Biochemical and Biophysical Research Communications 389 (2009) 112–116

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Ligand binding analyses of the putative peptide transporter YjdL from *E. coli* display a significant selectivity towards dipeptides

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ARTICLE INFO

Article history:
Received 14 August 2009
Available online 22 August 2009

Keywords:
E. coli
POTs
Transport
Peptides
Ligand specificity
YjdL

ABSTRACT

Proton-dependent oligopeptide transporters (POTs) are secondary active transporters that couple the inwards translocation of di- and tripeptides to inwards proton translocation. *Escherichia coli* contains four genes encoding the putative POT proteins YhiP, YdgR, YjdL and YbgH. We have over-expressed the previously uncharacterized YjdL and investigated the peptide specificity by means of uptake inhibition. The IC_{50} value for the dipeptide Ala-Ala was measured to 22 mM while Ala-Ala-Ala was not able to inhibit uptake. In addition, IC_{50} values of 0.3 mM and 1.5 mM were observed for Ala-Lys and Tyr-Ala, respectively, while the alanyl-extended tripeptides Ala-Lys-Ala, Ala-Ala-Lys, Ala-Tyr-Ala and Tyr-Ala-Ala displayed values of 8, >50, 31 and 31 mM, respectively. These results clearly indicate that unlike most POT members characterized to date, including YdgR and YhiP, YjdL shows significantly higher specificity towards dipeptides.

Introduction

Peptide uptake in *Escherichia coli* is mediated by three genetically different types of transport systems: the dipeptide permeases (Dpp), the tripeptide permeases (Tpp) and the oligopeptide permease (Opp). Common for all three is that in general, members of these families are able to transport both di- and tripeptides. In the case of Opp however, up to hexapeptides are translocated. Dpp and Opp are multicomponent primary transporters, which require the hydrolysis of ATP for transport. The Tpp in contrast consists of a single polypeptide and belongs to the group of secondary active transporters. The Tpps have been conserved onto mammals, in contrast to the Dpps and Opps [1].

Secondary active transporters constitute one of the largest groups of transporters. These transporters are able to utilize a secondary source of energy, *i.e.* an electrochemical ion gradient to accumulate vital substances such as saccharides, amino acids, nucleobases, ions, peptides etc. Proton-dependent oligopeptide transporters (POTs, http://www.tcdb.org/) are secondary active symporters that typically facilitate the uptake of a range of different di- and tripeptides by coupling it to the simultaneous energetically favorable uptake of protons. The POTs are found in a wide range of organisms from bacteria to mammals. Based on sequence analyses, the POTs have been categorized as a Major Facilitator Superfamily (MFS)-like family [2]. The MFS include the biochemically and structurally well-characterized transporter lactose permease [3]. The topology of the POTs has been shown experimentally to consist of 12 transmembrane helices with inwards facing N- and C-termini [4,5].

E. coli contains four genes encoding the putative POT proteins YhiP, YdgR, YjdL and YbgH. They all appear to have relatively low amino acid sequence identity with their human homolog peptide transporter 1 (hPept1; 21–23%), the most studied POT member, and mutually cluster in the pairs YdgR–YhiP (51% identity) and YjdL–YbgH (56% identity) with approximately 26–28% identity between them. While YhiP and YdgR have both been experimentally verified to be POTs and have substrate specificities homologous to hPept1 [6,7], the YjdL and YbgH remain uncharacterized.

In the study presented here we have cloned, over-expressed and characterized the ligand preference of the putative POT YjdL from *E. coli*. Our results clearly indicate that YjdL has a significantly higher specificity towards dipeptides compared to tripeptides.

