSo structure and outward-open PepTSo model falls somewhere between the last two levels; taken together, the positions of all the predicted peaks do not align exactly with the peaks in the DEER data nor do the distributions explain all the features of the experimental data. The latter suggests that the protein is sampling more than two distinct conformations, which complicates the validation. The spin-spin distance distributions predicted from the experimental inward-open PepTSo structure lie within the bounds of the DEER distance distributions for seven of the eight pairs of residues examined (Figure S3; the exception is the 141–500 distance, which has limited overlap). Of these seven, the positions and widths of the predicted peaks align reasonably for six of the seven remaining residue pairs, the exception being the 174–401 distance. For the outward-open model the predicted spin-spin distance distributions lie within the bounds of six of the DEER distance distributions, the exceptions being the 141–500 (again) and 201–364 distances (Figure S3). We shall address why the model poorly describes the 201–364 distance later. The predicted distributions align reasonably for five of the six remaining residue pairs, the exception being the 47–330 distance. The outward-open model therefore agrees slightly less well with the experimental DEER data than the inward-open PepTSo crystal structure and hence is only weakly quantitatively consistent with the experimental DEER data.

It is surprising, however, that the distance distributions predicted from the inward-open crystal structure of PepTSo do not better describe the experimental DEER distance
distributions. This suggests that our DEER-based approach would struggle to discriminate between good and poor models. There are several possible reasons for this. First, the experiments were carried out in detergent, which may have affected the dynamics of the protein. Second, our method assumes that the dynamics of the protein and the dynamics of the spin label (its rotamer states) are independent (although clashing rotamers are removed). Allowing the rotamers adopted by the MTSL spin label to be influenced by the conformation of the protein may improve the predicted distance distributions and hence the correspondence with the experimental DEER data (Roux and Islam, 2013).

Generating Outward-Open Conformations by Molecular Dynamics Simulation

We therefore attempted a second, independent approach to producing structures of both PepTSo and PepTSt in outward-open conformations. This was to run long molecular dynamics simulations of a single copy of each protein embedded in a lipid bilayer as described in the Experimental Procedures. Three simulations, each 200 ns long, were run for each protein. Since this is comparatively short, we did not expect to see transitions in all simulations. Inspecting how the arrangement of the transmembrane helices changed suggested that, in at least one of the simulations for both proteins, there was a partial transition toward the outward-open state due, we assume, to the stochasticity of the dynamics. We then categorized and clustered the conformations produced by the simulations based on the state of the cytoplasmic and periplasmic gates.

Determining the Conformational State of an MFS Transporter

Although a simple distance-based method for determining the conformational state of a structure of an MFS transporter has been recently proposed (Stelzl et al., 2014), it assumes which helices characterize the state of a transporter. We have extended the ideas of Stelzl et al. (2014) by considering all possible permutations of helices and thereby determining a priori which helices form the cytoplasmic and periplasmic gates of MFS transporters. To provide a reference data set we started by determining the maximum radius of a spherical probe that can be accommodated in the protein along the z axis, i.e. as the probe is moved from the cytoplasm, through the central cavity, and into the periplasm. The resulting pore profiles confirm that the central cavity in the new structure of PepTSo is accessible to the cytoplasm but inaccessible to the periplasm, hence the structure can be described as inward open (Figure 4A). Likewise, the central cavity of the model built using the repeat-swapping method is accessible to the periplasm but inaccessible to the cytoplasm (Figure 4B) and so can be described as outward open. Similar results were also obtained for PepTSt (Figure S4).
The same procedure was then applied to all currently known structures of MFS transport proteins (Figure S4), and the minimum value of the radius in both gate regions was determined. Plotting the minimum probe radius of the cytoplasmic gate against the same quantity for the periplasmic gate (Figure 4C) elegantly quantifies the conformational state of all known MFS transporter structures. As expected, the upper right quadrant in Figure 4C is empty since there are no crystal structures with both gates open; the coordinates instead describe an L-shaped locus. The MFS transporters in the top left quadrant have an open cytoplasmic gate but a closed periplasmic gate, and are therefore inward facing. This relationship is reversed in the bottom right quadrant (these are the outward-facing structures) while those in the bottom left quadrant are occluded. Analyzing the pore radius profiles in this way provides an intuitive and accurate way to characterize the conformation of an MFS transporter and has allowed us to assign, rigorously and without bias, each of the known MFS transporter structures to a specific state. This method is, however, both time consuming to apply to molecular dynamics trajectories and does not allow the helices forming the gates to be identified. To address these problems we shall develop a simple geometrical metric based on the minimum distance between the tips of the transmembrane helices in the protein (Stelzl et al., 2014).

We assume that both gates in MFS transporters are formed by the tips of two contiguous pairs of transmembrane helices comprising together, one pair from each half of the protein. This is consistent with previous suggestions about which helices contribute to one or other of the gates (Huang et al., 2003; Solcan et al., 2012). Since each half of the transporter is made up of six transmembrane helices and the N terminus of the protein is found in the cytoplasm, there are three helix pairs in each half of the transporter that could contribute to the periplasmic gate (Figure 5A: H1 & H2, H3 & H4, H5 & H6 and H7 & H8, H9 & H10, H11 & H12) and two helix pairs in each half of the transporter that could contribute to the cytoplasmic gate (H2 & H3, H4 & H5 and H8 & H9, H10 & H11). We only considered the Cα atoms of the tip of each helix, defined as the first or last ten residues of each helix on the cytoplasmic or periplasmic side of the protein. The minimum distance between each set of helix tip pairs was then calculated. This was repeated for all nine combinations of periplasmic helix tip pairs and four combinations of cytoplasmic helix tip pairs for all known MFS transporter structures. We then examined the correlation between these sets of distances and the previously determined minimum probe radius that can pass through each of the gates (Figure S5). The state of the periplasmic gate correlates best with the minimum distance between the tips of helix pairs H1 & H2 and H7 & H8 (r = 0.88, Figure 5B; Figure S5A), while the state of the cytoplasmic gate correlates well with the minimum distance between the tips of H4 & H5 and H10 & H11 (r = 0.78, Figure 5C; Figure S5B), thus suggesting that not only do these helices form the periplasmic and cytoplasmic gates but also that these simple distance-based metrics accurately capture the state of these gates. It is not surprising that these particular four helix pairs make up the periplasmic and cytoplasmic gates of MFS transporters since they are, in both cases, closest to the axis of symmetry that divides the N- from the C-terminal halves in any MFS transporter (Figure 5A).
We then examined how the conformations of PepTSo and PepTSt changed during the molecular dynamics simulations by projecting the density of states onto the 2D space defined by our new distance-based metrics (Figure 6; Figure S6). Both proteins sample inward-facing, occluded, and outward-facing conformations during each set of three simulations. We defined any conformation that has both distances less than 9 Å as being occluded and any conformation with the periplasmic distance ≥9 Å and the cytoplasmic distance <9 Å as being outward open (and the other way round for inward open). This allowed us to classify the ensemble of structures generated during the simulations as either outward facing, inward facing, or occluded. For both proteins, one of the three simulations explored parts of the outward-open region. The C-terminal half of both proteins was found to be more dynamic than the N-terminal half (Table S1), consistent with the differences between the crystal structures of PepTSo and PepTSt. This pattern continued when the repeat-swap units were analyzed, with C and D being more dynamic than repeat units A and B. Examining the individual transmembrane helices showed that within each repeat unit, the third helix was typically less dynamic than the first two. Each conformation generated by the simulations was then analyzed for the presence of salt bridges.

