Functional and Structural Characterization of a Prokaryotic Peptide Transporter with Features Similar to Mammalian PEPT1

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The ydgR gene of Escherichia coli encodes a protein of the proton-dependent oligopeptide transporter (POT) family. We cloned YdgR and overexpressed the His-tagged fusion protein in E. coli BL21 cells. Bacterial growth inhibition in the presence of the toxic phosphonopeptide alafosafalin established YgdR functionality. Transport was abolished in the presence of the proton ionophore carbonyl cyanide p-chlorophenylhydrazone, suggesting a proton-coupled transport mechanism. YdgR transports selectively only di- and tripeptides and structurally related peptidomimetics (such as aminocrotoporphorins) with a substrate recognition pattern almost identical to the mammalian peptide transporter PEPT1. The YdgR protein was purified to homogeneity from E. coli membranes. Blue native-polyacrylamide gel electrophoresis and transmission electron microscopy of detergent-solubilized YdgR suggest that it exists in monomeric form. Transmission electron microscopy revealed a crown-like structure with a diameter of ~8 nm and a central density. These are the first structural data obtained from a proton-dependent peptide transporter, and the YgdR protein seems an excellent model for studies on substrate and inhibitor interactions as well as on the molecular architecture of cell membrane peptide transporters.

Peptide transporters are integral membrane proteins that mediate the cellular uptake of di- and tripeptides and a variety of peptidomimetics (for review, see Refs. 1–4). They are found in bacteria, yeast, plants, invertebrates, and vertebrates. In vertebrates, the two peptide transporter proteins, PEPT1 (SLC15A1) and PEPT2 (SLC15A2), are expressed predominantly in brush border membranes of small intestine (PEPT1), kidney (PEPT1 and PEPT2), and lung (PEPT2). In these transport proteins substrate flux is coupled to proton movement down an electrochemical proton gradient with the membrane potential as the main driving force. PEPT1 and PEPT2 accept essentially all 400 possible dipeptides and 8000 possible tripeptides composed of -1,-2 amino acids as substrates. Moreover, they also transport a large spectrum of therapeutic drugs like -lactam antibiotics, selected angiotensin-converting enzyme inhibitors, and peptidase inhibitors and thereby determine their bioavailability and pharmacokinetics. Certain drugs with an intrinsic low oral bioavailability like L-DOPA and acyclovir have by coupling to an amino acid (L-DOPA-Phe and Val-acyclovir) turned into substrates of peptide transporters with markedly improved availability (5, 6). Peptide transporters are, therefore, considered as important and potent drug delivery systems. Although functionally characterized in detail, very little is known about the structure of peptide transporter proteins. Twelve transmembrane domains are predicted (7), and amino acid residues critical for transport activity have been identified in particular in transmembrane domains 2, 3, 4, and 10 by functional analysis of mutants (8–12).

Mammalian peptide transporters are part of the PTR2 family of membrane transporters characterized by two signatures that are conserved in all family members (13). The first is a region that begins at the end of the second putative transmembrane domain, including the following first cytoplasmic loop as well as the third transmembrane domain. The second motif corresponds to the core region of the fifth transmembrane region. Besides the mammalian PEPT1 and PEPT2 proteins, the PTR2 family includes the yeast peptide transporter PTR2, DtpT from Lactococcus lactis, and numerous “orphan” transporters for which function is not known yet. Most orphan transporters are found in prokaryotic organism, e.g. the four members ybgH, ydgR, yhlP, and yjdl in Escherichia coli. Although these gene sequences belong to the same family, the function of the corresponding proteins may be quite different.

Here we describe the cloning of the ydgR gene, which was identified by sequence analysis as the E. coli homologue of tppB from Salmonella typhimurium (14). Based on growth experiments with mutant bacterial strains, tppB was already identified in 1984 as a tripeptide permease (15, 16). However, neither TppB nor YdgR has been characterized biochemically nor with respect to mode of function. We overexpressed YdgR as a
fusion protein with the IPTG\textsuperscript{3}-inducible pET-expression system. YdgR encodes a proton-dependent peptide transporter with a broad substrate specificity ranging from di- and tripeptides to a variety of related peptidomimetics-like $\beta$-lactam antibiotics. The YdgR protein was purified to homogeneity and functionally reconstituted into proteoliposomes. Analysis by blue native (BN)-polyacrylamide gel electrophoresis and transmission electron microscopy (TEM) of detergent-solubilized YdgR suggests a monomeric state of the protein. In addition, TEM provided the first structural data on a proton-dependent di- and tripeptide transporter.