Materials and methods

Molecular biology. For construction of expression vector (pTTQ18-*yjdL*), the primers 5′CAGCAGAATTCGAAAACACCCTCACA GCCGCGCGCGATATACTATATCGTGGCGATCCA3′ and 5′ACCCAAGC TTTTAATGGTGATGGTGATGGTGATCGTTGCTCTCCTGTATCATATT3′

Abbreviations: POTs, proton-dependent oligopeptide transporters; MFS, major facilitator superfamily; Dpp, dipeptidepermeases; Tpp, tripeptidepermeases; Opp, oligopeptidepermease

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doi:10.1016/j.bbrc.2009.08.098

were used to amplify the ORF from the *E. coli* MG1655 genomic DNA using 'touchdown' PCR [8]. The EcoRI, and HindIII sites and oligonucleotides encoding hexa-Histag were introduced at the 5′ and 3′ ends of the *yjdL* gene, respectively. The PCR product, digested with EcoRI/HindIII, was then inserted into the corresponding sites of pTTQ18 [9] to generate a pTTQ18-*yjdL* construct. The construct was verified by DNA sequencing.

Expression. A single colony of *E. coli* BL21(DE3)pLysS cells harboring the plasmids pTTQ18-*yjdL* or pTTQ18 were inoculated in 5 mL LB-media containing 100 μg/mL Ampicillin and 40 μg/mL chloramphenicol and allowed to grow over night. Subsequently a 1:50 dilution of the overnight culture in 10 mL LB-media containing antibiotics as above was left to grow for approximately 3 h to reach an OD_{600} of 0.6–0.8 and hereafter induced by addition of 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). The cells were harvested after an additional growth of 3 h.

Isolation of solubilized membranes. Cells from 1 L expression culture were re-suspended in lysis buffer (20 mM Tris–HCl pH 7.6, 300 mM NaCl, 5 mM Imidazole, 30 μg/mL DNAse and one Complete Protease Inhibitor Cocktail tablet/50 mL buffer (Roche)) and lyzed by two passes through a cell disruptor (Constant Systems Ltd.) operating at 33 kpsi. Cell debris was removed by centrifugation at 30,000*g* for 25 min and membranes were subsequently isolated by centrifugation at 100,000*g* for 60 min. Membrane pellets were re-suspended in lysis buffer containing 2.5% *n*-dodecyl-β-D-maltoside (Anatrace) and solubilized under stirring for 30 min. Finally, the solution was cleared for insoluble material by centrifugation at 100,000*g* for 30 min.

Western blot. Solubilized membrane fractions were separated by SDS–polyacrylamide electrophoresis on a 10% Bis–Tris gel and subsequently blotted onto a polyvinylidenedifluoride membrane using an XCell II blotting module (Invitrogen). Immunodetection of the His-tagged recombinant protein was performed using the Penta-His HRP Conjugate kit (Qiagen) followed by SuperSignal® West Pico chemiluminescent substrate (Pierce). Signals were detected using a FluorChem® HD2 imaging system (Alpha Innotech).

Uptake Assay. The assay was performed essentially as described by Weitz et al. [6] with adjustments of the amount of cells and concentration of the dipeptide derivative β-Ala-Lys-AMCA (Biotrend). Briefly, the harvested cells were re-suspended in modified Krebs-buffer (25 mM Hepes/Tris pH 7.4, 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 5 mM glucose) to an optical cell density (600 nm) of 5×10^9 cells/mL. The assay volume of 100 μL contained 10 μL of 0.1 mM fluorescent β-Ala-Lys-AMCA stock solution, 50 μL of various concentrations of the competitor/substrate solutions or modified Krebs-buffer, and 40 μL of bacteria cells. All ligands (purchased from Sigma–Aldrich, Bachem or custom synthesized from CASLO) dilutions were prepared with Krebs-buffer. All uptake experiments were performed at 37 °C for a period of 60 s. After incubation, 500 μL ice-cold Krebs-buffer was added to stop uptake. The cells were pelleted and washed twice with ice-cold modified Krebs-buffer and subsequently dissolved in 100 μL modified Krebs-buffer. Nonspecific uptake control experiments with empty pTTQ18 vector transformed *E. coli* cells were performed under the same conditions and procedures as described above. Uptake was quantified by fluorescence measurements (excitation at 340 nm and emission 460 nm) using a Safire 2 fluorimeter. Specific β-Ala-Lys-AMCA uptake was determined by deducting unspecific uptake from total uptake.

All measurements were done in triplicates and all experiments were reproduced at least three times independently. Data analyses were performed using Graphpad Prism. All assay-related figures are a representative of three similar experiments.