Salt Bridges Stabilize the Intracellular Gate in the POT Family

Seven salt bridges were identified in the simulations of PepTSo (Figure 7; Figure S7). Two interactions (D136-K439 and K84-D79) were predicted to stabilize outward-facing conformations of PepTSo, one (R52-D328) was predicted to form in inward-facing and occluded conformations (Figure 7A), and the remaining four are discussed in the legend of Figure S7. The residues in the first two salt bridges are conserved in mammalian members of the POT family, but only the second salt bridge has the potential to form in PepTSt. The side chains of D136 and K439 are pointing away from one another in our outward-open model of PepTSo, and their Cα atoms are 1.5 Å further apart than when a salt bridge has formed in the simulations. The repeat-swapped model of PepTSo does not therefore predict the D136-K439 salt bridge, although only a small motion is required for it to form. Since the residues of the second salt bridge (K84-D79) are close in sequence, and therefore the distance between them is always small, it is not possible to say if the outward-open model predicts the K84-D79 interaction. Mutating any of these four residues to alanine abolishes transport or, in the case of K84A, reduces it significantly, which is consistent with (but does not prove) the hypothesis that these salt bridges stabilize outward-facing conformations of the POT family.

Finally, let us consider the putative R52-D328 interaction. Although these two residues are not interacting with one another in the inward-facing structure of PepTSo, again only comparatively small motions would be required to bring this about. These residues are not, however, conserved within the SLC15 family and mutating either residue to alanine merely reduces transport, suggesting that even if this interaction does stabilize inward-facing conformations, it is not essential and is only found in bacterial members of the POT family. Analysis of the PepTSt simulations identified five salt bridges (Figure S7), two of which (R33-E300 and R53-E312) have been previously suggested to stabilize inward-facing conformations (Solcan et al., 2012) and two of which (K126-E25 and K126-E22) are equivalent to those seen in PepTSo.

A Proline-Induced Kink in H8 Is Required for Transport in the POT Family

We previously noted the poor agreement between the DEER E201C-R364C distance distributions and our predictions from the outward-facing PepTSo model (Figure S3). This pair of residues reports the relative motions of H6 and H8, respectively (Figure 8A), which are part of repeat units B and C, respectively. A major structural difference between repeat units C and D is the kink in H8, which is absent in its symmetry-related partner, H11. Examining the structure of PepTSo suggests that the kink in H8 is due to two prolines, P345 and P353 (P329 and P345 in PepTSt), the latter being highly conserved across the POT family (Figure S8). Prolines are known to favor kinks in transmembrane helices (Fowler and Sansom, 2013). Mutating both prolines to alanine led to an altered spin-spin distance distribution in PepTSo with two peaks, one at a position similar to that of the single peak in PepTSt.
observed in wild-type, and another, more dominant, peak at a shorter distance (Figure 8D; Figure S3). This second peak overlaps with the distance distribution predicted from the outward-facing model and is likely due to a straightening of H8 caused by the removal of the two proline residues, resulting in a decrease in the distance between the intracellular ends of H6 and H8. We propose that the anomalously short distance predicted by the outward-open model of PepTSo is the result of the repeat-swapping process making H8 too straight. To support this hypothesis, mutating the first proline in either PepTSo (P345A) or PepTSt (P329A) reduced proton-driven transport (Figure 8E). The effect was more pronounced when the second, more conserved proline was mutated; the P353A PepTSo mutant abolished active transport and the P345A PepTSt mutant had only 20% of the level of transport activity of the wild-type. The PepTSo double mutant had no detectable transport activity, whereas the PepTSt double mutant had activity similar to that of the P329A mutant.

What about helix H11? It is approximately straight in the crystal structures but kinked in the outward-open models, since its sequence is threaded onto the structure of H8. The 174–466 and 141–438 residue pairs report the relative motions of helices H5 and H11 (Figure 3B). The distance distributions predicted for both pairs of residues appear to agree moderately well with the DEER data (Figure S3), although neither is shifted significantly by the conformational change. If the kink in H11 is an artifact of the repeat-swapping process then it is likely that this will bias both these predicted distance distributions. It is probable, however, that H11 bends to some extent as it has a central glycine (Gly453 in PepTSo, Gly434 in PepTSt), which is conserved across the POT family (Figure S8).

Our results suggest that (1) proline-induced kinks in transmembrane helices are important for the function of POT family transporters (Madej et al., 2012), (2) the repeat-swapping method captures the internal dynamics of a domain best when the repeat units are not too dissimilar to one another, and (3) taking into account the presence (or absence) of kink-forming residues in symmetry-related helices could further improve the repeat-swapping method.

DISCUSSION

Alternating access within secondary active transporters is currently understood to occur through the formation of three distinct sets of conformations: the outward-facing, occluded, and inward-facing states (Yan, 2013). As their names suggest, the three states are differentiated by whether gates permit or block access to the central cavity from either side (or both sides) of the membrane. We have studied outward-facing conformations of two members of the POT family using two independent methods; we built outward-open models of PepTSo and PepTSt using the repeat-swapping method and also ran molecular dynamics simulations starting from inward-facing crystal structures. The PepTSo outward-open model is only weakly consistent with a set of eight spin-spin distances measured by DEER spectroscopy. The DEER data suggest that apo PepTSo samples more than two conformations, which complicates the interpretation.