**EXPERIMENTAL PROCEDURES**

Cloning and Expression of the YdgR Transport Protein in E. coli—Genomic DNA from E. coli strain O157:H7 was prepared with the Qiagen DNAeasy kit. The ydgR gene was cloned from genomic DNA by PCR with primers (5'-3') AAAAAAGTTATGTCCTGCAAACAAAAAAC and AAACTCGAGCGCTACGGGTCTTTTGC. PCR products were digested with HindIII/XhoI and ligated into pET-21 vector (T7 promoter, C-terminal hexahistidine tag, Novagen). A ribosomal binding site (rbs) (AAGGAG) was added 7 bases 5' of the coding region to improve translation of the protein. The DNA construct (pET-21-rbs-YdgR-His) was verified by sequencing.

Expression experiments were carried out with freshly trans-struct (pET-21-rbs-YdgR-His) was verified by sequencing. Experiments were carried out with freshly transformed E. coli BL21(DE3)pLysS. Cultures were grown in LB medium supplemented with 100 $\mu$g/ml ampicillin to $A_{600}$ of ~1, and protein expression was induced by adding 0.1 mM IPTG (final concentration). After 3 h of incubation at 37 °C cells were harvested for biochemical or functional analysis. A second expression vector (pET-21b-rbs-YdgR-His) was constructed by cutting pET-21-rbs-YdgR-His with HindIII/Xhol and ligating the insert into pET-21b vector.

Western Blot Analysis—For Western blot analysis, cells from 1 ml of culture were pelleted and resuspended in lysis buffer (10 mM Hepes-NaOH, pH 7.4, 0.5 mM EDTA, 1 mM dithiothreitol, and protease inhibitor mixture 1:500 (Sigma)) with lysozyme (1 $\mu$g) added. After 1 h of incubation on ice, bacterial DNA was degraded for 30 min by 1 unit of benzonase in the presence of 3 mM MgCl$_2$. Membranes were pelleted by centrifugation at 18,000 $\times$ g for 30 min and solubilized in buffer containing 10 mM Hepes-NaOH, pH 7.4, 150 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol, and 1% n-dodecyl-$\beta$-d-maltoside (DDM). After 15 min on ice unsolubilized material was removed by centrifugation at 40,000 $\times$ g for 45 min. Proteins were separated by SDS-PAGE and blotted onto polyvinylidene difluoride membranes (Millipore). Filters were blocked by incubation for 1 h with 1% (w/v) milk powder in Tris-buffered saline (TBS; 137 mM NaCl, 3 mM KCl, and 25 mM Tris-Cl, pH 7.5) followed by incubation for 60 min with anti-His antibody (1:2000 dilution, Novagen) in TBS-T (TBS, 0.05% Tween 20). Filters were washed twice in the same buffer and then incubated for 30 min with secondary antibody (1:5000 dilution, goat anti-mouse-horseradish peroxidase; Santa Cruz). Filters were first washed twice in TBS-T and twice with TBS (10 min/wash). Labeled proteins were detected using the ECL system (Amersham Biosciences).

Transport Assays—Transport assays were performed in vivo with cells 3 h after induction with IPTG (see above) with the fluorescent dipeptide $\beta$-Ala-Lys-$N_7$-amino-4-methylcoumarin-3-acetic acid ($\beta$-Ala-Lys-AMCA) (custom-synthesis by Biotrend, Cologne, Germany). $\beta$-Ala-Lys-AMCA was previously established as a reporter substrate for peptide transport (17, 18). Approximately 5 $\times$ 10\textsuperscript{9} cells were harvested by centrifugation and resuspended in 1.5 ml of modified Krebs-Buffer (25 mM Hepes/Tris 7.4, 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl$_2$, 0.8 mM MgSO$_4$, and 5 mM glucose). The assay volume of 100 $\mu$l was made up with 40 $\mu$l of bacteria cells (1.3 $\times$ 10\textsuperscript{8} cells), 10 $\mu$l of a 500 $\mu$m $\beta$-Ala-Lys-AMCA stock solution (final concentration 50 $\mu$m), and 50 $\mu$l of Krebs buffer (control) or a competitor solution. Uptake was performed for 15 min at 37 °C and stopped by washing the cells twice with ice-cold Krebs buffer by centrifugation. Uptake of $\beta$-Ala-Lys-AMCA was quantified by fluorescence (excitation at 340 nm and emission at 460 nm, Thermo Varioscan).