Results

Our objective in this study was to over-express and investigate the substrate specificity of YjdL from *E. coli*. The preliminary results from uptake alanyl-peptide inhibition studies indicated that YjdL displays a lower specificity towards Ala-Ala-Ala compared to Ala-Ala. The subsequent experiments were designed to investigate this, among POTs, unusual property further.

Expression

YjdL was over-expressed in *E. coli* using the pTTQ18 vector. This vector has been shown to be particularly effective for over-expression of bacterial secondary transporters [10]. The final protein product is expressed as a fusion with a C-terminal hexa-His tag, in light of topology informed strategies for expression of membrane proteins in *E. coli* [11]. This tag was included for detection by Western blotting. Immunostaining of solubilized membrane fractions using a penta-HRP conjugate clearly showed a band at 37 kDa, not present in the membrane fractions of cells harboring the empty vector, corresponding to the over-expressed protein (Fig. 1A). Anomalous migration of secondary transporters in SDS-gels (calculated Mw of YjdL 53.9 kDa) is a common phenomenon, possibly caused by their hydrophobicity, high binding of SDS or the retention of secondary/tertiary structure facilitating their passage through the gel [12].

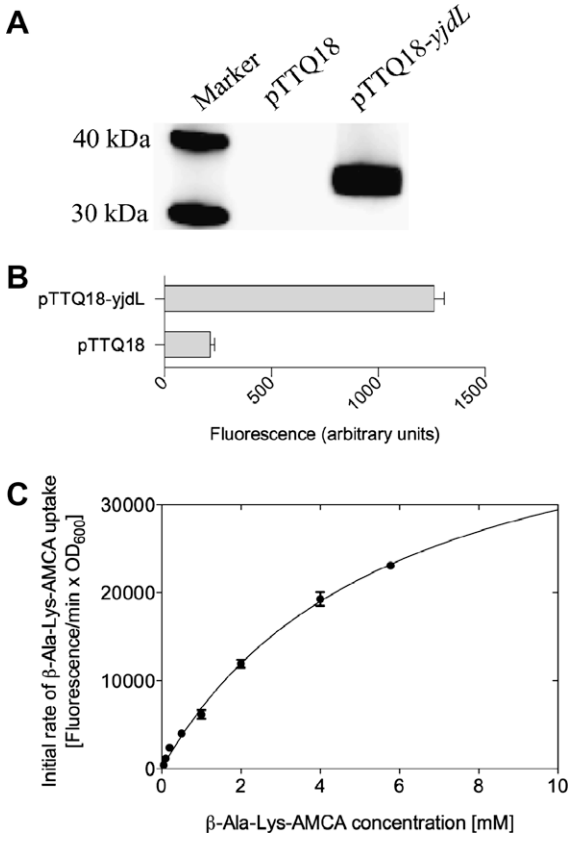


Fig. 1. (A) YjdL expression analyzed by Western blot showing the protein marker (MagickMark XP), solubilized membranes of IPTG induced *E. coli* BL21(DE3)pLysS cells harboring an empty pTTQ18 vector and the pTTQ18-*yjdL*, respectively. (B) β-Ala-Lys-AMCA uptake by IPTG induced *E. coli* BL21(DE3)pLysS cells harboring the plasmids pTTQ18 and pTTQ18-*yjdL*, respectively. The cells were incubated with modified Krebs-buffer containing 100 μM β-Ala-Lys-AMCA. (C) Saturation curve of β-Ala-Lys-AMCA specific uptake (normalized to number of cells) plotted as a function of substrate concentration. Uptake was measured at concentrations between 100 μM and 5.78 mM (maximal concentration achievable).