A Double Scissor-Switch Mode of Gating within the POT Family

Our experiments, models, and simulations suggest that in the POT family, the first six helices are less dynamic than the last
Mutating either or both prolines in PepT So or PepT St either reduces or abolishes proton-driven active transport. This observation is consistent with a previous analysis whereby H1 and H2 from repeat unit A and H7 and H8 from repeat unit C come together to form the periplasmic gate (and another are also likely to be important (Yaffe et al., 2013). Crucially, there is asymmetry in the relative magnitudes of how much each pair of scissors moves: the blades of the C-terminal scissors move more than the blades of the N-terminal scissors. This observation is consistent with a previous analysis comparing the asymmetric PepT So structure with structures of the lactose permease, LacY (Newstead et al., 2011). Our scissors analogy also extends the “rocker-switch” mechanism (Huang et al., 2003; Newstead et al., 2011; Solcan et al., 2012) to systematically demonstrate that the minimum distance between the tips of these helices correlates with the minimum radius of a spherical probe able to pass through the gate (Figure 5). This simple distance-based metric is also likely to apply across the whole MFS superfamily.

When we map these eight helices back onto the transmembrane topology, we find a surprising and pleasing symmetry (Figure 9C); the first two helices of each of the four repeat units are found to form half of one of the two gates. A picture emerges whereby H1 and H2 from repeat unit A and H7 and H8 from repeat unit C come together to form the periplasmic gate (and likewise the cytoplasmic gate is formed by H4 and H5 from repeat unit B and H10 and H11 from repeat unit D). By linking the concept of inverted-topology repeat units with our observation of which helices form the gates, we are therefore able to

Figure 8. The Kink Produced by the Conserved Prolines in H8 Is Important for Transport
(A) In the inward-occluded experimental structure of PepT So, H8 (in red) is kinked because of two prolines, P345 and P353 (in pink). We measured the relative motion of the C-terminal ends of H8 and H6 (pink) by attaching MTSL spin labels to the E364C R201C mutant of PepT So. (B) The same features are highlighted in blue on the outward-open model of PepT So, demonstrating that this model predicts a shorter distance between positions 201 and 364. (C) There is moderate overlap between the R201C E364C spin-spin distance distributions measured experimentally (black line) and those predicted from the inward-occluded crystal structure of PepT So (filled red bars). The outward-open model instead predicts a shorter distance between the ends of H6 and H8 (filled blue bars). (D) Mutating both prolines to alanines results in a more complex spin-spin distance distribution. We suggest that H8 in the R201C E364C P345A P353A is straighter than wild-type. Consistent with this, there is now reasonable agreement between the spin-spin distance distributions measured experimentally (black line) and those predicted from the model of the outward-facing conformation (filled blue bars). Error bars indicate the standard deviations from triplicate experiments.
suggest a putative gating topology of the MFS superfamily (Figure 9C).

Some of our observations, however, cannot be extended from the POT family to the wider MFS superfamily, reinforcing the view that individual families have evolved unique transport mechanisms. For example, we designed the DEER experiments so that five of the distances we studied were equivalent to five distances previously studied in the lactose permease, LacY (Smirnova et al., 2007). Unexpectedly, the spin-spin distances for PepTSo and LacY (Figure S9) were very different, suggesting that, despite both being bacterial proton-coupled symporters with hydrophilic substrates, the precise dynamics of these two MFS transporters are different. Likewise, it is unlikely that any of the salt bridges we have predicted and tested will be conserved outside the POT family, although the general concept may hold as similar stabilizing interactions have been proposed for VMAT2, another MFS transporter (Yaffe et al., 2013). While it is also likely that the role of the prolines in H8 is specific to the POT family, Brandl and Deber (1986) noticed nearly 30 years ago that prolines are overrepresented in the transmembrane α helices of transport proteins, the largest class of which are the MFS transporters. We anticipate that future studies will unravel the role of prolines and kinked helices in the functioning of MFS transporters.

**EXPERIMENTAL PROCEDURES**

The key methods are summarized here; detailed descriptions can be found in the Supplemental Experimental Procedures.
Both PepT So and PepT St sequences were constructed by superposing the structural repeats of each protein onto one another as described elsewhere (Radestock and Forrest, 2011). The additional helices present in both proteins, HA and HB, are not part of any repeat unit and so were omitted from all model building. Both preliminary alignments were then adjusted manually to remove gaps in the TM helices, and the sequences of individual helices were shifted to improve the sequence conservation. Using these sequence alignments the repeat-swapped models of PepT So and PepT St were constructed using Modeller 9.7 (Sali and Blundell, 1993). The inward-open PepT So (PDB: 4UVJ) and PepT St (PDB: 4APS) crystal structures were used as templates (Solcan et al., 2012). The 100 PepT So and PepT St models with the lowest scores were refined further. The two structures with the lowest Modeller (DOPE) scores were selected to be the representative, one for each protein. Both repeat-swapped models are available in the Supplemental Information.

Protein Purification and Crystallization, and Data Collection and Processing

Wild-type and mutant PepT So were purified to homogeneity (Newstead et al., 2011). Crystals were prepared as described previously (Lyons et al., 2014). X-ray diffraction data were collected on the I24 beamline at the Diamond Light Source, Oxford, UK. Molecular replacement search models were prepared from the inward-occluded PepT So model (PDB: 2XUT). More detail is given in the Supplemental Information, and the data collection and refinement statistics are shown in Table 1.

Molecular Dynamics Simulations

Molecular dynamics simulations of chain A from the experimental structure of apo PepT So (PDB ID 2XUT) were carried out as described previously by Newshead et al. (2011).

DEER data.

Received: September 26, 2014
Revised: November 21, 2014
Accepted: December 3, 2014
Published: February 3, 2015

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Supplemental Information

Gating Topology of the Proton-Coupled
Oligopeptide Symporters

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1 Supplemental Experimental Procedures

1.1 Materials.

(1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl)-methanethiosulfonate (MTSL) was obtained from Toronto Research Chemicals (North York, Canada).