Purification of the YdgR Protein—Cell pellets from 600 ml of culture were resuspended in 25 ml of lysis buffer (10 mM Hepes/Tris, pH 7.4, 1 mM dithiothreitol, 0.5 mM EDTA, and a Sigma protease inhibitor mixture 1:500) and broken by sonification (10 cycles of 30 s). After a short low speed centrifugation to separate unbroken cells (4500 $\times$ g, 8 min) and a short high speed centrifugation to pellet the outer membrane (120,000 $\times$ g, 5 min) the inner membranes from the supernatant were collected by centrifugation at 120,000 $\times$ g for 1.5 h (at 4 °C). The pellets were resuspended in buffer (20% glycerol, 10 mM Hepes/Tris, pH 7.4, 0.5 mM Tris-2-carboxyethylphosphine, frozen in liquid nitrogen, and stored at −80 °C. YdgR membranes were solubilized (60 min, 4 °C) in 20 mM Tris-HCl, pH 8, 300 mM NaCl, 1% DDM, 10% glycerol, 0.01% NaN$_3$ at protein concentrations of 1–3 mg/ml. After centrifugation (100,000 $\times$ g, 45 min), the supernatant was diluted 2-fold with 20 mM Tris-HCl, pH 8, 300 mM NaCl, 0.04% DDM, 2 mM histidine, 10% glycerol, 0.01% NaN$_3$ (wash buffer) and bound to Ni-NTA Superflow beads (2 h, 4 °C; Qiagen). The beads were then washed onto a spin column (Promega), washed with wash buffer, and eluted with buffer containing 200 mM histidine.

Reconstitution into Proteoliposomes—For functional reconstitution YdgR-containing membranes were solubilized in buffer (10 mM Hepes/Tris, pH 7.4, 150 mM NaCl, 30 mM imidazole, 1% DDM, 5% glycerol, 0.1 mM Tris-2-carboxyethylphosphine) at a protein concentration of 1 mg/ml for 30 min on ice. After centrifugation (40,000 $\times$ g, 30 min) the supernatant was loaded onto a Ni-NTA column (HisTrap FF, Amersham Biosciences) and washed with running buffer (10 mM Hepes/Tris, pH 7.4, 150 mM NaCl, 30 mM imidazole, 0.06% DDM, 5% glycerol, 0.1 mM Tris-2-carboxyethylphosphine). Protein was eluted with a gradient from 30 to 250 mM imidazole in running buffer. YdgR elutes in a sharp peak at about 150 mM imidazole. For reconstitution, E. coli lipids (500 $\mu$l, Avanti Polar Lipids) at a concentration of 20 mg/ml in CHCl$_3$ were dried under vac-

3 The abbreviations used are: IPTG, isopropyl 1-thio-$\beta$-d-galactopyranoside; BN, blue native; DDM, n-dodecyl-$\beta$-d-maltoside; TEM, transmission electron microscopy; TM, transmembrane regions; Ni-NTA, nickel-nitrilotriacetic acid; rbs, ribosomal binding site; TBS, Tris-buffered saline; AMCA, N$_7$-amino-4-methylcoumarin-3-acetic acid; MES, 4-morpholineethanesulfonic acid; M$_{app}$, apparent molecular mass.
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uum and resuspended in 1 ml of buffer (25 mM Hepes/Na, pH 7.4, 150 mM NaCl). Liposomes were destabilized with 10 mg of DDM and sonicated for 30 min. 400 μl of purified YdgR protein as eluted from the Ni-NTA column was added at a concentration of 250 μg/ml. After 10 min incubation on ice, detergent was removed by adding 500 mg of Bio-Beads SM-2 Adsorbants (Bio-Rad) overnight at 4 °C. The detergent removal step was repeated with 200 mg of Bio-Beads for 4 h at 4 °C.

**Electrical Measurements of Reconstituted YdgR with the SURFE2Rone Setup**—Electrical measurements of YdgR transport were based on the solid-supported membrane technology, which allows detection of capacitively coupled currents (19). YdgR-loaded sensors were prepared as described by Zuber et al. (20) using YdgR proteoliposomes (see above) and SURFE2Rone gold electrodes from IonGate Biosciences. The measurements of transporter-related currents on the chip are based on the shift of electrical charges as the transporters go through the transport cycle, and the shift can originate from the movement of charged substrates or of protein moieties carrying (partial) charges. YdgR-mediated transport was activated via rapid solution exchange from a so-called non-activating (off) (40 mM KCl, 50 mM Hepes, 50 mM MES, 2 mM MgCl2, pH 6.7) to an "activating" (on) solution (containing 30 mM glycine and 20 mM glycyl-glycine). In activating solutions with lower glycylglycine concentrations, the total osmolarity of the organic solutes glycine and glycyl-glycine together was adjusted to 50 mM. After a rapid fluid exchange to a peptide-containing solution, the charging of the proteoliposomes on the sensor driven by the H+/peptide symport is measured. Previous comparisons of the characteristics of rheogenic transporters employing this new cell-free electrophysiological technique with findings from patch clamp studies revealed a very good correlation in all features (21).

