scientific report

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* (PepT_{So2}), 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

EMBO reports (2013) 14, 804–810. doi:10.1038/embor.2013.107

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; PepT_{So} from *Shewanella oneidensis* in occluded conformation [9] and PepT_{St} 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*, PepT_{So2}, 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

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Fig 1 | Structure of the proton-dependent oligopeptide transporter PepT_{So2}. The transporter was crystallized in an inward open conformation in complex with the compound alafosfalin. (A) The overall structure of PepT_{So2} viewed from the plane of the membrane. N- and C-terminal subdomains are coloured yellow and blue respectively, helices HA and HB are coloured grey. Approximate dimensions of the molecule are presented and black bars depict the approximate location of the membrane. Alafosfalin, shown as red spheres, is buried in the central binding pocket located between the N- and C-terminal subdomains. (B) Schematic model of the MFS POT alternating access transport mechanism. The N- and C-bundles are coloured blue and red respectively. The substrate is presented as a black square located in the binding pocket. Available POT structures depicting different stages of the transport cycle are placed next to the schematic figures; helices HA and HB are coloured grey. (C) Overview of available POT structures including nomenclature, PDB codes and source organism. MFS, major facilitator superfamily; POT, proton-dependent oligopeptide transporter.

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 PepT_{So2}, a POT homologue from *S. oneidensis*, in complex with the anti-bacterial compound alafosfalin. PepT_{So2} shows 17% and 16% sequence identity to human PEPT1 and PEPT2, and 20% and 22% to the two previously characterized POTs, PepT_{So} and PepT_{St}. 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 R_{free} of 29.7% (supplementary Table I online). The transporter was captured in a ligand-bound inward open conformation with two molecules in the asymmetric unit, with virtually identical overall structure.

PepT_{So2} comprises 14 transmembrane helices; H1–6 and H7–12 constitute the amino- and carboxy-terminal six-helical bundles characteristic of the MFS fold (Fig 1A). HA–HB correspond to two helices inserted in the cytoplasmic linker connecting the two subdomains and is a common feature of bacterial POTs—similar

insertions are also present in the previously reported POT structures [9,10]. The functional role of these helices is unclear, but owing to their low-sequence conservation and absence in eukaryotic homologues, it is likely that they are not playing an essential role in the transport mechanism. Indeed the position of these additional helices is quite different in the three known POT structures (supplementary Fig 2 online). The two six-helical bundles, representing the core of the transporter, share low-sequence identity (15%) with each other, but the overall structure of their main chains is similar (root-mean-square deviation $C\alpha$ of 3.02 Å over 581 atoms), as seen for other MFS transporters [11]. In the inward open conformation, the N- and C-terminal bundles interact tightly at the periplasmic side, whereas a large central hydrophilic crevice hosting the substrate is facing the intracellular side of the membrane.

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

Fig 2 | PepT_{S02}-binding pocket and alafosfalin coordination. (A) The omit density, Fo-Fc, of alafosfalin is shown in green and contoured at 3 σ . Highly conserved amino-acid residues in the binding pocket are labelled and shown as sticks. (B) 2Fo-Fc density for alafosfalin and the conserved residues in the binding pocket; electron density map is shown in blue and contoured at 1 σ . The flexible nature of K121 resulted in poor electron density for the side chain, this is illustrated as a dashed circle. (C) Electrostatic surface of PepT_{S02} showing the location and dipole like charge distribution of the binding site. The alafosfalin phosphate head group is pointing towards the positively charged surface of the binding pocket whereas the N-terminus is oriented closer towards the negative surface. (D) Chemical formula of alafosfalin. (E) Thermal shift assay monitoring the degree of precipitation using centrifugation after unfolding (see supplementary information online). Coomassie stained gels of PepT_{S02} incubated with $\pm$ 10 mM alafosfalin and heated up to 70 °C. (F) Resulting melting curves show a stabilization of PepT_{S02} incubated with alafosfalin compared to the control samples, indicative of ligand binding. (G) Stabilization of PepT_{S02} in the presence of different peptides and compounds. The stabilization effect is normalized against the stability of apo PepT_{S02}. The sugar transporter XylE, which has a similar thermal stability as PepT_{S02}, was used as a control protein and the results are shown. Error bars represent standard deviation from triplicate experiments.

data [9,10,12] and a spurious density in the PepT_{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 (50mM). 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 [16,17]. We employed a precipitation-after-unfolding based thermal shift assay to demonstrate binding of alafosfalin to detergent solubilized PepT_{S02} (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_{S02} in the presence of the dipeptide Gly-Asp was thus very similar to the one used for the alafosfalin co-crystallization experiment,

Fig 3 | Gating residues and structural comparison of PepT_{So2}, PepT_{So} and PepT_{St}. (A) Two conserved networks of hydrogen bonds and salt bridges (coloured blue and red), which might act as potential gates are found on the periplasmic side of PepT_{So2}. Y37 (yellow) mediates more interactions between the N- and C-terminal bundles, blocking access to the binding site from the periplasm. Corresponding residues are labelled and shown as sticks. (B) Conformational changes of H11 between occluded and inward open structures. The position of the respective methionine residues, regulating access to the binding site from the cytoplasm, is presented as sticks. H11 of PepT_{So2} is shown in blue, PepT_{So} in green and PepT_{St} in brown. (C, D) Cartoon representation of PepT_{So2} in blue, PepT_{So} in green and PepT_{St} in brown. N- and C-terminal bundles and individual helices are labelled. The structures were superimposed on the N-terminal subdomain and shown from the periplasmic side (C) and cytoplasmic side (D). Arrows denote movements of individual helices in the C-subdomain relative to the PepT_{So} occluded-state structure. The additional linker helices HA and HB are omitted for clarity.

