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Structural insights into substrate recognition in proton-dependent oligopeptide transporters

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Short-chain peptides are transported across membranes through promiscuous proton-dependent oligopeptide transporters (POTs)—a subfamily of the major facilitator superfamily (MFS). The human POTs, PEPT1 and PEPT2, are also involved in the absorption of various drugs in the gut as well as transport to target cells. Here, we present a structure of an oligomeric POT transporter from Shewanella oneidensis (PepTSo2), which was crystallized in the inward open conformation in complex with the peptidomimetic alafosfalin. All ligand-binding residues are highly conserved and the structural insights presented here are therefore likely to also apply to human POTs.

Keywords: alafosfalin; major facilitator superfamily; proton-dependent oligopeptide transporter; substrate recognition; x-ray structure

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INTRODUCTION

Cell membranes compartmentalize metabolic processes and present a selective barrier for permeation. Therefore, nutrient transport through the plasma membrane is essential to maintain homeostasis within the cell. Most nutrient transport pathways in bacteria, yeast and plants are energized by an electrochemical proton gradient providing a powerful driving force for transport and accumulation of nutrients above extracellular concentrations [1]. Proton-dependent oligopeptide transporters (POTs) are representatives of such secondary active H+ -dependent transporters. The POT transporter family belongs to the major facilitator superfamily (MFS) and is characterized by the presence of two highly conserved sequence stretches known as the peptide transporter motifs. Together with the ABC transporters, MFS transporters represent the largest transport family found in nature [2]. They function by an alternate access mechanism [3], where transport is mediated through conformational changes, which allow the substrate-binding site to face either side of the membrane. A full reaction cycle (Fig 1B) involves at least three different conformational states (inward open, occluded and outward open), with each of them in a ligand-bound and ligand-free form [4]. Two POT transporters, PEPT1 and PEPT2, are found in humans. They share the canonical fold of MFS transporters with 12 predicted transmembrane helices each. PEPT1 is mainly localized to the intestinal brush border membrane, whereas PEPT2 is found in lungs, kidney and the central nervous system [5]. POTs accept most di- and tripeptides but do not transport longer peptides [6]. They also recognize and transport compounds with similar stereochemical properties to small peptides, such as β-lactam antibiotics, angiotensin converting enzyme inhibitors and antiviral nucleoside prodrugs [7]. Furthermore, owing to their promiscuous substrate portfolio, many additional pharmacologically active compounds can potentially be converted into substrates for PEPT1 and PEPT2 and thus utilize these proteins as drug delivery systems [8].

There are currently no crystal structures available for any of the human POT transporters, but two bacterial POT structures have recently been reported; PepTSo from Shewanella oneidensis in occluded conformation [9] and PepTSt from Streptococcus thermophilus in inward open conformation [10] (for nomenclature see Fig 1C). These structures revealed the overall architecture of these proteins and provided insights into the intracellular gating mechanism, emphasizing the role of conserved salt bridge interactions.

Here we report the inward open structure of another POT transporter from S. oneidensis, PepTSo2, co-crystallized with the anti-bacterial peptidomimetic alafosfalin and refined at 3.2 Å maximum resolution. Clear electron density demarks the ligand-binding site of the transporter, allowing us to model
the principle substrate-binding mode. This is to our knowledge the first POT transporter where a complex with a substrate has been structurally characterized. Owing to the high conservation of the binding site, this is also likely to provide a good model for ligand binding to human POT transporters.

RESULTS AND DISCUSSION

Overall structure

We have solved the structure of PepTSo2, a POT homologue from S. oneidensis, in complex with the anti-bacterial compound alafosfalin. PepTSo2 shows 17% and 16% sequence identity to human PEPT1 and PEPT2, and 20% and 22% to the two previously characterized POTs, PepTSo and PepTSt. Residues forming the proposed peptide-binding site are highly conserved among the different species (supplementary Fig 1 online; supplementary Table II online). The structure was solved by selenomethionine SIRAS phasing and refined at 3.2 Å resolution using non-crystallographic symmetry restraints yielding a final Rfree of 29.7% (supplementary Table I online). The transporter was captured in an inward open conformation in complex with the compound alafosfalin. Alafosfalin, shown as red spheres, is buried in the central binding pocket located between the N- and C-terminal subdomains.