1.2 Constructing the preliminary sequence alignments.

Preliminary pair-wise sequence alignment between the two halves of the PepTS0 and PepTS1 sequences were constructed by superposing the structural repeats of each protein onto one another using the structure alignment program SKA (Petrey et al., 2003). Hence, repeat unit A of PepTS0 (residues 11-107) was superposed on repeat unit B (residues 108-201), and repeat unit C (residues 287-400) was superimposed on repeat unit D (residues 402-512) resulting in two alignments. The RMSD values between repeat units A & B and C & D are 2.1 and 4.0 Å, respectively. Likewise, repeat unit A of PepTS1 (residues 14-105) was superposed on repeat unit B (residues 106-201), and repeat unit C (residues 280-379) was superimposed on repeat unit D (residues 380-471) with RMSD values of 2.6 Å (A & B) and 3.5 Å (C & D). Each set of two sequence alignments was then spliced together to obtain a pair-wise alignment between the model sequence (either PepTS0 or PepTS1) and the corresponding template, which is the PepTS0 or PepTS1 sequence whose repeats have been rearranged so that they have the order BADC. The additional helices present in both protein, HA & HB, are not part of any repeat unit and so were omitted from all model building. Both preliminary alignments were then adjusted manually to remove gaps in the TM helices and the sequences of individual helices were shifted to improve the sequence conservation, resulting in the final alignments (Figure S10, S11). Even before these further adjustments to the preliminary alignment, the corresponding models are consistent with outward-facing conformations of PepTS0 and PepTS1.

1.3 Building the repeat-swapped models.

Using these sequence alignments the repeat-swapped models of PepTS0 and PepTS1 were constructed using Modeller 9.7 (Sali and Blundell, 1993). The inward-open PepTS0 (PDB:4UVM) and PepTS1 (PDB:4APS) crystal structures were used as templates (Solcan et al., 2012). Structural restraints were added to preserve the secondary structure of the models. Specifically, for PepTS0 we constrained the N-terminal tips of H2, H4, H5, H6, H7 and H11 and the C-terminal tips of H1, H2, H3 and H5 to be helical (for PepTS1 the equivalent helices were H5, H6, H9 & H10 and H1, H7, H8, H9, H10 and H11, respectively). In addition, the distance between the guanidine carbon and the side chain nitrogen of the charged pair R25 and K127 (R26 and K128 in PepTS1) was constrained to be 4.5 ± 0.5 Å. This prevented the arginine side chain from pointing into the hydrophobic lipid core. One thousand models were created of each protein and the models were then ordered according to the Modeller (DOPE) score.

The 100 PepTS0 and PepTS1 models with the lowest scores were selected for further refinement. The energy of the PepTS0 models was minimised prior to analysis and hydrogens were added using GROMACS (version 4.6) (Prónk et al., 2013). The energy minimisation was performed in three stages, each with 250 steps of steepest decent minimization, followed by 250 steps of conjugated gradient minimization. In the first stage, only the hydrogen atoms were allowed to move. In the second stage, only the hydrogen and side chain atoms were allowed to move. In the final stage, no constraints were applied. The minimization was performed using the CHARMM22 force field (MacKerell et al., 1998). To account for the fact that the central cavity is filled with water, a high dielectric constant (ε = 80) was used for the calculation of electrostatic interactions. The same procedure was repeated for the PepTS1 models, except CHARMM (version 3.4) (Brooks et al., 1983) was used to minimise the energy and the positions of hydrogen atoms were predicted using REDUCE (version 2.21) (Word et al., 1999). The two structures with the lowest Modeller (DOPE) scores were selected to be the representative, one for each protein. Both models had reasonable quality according to Procheck (Laskowski et al., 1993), with the PepTS0 model having only three and four residues (two and three for PepTS1) occupying generously allowed or disallowed regions of the Ramachandran plot, respectively.

1.4 Protein purification and crystallization.

Wild-type and mutant PepTS0 were purified to homogeneity as described previously (Newstead et al., 2011).

The protein-laden mesophase was prepared by homogenizing 7.8 MAG and 10 mg/ml protein solution in a 1:1 ratio by weight using a dual syringe mixing device at 20 °C (Caffrey and Cherezov, 2009). Crystallisation trials were carried out at 20°C.
Pulse was 12 ns. A two-step phase cycle (were both used. For both the 3p and 4p DEER experiments, the observer and the three-pulse (3p) DEER with the sequence (Q All measurements were carried out on a Bruker Elexsys 680 at X-band (water crystallization. Stedium). For pulsed experiments, samples were loaded into 3 mm quartz tubes and flash frozen in liquid nitrogen to avoid and the sample concentrated to 200-300 µm labeling site, and incubated with the sample for three hours at room temperature. The spin label was removed as before, DTT (5 ml HiTrapTM, GE healthcare). MTSL dissolved in DMSO (10mg/ml) was added at a 10X molar ratio to each pH 7.5, 150 mM NaCl, 0.03% DDM, 30% glycerol). The solution was diluted 10X and salt-exchanged to remove excess The PepT1.92011 and flash-frozen in liquid nitrogen for storage. Protein purification was carried out as described previously (and cell membranes were separated by ultracentrifugation at 130,000g for 2h at 4°C harvested, resuspended in 1x PBS and lysed in a cell disruptor at 30Kpsi. Cell lysates were centrifuged at 25,000g for 30min, and expression was induced with 0.2mM IPTG at OD600 =0.6. The temperature was dropped to 25°C overnight. Cells were harvested, resuspended in 1x PBS and lysed in a cell disruptor at 30Kpsi. Cell lysates were centrifuged at 25,000g for 30min, and cell membranes were separated by ultracentrifugation at 130,000g for 2h at 4°C. Membranes were resuspended in 1x PBS and flash-frozen in liquid nitrogen for storage. Protein purification was carried out as described previously (Newstead et al., 2011). 1.7 Transport assay Both PepTSo and PepTSt were reconstituted into Escherichia coli total lipids with egg PC liposomes and assayed using a proton-driven system as previously described (Solcan et al., 2012). 1.8 Preparation of PepTSo double-cysteine mutants. E. coli C43 (DE3) cells overexpressing the PepTSo-pWaldo-GFPe construct (Drew et al., 2006) were grown in TB at 37°C and expression was induced with 0.2mM IPTG at OD600=0.6. The temperature was dropped to 25°C overnight. Cells were harvested, resuspended in 1x PBS and lysed in a cell disruptor at 30Kpsi. Cell lysates were centrifuged at 25,000g for 30min, and cell membranes were separated by ultracentrifugation at 130,000g for 2h at 4°C. Membranes were resuspended in 1x PBS and flash-frozen in liquid nitrogen for storage. Protein purification was carried out as described previously (Newstead et al., 2011). The PepTSo double-cysteine mutants were reduced for one hour at room temperature in 10 mM DTT in buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 0.03% DDM, 30% glycerol). The solution was diluted 10X and salt-exchanged to remove excess DTT (5 ml HiTrapTM, GE healthcare). MTSL dissolved in DMSO (10mg/ml) was added at a 10X molar ratio to each labeling site, and incubated with the sample for three hours at room temperature. The spin label was removed as before, and the sample concentrated to 200-300 µM using a vivapsin concentrator with a 50 kDa molecular weight cut-off (Sartorius Stedium). For pulsed experiments, samples were loaded into 3 mm quartz tubes and flash frozen in liquid nitrogen to avoid water crystallization. 1.10 Pulsed DEER spectroscopy. All measurements were carried out on a Bruker Elexsys 680 at X-band (~9.5 GHz) between 50 and 80 K using an overcoupled (Q ≈ 100) 3 mm ER4118X-MS3 resonator. The four-pulse (4p) DEER experiment with the sequence (Pannier et al., 2000)