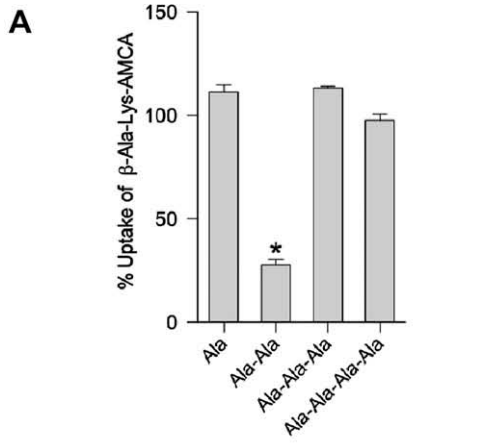
Uptake of β-Ala-Lys-AMCA

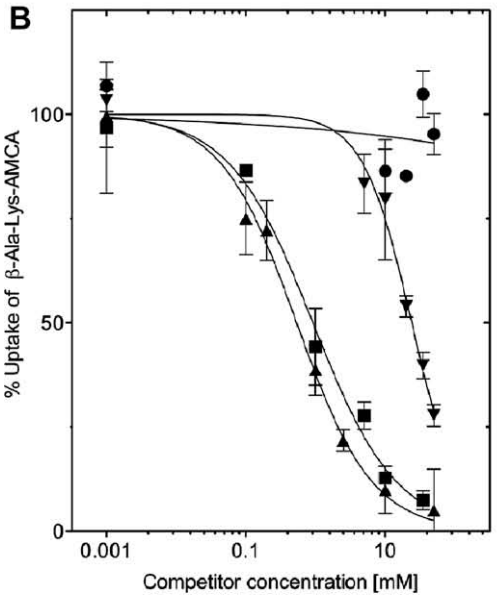
The activity of YjdL was assessed using the fluorescent dipeptide β-Ala-Lys-AMCA, which has been shown in several previous studies to be a substrate of the POTs [6,13]. Under the given experimental conditions, typically, a 5-fold increase in uptake relative to cells harboring an empty pTTQ18 vector or pTTQ18-*yjdL* without IPTG induction was observed (Fig. 1B). The rate of uptake did not show any significant variation due to pH changes in the range 6–8, and was linear up to 90 s at all measured β-Ala-Lys-AMCA concentrations (data not shown). Apparent saturable concentration dependent β-Ala-Lys-AMCA uptake was observed (Fig. 1C), corresponding to an apparent K_t of 5.2 ± 0.3 mM. Under the given experimental setup it was not possible to obtain data significantly beyond a concentration of 5.78 mM, to reach full saturation. In comparison, the *E. coli* POT YdgR showed a much lower K_t of 0.5 mM for β-Ala-Lys-AMCA [6], while YhiP was reported incapable to transport this peptide [7].

Peptide specificity

Initially, the ligand specificity of YjdL was probed by measuring the inhibitory effect of the mono-, di-, tri-, and tetra L-alanyl peptides, all at a concentration of 50 mM, on β-Ala-Lys-AMCA uptake (Fig. 2A). Only Ala-Ala showed an ability to inhibit the uptake by more than 50%, suggesting that YjdL has a significant dipeptide preference. This was supported by determination of the IC_{50} values of Ala-Ala (22 mM) and Ala-Ala-Ala (no significant inhibition up to a concentration of 50 mM) (Table 1). The lack of inhibitory effect of 50 mM of D-Ala-Ala, Ala-D-Ala or D-Ala-D-Ala as competitors (data not shown) furthermore indicated stereo specificity for amino acids with L-configuration. In comparison, both YdgR and YhiP can be inhibited almost equally well by both di- and trialanyl peptides (IC_{50} values of 0.52 and 0.24 mM for YdgR) [6,7], and YdgR tolerates both L- and D-Ala at the N-terminal position. To investigate possible charge or size preferences of YjdL, we tested the peptides Ala-Asp, Asp-Ala, Ala-Lys, Lys-Ala, Ala-Gln, Ala-Tyr, and Tyr-Ala (summarized in Table 1). Introduction of a negatively charged side chain essentially did not alter the affinity compared to Ala-Ala, and no notable positional preference was seen: Ala-Asp (IC_{50} of 15 mM) and Asp-Ala (IC_{50} of 24 mM). In contrast, a positively charged lysine side chain dramatically improved the affinity, but only when placed in the C-terminal position: Ala-Lys (IC_{50} of 0.3 mM) and Lys-Ala (IC_{50} of 32 mM). In comparison, YdgR and YhiP prefer a negatively charged residue in the C-terminal position and a positively charged residue at the N-terminal position [6,7]. Tyr-Ala (IC_{50} of 1.5 mM) and Ala-Gln (IC_{50} of 4 mM) showed that side chain bulk is preferred in both the positions; however, comparatively poor binding of Ala-Tyr (IC_{50} > 20 mM) suggested that the C-terminal binding pocket prefers a large but flexible side chain.