**Blue Native Gel Electrophoresis**—Linear 5–12% gradient gels for BN-PAGE were prepared and run as previously described by Schägger and von Jagow (22). Thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), and bovine serum albumin (66 kDa) were used as standard proteins.

**Transmission Electron Microscopy**—DDM-solubilized YdgR protein as eluted from the Ni-NTA column was adsorbed for 10 s to parlodion carbon-coated copper grids rendered hydrophilic by glow discharge at low pressure in air. Grids were washed with four drops of double-distilled water and stained with 2 drops of 0.75% uranyl formate. Images were recorded on Eastman Kodak Co. SO-163 sheet films with a Hitachi H-7000 electron microscope operated at 100 kV.

**Data Analysis**—All experiments were performed for the indicated number of observations (n). IC50 values were obtained by nonlinear regression, and the value is given ± S.E. The Kf were calculated from the IC50 using the equation from Cheng and Prusoff (23).

**RESULTS**

**Cloning and Functional Expression of the YdgR Protein from E. coli**—The ydgR gene from E. coli was amplified from genomic DNA by PCR using gene-specific primers. The gene was cloned into the pET-21 vector fused with a C-terminal hexahistidine tag (pET-21-ysgR-His). The expression of the YdgR protein in E. coli BL21(DE3)pLysS was tested by Western blot analysis with an antibody derived against the hexahistidine tag (Fig. 1A). Proteins from non-induced (control, Fig. 1A, lanes 1–3) and induced E. coli (Fig. 1A, lanes 4–6) were fractionated in (i) soluble proteins, (ii) membrane proteins solubilized in 1% DDM, and (iii) insoluble proteins and separated by SDS-PAGE. The anti-His antibody detected a protein in the membrane protein fraction of induced cells (lane 5) with an apparent molecular mass of 39 kDa. Small amounts of the protein were detected in the insoluble protein fraction (lane 6), indicating some protein not properly solubilized or from inclusion bodies. Because no band was detected from extracts obtained from control cells, the 39-kDa protein band represents the YdgR protein. The discrepancy between the expected molecular mass (55 kDa) as deduced from the amino acid sequence of YdgR and the apparent molecular mass as determined by SDS-PAGE might

![FIGURE 1. Functional expression of YdgR. A, Western blot analysis of YdgR protein expressed in E. coli BL21(DE3)pLysS with pET-21-rbs-YdgR-His. Soluble and membrane fractions of uninduced (lane 1–3) and IPTG-induced (lane 4–6) E. coli cells were separated on a 12.5% SDS-polyacrylamide gel, subsequently transferred on polyvinylidene difluoride membrane, and probed with an anti-His antibody (Novagen). Each lane contains protein from an equivalent of 40 μl of bacterial culture: lanes 1 and 4, soluble proteins (sol); lanes 2 and 5, DDM-solubilized membrane proteins (ddm); lanes 3 and 6, non-solubilized proteins (pellet). B, Western blot analysis of YdgR protein expressed with a C-terminal hexahistidine tag compared with YdgR exhibiting a C-terminal hexahistidine tag and an N-terminal T7 tag. Membrane proteins of IPTG-induced E. coli cells were separated on a 12.5% SDS-polyacrylamide gel, subsequently transferred on a polyvinylidene difluoride membrane, and probed with an anti-His antibody (Novagen, lanes 1 and 2) or an anti-T7 antibody (Novagen, lanes 3 and 4). Each lane contains protein from an equivalent of 100 μl of bacterial culture. C, growth curve in the presence of 200 μg/ml alafosfalin. E. coli BL21 transformed with pET-21-rbs-YdgR-His were grown in the absence (a) and presence (b and c) of alafosfalin. At A600 = 1 expression of YdgR was induced by 0.1 mM IPTG (a and c).
be due to the known abnormal migration behavior of several membrane proteins. This often causes an underestimation of the true molecular mass. To test this hypothesis and to verify that the protein is not degraded, we constructed a second expression vector with an N-terminal T7 tag (11 N-terminal amino acids of the T7 gene 10 plus 10 amino acids spacer) in addition to the C-terminal hexahistidine tag (pET-21b-rbs-T7-ydgR-His). The apparent molecular mass of the two proteins YdgR-His and T7-YdgR-His were compared by Western blot analysis with the anti-His antibody (Fig. 1B, lanes 1 and 2) and the anti-T7 antibody (Fig. 1B, lanes 3 and 4). As expected, both proteins were detected by the anti-His antibody (Fig. 1B, lanes 1 and 2), whereas the anti-T7 antibody detected only T7-YdgR-His (Fig. 1B, lane 4). Because both antibodies detected the same protein (T7-YdgR-His) and the apparent molecular mass of this protein is only slightly increased compared with YdgR-His, we conclude that the overall calculated low apparent molecular mass is due to an abnormal migration behavior but not proteolysis. All further experiments were performed with the pET-21-rbs-ydgR-His construct.