$$\pi/2(v_{obs}) - \tau_1 - \pi(v_{obs}) - \tau' - \pi(v_{pump}) - (\tau_1 + \tau_2 - \tau') - \pi(v_{obs}) - \tau_2 - \text{echo}$$

and the three-pulse (3p) DEER with the sequence (Milov et al., 1981)

$$\pi/2(v_{obs}) - \tau - \pi(v_{pump}) - (\tau_1 - \tau) - \pi(v_{obs}) - \tau_1 - \text{echo}$$

were both used. For both the 3p and 4p DEER experiments, the observer \(\pi\) and \(\pi/2\) pulses were 32 ns, and the pump \(\pi\) pulse was 12 ns. A two-step phase cycle (+x −x) was applied to the first observer pulse, and \(\tau\) was incremented in 8 ns
steps. The observer frequency coincided with the maximum of the nitroxide spectrum and the center of the microwave mode of the resonator, while the pump pulse was 65 MHz upfield. Accumulation times were between approximately four and sixteen hours, and the repetition rate adjusted to avoid signal saturation. \( \tau \) was varied between 2000-3400 ns in the 3p DEER experiment, and between 800 and 2500 ns in the 4p DEER experiment to maximize signal to noise. For samples where both 3p and 4p DEER were collected, the DEER datasets were computationally combined such that one has to access both the zero time in the 4p DEER data, as well as longer evolution times due to the increased sensitivity in the 3p DEER experiment (Lovett et al., 2012). Data were processed and analyzed using DeerAnalysis2011 (Jeschke et al., 2006).

### 1.11 Least squares fit method for combining 3p and 4p DEER.

To combine the two datasets, the 3p and 4p DEER raw data were phased in DEERAnalysis 2011 to correct for any experimental data collected in the imaginary channel. Next, the zero times for the 3p and 4p datasets were defined. Distorted data points were removed from the end of the 4p DEER dataset, and the beginning of the 3p DEER dataset. Finally, each dataset was background-corrected simultaneously assuming a homogenous, spatially confined background,

\[
B(t) = \exp(-kt^{(d/3)})
\]

where \( k \) quantifies the concentration of the spins, and \( d \) is the background dimensions (typically two for membrane proteins in bilayers, and three when in detergent micelles). \( k \) is varied such that differences between the background decay of the two datasets are minimized to obtain the best fit between the two experimental datasets.

### 1.12 Determining the conformational state of MFS transporters.

The high resolution crystal structure of PepT\(_{So}\) was manually arranged so that the \( z \)-axis was parallel to the membrane normal and the origin of the coordinate system was located in the central cavity. HOLE (version 2.2) (Smart et al., 1996) was then run to find the maximum radius of a spherical probe that could be fitted at each value of \( z \) (Figure S4). This analysis was repeated for all other current structures of MFS transporters. All structures were fitted onto the structure of PepT\(_{So}\) using LOVOALIGN (Martinez et al., 2007). The minimum values of the probe radius in the two regions defined by the \( z \) values of the top or bottom two turns of the transmembrane helices was calculated, thereby identifying the constrictions that would create the periplasmic or cytoplasmic gates, respectively. Since the probe radius can vary rapidly with \( z \), creating sharp peaks, we arbitrarily defined the probe radius for each gate as the average probe radius over a window 4 \( \text{Å} \) wide centred on the minimum value.

We determined which pairs of helices constituted the periplasmic and cytoplasmic gates by correlating the smallest distance between each pair of pairs and the gate probe radius defined above. Each gate was assumed to consist of two pairs of helices with the N- and C-terminal halves of the protein contributing one pair each. Due to the topology of MFS transporters, there are three and two helix pairs on the periplasmic and cytoplasmic sides of each half of the protein, respectively, and hence there are nine and four possible combinations for the periplasmic and cytoplasmic gates. We wrote a python program, using the MDAnalysis module (Michaud-Agrawal et al., 2011), to calculate the minimum \( \text{C}_\alpha-\text{C}_\alpha \) distances between the pairs of helices. To ensure that the closest distance occurs on the periplasmic or cytoplasmic side of the protein, only the first (or last, as appropriate) ten residues were considered. The locations of the transmembrane helices were defined using STRIDE (Frischman and Argos, 1995).

### 1.13 Molecular dynamics simulations.

Molecular dynamics simulations of chain A from the experimental structure of apo PepT\(_{So}\) (PDB:2XUT) were carried out as described previously by Newstead et al. (2011). All amino acid side chains were set to their standard protonation states as there was no information about which side chains may become protonated during transport. Waters and neutralising ions were added creating a simulation unit cell containing 205 POPC lipids, 7 chloride ions and 13 711 water molecules making a total of 76 245 atoms. This procedure was repeated for PepT\(_{St}\) (PDB:4APS) (Solcan et al., 2012) resulting in a simulation unit cell containing 250 POPC lipids, 6 chloride ions and 12 429 water molecules making a total of 78 033 atoms. Three independent molecular dynamics simulations were run for each protein, each 200 ns long, making a total of 1.2 \( \mu \text{s} \). All coordinates were recorded every 10 ps.
1.14 Mapping MTSL spin labels onto structures and trajectories.