Ala-Lys, Ala-Gln and Tyr-Ala were the only dipeptides in our analysis that showed significantly higher inhibitory effects (10-fold) than Ala-Ala. To further investigate the possible preference of YjdL for di- over tripeptides, we pursued the results obtained on the most potent competitors in our study, Ala-Lys and Tyr-Ala, and tested the corresponding alanyl tripeptides. Alanyl extensions were chosen as they would minimize the influence of the side chain on the tripeptide conformation and because alanyl-containing dipeptides in general showed higher affinity than their glycine containing analogs (data not sown). Ala-Ala-Lys and Ala-Lys-Ala (IC_{50} of >50 mM and 8 mM, respectively), and Ala-Tyr-Ala and Tyr-Ala-Ala (IC_{50} of 31 mM and 31 mM, respectively), all showed significant higher IC_{50} values than their parent dipeptide. Thus, addition of an Ala residue in either end leads to a significant (almost 10-fold) reduction in competitive ability.





C

POT consensus	FYxxINxG
L104437	FVVGINMG
YdgR	YYMSVNIG
YhiP	FYMSVNIG
YbgH	MYAAGNVG
SF0588	MYAAGNVG
STY0750	LYAAGNIG
YjdL	^{142}LYAAGNIG^{149}
STY2807	LYAAGNIG
hPept1	FYLAINAG

Fig. 2. (A) Ligand inhibition profiles of mono-, di-, tri-, and tetraalanylpeptides. The cells were incubated with modified Krebs-buffer containing 100 μM β-Ala-Lys-AMCA and 50 mM of the ligand (asterisks indicate that values are significantly different, $P < 0.05$, from uninhibited YjdL). (B) Inhibition curves of Ala-Ala (downwards pointing triangles), Ala-Ala-Ala (circles), Ala-Lys (upwards pointing triangles) and Tyr-Ala (squares). The cells were incubated with modified Krebs-buffer containing 100 μM β-Ala-Lys-AMCA and increasing concentrations of the ligand. (C) Conserved region III sequences of a subset of POT members (*Lactococcus lactis*: L104437, *E. coli*: YdgR, YhiP, YbgH, YjdL, *Shigella flexneri*: SF0588, *Salmonella typhimurium*: STY0750, STY2807, *Homo sapiens*: hPept1) with a particularly high sequence similarity to YjdL are shown.

It is important to note that the results show how well certain peptides displace β-Ala-Lys-AMCA, and therefore do not demonstrate if the tested di- or tripeptides are actual substrates of YjdL.

Table 1
IC_{50} (mM) values with SEM ($n = 3$) for selected di- and tripeptides.

	IC_{50}
Ala-Ala	22 ± 2
Ala-Gln	4 ± 1
Ala-Asp	15 ± 2
Ala-Lys	0.3 ± 0.1
Ala-Tyr	>20
Asp-Ala	24 ± 3
Lys-Ala	32 ± 5
Tyr-Ala	1.5 ± 0.4
Ala-Ala-Ala	>50
Ala-Lys-Ala	8 ± 1
Ala-Ala-Lys	>50
Tyr-Ala-Ala	31 ± 2
Ala-Tyr-Ala	31 ± 2

Discussion

To date, several different POTs from bacteria, fungi, plants, and mammals have been characterized. The general consensus from the analyses is that these transporters are able to translocate di- and tripeptides albeit with differing affinities depending on the nature and position of the amino acid residues (reviewed by Daniel et al. [14]). Also, a peptide backbone does not seem to be a strict requirement for translocation [15].