To assess the functionality of the expressed YdgR protein, simple growth experiments were conducted (Fig. 1C). In S. typhimurium the toxic phosphonopeptide alafosfalin is taken up by the tripeptide permease TppB, and mutants in TppB show resistance to alafosfalin (16). We, therefore, grew E. coli carrying the vector (pET-21-rbs-YdgR-His) in the presence of a non-toxic concentration of alafosfalin (200 μg/ml, Fig. 1C, curve b). As compared with E. coli grown in the absence of alafosfalin (curve a), growth rates were only slightly reduced. When expression of YdgR was induced at an A600 of about 1 (curve c), the peptide then caused cell death. This indicates that YdgR was functionally expressed in the membrane, loading the cells with the toxic agent. Transport function of YdgR was also determined using the fluorescent dipeptide reporter β-Ala-Lys-AMCA as a substrate (Fig. 2A). Cells expressing YdgR showed only minor autofluorescence in the absence of β-Ala-Lys-AMCA (bar 4), but fluorescence increased significantly after incubation of cells with 50 μM β-Ala-Lys-AMCA (bar 5). Fluorescence was abolished by the addition of an excess amount of the non-labeled dipeptide Gly-Gln (bar 6). When β-Ala-Lys-AMCA uptake was determined as a function of its concentration, transport was found to be saturable with an apparent Kt of 0.44 ± 0.05 mM (Fig. 2C). Control experiments with pET-21 vector transformed E. coli showed no uptake of β-Ala-Lys-AMCA (bars 1–3), indicating that no other endogenous transport system in E. coli mediates β-Ala-Lys-AMCA uptake. For further characterization of transport, we studied the requirements of Na+ and H+ as cotransport ions (Fig. 2B). The replacement of Na+ by choline had no significant effect on the uptake of β-Ala-Lys-AMCA, but the presence of CCCP, a proton ionophore, caused complete inhibition of transport. Thus, peptide uptake via YdgR depends on the proton-motive force and is most likely as described for the other family members mediated by a coupled proton-substrate cotransport.

Studies on Substrate Specificity of YdgR—To determine the transporter’s substrate specificity, E. coli cells overexpressing YdgR were incubated with 50 μM β-Ala-Lys-AMCA in the presence of an excess (200-fold) of l-alanine either as free amino acid or as a di-, tri-, and tetrapeptide (Fig. 2D). Alanine and tetra-alanine did not inhibit uptake of β-Ala-Lys-AMCA, whereas di- and tri-alanine completely inhibited β-Ala-Lys-AMCA uptake. Inhibition of transport by increasing concentrations of di- and tri-alanine allowed apparent affinities (IC50 values) of 0.52 ± 0.03 and 0.24 ± 0.01 mM to be determined (Fig. 3A). Stereospecificity of YdgR-mediated flux was assessed by determining IC50 values of dipeptides carrying L- or D-alanine residues (Fig. 3B). Substitution of the N-terminal l-Ala for the d isomer did not alter affinity significantly, but when the C-terminal l-Ala was replaced by a d isomer, affinity of the competitor was reduced about 8-fold (Fig. 3B). d-Ala-d-Ala did not show detectable affinity for interaction with YdgR (Fig. 3B).