We used a program to fit MTSL rotamers onto protein structures and MD trajectories (Stelzl et al., 2014). This incorporates a published rotamer MTSL library (Polyhach et al., 2011) and is implemented in python using the MDAnalysis module (Michaud-Agrawal et al., 2011). The python code is available from the MDAnalysis website (http://code.google.com/p/mdanalysis/). All images were produced with VMD (Humphrey et al., 1996) using the bendix plugin (Dahl et al., 2012) and all graphs produced with gnuplot.
Figure S1: Related to Figure 1. Major Facilitator Superfamily transporters comprise two bundles of six helices. The central binding site is exposed to either side of the membrane (or neither, the occluded state). The known structures are pictured here ordered by conformation from (A) inward-facing (LacY (Abramson et al., 2003), PepT_{So}(Newstead et al., 2011), PepT_{St}(Lyons et al., 2014; Solcan et al., 2012), PepT_{So2}(Guettou et al., 2013), NRT1.1 (Parker and Newstead, 2014), GkPOT (Doki et al., 2013), GlpT (Huang et al., 2003), GLUT1 (Deng et al., 2014)) through (B) occluded (PiPT (Pedersen et al., 2013), EmrD (Yin et al., 2006), NarU (Yan et al., 2013), XylE (Quistgaard et al., 2013; Sun et al., 2012)) to (C) outward-facing (YarJ (Jiang et al., 2013) and FucP (Dang et al., 2010)). Although several proteins have been captured in more than one conformation we only show one example of each here for clarity. The colour scheme is in the same as used in Figure 1.
|                      | RMSD between inward-open structure and ... | RMSD between outward-open model and ... |
|---------------------|------------------------------------------|----------------------------------------|
|                      | inward-facing conformations               | outward-facing conformations            |
|                      | inward-facing conformations               | outward-facing conformations            |
| **PepTSo MD sims**   |                                          |                                        |
| 6-TM helix bundles   |                                          |                                        |
| half 1               | 2.0 ± 0.1                                 | 2.2 ± 0.0                              | 2.9 ± 0.1 | 2.8 ± 0.1 |
| half 2               | 2.4 ± 0.0                                 | 2.8 ± 0.0                              | 4.9 ± 0.1 | 4.7 ± 0.0 |
| **Inverted topology repeats** | | | | |
| unit 1               | 1.7 ± 0.1                                 | 1.9 ± 0.1                              | 2.5 ± 0.1 | 2.5 ± 0.0 |
| unit 2               | 1.9 ± 0.1                                 | 2.2 ± 0.1                              | 2.4 ± 0.1 | 2.8 ± 0.0 |
| unit 3               | 2.4 ± 0.1                                 | 2.8 ± 0.0                              | 4.1 ± 0.1 | 4.0 ± 0.0 |
| unit 4               | 1.7 ± 0.1                                 | 2.4 ± 0.1                              | 4.1 ± 0.1 | 4.8 ± 0.1 |
| **Transmembrane helices** | | | | |
| helix 1              | 1.3 ± 0.1                                 | 1.8 ± 0.0                              | 1.5 ± 0.1 | 1.6 ± 0.0 |
| helix 2              | 1.9 ± 0.2                                 | 1.3 ± 0.0                              | 3.1 ± 0.2 | 2.3 ± 0.1 |
| helix 3              | 0.7 ± 0.0                                 | 0.5 ± 0.0                              | 1.1 ± 0.0 | 0.9 ± 0.0 |
| helix 4              | 1.0 ± 0.1                                 | 1.1 ± 0.1                              | 1.5 ± 0.1 | 1.4 ± 0.0 |
| helix 5              | 2.1 ± 0.1                                 | 1.6 ± 0.1                              | 2.6 ± 0.1 | 2.8 ± 0.1 |
| helix 6              | 0.7 ± 0.1                                 | 0.6 ± 0.1                              | 1.0 ± 0.1 | 1.0 ± 0.0 |
| helix 7              | 2.0 ± 0.1                                 | 2.1 ± 0.1                              | 4.0 ± 0.1 | 3.3 ± 0.0 |
| helix 8              | 0.8 ± 0.1                                 | 0.9 ± 0.0                              | 0.9 ± 0.1 | 0.8 ± 0.0 |
| helix 9              | 1.3 ± 0.1                                 | 1.7 ± 0.0                              | 3.7 ± 0.1 | 3.8 ± 0.0 |
| helix 10             | 2.1 ± 0.1                                 | 1.6 ± 0.1                              | 2.6 ± 0.1 | 2.8 ± 0.1 |
| helix 11             | 0.7 ± 0.1                                 | 0.6 ± 0.0                              | 0.9 ± 0.1 | 0.8 ± 0.0 |
| helix 12             | 0.7 ± 0.1                                 | 0.6 ± 0.0                              | 0.9 ± 0.1 | 0.8 ± 0.0 |

| **PepTSt MD sims**   |                                          |                                        |
| 6-TM helix bundles   |                                          |                                        |
| half 1               | 2.0 ± 0.1                                 | 2.2 ± 0.0                              | 2.9 ± 0.1 | 2.8 ± 0.1 |
| half 2               | 2.4 ± 0.0                                 | 2.8 ± 0.0                              | 4.9 ± 0.1 | 4.7 ± 0.0 |
| **Inverted topology repeats** | | | | |
| repeat unit 1        | 1.7 ± 0.1                                 | 1.9 ± 0.1                              | 2.5 ± 0.1 | 2.5 ± 0.0 |
| repeat unit 2        | 1.9 ± 0.1                                 | 2.2 ± 0.1                              | 2.4 ± 0.1 | 2.8 ± 0.0 |
| repeat unit 3        | 2.4 ± 0.1                                 | 2.8 ± 0.0                              | 4.1 ± 0.1 | 4.0 ± 0.0 |
| repeat unit 4        | 1.7 ± 0.1                                 | 2.4 ± 0.1                              | 4.1 ± 0.1 | 4.8 ± 0.1 |
| **Transmembrane helices** | | | | |
| helix 1              | 1.3 ± 0.1                                 | 1.8 ± 0.0                              | 1.5 ± 0.1 | 1.6 ± 0.0 |
| helix 2              | 1.9 ± 0.2                                 | 1.3 ± 0.0                              | 3.1 ± 0.2 | 2.3 ± 0.1 |
| helix 3              | 0.7 ± 0.0                                 | 0.5 ± 0.0                              | 1.1 ± 0.0 | 0.9 ± 0.0 |
| helix 4              | 1.0 ± 0.1                                 | 1.1 ± 0.1                              | 1.5 ± 0.1 | 1.4 ± 0.0 |
| helix 5              | 2.1 ± 0.1                                 | 1.6 ± 0.1                              | 2.6 ± 0.1 | 2.8 ± 0.1 |
| helix 6              | 0.7 ± 0.1                                 | 0.6 ± 0.1                              | 1.0 ± 0.1 | 1.0 ± 0.0 |
| helix 7              | 2.0 ± 0.1                                 | 2.6 ± 0.1                              | 4.0 ± 0.1 | 3.3 ± 0.0 |
| helix 8              | 2.2 ± 0.0                                 | 2.1 ± 0.1                              | 2.4 ± 0.1 | 2.3 ± 0.0 |
| helix 9              | 0.8 ± 0.1                                 | 0.9 ± 0.0                              | 0.9 ± 0.1 | 1.2 ± 0.0 |
| helix 10             | 1.3 ± 0.1                                 | 1.7 ± 0.0                              | 3.7 ± 0.1 | 3.8 ± 0.0 |
| helix 11             | 1.1 ± 0.1                                 | 0.8 ± 0.0                              | 1.3 ± 0.1 | 1.4 ± 0.0 |
| helix 12             | 0.7 ± 0.1                                 | 0.6 ± 0.0                              | 0.9 ± 0.1 | 0.8 ± 0.0 |