Ligand-binding pocket

The substrate-binding site is located in a hydrophilic cavity found in the centre of the cytoplasmic crevice made up by an equal contribution of the N- and C-terminal helix bundles. Helices contributing to the binding crevice are H1, H2, H4 and H5 from the N-terminal bundle and H7, H8, H10 and H11 from the C-terminal. Upon refinement of the structure, extra electron density was observed, which could not be accounted for by the protein, in a region, which has previously been indicated as the potential ligand-binding site based on the mutagenesis

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<th>Protein</th>
<th>PDB code</th>
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<td>PepTSo2</td>
<td>4LEP</td>
<td>Shewanella oneidensis</td>
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<tr>
<td>PepTSo</td>
<td>2XUT</td>
<td>Shewanella oneidensis</td>
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<tr>
<td>PepTSt</td>
<td>4APS</td>
<td>Streptococcus thermophilus</td>
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data [9,10,12] and a spurious density in the PepT\textsubscript{S0} structure originating from an unknown ligand (Fig 2). The electron density accounted well for the dipeptide analogue alafosfalin, which was present at high concentrations in the crystallization condition (50 mM). This compound has been described as a substrate for pro- and eukaryotic POT transporters with expected affinities in the mM range [13–15]. Thermal shift assays have emerged as a valuable method to monitor ligand binding to membrane proteins \cite{16,17}. We employed a precipitation-after-unfolding based thermal shift assay to demonstrate binding of alafosfalin to detergent solubilized PepT\textsubscript{S0} (see supplementary Methods online). As expected, the transporter was stabilized against heat denaturation in the presence of alafosfalin, di- and tripeptides, but not with single amino acids or longer peptides (Fig 2E–G). These data are consistent with the described transport activity of di- and tripeptides for this class of transporters. We also obtained crystals with the dipeptide Gly-Asp, however, here the density in the ligand-binding pocket was much weaker and the ligand could therefore not be modelled. Nonetheless, we could use this data set to support the fact that the ligand density in the alafosfalin data does indeed represent alafosfalin and not a buffer molecule: The crystallization condition for PepT\textsubscript{S0} in the presence of the dipeptide Gly-Asp was thus very similar to the one used for the alafosfalin co-crystallization experiment,
but yet, refining the Gly-Asp data set with alafosfalin in the ligand-binding pocket resulted in very strong negative difference density peaks (7 sigma) around the alafosfalin molecule (supplementary Fig 5 online).

Guided by the stronger density for the phospho-moiety in the 2Fo-Fc and Fo-Fc omit maps in the alafosfalin data set, the ligand could be modelled (Fig 2A,B). After refinement, the N-terminal amine group of alafosfalin is interacting in a narrow polar pocket formed by Asn151, Asn329 and Glu402. The phospho-moiety, which is an analogue of the C-terminus of a dipeptide, is positioned so that it can form a hydrogen bond to the hydroxyl group of the Tyr29 side chain and a salt bridge with the side chain of Arg25, (which in turn is stabilized by an interaction with Glu24). The side chain of Tyr291 is also within the hydrogen bonding distance of the ligand, possibly to the alafosfalin carbonyl, but at the present resolution, this interaction remains speculative. The ligand-binding cavity of PepTSo2 is highly similar in terms of size and amino-acid arrangements to the ones in the structures of PepTS6 and PepTS6. The alignment of the peptide in the pocket appears to be guided by the tight coordination with the N-terminus, but there is sufficient space for interactions of the C-terminus of both a di- and tri-peptide with Arg25. Mutation of either R25 or E402 to alanine in PepTSo2 abolished the stabilization effects of alafosfalin and the dipeptide Gly-Asp in the thermal shift assay (Fig 2G), supporting the notion that both are key residues in coordination of the substrate.

A characteristic feature of the binding pocket is the presence of several highly conserved tyrosine residues pointing towards the centre of the cavity. Tyrosine residues are known to be versatile interaction residues because they can form hydrogen bonds, hydrophobic as well as electrostatics interaction. Hence, they can interact favourably in both hydrophobic and hydrophilic environments, which could be a key aspect for the broad substrate specificity of POTs. The principle binding mode of the ligand is consistent with the mutagenesis data on PEPT1, the prokaryotic POTs PepTSo, PepTS6 and two transporters from *Escherichia coli* [9,10,12,13,15,18,19], indicating that these invariant residues have a key role in the recognition of the N- and C-terminus of peptide substrates. For the recognition of a large variety of di- and tripeptides, further interactions could be formed with the invariant backbone CO- and NH-groups of the peptide via the tyrosine cluster consisting of residues 29, 147 and 291.