The results presented here show that YjdL displays IC_{50} values at least 20-fold higher for the tested tripeptides compared to their structurally related dipeptides. To our knowledge the only POT member that has been reported to have higher specificity towards dipeptides compared to tripeptides is DtpT from *Lactococcus lactis* [16], though generally with a much higher affinity towards its ligands than YjdL. Fang et al. [16] showed that the tripeptides Ala-Ala-Ala, Leu-Leu-Leu, Arg-Arg-Arg and Phe-Phe-Phe all had at least 10-fold higher IC_{50}values than their corresponding dipetides. In the present study, we adopted a different approach, which involved the identification of alanyl dipeptides that would bind with higher affinity to YjdL than Ala-Ala. Dipeptides were identified with the affinity-improving residues being located in the N- or C-terminal ends, respectively: Ala-Lys (70-fold), Tyr-Ala (15-fold) and Ala-Gln (5-fold) showed an increase in affinity (Table 1). Although a more extensive screening of dipeptides is required for a fully understanding of side chain preferences, it is clear that YjdL has an unusually high dependency on side chain type for affinity. For the human peptide transporter 2 (hPept2), the dipeptides with the highest affinity show at most a 2-fold [17] and for hPept1 at most a 12-fold [18] increase in binding compared to Ala-Ala.

We subsequently extended the most potent dipeptides, Tyr-Ala and Ala-Lys, with an alanyl residue (Table 1). However, the affinity for these tripeptides at YjdL decreased significantly upon elongation with an alanyl residue on either side (8 to >50 mM). This suggests that tripeptides are accommodated poorly in the active site of YjdL, a feature quite different from its homologs from *E. coli*. Of note, DppA from *E. coli* has also been shown to have a significantly higher specificity towards dipeptides (excluding Gly-Gly) [19]. Although this apparently seems similar to YjdL, there are major differences: YjdL binds Ala-Ala with very low affinity and binding increases dramatically upon specific changes of residues, whereas DppA binds Ala-Ala with high affinity and the nature of the residues (except Gly) effects the affinity to a lesser extent.

Sequence analyses show that the POT family contains three regions with a high degree of conservation [14]. Region I, ExxERFxYYG, is located in the N-terminal region of helix I, and region II, GxxxADxxxGKxxTI, is located in between the C-terminal half of transmembrane helix II and the N-terminal end of transmembrane helix III. Region III, FYxxINxG, is located in transmembrane helix V and is the most conserved of the three regions. Mutational studies of the *Saccharomyces cerevisiae* POT in and around this latter region have shown that this transporter can be engineered to change preferences towards either di- or tripeptides [20]. Interestingly, the FYxxINxG is considerably different, L/MYxxGNxG, in a subset of bacterial POTs, which includes YjdL and YbgH (Fig. 2C). In the *L. lactis* POT this motif is FVxxINxG.

In the absence of an atomic structure of a POT it is difficult to visualize the reasons for the dipeptide preference and the significance, if any, of changes in conserved sequence motifs. However, the distant relation of the POTs to the MFS transporters suggests a similar structural organization: two six-helix domains connected by a cytoplasmic loop as seen from the crystal structures of the MFS proteins lactose permease [21], glycerol–phosphate transporter [22] and EmrD [23]. For both lactose permease and the glycerol–phosphate transporter transmembrane helix V has been shown to be functionally important [24,25]. In the case of lactose permease, a structure solved with an inhibitor shows that two functionally essential residues Arg144 and Trp151 are located in the N-terminal half of helix V and that Arg144 may be involved in substrate specificity [21].

In conclusion, we have shown that YjdL displays a ligand specificity different from the prototypical POTs, exemplified by its *E. coli* homologs YdgR and YhiP and the human hPept1. Further studies involving a broader range of dipeptides will enhance our view on the preference of residues in the YjdL active site. In addition, tripeptide translocation studies will be required to confirm whether tripeptides indeed are low affinity substrates or inhibitors of YjdL.

Acknowledgments

The authors would like to thank Professor Peter J.F. Henderson (Leeds) for the donation of the pTTQ18 vector and the Alfred Benzon Foundation, the Lundbeck Foundation and the Danish Research Council for financial support.

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