Table S1: Relates to Figure 6. The C-terminal half of PepTSt is more dynamic than the N-terminal half. The inward-open and outward-open structures in the ensemble of structures generated by the 600 ns molecular dynamics of both proteins were identified. Components of each structure were aligned back onto both the relevant inward-open crystal structure (or outward-open repeat swapped model) and the Cα RMSD calculated (in Å).
Figure S2: Related to Figure 2. (A) An overlay of the new and existing structures of PepT₆₀ showing that although the structures are similar there are key differences in the positions of the residues that make up the cytoplasmic ‘thin’ gate. (B) The previous occluded structure of PepT₆₀ (2XUT) contained a hydrophilic cavity adjacent to the E21xxERF motif on helix H1. In the new inward open structure (4UVM) this cavity is also observed. (C) Interestingly two well coordinated water molecules sit at the far end of the cavity, making hydrogen bonds to Ser20 (H1) and Ser92 (H3). Previously we showed that the ExxERF motif on helix H1 plays an important role in coupling peptide transport to the inward movement of protons (Solcan et al., 2012). The inward open structure of PepT₆₀ now provides a mechanism by which Glu21 and/or Glu24 could exchange protons with the bulk solvent. (D) A structural overlay with the occluded structure (2XUT, grey) and inward open structure (4UVM, coloured helices) reveals that in the inward-occluded 2XUT structure the tunnel is closed through the close positioning of Arg25 (H1) and Lys126 (H4). The functional significance of this with respect to the transport mechanism however is currently unclear, but may suggest a dynamic mechanism allowing water access to this region of the transporter. (E) The new structure of PepT₆₀ contains an unknown ligand sitting in the peptide binding site. An unidentified ligand is observed sitting in the central peptide binding site, as revealed by the positive difference density map (Fᵣ-Fᵢ, green/red, contoured at ±3.5 σ). This observation suggests that the current structure (PDB: 4UVM) represents a possible ligand bound inward open state, whereas the previously reported structure (PDB: 2XUT) represents an occluded inward open state. The second panel shows the same view but with the 2Fᵣ-Fᵢ electron density map, contoured at 1 σ. (F) The inward-open structure of PepT₆₀ with the helices rendered using curved cylinders (Dahl et al., 2012) to illustrate their intrinsic kinks and bends (Solcan et al., 2012). The two additional helices found in the bacterial proton oligopeptide transporters, HA and HB, are coloured light grey. (G) An outward-open model of PepT₆₀, built using the repeat-swapping method. The transmembrane helices are coloured according to the same scheme throughout. Equivalent images to panels F and G for PepT₆₀ can be found in Figure 2.
Figure S3: Related to Figure 3. The outward-facing model of PepTSo can be validated against the spin-spin distance distributions inferred from the DEER experiments. (A) Time-domain data for the three periplasmic and five cytoplasmic distances studied by DEER, including the double proline mutant of the 201-364 distance. (B) Pake patterns obtained after Fourier transformation of the time-domain data. (C) Measured DEER spin-spin distance distributions, following Tikhonov regularization. (D) A comparison between the predicted spin-spin distance distribution (in red) for the inward-open crystal structure (PDB:4UVM) and the measured DEER distance distributions. A library of MTSL rotamers was mapped onto each position and then any that clashed with the protein were discarded. (E) A comparison between the predicted spin-spin distance distributions (in blue) for the outward-open repeat-swapped model (RSM) and the DEER distance distributions.
Figure S4: Related to Figure 4. The percolation surfaces and pore profiles for all known structures of MFS transport proteins. This was calculated using HOLE (Smart et al., 1996) as described in the Experimental Procedures. The surface is coloured according to the maximum radius of the spherical probe; less than 1.15 Å is coloured red, greater than 2.30 Å yellow and in between, orange. The pore profile (the variation in the maximum radius of a spherical probe as a function of z) can be used to identify constrictions. The maximum radius of a probe that can pass any constriction is estimated as the average of the probe radius over a window 4 Å wide centred on the constriction (i.e. the minimum value). The periplasmic and cytoplasmic gate regions in the pore profile are coloured light green and cyan and the 4 Å windows coloured dark green and dark blue, respectively.

(a) The percolation surfaces through 16 structures of members of the MFS. The PDB code of each structure is given in parentheses.
(b) The percolation surfaces through the remaining 16 structures of members of the MFS. The PDB code of each structure is given in parentheses.