Transport Characteristics of Differently Charged Dipeptide Substrates—To further characterize substrate specificity of YdgR, we determined IC50 values of various other compounds ranging from differently charged dipeptides consisting of L-α-amino acids to an ω-α-amino fatty acid and a known inhibitor of mammalian peptide transporters (see Table 1). Neutral dipeptides represented by Gly-Gln or cationic peptides such as Lys-Gly showed relatively high affinities with IC50 values of 0.51 ± 0.06 mM and 0.43 ± 0.02, respectively. Introducing the posi-
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A

B

C

FIGURE 3. Substrate specificity of the YdgR protein. A, inhibition of β-Ala-Lys-AMCA uptake by the di- and tripeptides of L-Ala. Uptake of β-Ala-Lys-AMCA (50 μM) was determined in the presence of increasing concentrations (0.05–10 mM) of either L-Ala–L-Ala (circles) with an IC_{50} of 0.52 ± 0.03 mM or L-Ala–L-Ala–L-Ala (triangles) with an IC_{50} of 0.24 ± 0.01 mM. B, stereoselectivity of transport was determined by inhibition of β-Ala-Lys-AMCA (50 μM) uptake by 0.1–10 mM D-Ala–L-Ala (triangles) with an IC_{50} of 0.48 ± 0.05 mM, or 0.8–80 mM L-Ala–D-Ala (circles) with an IC_{50} of 4.10 ± 0.33 mM, or 5–80 mM L-Ala–D-Ala (squares). C, interaction of peptidomimetics with YdgR was tested by uptake (n = 4, * n = 2) of 50 μM β-Ala-Lys-AMCA in the absence (bar 1) and presence of 10 mM concentrations of the competitors Gly-Gln (bar 2), cefadroxil (bar 3), cefalexin (bar 4), cephradine (bar 5), cephapram (bar 6), cefuroxime (bar 7), ampicillin (bar 8), amoxicillin (bar 9), captopril (bar 10), or enalapril (bar 11).

TABLE 1
Summary of IC_{50} values (mM) and K_{i} values

| Substrate | IC_{50}  | K_{i}   |
|-----------|---------|---------|
| Gly-Gln   | 0.21 ± 0.06 (7) | 0.46 ± 0.05 |
| Lys-Gly   | 0.03 ± 0.02 (4) | 0.59 ± 0.02 |
| Gly-Lys   | 2.83 ± 0.27 (5) | 2.54 ± 0.24 |
| Asp-Gly   | 4.46 ± 0.45 (6) | 4.00 ± 0.40 |
| Gly-Asp   | 1.92 ± 0.13 (12) | 1.72 ± 0.12 |
| Gly-Ala   | 0.77 ± 0.05 (4) | 0.69 ± 0.04 |
| Lys-Ala   | 1.27 ± 0.10 (4) | 1.14 ± 0.09 |
| Ala-Ala   | 0.52 ± 0.03 (6) | 0.47 ± 0.03 |
| Ala-D-Ala | 4.10 ± 0.33 (4) | 3.68 ± 0.30 |
| d-Ala-d-Ala| 0.84 ± 0.05 (4) | 0.43 ± 0.04 |
| Ala-Ala   | 0.24 ± 0.01 (8) | 0.22 ± 0.01 |
| Gly-Sar   | 1.16 ± 0.09 (10) | 1.04 ± 0.08 |
| Alafosfalin| 0.28 ± 0.03 (4) | 0.25 ± 0.03 |
| Lys-Z-Nitro-Pro| 0.033 ± 0.006 (4) | 0.03 ± 0.01 |
| 5-Aminolevulinic acid | 1.60 ± 0.14 (6) | 1.52 ± 0.13 |

instead of Lys was placed in the first position affinity decreased by 10-fold (Asp-Gly, IC_{50} = 4.46 ± 0.45 mM), whereas when placed in the second position, affinity increased again when compared with a Lys residue (Gly-Asp, IC_{50} = 1.92 ± 0.13 mM). Modifying the peptide bond nitrogen by a CH_{3} group like in glycyl-sarcosine yielded moderate affinity (IC_{50} = 1.16 ± 0.09 mM). The highest affinity of all test compounds displayed the inhibitor of mammalian peptide transporters Lys-Z-nitro-Pro with an IC_{50} value of 0.033 ± 0.006 mM. Mammalian peptide transporters do not require a peptide bond for recognition of a substrate (25). To test whether this holds true also for the YdgR protein, we used 5-aminolevulinic acid as a substrate that carries only the two oppositely charged head groups separated by four carbon units and a backbone carbonyl. Its apparent affinity was 1.69 ± 0.14 mM and, therefore, higher than that of most charged dipeptides.