**Clues to the gating mechanism—conformational changes**

For upload and release of substrates across a lipid bilayer, a transporter has to undergo distinct conformational changes.
Residues controlling the access to the binding site from either side of the membrane, also known as gating residues, are important for function and activity of many secondary active transporters. Typically, a network of hydrogen bonds and salt bridges is formed and broken during a reaction cycle between the outward open and inward open states [10]. We recently provided a detailed view of such gating in a MFS sugar transporter [20]. A salt bridge found between H2 and H7 has been proposed to be involved in gating in PepTSt (R53 and E312), which is also present in PepTSo (R52 and D328) [9,10]. This salt bridge is conserved in PepTSo2 (D47 and R304), but here the charges are swapped, indicating conservation of the salt bridge rather than of the individual residues (Fig 3A). A second conserved interaction network between the N- and C-terminal subdomains in PepTSo2 is formed by K165, D166 and S321 between H5 and H8 and is also present in the other known POT structures (Fig 3A). More van der Waals and hydrophobic interactions help to stabilize the periplasmic interface in the inward open conformation. Here Y37 probably has a key role in sealing the N- and C-terminal bundle and concomitantly blocking access to the binding pocket.

A comparison of all known POT structures reveals that the intracellular part of H11, which has been identified as part of the intracellular gate (M443 in PepTSo, M424 in PepTSt and M426 in PepTSo2) in the occluded state structure of PepTSo, is displaced by ~6Å in PepTSo2 as compared to the position in PepTSo, which is even more than the displacement observed in PepTSt relative to PepTSo (Fig 3B). In addition, small differences in the positions of H9 and H12 on the periplasmic side between the two inward open structures, PepTSo2 and PepTSt can be observed (Fig 3C). Conformational changes on the cytoplasmic side between the inward open and occluded state structures have recently been described for PepTSo and PepTSt [10] and are consistent with the analysis of the inward open structure of PepTSo2 (Fig 3D).
Oligomeric state

Most studied MFS transporters are considered to be monomers [21], although for only a few family members the oligomeric structure has been assessed experimentally [22–25]. While screening various POTs for structural studies, we realized that PepTSo2 elutes after significantly shorter retention times on an analytical gel filtration column compared to previously characterized POTs (Fig 4A), indicative of a higher oligomer assembly. Further characterization including Blue Native PAGE, cross-linking and negative stain electron microscopy supports a tetrameric assembly of PepTSo2 in detergent solubilized form (Fig 4B–D). In contrast, PepTSo and two well-characterized POT members from E. coli show the expected monomeric behaviour. For PepTSo, we found evidence that the protein exists as a mixture of monomers and dimers. The size and shape of PepTSo2 revealed by negative stain class averages is congruent with a homo-tetrameric arrangement. The crystal form used for the structure determination of PepTSo2 is in a dimeric conformation. This dimer interface appears to be stabilized by the presence of a zinc ion and is most likely a crystal-packing artefact (supplementary Figs 6 and 7 online). However, a second crystal form of PepTSo2 in spacegroup P3121, diffracting to 4.6 Å resolution, was obtained in a crystallization condition in the absence of zinc, and molecular replacement into this form did indeed show a tetrameric assembly in the crystal lattice, which might correspond to the tetramer visible in the EM experiments (Fig 4E). Both structures mainly differ in the position of TM12, which mediates key interactions in the oligomeric assembly (Fig 4E,F). Higher resolution data of this crystal form and more functional data will be necessary to unravel potential structural differences of transporter units within the tetramer and the relevance of the oligomerization.

METHODS

Molecular cloning and protein preparation. The cDNA of full-length PepTSo2 (Q8EHE6) was amplified from genomic DNA and cloned into a pNIC-CTHF-vector carrying a C-terminal His-tag [26,27] with a tobacco etch virus cleavage site. PepTSo2 was identified as prime candidate for structural studies as recently described [28]. Detailed expression and purification of PepTSo2 is described in supplementary information online.

Crystallization and structure determination. Before crystallization, PepTSo2 was incubated with 50 mM alafosfalin for 30 min at 4°C. Native crystals grew in 40% PEG 300, 0.1 M phosphate citrate pH 4.5, 0.12 M ZnCl2 and 3% trimethylamine N-oxide dehydrate pH 11 at 20°C. Crystals appeared after 21 days with dimensions of ~100 × 50 × 10 μm. Selenomethionine crystals grew in 38% PEG 300, 0.1 M phosphate citrate pH 5.2 and 0.01 M ZnCl2. For the P3121 data set, crystals grew in 0.1 M citric acid pH 4.2, 0.1 M sodium hydrogen phosphate and 40% PEG 300. The data sets for PepTSo2 in complex with alafosfalin and the dipeptide Gly-Asp were both collected at the ESRF beamline ID29 in Grenoble. The data sets used for phasing were collected at BESSY beamline MX 14.3 in Berlin (native data) and SLS beamline PXIII in Villigen (selenomethionine data). The P3121 tetramer data set were collected at ESRF beamline ID14-4. Data processing, phase determination by single-wavelength anomalous diffraction, model building and refinement are described in supplementary information online.
the prokaryotic peptide transporter YdgR identifies potential periplasmic gating residues. J Biol Chem 286: 23121–23131