(c) The percolation surfaces through the structures of PepTSt and its repeat-swapped model.
Figure S5: Related to Figure 5. MFS transporters are made up of two bundles of six helices with the N-terminus of the first helix starting in the cytoplasm. In each half of the transporter there are therefore three helix pairs that could contribute to the periplasmic gate (H1 & H2, H3 & H4, H5 & H6 and H7 & H8, H9 & H10, H11 & H12) and two helix pairs that could contribute to the cytoplasmic gate (H2 & H3, H4 & H5 and H8 & H9, H10 & H11) – see Figure 5A. If we assume that each gate is made up of one helix pair from the first six helices and one helix pair from the second six helices, then there are (A) nine possible combinations for the periplasmic gate and (B) four for the cytoplasmic gate. To determine which helices form the gates, we have plotted the minimum Cα-Cα distance as described in the Methods against the (cytoplasmic or periplasmic) minimum probe radius as determined by HOLE (see Figure 4, S4). The Pearson correlation coefficient is calculated for each set of data: this indicates that the minimum helix tip distance between H1 & H2 and H7 & H8 correlates best with the state of the periplasmic gate (R=0.88) and the minimum helix tip distance between H4 & H5 and H10 & H11 correlates best with the state of the cytoplasmic gate (R=0.78. Twenty nine different MFS structures were used.
Figure S6: Related to Figure 6. During the molecular dynamics simulations PepT\textsubscript{St} explores inward-inward and some partially outward-facing conformations, as defined by the minimum distance between the C\textsubscript{a} atoms of the relevant pairs of helix tips. 

The density of states explored during the simulations are plotted in blue and two representative inward-facing and outward-facing structures are shown. The coordinates of known MFS structures are plotted to provide some context and the different quadrants of the coordinate space are labelled. The coordinates of the PepT\textsubscript{So} and PepT\textsubscript{St} crystal structures and repeat-swapped models (RSM) are labelled in red and blue, respectively. The results from the PepT\textsubscript{So} simulations can be found in Figure 6.
Figure S7: Related to Figure 7. The molecular dynamics simulations predict several salt bridges in the different conformations of PepTSo and PepTSst. (A) Four salt bridges additional to those given in Figure 7 were identified in PepTSo. The propensity of three of these (K127-E24, R25-E21 and R32-D316) was found to be uniformly high across all the conformations sampled and K127-E21 was predicted to form only occasionally in inward and occluded conformations. Two of these interactions (K127-E24 and K127-E21) join helices (H1 & H4) involved in the periplasmic and cytoplasmic gates. Asp316 forms part of the GDQF signature motif on H4, except in PepTSst which does not possess this motif. Several of the other residues belong to the conserved ExxERFxYY motif on H1, which has been identified as playing an important role in both proton-coupling and peptide recognition in PepTSst (Solcan et al., 2012). Apart from the exception mentioned above, all of the residues are conserved from PepTSo to mammalian PepT1 (Figure S8). These observations are consistent with (but do not prove) the hypothesis that these salt bridges are essential for the function of PepTSo. (B) Mutating any of the residues involved in these salt bridges abolishes transport, with the exception of R32A which has increased active transport compared to wild-type. (C) Five salt bridges were identified in the simulations of PepTSst. Two of these (R33-E300 and R53-E312) have been previously suggested to stabilise inward-facing conformations (Solcan et al., 2012). We find that this salt bridge only occurs in ~10% of inward and occluded conformations and hence is unlikely to be critical, consistent with the transport data. The equivalent interaction (R52-D328) was detected in the simulations of PepTSo – it too, only occurred in a minority of inward and occluded conformations and mutation to alanine also reduced but did not abolish active transport. Of the remaining three salt bridges, two (E22-K126 and E25-K126) are equivalent to those seen in PepTSo. This leaves R26-E300 which is predicted to occur in occluded conformations of PepTSst. Mutating any of E22, E25, R26, R33, K126 or E300 has been previously shown to abolish or significantly reduce transport (Solcan et al., 2012), again consistent with (but not proving) the hypothesis that these salt bridges stabilise PepTSst.
Figure S8: Refers to Figure 8. PepT<sub>E</sub> and PepT<sub>S</sub> are homologous to human PepT1. A multiple sequence alignment of PepT1 from four mammalian species and two bacterial peptide transporters, PepT<sub>E</sub>, Newsstead et al. (2011) and PepT<sub>S</sub>, Solcan et al. (2012). Conserved residues are highlighted and key features, derived from the structure of PepT<sub>E</sub>, are labeled Newsstead et al. (2011). These include all twelve transmembrane helices and several residues (in red) referred to in the main body of the manuscript. All residue numbering is based on PepT<sub>E</sub>, as are the definitions of the transmembrane helices. For clarity two regions are not shown: these are the extracellular domain (ECD) in PepT1 that has no equivalent in the bacterial peptide transporters and the additional two helices, HA and HB, in the bacterial peptide transporters that have no equivalent in the mammalian peptide transporters.
Figure S9: Related to Figure 9. Images of the current structures of MFS transporters coloured with the helices making up the periplasmic and cytoplasmic gates highlighted. Helices H1, H2, H7 & H8 are coloured green, whilst helices H7, H8, H10 & H11 are coloured cyan. The known structures are pictured here ordered by conformation from (A) inward-facing (LacY (Abramson et al., 2003), PepTSo (Newstead et al., 2011), PepTSf (Lyons et al., 2014; Solcan et al., 2012), PepTSo2 (Guettou et al., 2013), NRT1.1 (Parker and Newstead, 2014), GkPOT (Doki et al., 2013), GlpT (Huang et al., 2003), GLUT1 (Deng et al., 2014)) through (B) occluded (PiPT (Pedersen et al., 2013), EmrD (Yin et al., 2006), NarU (Yan et al., 2013), XyIE (Quistgaard et al., 2013; Sun et al., 2012)) to (C) outward-facing (YarJ (Jiang et al., 2013) and FucP (Dang et al., 2010)). Although several proteins have been captured in more than one conformation we only show one example of each here for clarity. (D) Five of the eight distances studied with DEER are structurally equivalent to distances studied in LacY, the canonical member of the MFS (?). The LacY DEER study used the same spin label and a non-binding sugar, 4-nitrophenyl-α-D-glucopyranoside (NPGlc), was added. The DEER distance distributions for LacY (shown as a grey shaded area) are very different to those of PepTSo, with not only the positions of peaks, but also the number of peaks varying between the two transporters.
Figure S10: Related to the Experimental Procedures. Wildtype PepTSo aligned onto PepTSo with its alternating repeats swapped (ABCD to BACD). This alignment was used to construct the outward-open model of PepTSo. The helices are coloured as in Fig. 1.
Figure S11: Related to the Experimental Procedures. Wildtype PepT₅ St aligned onto PepT₅ with its alternating repeats swapped (ABCD to BACD). This alignment was used to construct the outward-open model of PepT₅. The helices are coloured as in Fig. 1.
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