Transport of Peptidomimetics—
Beside di- and tripeptides, mammalian peptide transporters accept a broad spectrum of peptidomimetics like β-lactam antibiotics and ACE inhibitors as substrates. We have tested selected peptidomimetics as known substrates of mammalian peptide transporters for analysis of their interaction with the YdgR protein in competition assays (Fig. 3C). Compared with the dipeptide Gly-Gln (bar 2), which inhibited β-Ala-Lys-AMCA uptake by 97%, similar inhibition rates of 86, 79, and 79%, respectively, were observed for the three aminocephalosporins cefadroxil (bar 3), cefalexin (bar 4), and cephradine (bar 5) at 10 mM concentrations. Cefuroxime (bar 6) and cefamandole (bar 7) showed markedly reduced affinities with modest inhibition of only 21 and 32%, respectively, which results from the lack of an α-amino group important for high affinity. Whereas the two aminopenicillins ampicillin (bar 8) and amoxicillin (bar 9, 5 mM concentration) seemed not to serve as substrates, the angiotensin I-converting enzyme inhibitors captopril (bar 10) and enalapril (bar 11) also showed only low affinity type inhibition of β-Ala-Lys-AMCA uptake.

Purification and Functional Reconstitution of YdgR—Inner membranes of E. coli were solubilized with DDM, and YdgR was purified by nickel affinity chromatography. The SDS-polyacrylamide gel displayed in Fig. 4A summarizes the different purification steps. The purified YdgR protein migrated as a single band with an apparent mass of ~39 kDa that is identical to that observed by Western blot analysis for the non-purified tively charged amino acid at the C-terminal position (Gly-Lys) resulted in a 7-fold reduction of affinity represented by an IC_{50} of 2.83 ± 0.27 mM as compared with 0.43 mM for Lys-Gly. This indicates an asymmetric substrate binding site in YdgR similar to that described for the mammalian peptide transporters (24). This observation is strengthened by experiments with the anionic dipeptide Asp-Gly. When Asp
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The genome of *E. coli* contains four yet uncharacterized members of the family of proton-dependent oligopeptide transporter (POT family) named *ydgR*, *ybgH*, *yhiP*, and *yjdL* as identified by sequence analysis. Because of a lack of functional data, they are still classified as hypothetical proteins. We have cloned the *ydgR* gene from genomic DNA and overexpressed the protein in *E. coli* BL21 cells. Coomassie-stained SDS-PAGE and Western blot analysis identified the *YdgR* protein, and uptake experiments with the fluorescent dipeptide β-Ala-Lys-AMCA in bacterial cells demonstrated its function as a dipeptide transporter with features similar to mammalian peptide transporters. Moreover, employing the SURFE²Rone sensor technology we demonstrate for the first time rheogenic transport by a prokaryotic peptide transporter.

The bacterial peptide transport system tppB (tripeptide permease) was genetically characterized in mutants of *S. typhimurium*. Deficiency in the locus for tppB conferred resistance to the toxic phosphonopeptide alafosfalin, and this was classified as a characteristic tppB activity (16). By locus analysis, the *ydgR* gene of *E. coli* was identified as a similar tripeptide permease (tppB) (14) but had not been functionally characterized. By growth experiments with the toxic phosphonopeptide alafosfalin we could experimentally confirm that *YdgR* represents the *of E. coli* homolog of tppB. However, our functional data on substrate specificity of *YdgR* reveal that not only tripeptides but also dipeptides serve as substrates. Moreover, transport was proven to be Na⁺-independent and completely abolished in the presence of the proton ionophore CCCP, suggesting a proton-coupling of substrate transport in analogy to other members of the family such as PEPT1 or PEPT2. These two proteins operate as electrogenic proton-coupled symporters with a variable flux-coupling stoichiometry for proton to substrate cotransport and the main driving force being provided by membrane voltage. Via the SURFE²Rone measure-
ments of YdgR reconstituted into proteoliposomes, we could experimentally verify that dipeptide transport by YdgR is also of an electrogenic nature and occurs in the absence of Na\(^+\)/H\(^+\) ions, most likely in analogy to PEPT1 and PEPT2 as proton-peptide symport.