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 PepT_{So2} is highly similar in terms of size and amino-acid arrangements to the ones in the structures of PepT_{So} and PepT_{St}. 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-termini of both a di- and tri-peptide with Arg25. Mutation of either R25 or E402 to alanine in PepT_{So2}

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 PepT_{So}, PepT_{St} 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.

Fig 4 | Oligomeric structure of PepT_{S02}. (A) Analytical size-exclusion chromatograms of four POT transporters suggest that PepT_{S02} has the largest molecular weight. Elution profiles for different POTs are color coded according to the legend displayed on the right part of the panel. (B) SDS-PAGE of glutaraldehyde cross-linked PepT_{S02}, PepT_{S0} and PepT_{St} purified in the detergent DDM. The different oligomeric states are indicated by arrows and asterisks. The incubation time ranged from 0–15 min. (C) BN-PAGE of five different prokaryotic POT transporters indicates different oligomeric arrangements. (D) Negative stain EM of PepT_{S02} tetramers. In the upper panel, the tetrameric nature of the particles is visible in an otherwise clearly monodisperse preparation. The scale bar represents 200 nm. The lower panel shows a gallery of 11 classes viewing the tetramer slightly tilted compared to or parallel to the membrane plane with dimensions of about 12.4 × 12.4 nm. The frame size of the boxed, magnified particles is 21.4 nm. (E) Surface representation of a potential tetramer arrangement obtained from a different crystal form (P3₁21) at lower resolution. The helices HA and HB are coloured grey to facilitate the visualization of four-fold symmetry. (F) The P3₁21 tetramer is shown as cylinders in magenta, H12 is coloured orange. A PepT_{S02} monomer derived from the P2₁2₁2₁ crystal form (yellow cylinders) is aligned on one monomer of the tetramer structure to visualize the change in tilt angle of H12. In the tetrameric arrangement, H12 enables tighter packing between the monomers. BN-PAGE, Blue Native PAGE; DDM, *n*-dodecyl-β-D-maltoside; EM, electron microscopy; POT, proton-dependent oligopeptide transporter; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

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 PepT_{St} (R53 and E312), which is also present in PepT_{S0} (R52 and D328) [9,10]. This salt bridge is conserved in PepT_{S02} (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 PepT_{S02} 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 PepT_{S0}, M424 in PepT_{St} and M426 in PepT_{S02}) in the occluded state structure of PepT_{S0}, is displaced by ~6 Å in PepT_{S02} as compared to the position in PepT_{S0}, which is even more than the displacement observed in PepT_{St} relative to PepT_{S0} (Fig 3B). In addition, small differences in the positions of H9 and H12 on the periplasmic side between the two inward open structures, PepT_{S02} and PepT_{St} can be observed (Fig 3C). Conformational changes on the cytoplasmic side between the inward open and occluded state structures have recently been described for PepT_{S0} and PepT_{St} [10] and are consistent with the analysis of the inward open structure of PepT_{S02} (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 PepT_{So2} 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 PepT_{So2} in detergent solubilized form (Fig 4B–D). In contrast, PepT_{So} and two well-characterized POT members from *E. coli* show the expected monomeric behaviour. For PepT_{St}, we found evidence that the protein exists as a mixture of monomers and dimers. The size and shape of PepT_{So2} revealed by negative stain class averages is congruent with a homo-tetrameric arrangement. The crystal form used for the structure determination of PepT_{So2} 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 PepT_{So2} 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 PepT_{So2} (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. PepT_{So2} was identified as prime candidate for structural studies as recently described [28]. Detailed expression and purification of PepT_{So2} is described in supplementary information online.

Crystallization and structure determination. Before crystallization, PepT_{So2} 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. Seleno-L-methionine 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 PepT_{So2} 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 (seleno-L-methionine 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.

Oligomeric state. We used crosslinking, electron microscopy, analytical gel filtration and Blue Native PAGE to determine the oligomeric state of PepT_{So2} (details in supplementary information online).

Accession numbers. Coordinates and structure factors for PepT_{So2} in complex with alafosfalin has been deposited in the protein data bank with accession number 4LEP.

Supplementary information is available at EMBO reports online (<http://www.emboreports.org>).

ACKNOWLEDGEMENTS

We thank Ramakrishnan Balakrishnan Kumar for the BN-PAGE data and the members of our group for discussions on the manuscript. E.M.Q. was supported by The Danish Council for Independent Research. C.L. was supported by an EMBO postdoctoral fellowship. This research was further supported by grants from the Swedish Research council, Swedish Cancer Society and the integrated EU project EDICT, as well as a Singapore NRF-CRP grant. We would like to thank ESRF for access to beamline ID 14-4 and ID29 (proposal MX-1127 and MX-1416), SLS for access to beamline PXIII (proposals 20110031 and 20110372) and BESSY for access to beamline MX 14.3 (proposal 2012_1_111073). We acknowledge the Protein Science Facility at the Karolinska Institutet for providing crystallization infrastructure. The research leading to these results has furthermore received funding from the European Community's Seventh Framework Programme (FP7/2007–2013) under BioStruct-X (grant agreement N°783).

Author contributions: Performed experiments: F.G., P.M., C.J., L.Z., A.J.O.J., C.L. Analysed the data: F.G., E.M.Q., L.T., C.J., P.N., C.L. Wrote the manuscript: F.G., E.M.Q., P.N., C.L. Supervised the project: P.N. and C.L.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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