The substrate recognition pattern of YdgR shows a remarkable and unexpected similarity to the mammalian PEPT1 in various aspects. All known substrates of PEPT1 that were tested with YdgR also interact with the substrate binding site of the bacterial protein with very similar affinities. Moreover, the observed stereoselectivity of transport and differences in affinities of charged peptides with identical side chains but in different spatial position (N- versus C-terminal) are also characteristic for PEPT1. Finally, even peptidomimetics such as the aminoccephalosporins interact with the YdgR substrate binding site with similar affinities as determined for PEPT1. YdgR represents in its substrate recognition pattern in all aspects a mammalian PEPT1-phenotype. This suggests that YdgR possesses a similar architecture in the substrate binding domain.

There is a moderate degree of sequence homology between YdgR and mammalian PEPTs. We have projected the YdgR amino acid sequence onto the proposed topology model of PEPT1 (Fig. 6) on the basis of a Clustal W analysis (26). Highest sequence identity can be found in the first half of the protein especially in the transmembrane regions (TM) 1–6 and the first extracellular loop. A modest sequence identity is also found in TM10–12 and as suggested by functional analysis of mammalian PEPTs based on chimeric proteins and by site-directed mutagenesis, these regions are involved in substrate binding and transport (8–12, 27). Some of the amino acid residues in these regions were identified as essential for transport by PEPT1 and are well conserved in the YdgR protein including Trp\(^{295}\) in TM7 and Glu\(^{595}\) in TM10. The amino acid His\(^{121}\) in TM4 is not conserved, but there is an alternative His residue nearby in the primary structure of YdgR. Surprisingly the His\(^{57}\) residue that is described as essential for proper function in the mammalian peptide transporters is not conserved in YdgR and is replaced by a serine residue. The lowest homology found between the mammalian proteins is in transmembrane regions 7–9 together with the loops in between. These regions are thought to contribute to the different kinetic phenotypes of PEPT1 and PEPT2 (28). Most strikingly, YdgR does not possess the large extracellular loop between TM 9 and TM 10 found in PEPT1 and PEPT2, which suggests that the loop domain is not important at all for the transport process.

Nothing is known about the three-dimensional structure of any peptide transporter protein of the PTR family. Protein expression is low in native cells and tissues, and heterologous expression of mammalian PEPT1 in Pichia pastoris did not yield enough protein for structural analysis (29). The discovery of a bacterial homologue with essentially identical transport characteristics to the mammalian proteins that can be
expressed and purified with high yield represents, therefore, an important step toward structural analysis of the protein and insights into the transport mechanism. We were able to obtain 2–4 mg of pure and stable YdgR membrane protein from 1 liter of bacterial culture. BN-PAGE of purified YdgR displayed a strong band with a $M_{obs}$ of $\sim 85$ kDa (Fig. 4B). This $M_{obs}$ is composed of the molecular mass of YdgR ($\sim 55$ kDa) and the mass of the DDM-Coomassie Brilliant Blue G-250 micelle and lipids attached to the protein. Thus, the $M_{obs}$ indicates that YdgR may exist as a monomer. The use of the conversion factor determined by Heuberger et al. (30) to estimate the mass of membrane proteins from the $M_{obs}$ also supports the monomeric nature of YdgR. Chemical cross-linking experiments performed with the amino-specific reagents disuccinimidyl suberate and bisulfo-succinimidyl suberate did not yield specific cross-linking products (data not shown), in line with the results from BN-PAGE.

TEM of negatively stained DDM-solubilized YdgR proteins revealed a crown-like structure with a central density. The measured diameter of the YdgR particles was $\sim 8$ nm. Assuming a boundary layer of $\sim 1.5$ nm (31) for DDM attached to the hydrophobic part of the protein, the resulting diameter is similar to that of the red permease monomer when embedded into the lipid bilayer; Red permease is a lactose permease fusion protein that forms monomers and trimers. It consists of 12 transmembrane helices and has a similar molecular mass to YgdR (32).

In summary, we have cloned, overexpressed, and characterized biochemically, functionally, and structurally the prokaryotic proton-dependent peptide transporter YdgR. The substrate recognition pattern of YdgR shows a remarkable similarity to the mammalian PEPT1 protein, and therefore, YdgR could serve as a paradigm for the mammalian peptide transporters. To understand the biophysical principles underlying transmembrane peptide transport, a high resolution structure is indispensable. Our present work with YdgR sets the basis for further structural analysis using two-dimensional and three-dimensional crystals and electron and X-ray crystallography studies of the first peptide transporter protein. Obtaining a high resolution structure of YdgR is crucial for any exploitation of the architecture of this class of transport proteins that are unique among the solute carriers with respect to their ability to transport literally thousands of substrates differing in size, polarity, and charge.

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